British Journal of Pharmacology

January 1994

Volume 111 Number 1 pages 1–388

Br. J. Pharmacol. (1994), **111**, 1–3

SPECIAL REPORT

Natriuretic action of angiotensin(1-7)

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Evidence that angiotensin(1-7) (Ang(1-7)) is biologically active and can be synthesized by the kidney prompted us to examine its actions in the rat, isolated kidney. Ang(1-7) had three major effects producing, (1) a substantial natriuresis and diuresis, (2) an increase in urinary sodium concentration associated with a fall in potassium concentration and (3) an increase in glomerular filtration rate without affecting renal vascular resistance. Thus, Ang(1-7) may participate in the renal effects of the renin-angiotensin system.

Keywords: Angiotensin(1-7); electrolyte excretion; GFR; rat isolated kidney; angiotensin II

Introduction Angiotensin(1-7), (Ang(1-7)) has generally been regarded as an inactive fragment of angiotensin I (Ang I) or angiotensin II (Ang II) degradation (Erdös & Skidgel, 1990). However, recent studies have demonstrated that Ang(1-7) has biological activity, distinguishable from that of Ang II (Ferrario *et al.*, 1991).

Enzymes necessary for Ang(1-7) formation are abundant in the kidney and have also been found in urine (Erdös & Skidgel, 1990). Moreover, Ang(1-7) is the major product of Ang I metabolism by proximal tubule membranes (Stephenson & Kenny, 1987). We therefore examined the renal actions of Ang(1-7) using the isolated kidney of the rat in which tubular function is preserved due to perfusion with oncotic agents. The use of this preparation excludes systemic influences, such as alterations in release of vasopressin and sympathetic nervous system activity provoked by Ang(1-7). Additional influences such as changes in extracellular fluid volume or the activity of the renin-angiotensin-aldosterone system are also obviated. At a dose as low as 0.1 pmol ml^{-1}, Ang(1-7) provoked a diuretic-natriuretic response without altering renal vascular resistance (RVR).

Methods Right kidneys from male Sprague Dawley rats (325–400 g, Charles River, Wilmington, MA, U.S.A.) were perfused *in situ* as described previously (Quilley & McGiff, 1990) with the following changes; 10 mM NaCl was replaced with 10 mM $NaCH_2COOH$ and bovine serum albumin was obtained from a different source (Bovimar, Purchase, N.Y., U.S.A.). Flow rate was adjusted throughout to give a mean renal perfusion pressure of 90 mmHg. Thus, changes in glomerular filtration rate (GFR) and electrolyte excretion were independent of changes in perfusion pressure.

Following equilibration for 15 min and a control 10 min, pretreatment clearance period, kidneys were infused with either Ang(1-7), kindly provided by Dr Mahesh C. Khosla, (Cleveland Clinic Foundation, Cleveland, OH, U.S.A.) or Ang II (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 5 consecutive experimental clearance periods. Infusion rates were adjusted in accordance with perfusate flow rate changes so that peptide concentrations remained constant.

In pilot studies the dose-range over which Ang(1-7) produced an effect was compared to that of Ang II ($n = 3$ per group) by infusing the peptides incrementally to achieve rates of 0.1–10.0 pmol ml^{-1}. Having established the effective dose-range, the effect of continuous exposure to Ang(1-7) at a single dose of 3 pmol ml^{-1} ($n = 7$) was compared to that of control kidneys infused with an equivalent volume of the 0.9% w/v saline vehicle throughout ($n = 7$). Perfusate and urine flow rates, GFR, electrolyte excretion and statistical analyses were determined as described previously (Quilley & McGiff, 1990).

Results At the lowest dose tested (0.1 pmol ml^{-1}) Ang(1-7) produced significant ($P<0.05$) diuretic and natriuretic responses of 58 ± 2 µl min^{-1} and 3.6 ± 0.7 µEq min^{-1} compared to pretreatment values of 36 ± 4 µl min^{-1} and 1.6 ± 0.2 µEq min^{-1}, respectively. As the dose was increased, further increments were observed. RVR was unchanged, irrespective of dose. In contrast, graded-dose infusion of Ang II produced the expected dose-dependent elevation in RVR which was significantly increased ($P<0.05$) at 0.3 pmol ml^{-1} to 3.9 ± 0.5 mmHg ml^{-1} min^{-1} versus 2.8 ± 0.1 mmHg ml^{-1} min^{-1} in the control and began to level off at 3 pmol ml^{-1} and 10 pmol ml^{-1} being 12.6 ± 1.1 and 14.1 ± 1.3 mmHg ml^{-1} min^{-1}, respectively. In addition, GFR was reduced significantly ($P<0.05$) by Ang II at doses of 1–10 pmol ml^{-1} falling to 54–67% of the respective time-control values. Water and electrolyte excretion were minimally affected by 0.1–1 pmol ml^{-1} Ang II. However, at the two highest doses there was an abrupt increase in excretion rates to values similar to those evoked by Ang(1-7), i.e. Ang II increased sodium excretion to 6.9 ± 0.6 and 16.6 ± 1.3 µEq min^{-1}, respectively compared to 11.2 ± 3.1 and 13.0 ± 2.7 µEq min^{-1}, respectively for 3 and 10 pmol ml^{-1} Ang(1-7).

Having established the potency range, Ang(1-7) was infused throughout at a dose of 3 pmol ml^{-1}. RVR (Table 1) and therefore, perfusate flow rate were not significantly altered. However, GFR tended to be higher being significantly different from control ($P<0.05$) in three of the five experimental clearance periods (Table 1).

Ang(1-7) produced a prompt diuretic response with a doubling of urine volume in the first experimental clearance period. Continued infusion elicited further increases which stabilized by the end of the third clearance period (Table 1). Even greater increments in the excretion of sodium were observed (Table 1) as its urine concentration also increased (Table 1). Potassium excretion increased (Table 1) to a lesser extent than sodium, and tended to decline with long-term Ang(1-7) infusion as the urinary concentration of potassium was reduced (Table 1).

The effects of Ang(1-7) on water and electrolyte excretion could be dissociated from the increase in GFR. For example, fractional water excretion was significantly higher ($P<0.05$) than control in all but the first experimental clearance period reaching a maximum of 12.5 ± 1.3% compared to the control of 6.4 ± 1.5%. Fractional sodium excretion increased ($P<0.05$) in periods four and five to 6.2 ± 0.9 and 6.8 ± 0.7% versus control values of 2.2 ± 0.8 and 3.0 ± 0.9%, respectively.

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Table 1 Effect of single dose infusion of angiotensin(1-7) (Ang(1-7)) at 3 pmol ml^{-1} on glomerular filtration rate, water and electrolyte excretion rates and urinary electrolyte concentrations of the rat, isolated perfused kidney

Treatment group	*Pretreatment*	*Clearance period (10 min each) Post treatment* 1	2	3	4	5
			Renal vascular resistance (mmHg ml^{-1} min^{-1})			
Control	2.9 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
Ang(1-7)	2.9 ± 0.2	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
			Glomerular filtration rate (ml^{-1} min^{-1})			
Control	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
Ang(1-7)	1.2 ± 0.1	1.6 ± 0.1*	1.4 ± 0.1	1.2 ± 0.1*	1.1 ± 0.1	1.1 ± 0.1*
			Urine volume (μl min^{-1})			
Control	20 ± 2	27 ± 4	36 ± 8	38 ± 9	44 ± 8	43 ± 7
Ang(1-7)	31 ± 3	62 ± 10*	96 ± 12*	112 ± 15*	124 ± 14*	130 ± 13*
			Sodium excretion rate (μEq min^{-1})			
Control	0.8 ± 0.2	1.1 ± 0.3	1.8 ± 0.6	2.2 ± 0.7	2.6 ± 0.7	2.8 ± 0.7
Ang(1-7)	1.1 ± 0.3	3.4 ± 0.9*	6.0 ± 1.4*	9.7 ± 1.4*	9.9 ± 1.3*	10.2 ± 1.0*
			Potassium excretion rate (μEq min^{-1})			
Control	0.9 ± 0.1	1.3 ± 0.3	1.7 ± 0.4	1.5 ± 0.2	1.5 ± 0.2	1.2 ± 0.2
Ang(1-7)	1.8 ± 0.3	3.4 ± 0.4*	3.1 ± 0.2*	2.7 ± 0.1*	2.3 ± 0.1*	2.4 ± 0.3*
			Sodium concentration (μEq ml^{-1})			
Control	38 ± 11	41 ± 11	43 ± 11	50 ± 10	52 ± 9	56 ± 11
Ang(1-7)	32 ± 6	48 ± 7	63 ± 6	75 ± 3*	79 ± 2*	80 ± 5*
			Potassium concentration (μEq ml^{-1})			
Control	42 ± 4	47 ± 4	48 ± 4	46 ± 4	39 ± 4	33 ± 4
Ang(1-7)	55 ± 4	56 ± 3	41 ± 6	20 ± 5*	20 ± 2*	19 ± 2*

Statistical significance of the difference at $P < 0.05$ between control ($n = 7$) and Ang(1-7) treated ($n = 7$) kidneys was determined by analysis of covariance against the pretreatment values to take account of possible differences prior to drug infusion

Discussion The observation that Ang(1-7) had a positive effect on GFR and increased water and electrolyte excretion in a dose-dependent fashion is the first evidence that this heptapeptide fragment of the renin-angiotensin system can affect function of the whole kidney. Moreover, Ang(1-7) had a unique profile of activity producing sustained effects on excretion which occurred at doses lower than those for Ang II and were not accompanied by increases in RVR, in contrast to the dose-dependent vasoconstrictor action of Ang II, observed in this and other studies using the isolated kidney preparation (Bell-Quilley *et al.*, 1993). As Ang(1-7) increased GFR in the absence of a change of RVR it may modify determinants of the ultrafiltration coefficient or affect discreet segmental resistance changes in the renal vasculature. The latter, in turn, may contribute to the increased excretion rates as a consequence of alterations in peritubular pressures.

Given the magnitude of the natriuretic response to Ang(1-7), coupled with the increase in urinary sodium concentration and fall in potassium concentration, it is likely that Ang(1-7), like Ang II, has direct effects at both proximal and distal nephron sites. Studies on the tubular actions of Ang II, using several different experimental approaches, have shown that in the proximal tubule Ang II has a dose-dependent, biphasic effect on sodium reabsorption (Harris & Navar, 1985), where higher doses inhibit sodium transport. In addition, Ang II inhibits sodium reabsorption distally (Harris & Navar, 1985). Likewise in the rat, isolated kidney the response is complex as Ang II elicits a biphasic natriuretic response as a function of dose (personal observation) as well as having multiple haemodynamic effects (Harris & Navar, 1985, Bell-Quilley *et al.*, 1993). However, unlike Ang(1-7) the natriuretic phase of the response to Ang II is consistantly accompanied by a marked vasoconstriction suggesting that the renal effects of the two peptides are due to activation of different receptor subtypes. In this regard, it has been suggested that Ang(1-7) stimulation of prostaglandin release in non-renal tissue is predominantly through AT_2 receptor activation based on blockade by AT_2 selective antagonists (Jaiswal *et al.*, 1992), some of which have preferential affinity for the AT_{1B} receptor e.g. PD123177 (Ernsberger *et al.*, 1992). Although many of the actions of Ang II have been attributed to AT_1 receptor activation it is also known that Ang II binds to multiple receptor subtypes. It is possible therefore, that the increase in RVR and the antinatriuretic effect of Ang II results from activation of receptors not stimulated by Ang(1-7) but that the two peptides interact with the same receptor to increase excretion rates. Alternatively, the natriuretic effect of Ang II could result from its conversion to Ang(1-7) prior to receptor activation (Erdös & Skidgel, 1990).

Ang(1-7) is the earliest and also the major product of Ang I metabolism in various tissues (Ferrario *et al.*, 1991), including the proximal tubule (Stephenson & Kenny 1987). Moreover, in rat kidney, Ang(1-7) concentrations are 6 fold higher than levels in the systemic circulation (Campbell *et al.*, 1991) suggesting its intrarenal formation. Ang(1-7) continues to be formed during angiotensin converting enzyme (ACE) inhibition and plasma levels of Ang(1-7) in the rat are elevated 25 fold by ACE inhibition (Campbell *et al.*, 1991). In view of its natriuretic effect, it is therefore possible that increased Ang(1-7) participates in the antihypertensive effect of these drugs. Interestingly, neutral endopeptidase 24.11, a major enzyme for Ang(1-7) formation, has the same renal distribution as ACE being richest in the proximal brush border (Erdös & Skidgel, 1990). Thus, the requisite enzymes are strategically placed to generate both Ang(1-7) and Ang II in the nephron segment with the greatest concentration of angiotensin receptors and greatest capacity for sodium reabsorption.

In conclusion, the demonstration that Ang(1-7) has potent natriuretic activity coupled with evidence for its formation in the kidney lend support to an endogenous role. In view of the high levels of neutral endopeptidase 24.11 and ACE in the proximal tubule, sodium chloride reabsorption at this site may be regulated by the relative concentration of Ang(1-7) to Ang II. Subsequently, delivery of solute to more distal parts of the nephron, such as the macula densa, also may be regulated. We suggest that the expression of the renal response to activation of the renin-angiotensin system may not

only be due to the actions of Ang II as the major effector peptide, but may also include effects mediated by Ang(1-7). A recent preliminary report has described similar responses of the rat kidney to Ang(1-7) *in vivo* (Handa *et al.*, 1993).

Supported by American Heart Association Grant 91-014G and National Institutes of Health Research Grant RO1-HL 25394. We thank Dr John C. McGiff, Dr John Quilley and Dr Alberto Nasjletti for their comments. We are extremely grateful to Jennifer Brown for her excellent secretarial assistance. Part of this work has been presented as a preliminary report (*Pharmacologist* (1992), **34**, 326).

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(Received September 18, 1993
Accepted October 22, 1993)

Br. J. Pharmacol. (1994), **111**, 4–6

SPECIAL REPORT

[^{125}I]-PD151242: a selective radioligand for human ET_A receptors

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Our aim was to synthesize a new endothelin ET_A selective radioligand, [^{125}I]-PD151242 and characterize the compound in human vascular tissue. Binding of [^{125}I]-PD151242 to sections of human aorta was time-dependent and reached equilibrium after 120 min at 23°C with an association rate constant of $1.26 \pm 0.17 \times 10^8$ M^{-1} min^{-1} ($n = 3$ individuals ± s.e.mean). The binding was reversible at 23°C with an observed dissociation rate constant of 0.0025 ± 0.0006 min^{-1} ($n = 3$). Saturation binding assays using [^{125}I]-PD151242 revealed a single population of high affinity ET receptors ($n = 3$) in aorta ($K_D = 0.76 \pm 0.17$ nM; $B_{max} = 5.98 \pm 1.56$ fmol mg^{-1} protein), pulmonary ($K_D = 1.75 \pm 0.20$ nM; $B_{max} = 12.78 \pm 1.39$ fmol mg^{-1} protein) and coronary arteries ($K_D = 0.51 \pm 0.07$ nM; $B_{max} = 44.9 \pm 1.67$ fmol mg^{-1} protein). ET_A selective ligands competed for [^{125}I]-PD151242 binding in aorta with nanomolar affinity (BQ123, $K_D = 0.41 \pm 0.26$ nM; FR139317, $K_D = 0.55 \pm 0.11$ nM) whereas the ET_B selective compound, BQ3020, competed with micromolar affinity ($K_D = 1.36 \pm 0.25$ μM). In isolated coronary arteries, PD151242 was a functional antagonist and caused a significant, parallel rightward shift of the ET-1 dose-response curve with a pA_2 value of 5.92 ($n = 5$) and a slope of unity. The high affinity and selectivity of [^{125}I]-PD151242 for ET_A receptors will facilitate the characterization of this sub-type in human tissues.

Keywords: PD151242; endothelin; FR139317; BQ123; BQ3020; ET_A receptor; human aorta; pulmonary artery; coronary artery

Introduction Two endothelin (ET) receptor sub-types, ET_A and ET_B have been isolated and cloned from human tissue. They are classified at present by the relative potency of the three ET isoforms. ET-1 and ET-2 have similar affinities for the ET_A receptor whereas that of ET-3 is much lower. All three isoforms are thought to be equipotent for the ET_B sub-type (see Miller *et al.*, 1993). Classification of receptors has been advanced by the development in animals of sub-type selective agonists for the ET_B receptor (Doherty, 1992). Radiolabelled versions of these compounds, [^{125}I]-[Ala^{1,3,11,15}]ET-1 and [^{125}I]-BQ3020, are selective for human ET_B receptors (Molenaar *et al.*, 1992) and the peptides have been used to determine the distribution of this sub-type in human tissues (Molenaar *et al.*, 1993; Davenport *et al.*, 1993; Karet *et al.*, 1993).

ET_A-selective antagonists have also been developed in animal models including BQ123 (Ihara *et al.*, 1992) and FR139317 (Nirei *et al.*, 1993) but these are unsuitable for direct iodination. We have therefore synthesized PD151242, which contains a tyrosine residue and has structural similarity to FR139317, and tested the ability of the iodinated compound to bind to ET receptors in human blood vessels. *In vitro* pharmacological assays were used to test for functional antagonism in human isolated blood vessels.

Methods Cardiovascular tissue was obtained from recipient patients (age range 43–58 years) undergoing heart transplants for ischaemic heart disease. Immediately on removal from the patient, the endothelial layer and intima were removed from the vessels (aorta, pulmonary and epicardial coronary arteries) and the media snap frozen in liquid nitrogen and stored at −70°C until use. Sections (10 μm thick) were cut on a cryostat microtome and mounted onto gelatin coated microscope slides.

For saturation experiments, tissue sections were pre-incubated for 15 min in HEPES buffer as previously described (Davenport *et al.*, 1989). Sections were then incubated with increasing concentrations (8 pM–8 nM) of [^{125}I]-PD151242 in incubation buffer for 2 h at 23°C. Non-specific binding was defined by use of 1 μM unlabelled PD151242. Sections were rinsed in Tris-HCl buffer (0.05 M, pH 7.4) at 4°C (3 × 5 min) and the amount of radioactivity measured in a gamma counter. Under these washing conditions (4°C), used to separate tissue bound [^{125}I]-PD151242 from free, less than 7% of the label had dissociated after 15 min. Association experiments were carried out using aorta as described above, except that sections were incubated for increasing time periods (0–240 min) with [^{125}I]-PD151242. For dissociation experiments, sections were incubated with [^{125}I]-PD151242 for 2 h at 23°C before incubating sections in an excess of buffer at 23°C for increasing time periods (0–240 min). In competition assays, sections were incubated with 100 pM [^{125}I]-PD151242 or [^{125}I]-ET-1 and increasing concentrations of unlabelled peptides (20 pM–100 μM). Non-specific binding was defined by use of 1 μM unlabelled PD151242 or ET-1 respectively.

The results of binding experiments were analysed using EBDA and LIGAND programmes as previously described (Molenaar *et al.*, 1992; 1993; Davenport *et al.*, 1993). The presence of 1 or 2 sites was tested by the *F*-ratio test in LIGAND. The model adopted was that which provided the best fit ($P < 0.05$).

To test for functional antagonism of PD151242, endothelium-denuded rings (2 mm) of epicardial coronary arteries were mounted in 25 ml organ baths in continuously oxygenated Krebs solution at 37°C as previously described (Davenport *et al.*, 1993). Dose-response curves were determined for ET-1 (100 pM–1 μM) in the absence (control) and presence of PD151242 (1–30 μM) added to the medium 30 min prior to addition of ET-1. The responses to ET-1 were expressed as a percentage of the maximum contraction to 50 mM KCl used to terminate the experiment and pA_2 values calculated by Schild analysis.

PD151242, (N-[(hexahydro-1-azepinyl)carbonyl])L-Leu(1-Me) D-Trp-D-Tyr; FR139317 (N-[(hexahydro-1-azepinyl)

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carbonyl)L-Leu(1-Me)D-Trp-3(2-pyridyl)-D-Ala; BQ123, cyclo [D-Asp-L-Pro-D-Val-L-Leu-D-Trp-] and BQ3020, [Ala^{11,15}]Ac-ET-1$_{(6-21)}$, were synthesized by solid phase t-Boc chemistry. Peptide concentration was determined by u.v. spectrophotometry. [^{125}I]-PD151242 (2000 Ci mmol^{-1}) was synthesized (Amersham International plc, Amersham, Bucks) from the unlabelled material by mild oxidation using an enzymatic method and purified to be carrier-free. [^{125}I]-ET-1 (2000 Ci mmol^{-1}) was obtained from Amersham and unlabelled ET-1 from Novabiochem, Nottingham.

Results Binding of [^{125}I]-PD151242 to sections of human aorta was time-dependent and reached equilibrium after 120 min at 23°C with an association rate constant of $1.26 \pm 0.17 \times 10^8$ M^{-1} min^{-1} ($n = 3 \pm$ s.e.mean). The binding was reversible at 23°C with an observed dissociation rate constant of 0.0025 ± 0.0006 min^{-1} ($n = 3$). [^{125}I]-PD151242 binding to sections of aorta, pulmonary and coronary arteries was concentration-dependent and saturable (Table 1) with affinities ranging from 0.5–1.8 nM. The Hill coefficients were close to unity, and in each case a one site was preferred to a two site model.

Unlabelled PD151242 competed for the binding of 0.1 nM [^{125}I]-ET-1 to aorta in a biphasic manner: K_D $ET_A = 3.27 \pm 0.93$ nM and $K_D ET_B = 4.85 \pm 1.65$ μM with a ratio of ET_A:ET_B of 84:16%. ET_A and ET_B-selective compounds were tested further in human aorta for their ability to compete for the binding of [^{125}I]-PD151242. The ET_A-selective ligands, FR139317 and BQ123, competed with [^{125}I]-PD151242 binding to aorta with high affinity in the sub-nanomolar range (Table 1). The ET_B-selective ligand, BQ3020 competed only at high concentrations. In all cases, a one site fit was preferred to a two site model. Vasoactive non-endothelin peptides (including calcitonin gene-related peptide, angiotensin II, bradykinin and atrial natriuretic peptide) did not compete for binding of [^{125}I]-PD151242 when tested at a concentration of 1 μM (data not shown). Binding of [^{125}I]-PD151242 (up to 0.1 nM) could not be detected to cultures of human umbilical vein endothelial cells that have previously been shown by reverse-transcriptase polymerase chain reaction assays to express only ET_B mRNA (Molenaar *et al.*, 1993).

In human isolated epicardial coronary arteries, PD151242 (1–30 μM) produced significant, parallel rightward shifts of the ET-1 dose-response curve. A pA_2 value of 5.92 ($n = 5$ individuals) was derived by Schild analysis and the slope of the regression was not significantly different ($P > 0.05$) from unity.

Discussion [^{125}I]-PD151242 has a high affinity and selectivity for human ET_A receptors in aorta, pulmonary and coronary arteries. Hill slopes were close to unity suggesting that the ligand binds to a single population of receptors or to multiple binding sites with similar affinities. In each vessel, affinities were comparable to those previously obtained for [^{125}I]-ET-1 in saturation binding assays: aorta ($K_D = 0.51$ nM), pulmonary ($K_D = 0.85$ nM) and coronary arteries ($K_D = 0.14$ nM) (Davenport *et al.*, 1993). We have previously shown that the media of all three vessels express mRNA encoding both ET_A and ET_B sub-types by reverse-transcriptase polymerase chain reaction and *in situ* hybridization assays. Competition binding assays have confirmed that human vascular tissue contains predominantly the ET_A subtype with a smaller population of ET_B receptors. For example, BQ123 competes with [^{125}I]-ET-1 binding to human coronary artery in a biphasic manner with $K_D ET_A = 0.85$ nM, $K_D ET_B = 7.6$ μM, ratio 87%:13% (Davenport *et al.*, 1993). In support of these results, the density of ET_A receptors estimated by [^{125}I]-PD151242 (Table 1) was lower than those obtained with the non-selective ligand [^{125}I]-ET-1 where B_{max} values of 9, 15 and 71 fmol mg^{-1} protein were found in aorta, pulmonary and coronary arteries respectively (Davenport *et al.*, 1993). Bax *et al.* (1993) have observed that in *post-mortem* coronary artery, both BQ123 and [Ala^{1,3,11,15}]ET-1 compete monophasically for [^{125}I]-ET-1 binding suggesting an atypical ET receptor but our results thus far are consistent with the presence of the two known receptor sub-types.

PD151242 antagonized ET-1 constrictor responses in human isolated coronary arteries and the regression slope of unity indicated the compound acts in a competitive manner. These data support results obtained with other ET_A antagonists, suggesting that vasoconstriction in the human vasculature is predominantly mediated via the ET_A sub-type (Maguire & Davenport, 1993). The difference between the affinity of [^{125}I]-PD151242 measured by ligand binding and the potency of the unlabelled compound observed *in vitro* is intriguing but consistent with results from other studies using established ET_A-selective antagonists. For example, the K_D for BQ123 competing for [^{125}I]-ET-1 binding in the media of human coronary artery was 0.85 nM (Davenport *et al.*, 1993) but has a pA_2 value of 7.0 in the same tissue *in vitro* (Maguire & Davenport, 1993). Similar pA_2 values of BQ123 have been reported for example, in pig coronary artery (Ihara *et al.*, 1992) and rat thoracic artery (Summer *et al.*, 1992).

The results show the new iodinated ligand, PD151242, has high affinity and selectivity for ET_A receptors and the availability of this radioligand will facilitate the further characterization of this sub-type in human tissues.

Table 1 **A** Saturation binding experiments: dissociation constants (K_D), maximal density of receptors (B_{max}) and Hill coefficients (nH) for [^{125}I]-PD151242 binding to the media from human arteries

	n	K_D (nM)	B_{max} (fmol mg^{-1} protein)	*nH*
Aorta	3	0.76 ± 0.17	5.98 ± 1.56	1.00 ± 0.01
Pulmonary	3	1.75 ± 0.20	12.78 ± 1.39	1.00 ± 0.03
Coronary	3	0.51 ± 0.07	44.9 ± 1.67	0.84 ± 0.09

B Competition binding experiments: comparison of ET_A and ET_B ligands competing for the binding of [^{125}I]-PD151242 to the media of human aorta

	n	K_D
ET_A		
PD151242	3	1.20 ± 0.88 nM
BQ123	3	0.41 ± 0.26 nM
FR139317	3	0.55 ± 0.11 nM
ET_B		
BQ3020	3	1.36 ± 0.25 μM

Values are the mean ± s.e.mean of three individuals in both **A** and **B**.

This work was supported by grants from the British Heart Foundation, SERC, Isaac Newton Trust and the Royal Society. We thank the consultant and theatre staff of Papworth Hospital, Cambridge, for their permission to obtain cardiovascular tissue.

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(Received October 6, 1993
Accepted November 3, 1993)

Br. J. Pharmacol. (1994), **111**, 7–12

Effect of PF 10040 on PAF-induced airway responses in neonatally immunized rabbits

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1 PF 10040 displaced [^{3}H]-PAF from binding sites on rabbit platelets with an $IC_{50} = 1.07 \times 10^{-5}$ M, which was approximately three orders of magnitude below that of a standard PAF antagonist WEB 2086 ($IC_{50} = 4.23 \times 10^{-9}$ M).

2 PF 10040 at doses of 5 and 10 mg (direct intratracheal administration) had no effect on the acute bronchoconstriction induced by PAF in neonatally immunized rabbits (airway resistance R_L or dynamic compliance C_{dyn}). However, the PAF-induced increase in airway responsiveness to inhaled histamine was significantly inhibited (R_L and C_{dyn}) by both doses of PF 10040.

3 PF 10040 (5 and 10 mg) significantly inhibited the total pulmonary cell infiltration and neutrophil influx induced by PAF as assessed by bronchoalveolar lavage. PAF-induced eosinophil infiltration into the airways was significantly inhibited in rabbits that received only 10 mg PF 10040.

4 We suggest from the results of the present study that PF 10040 does not exert an inhibitory effect on PAF-induced airway responses solely via antagonism of the PAF receptor located on platelets, as PF 10040 significantly inhibited PAF-induced airway hyperresponsiveness in the absence of an effect on the acute bronchospasm induced by PAF.

5 We provide further evidence that pulmonary eosinophil infiltration and the development of airway hyperresponsiveness are not causally related events as the lower dose of PF 10040 (5 mg) significantly inhibited PAF-induced airway hyperresponsiveness yet was without effect on the eosinophil influx.

Keywords: PAF; airways; inflammation; airway hyperresponsiveness; neonatally immunized rabbit

Introduction

Bronchoconstriction, airway hyperresponsiveness and the recruitment of leucocytes, in particular eosinophils, into the airways are well known characteristics of bronchial asthma. Platelet activating factor (PAF) has been suggested as a mediator of this disease as it can induce many of these features, both in experimental animals and in man (reviewed in Page, 1988).

The mechanism by which PAF produces bronchoconstriction and airway hyperresponsiveness has yet to be determined. A role for the lipoxygenase products of arachidonic acid, particularly leukotrienes formed by the 5-lipoxygenase pathway, has been suggested to account for a number of PAF-induced effects in rabbits, including bronchoconstriction and the increase in airway responsiveness to histamine following PAF exposure (Herd *et al.*, 1992).

In the present study, the ability of PF 10040 (1-3,4-dimethoxyphenylethyl)-6-methyl-3,4-dihydroisoquinoline hydrochloride), a substance recently reported to inhibit PAF-induced oedema in rabbit skin (Rossi *et al.*, 1992), has been investigated for its ability to act as a PAF antagonist on rabbit platelets in comparison with the triazolodiazepine, WEB 2086 (Casals-Stenzel *et al.*, 1987). Furthermore, we have investigated the ability of PF 10040 to influence bronchoconstriction, pulmonary cell infiltration and airway hyperresponsiveness induced by PAF in spontaneously breathing rabbits. Immunized rabbits have been used as we have previously shown that while aerosolized PAF will induce airway hyperresponsiveness to inhaled histamine in only a proportion of normal rabbits, it is effective in all rabbits that have been neonatally immunized with antigen (Herd *et al.*, 1992).

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Methods

Animals

New Zealand White (NZW) rabbits (Froxfield Farms, Petersfield, Hampshire) of either sex were used throughout the study. The immunization procedure of neonatal rabbits was as previously described (Minshall *et al.*, 1993). Rabbits were injected intraperitoneally (0.5 ml) within 24 h of birth with *Alternaria tenuis* extract in aluminium hydroxide ($Al(OH)_3$) moist gel and saline in the ratio of 2:1:1. The i.p. administration of antigen and adjuvant was repeated weekly for the first month and then biweekly for the following 2 months. At 3 months of age, the adult animals were transferred from the breeding unit to our laboratory. The methodology described in this study was subject to Home Office approval and performed under the Animals (Scientific Procedures) Act 1986.

^{3}H-labelled platlet activating factor receptor binding studies

Blood (100 ml) was obtained via cardiac puncture of pentobarbitone-overdosed rabbits and collected into 16 ml anticoagulant (g 50 ml^{-1}: sodium citrate 11.75, citric acid 6.85, dextrose 2.0). The citrated blood was immediately diluted 1:1 in buffer A (composition mM: NaCl 140, KCl 2.6, NaH_2PO_4 0.42, $MgCl_2$ 1, HEPES 17, EDTA 5, pH 7.4, 4°C) and centrifuged at 200 *g* for 15 min at 2°C. The platelet-rich plasma was removed and replaced with buffer A. Centrifugation of the rediluted plasma-depleted blood was repeated and the platelet-rich supernatant fractions were combined and pelleted by centrifugation at 2,800 *g* for 10 min at 2°C. The final cell pellet was resuspended in buffer B (composition mM: NaCl 140, KCl 2.7, NaH_2PO_4 0.4, $MgCl_2$ 2.0, dextrose 6.2, HEPES 17, EDTA 0.2 and BSA 0.1%). Displacement of

1-O-[^{3}H] alkyl platelet activating factor ([^{3}H]-PAF) was examined in rabbit platelets as previously described (Ukena *et al.*, 1988). Cells (1×10^8) were incubated in buffer B in a final volume of 0.9 ml with [^{3}H]-PAF (30 *p*M) in the presence or absence of PF 10040 (3–500 μM) or WEB 2086 (0.01–10 μM). Reactions were conducted for 90 min at 22°C and terminated by filtration through Whatman GF/C filters. Filters were washed three times with 3 ml buffer C (composition mM: NaCl 140, $MgCl_2$ 10, EDTA 2 and Tris 10) and counted in a beta-counter.

Pulmonary function measurements

Rabbits (1.95–3.6 kg) were pre-medicated with diazepam (2.5 mg kg^{-1}, i.p.) and subsequently anaesthetized with Hypnorm (0.4 ml kg^{-1}, intramuscularly), a regime which produces neuroleptanalgesia and is recommended for recovery anaesthesia in laboratory rabbits (Flecknall, 1987). Neuroleptanalgesia was maintained throughout the course of the experiment by administration of Hypnorm i.m. approximately every 30 min (Flecknall, 1987). Animals were placed in a supine position and intubated with a cuffed endotracheal tube (3.0 mm internal diameter; Mallinckrodt Laboratories, Athlone, Ireland) attached to a heated (37°C) Fleisch pneumotachograph (size 00). Flow was measured with a Validyne differential pressure transducer (model MP 45-14-871; Validyne Engineering Corp., Northridge, CA, U.S.A.). Pleural pressure was estimated by placing an oesophageal balloon in the lower third of the oesophagus to obtain the maximum expiratory pressure. Transpulmonary pressure, the difference between thoracic and pleural presure, was measured with a second Validyne differential pressure transducer (model MP 45-24-871) connected between the oesophageal balloon and atmospheric air. The flow was integrated to obtain a continuous recording of tidal volume. Measurements of total lung resistance (R_L) and dynamic compliance (C_{dyn}) were calculated by an online respiratory analyser (PMS Version 5.1, Mumed Ltd., London) as previously described (Minshall *et al.*, 1993).

Measurement of airway responsiveness to histamine

After measurement of baseline lung function, rabbits were exposed to an aerosol of saline for 2 min and lung function parameters recorded. Airway responsiveness was determined by exposing animals to cumulative concentrations of aerosolized histamine (1.25–80 mg ml^{-1}; 2 min per concentration) administered directly to the lungs via the endotracheal tube. Pulmonary function was recorded following each 2 min exposure. Aerosols were generated by an ultrasonic nebuliser (Ultra-Neb 99, DeVilbiss Health Care Ltd., Heston, Middlesex) which has previously been demonstrated to generate particles of which the majority are in the 0.5–5 μm diameter range (DeVilbiss data). The provocation concentration (PC) of histamine which produced a 50% increase in R_L (PC_{50}) and 35% decrease in C_{dyn} (PC_{35}) was determined for each animal by linear interpolation and used as indices of airway responsiveness.

PAF challenge and drug administration

On day 2, animals were re-anaesthetized and challenged with either PAF or 0.25% bovine serum albumin (BSA) (the carrier vehicle for PAF). After exposure to an aerosol of BSA for 2 min, rabbits were exposed to PAF (80 μg ml^{-1}) or BSA over a 1 h period, after which time respiratory parameters were recorded. On day 3, increasing concentrations of histamine were administered to the anaesthetized rabbits as on day 1 and the PC_{50} (R_L) and the PC_{35} (C_{dyn}) values determined.

Drug studies

A solution of PF 10040 (5 mg or 10 mg in a volume of 0.5 ml) or saline vehicle was instilled directly into the lung via a cannula passed into the airways to the point of the bifurcation, via the endotracheal tube, 30 min prior to the commencement of the PAF aerosol. Similarly, a solution of PF 10040 (5 mg or 10 mg) was instilled into the airways 30 min before a corresponding aerosol of 0.25% BSA. In these latter experiments respiratory parameters were recorded prior to, and 1, 15 and 30 min following the drug administration, then as previously described for the BSA challenge.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavages were performed immediately following completion of the histamine aerosol challenge. The airways were lavaged by use of a polyethylene catheter inserted into the lung via the endotracheal tube. Five ml saline was injected into the lungs, then immediately aspirated into a collection trap, with approximately 50% recovery of fluid. Total cell counts were determined under light microscopy using an improved Neubauer haemocytometer. For differential cell counts, 75 μl aliquots were used for centrifugation (Shandon Cytospin 2; Shandon Southern Instruments, Sewickley, PA, U.S.A.) and the cells were stained with Lendrum's stain (active constitutuents haematoxylin and chromotrope 2R) to facilitate the discrimination of eosinophils as previously described (Lendrum, 1944). A total of 200 cells were counted differentially and classified as either neutrophils, eosinophils or mononuclear cells based on standard morphological criteria.

Analysis of results

Results of the lung function studies are expressed as mean ± s.e.mean. *In vivo* histamine potency values were derived from measurements of airway resistance (R_L) (PC_{50}) and dynamic compliance (C_{dyn}) (PC_{35}) and are expressed as the geometric means together with upper and lower values for s.e.mean. For statistical purposes PC_{50} and PC_{35} values were $\log_{10}$ transformed. One-way analyses of variance were used to analyse the acute bronchoconstriction data (R_L and C_{dyn}) (expressed as maximal percentage change) and airways responsiveness to histamine in the three groups pre drug treatment. Paired *t* tests were employed to analyse the histamine potency data prior to and 24 h following PAF administration within treatment groups. The total cell, neutrophil, eosinophil and mononuclear cell counts obtained from BAL before and 24 h after PAF challenge were subjected to Kruskall-Wallis analysis of variance by ranks as the variances were found to be non-homogeneous. Distribution-free multiple comparisons were used to determine differences in means when multiple comparisons were made. Results were considered significant if $P < 0.05$.

Drugs

The drugs and chemicals used were: *Alternaria tenuis* extract (Batch No. M1-147-10P20; 40,000 PNU ml^{-1}, 1 mg ml^{-1}; Greer Laboratories Inc. Lenoir, NC, U.S.A.); aluminium hydroxide ($Al(OH)_3$) moist gel (FSA Laboratory Supplies, Loughborough, Leicestershire); histamine diphosphate, bovine serum albumin (low endotoxin) and chromotrope 2R (Sigma Chemical Co., Poole, Dorset); haematoxylin (BDH Chemicals, Poole, Dorset); platelet activating factor (PAF C_{16},1-0-hexadecyl-2-0-acetyl-sn-glycero-3-phosphocholine; Novabiochem, Nottingham, Nottinghamshire); diazepam (Valium 5 mg ml^{-1}; Roche Products Ltd., Welwyn Garden City, Hertfordshire); Hypnorm (a mixture of fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}; Janssen Pharmaceutical Ltd., Grove, Oxfordshire); PF 10040 (1-3,4-

dimethoxyphenylethyl)-6-methyl-3,4-dihydroisoquinoline hydrochloride) (a gift from The Purdue Frederick Company, Norwalk, CT, U.S.A.); WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanon) (a gift from Boehringer Ingelheim, Ingelheim-am-Rhein, Germany); 1-O-[^{3}H] alkyl platelet activating factor (Amersham International, Amersham, Buckinghamshire); sterile pyrogen-free 0.9% sodium chloride solution (saline; Baxter Healthcare Ltd., Thetford, Norfolk). All reagents were of analytical grade. For *in vivo* studies all solutions were prepared in saline.

Results

Displacement of [^{3}H]-PAF

Specific binding, defined by inclusion of a saturating concentration of WEB 2086 (10 μM), was approximately 50% of total binding. WEB 2086 and PF 10040 displaced [^{3}H]-PAF in a dose-dependent fashion (Figure 1). WEB 2086 exhibited an apparent affinity approximately two orders of magnitude above that of PF 10040 (IC_{50}; WEB 2086, 4.23×10^{-9} M 95% confidence interval: $1.09-16.50 \times 10^{-9}$ M; PF 10040, 1.07×10^{-5} M (95% confidence interval: $0.57-2.03 \times 10^{-5}$ M)).

Baseline lung function

Single doses of saline vehicle or PF 10040 10 mg instilled directly into the lungs of immunized rabbits had no significant effect on baseline lung function (R_L or C_{dyn}) measured 1, 15 and 30 min following administration (data not shown; $n = 3$).

Airway responses

Airway responsiveness to inhaled histamine (both R_L PC_{50} and C_{dyn} PC_{35}) prior to PAF challenge was not significantly different in rabbits that were to receive either vehicle, PF 10040 5 mg or 10 mg (R_L: $F = 0.127$, d.f. = 18; C_{dyn}: $F = 1.189$, d.f. = 18; Table 1a and b). The combined mean values were R_L: 22.96 ± 1.30 mg ml^{-1} ($n = 21$); C_{dyn}: 11.54 ± 1.25 mg ml^{-1} ($n = 21$).

PAF-induced bronchoconstriction

Acute bronchoconstriction induced by PAF was not significantly different in groups of immunized rabbits pretreated with saline vehicle or with PF 10040 at doses of 5 mg or 10 mg directly administered into the airway (R_L: $F = 1.117$, d.f. = 18; C_{dyn}: $F = 3.809$, d.f. = 18; Table 2, Figure 2).

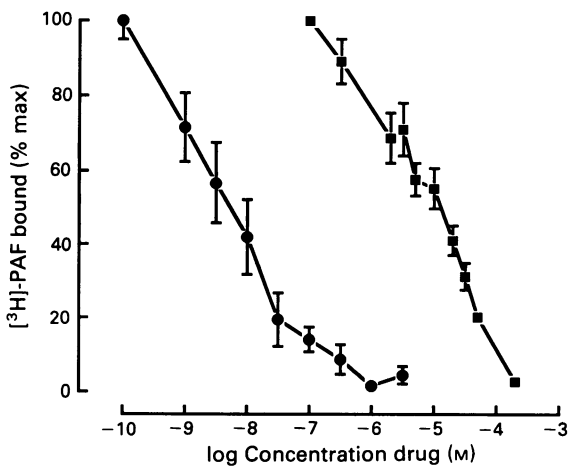

Figure 1 Displacement of [^{3}H]-PAF binding in rabbit platelets by PF 10040 (■) and WEB 2086 (●). Each point represents the mean ± s.e.mean of 4 experiments.

Table 1 Effect of PF 10040 (vehicle, 5 mg and 10 mg) on airway responsiveness to inhaled histamine prior to and 24 h following exposure to PAF aerosol (80 μg ml^{-1}) in immunized rabbits

(a)		*Histamine* PC_{50} (mg ml^{-1}) Pre	Post	*P*
Vehicle	$n = 9$			
	mean ± s.e.mean	19.77 ± 1.51	7.89 ± 1.37*	0.0036
5 mg	$n = 6$			
	mean ± s.e.mean	27.35 ± 1.76	23.50 ± 1.62	NS
10 mg	$n = 6$			
	mean ± s.e.mean	24.10 ± 1.84	19.50 ± 1.66	NS

(b)		*Histamine* PC_{35} (mg ml^{-1}) Pre	Post	*P*
Vehicle	$n = 9$			
	mean ± s.e.mean	16.83 ± 1.48	4.86 ± 1.45*	0.0001
5 mg	$n = 6$			
	mean ± s.e.mean	7.59 ± 1.61	14.13 ± 1.84	NS
10 mg	$n = 6$			
	mean ± s.e.mean	9.91 ± 1.43	9.16 ± 1.47	NS

(**a**) PC_{50} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 50% increase in airway resistance (R_L); (**b**) PC_{35} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 35% fall in dynamic compliance (C_{dyn}). *$P < 0.05$ compared with Pre value (paired *t* test).

Table 2 Percentage change in airway resistance (R_L) and dynamic compliance (C_{dyn}) following PAF aerosol (80 μg ml^{-1}) in immunized rabbits pretreated with PF 10040 vehicle, 5 mg and 10 mg

			R_L	C_{dyn}
Vehicle ($n = 9$)	Saline	mean	59.34	− 43.90
		s.e.mean	5.39	4.36
PF 10040 ($n = 6$)	5 mg	mean	43.40	− 42.89
		s.e.mean	11.61	4.20
PF 10040 ($n = 6$)	10 mg	mean	51.61	− 28.76
		s.e.mean	6.59	3.22

Airway hyperresponsiveness

Airway responsiveness to inhaled histamine (both R_L PC_{50} and C_{dyn} PC_{35}) was not significantly different in animals treated with PF 10040 (10 mg) prior to and 24 h following BSA challenge (data not shown). However, single doses of 5 mg and 10 mg PF 10040 directly instilled into the lungs of immunized rabbits were sufficient to significantly inhibit PAF-induced airway hyperresponsiveness at 24 h following PAF challenge for both R_L and C_{dyn} compared with the vehicle-treated control group ($P < 0.05$) (Tables 1a and b, Figures 3a and b).

Bronchoalveolar lavage

In rabbits that received vehicle alone, PAF induced a significant increase in the total number of inflammatory cells recovered in BAL fluid 24 h following challenge. This increase in total cell counts was reflected as an increase in total neutrophil and eosinophil counts (Table 3). PF 10040, 5 mg and 10 mg, significantly inhibited the increase in the total cell number and number of neutrophils ($P < 0.05$). Only in rabbits pretreated with PF 10040 10 mg was eosinophil infiltration significantly inhibited (Table 3).

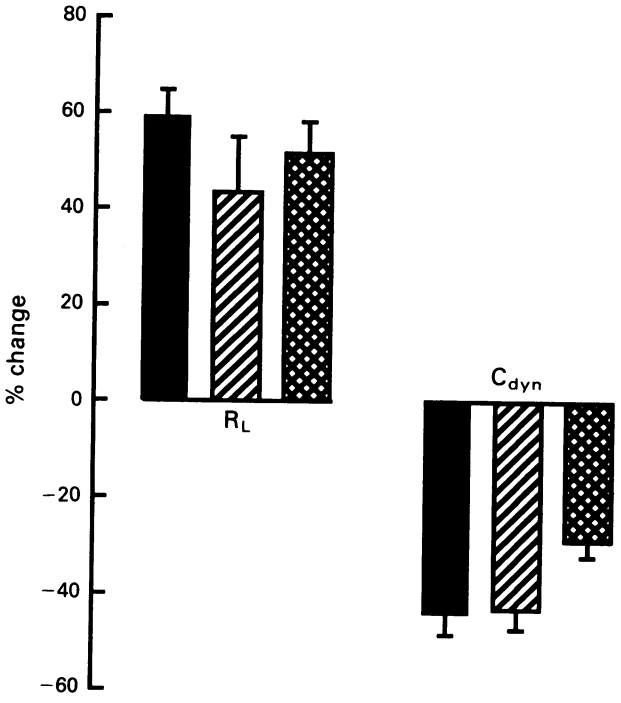

Figure 2 Percentage change in airway resistance (R_L) and dynamic compliance (C_{dyn}) following PAF aerosol (80 $\mu g\ ml^{-1}$) in immunized rabbits pretreated with PF 10040 vehicle (solid columns), 5 mg (hatched columns) and 10 mg (cross-hatched columns).

Discussion

The local administration of PF 10040 into rabbit skin has recently been shown to inhibit oedema formation induced by PAF (Rossi *et al.*, 1992) and it has therefore been suggested that PF 10040 is a novel PAF antagonist. No further evidence of the ability of PF 10040 to inhibit the binding of PAF however was provided in this study. The present experiments show that PF 10040 can act as a competitive antagonist of PAF binding to rabbit platelets, with apparent affinity approximately 3 orders of magnitude less than the triazolodiazepine, WEB 2086 (Casals-Stenzel *et al.*, 1987). The IC_{50} value obtained for WEB 2086 in the present study is in close agreement with that obtained by other investigators using similar methodology (Ukena *et al.*, 1988). However, despite PF 10040 acting as a PAF antagonist *in vitro*, acute bronchoconstriction induced by aerosolized PAF was unaffected by the prior treatment of rabbits with PF 10040 at doses of 5 mg or 10 mg, administered directly into the airways. All PAF antagonists so far described that inhibit PAF binding to platelets *in vitro* have been shown also to inhibit PAF-induced bronchoconstriction *in vivo* (Hosford *et al.*, 1989) as this biological response induced by PAF is known to be a platelet dependent phenomenon (Coyle *et al.*, 1990). It is plausible that we failed to inhibit PAF-induced bronchoconstriction with PF 10040 because we did not achieve high enough local concentrations of the drug in the airways. However, this explanation seems unlikely as we were clearly able to inhibit PAF-induced pulmonary cell infiltration and airway hyperresponsiveness with PF 10040. Both of these responses have also been shown previously to be platelet-dependent in the rabbit (Coyle *et al.*, 1990), making our present results somewhat surprising.

In the present study, PAF aerosol induced airway hyperresponsiveness to inhaled histamine 24 h after challenge. Following single doses of 5 and 10 mg PF 10040, PAF was unable to alter significantly the responsiveness of the airways to histamine (either airway resistance (R_L) or dynamic compliance (C_{dyn})). The effect of PF 10040 on the R_L component, considered to be a measure of larger, upper airway function, was similar for both doses of the drug. However, the effect of PF 10040 on the C_{dyn} component, a measure of smaller airway function, is not as clear, as the PC_{35} value pre PAF is unexpectedly small in comparison with that of the vehicle and PF 10040 10 mg-treated groups. The large range of PC_{35} values obtained in this study implies that measurement of

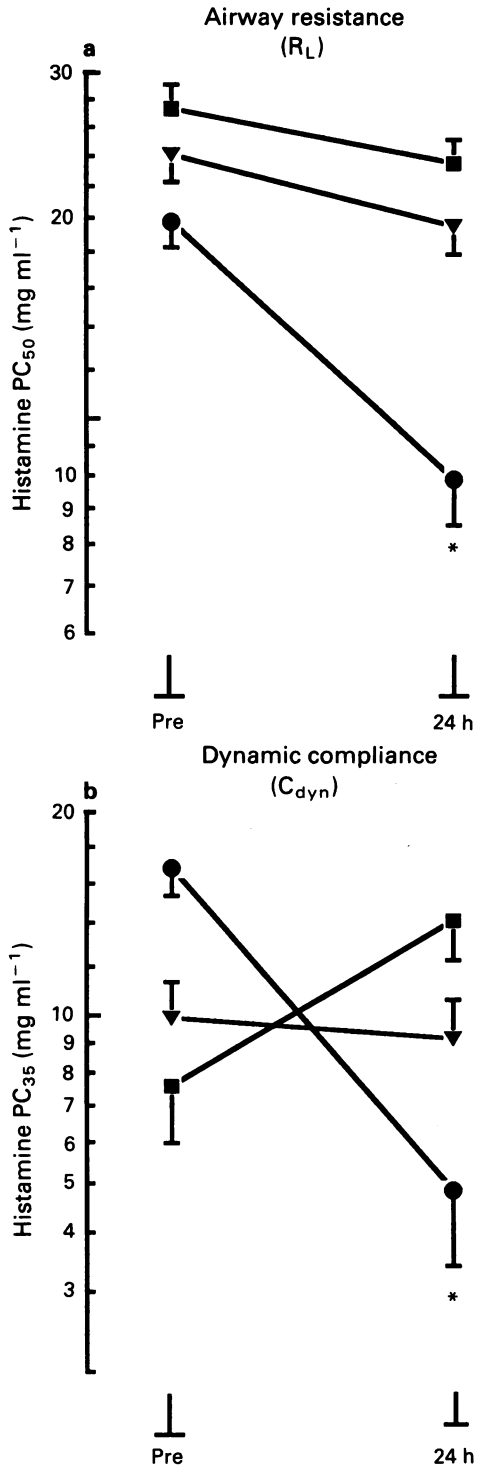

Figure 3 Effect of PF 10040 vehicle (●) ($n = 9$), 5 mg (■) ($n = 6$) and 10 mg (▲) ($n = 6$) on PAF-induced airway hyperresponsiveness in immunized rabbits (a) histamine PC_{50} is the concentration of histamine required to cause a 50% increase in airway resistance (R_L); (b) histamine PC_{35} is the concentration of histamine required to cause a 35% decrease in dynamic compliance (C_{dyn}). $^*P < 0.05$ compared with pre-PAF control.

Table 3 Total and differential cell numbers recovered from bronchoalveolar lavage (BAL) fluid before (Pre) and 24 h (Post) following exposure to PAF aerosol (80 μg ml^{-1}) in immunized rabbits pretreated with PF 10040 vehicle ($n = 9$), 5 mg ($n = 6$) and 10 mg ($n = 5$)

		× 10^5 cells ml^{-1}			
		Total	*Neutrophils*	*Eosinophils*	*Mononuclear cells*
0 mg (vehicle)	Pre	2.15 (0.40–5.25)	0.373 (0.014–1.864)	0.0018 (0–0.0160)	1.78 (0.32–3.39)
	Post	6.92 (1.30–21.5)*	5.090 (0.514–19.35)*	0.2619 (0.0900–0.5040)*	1.57 (0.74–1.84)
5 mg	Pre	3.02 (0.35–6.65)	0.408 (0.033–0.865)	0.0000	2.61 (0.25–5.79)
	Post	2.95 (0.12–5.55)†	1.126 (0.344–2.664)*†	0.1309 (0.0203–0.2775)*	1.70 (0.50–3.90)
10 mg	Pre	1.84 (0.30–2.95)	0.087 (0–0.162)	0.0089 (0–0.0443)	1.74 (0.30–2.74)
	Post	2.51 (0.65–10.95)†	1.462 (0.176–3.116)*†	0.0265 (0–0.0615)†	1.02 (0.47–1.78)

Values represent mean with range in parentheses.
*$P < 0.05$ compared with Pre control.
†$P < 0.05$ compared with Post vehicle control.

C_{dyn} are more variable and less reproducible than those of R_L.

As PF 10040 had no effect on the airway responsiveness to histamine following BSA challenge, the inhibitory action of PF 10040 on PAF-induced airway hyperresponsiveness is not attributable to histamine H_1 antagonism or via some non-specific effect on airway hyperresponsiveness. PF 10040 has recently been shown to protect against experimental NSAID-gastritis (Wallace *et al.*, 1993) and it remains to be determined whether a common mechanism of action may account for the ability of PF 10040 to inhibit PAF-induced airway hyperresponsiveness in the present study.

In agreement with previous studies PAF was found to induce an influx of inflammatory cells into the airways (predominantly neutrophils and eosinophils) as assessed by bronchoalveolar lavage (BAL) (Herd *et al.*, 1992). The accumulation and activation of inflammatory cells within the airways has been suggested to lead to epithelial damage causing the exposure of nerve endings in the bronchial lumen, which are thought to initiate an increase in airway responsiveness (Barnes, 1986). Whilst PF 10040 (5 mg and 10 mg) inhibited both the PAF-induced total cell infiltration and the development of airway hyperresponsiveness, only in rabbits pretreated with the higher dose of PF 10040 (10 mg) was eosinophil recruitment significantly inhibited. In rabbits that received the lower dose of PF 10040 (5 mg), eosinophil infiltration persisted despite airway hyperresponsiveness being inhibited. This observation supports previous findings in this model suggesting that eosinophil infiltration and airway hyperresponsiveness may be unrelated events. The 5-lipoxygenase activating protein (FLAP) inhibitor, PF 5901, did not inhibit PAF-induced cell infiltration despite inhibiting the associated airway hyperresponsiveness (Herd *et al.*, 1992) and pretreatment of rabbits with capsaicin has been shown to inhibit airway hyperresponsiveness but not the pulmonary eosinophil infiltration induced by PAF (Spina *et al.*, 1991). Furthermore, in guinea-pigs, capsaicin will inhibit allergen-induced airway hyperresponsiveness without modifying pulmonary eosinophil influx (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991) and certain cytokines have been shown to cause pulmonary eosinophilia without eliciting airway hyperresponsiveness (Kings *et al.*, 1990). Exposure of allergic guinea-pigs to low doses of antigen has been reported to induce eosinophil accumulation in BAL in the absence of any alteration in airway responsiveness, and at a higher dose of antigen the associated airway hyperresponsiveness was not inhibited by ketotifen, the phosphodiesterase inhibitors AH 21132 and aminophylline, or dexamethasone despite the finding that these drugs inhibited the pulmonary eosinophilia (Sanjar *et al.*, 1990). Together these results suggest that the actual presence of eosinophils within the airway lumen may not be a prerequisite for the development of airway hyperresponsiveness. It is relevant that in asthmatic subjects airway hyperresponsiveness may be present in the absence of an observed eosinophil infiltrate (Lundgren *et al.*, 1988) and conversely, chronic eosinophilic bronchitis is not always associated with airway hyperresponsiveness (Gibson *et al.*, 1989). However, in the absence of data on the activation state of the inflammatory cells in the present study, the participation of eosinophils in the pathogenesis of airway hyperresponsiveness cannot be completely excluded.

In conclusion, PAF-induced airway hyperresponsiveness in neonatally immunized rabbits can be inhibited by PF 10040, an effect which cannot be explained solely by the ability of this drug to act as a modest antagonist at the PAF receptor located on platelets. In addition, these experiments provide further evidence that pulmonary infiltration of eosinophils is not a prerequisite for the exacerbation of airway hyperresponsiveness.

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(Received July 13, 1993
Revised August 23, 1993
Accepted August 25, 1993)

Br. J. Pharmacol. (1994), **111**, 13–20

The effects of perfusion rate and N^G-nitro-L-arginine methyl ester on cirazoline- and KCl-induced responses in the perfused mesenteric arterial bed of rats

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1 The purpose of this study was to characterize the effects of N^G-nitro-L-arginine methyl ester (L-NAME) on the perfusion rate/pressure relations, and on the pressor responses induced to cirazoline and KCl in isolated, perfused mesenteric arterial beds from normotensive and spontaneously hypertensive rats.

2 The basal perfusion pressure of arterial beds perfused with either physiological salt solution (PSS) or PSS containing 1% polyvinylpyrrolidone increased as the perfusion rate increased. L-NAME, in concentrations up to 100 μM, failed to alter the basal pressure regardless of the perfusion rate and viscosity; however, at 5 μM, it potentiated cirazoline-induced vasoconstriction at each of the perfusion rates.

3 L-NAME but not D-NAME caused a leftward shift of cirazoline concentration-response curves with a marked increase in the maximal response. The potentiating action of L-NAME was abolished in arterial beds perfused with a Ca^{2+}-free physiological salt solution and also in beds denuded of endothelium by an infusion of distilled water for 5 min.

4 In endothelium-intact and -denuded preparations, L-NAME potentiated KCl pressor responses; the endothelium-independent potentiation of KCl pressor activity was stereospecific, time-independent and was not prevented by the presence of dexamethasone (0.5 μM) in the perfusion medium. However, L-NAME failed to potentiate vasoconstriction obtained to KCl in arterial beds denervated by cold storage (4–5°C) for 2 days.

5 The absence of K^+ in the perfusate did not inhibit the ability of L-NAME to potentiate α-adrenoceptor-mediated pressor responses, and nor did L-NAME inhibit KCl-induced vasodilatation in preconstricted arteries. It was thus concluded that L-NAME does not affect Na^+/K^+-ATPase activity.

6 No differences in the potentiating ability of L-NAME on either cirazoline- or KCl-mediated pressor responses were apparent between normotensive Sprague Dawley (SD), Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats.

7 Our data thus provide evidence that: the presence of a vasoconstrictor is required for basal nitric oxide (NO) release in the mesenteric arterial bed from either normotensive or spontaneously hypertensive rats; L-NAME causes potentiation of cirazoline- and KCl-induced vasoconstriction respectively by inhibiting endothelial and neuronal NO synthase(s). Furthermore, our data indicate that NO synthase activity is not impaired in the mesenteric arterial bed of spontaneously hypertensive rats.

Keywords: N^G-nitro-L-arginine methyl ester; nitric oxide; nitric oxide synthase; perfused mesenteric arterial bed; hypertension; α-adrenoceptors; potassium chloride

Introduction

The ability of endothelial cells to modulate vascular smooth muscle tone via the synthesis and release of one or more endothelium-derived relaxing factors (EDRFs) is now well recognized (Furchgott & Zawadzki, 1980; Moncada *et al.*, 1991). Nitric oxide (NO), or a labile nitroso compound, produced from L-arginine has been demonstrated to be an important EDRF in several vascular tissues (Palmer *et al.*, 1988; Ignarro, 1989). Several L-arginine analogues have been developed as nitric oxide synthase inhibitors, for example, N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO), and N^G-nitro-L-arginine, or its methyl ester (L-NAME), (Dubbin *et al.*, 1990; Moore *et al.*, 1990; Moncada *et al.*, 1991); all these have been shown to inhibit NO biosynthesis and also endothelium-dependent responses *in vitro* and *in vivo*. In addition to inhibiting endothelium-dependent relaxations, L-arginine analogues can also cause endothelium-dependent contractions of isolated vascular rings (Gold *et al.*, 1990), increases in coronary perfusion pressure in isolated perfused hearts of rabbit (Amezcua *et al.*, 1989) and guinea-pig (Levi *et al.*, 1990) and marked hypertension and regional vasoconstriction when administered to conscious rats (Gardiner *et al.*, 1990; Moncada *et al.*, 1991). These actions of L-NAME, and the other L-arginine derivatives, have been ascribed to prevention or inhibition of basal or stimulated EDRF (NO)-elicited activation of cytosolic guanylate cyclase.

In the perfused mesenteric arterial bed isolated from either the normotensive (Criscione *et al.*, 1984) or spontaneously hypertensive (Dohi *et al.*, 1990) rats, endothelial denudation augmented α_1-adrenoceptor mediated vasoconstriction; however, neither L-NMMA, methylene blue (Ebeigbe *et al.*, 1990) nor L-NAME (Adeagbo & Triggle, 1992; 1993) caused vasoconstriction as would be expected if basal nitric oxide release, and subsequent elevation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), maintains resting tone. In our latter studies, we demonstrated that a putative endothelium-derived hyperpolarizing factor (EDHF), causing vascular smooth muscle hyperpolarization and vasodilatation via the activation of apamin-sensitive K^+ channels plays a dominant role in the maintenance of vascular tone in this arterial bed (Adeagbo & Triggle, 1992; 1993). However, the possible influence of shear stress induced by the perfusion rate on the

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release of nitric oxide deserves to be addressed. Thus, assuming the failure of L-NAME to induce vasoconstriction of this vascular bed under *in vitro* basal conditions was due to an inadequate shear stress generated in our perfusion system, then, increasing the flow rate and/or viscosity of our perfusion salt solution should result in the activation of the endothelial nitric oxide synthase, and, its subsequent inhibition by L-NAME. Accordingly, the first objective of the present study was to assess the effects of L-, and D-isomers of N^G-nitro-arginine methyl ester on the basal perfusion pressure at different flow rates during perfusion with physiological salt solution (PSS) or PSS plus the plasma protein substitute polyvinylpyrrolidone (PVP), at 1%.

Second, we have also examined the interactions of L-NAME with α-adrenoceptor agonists- and KCl-induced responses with a view to: (a) determining the source (endothelial, neuronal or smooth muscle) of nitric oxide that modulates vasoconstriction to these agonists in this vascular bed; and, (b) comparing the nitric oxide-mediated modulation of vasoconstriction in normotensive Sprague-Dawley (SD) and Wistar-Kyoto (WKY) rats with spontaneously hypertensive rats (SHR). There are conflicting reports about the status of endothelial function in resistance vasculature during hypertension. Dohi *et al.* (1990) noted that the release of EDRF(s) in the mesenteric vascular bed is impaired in hypertension, while Randall *et al.* (1991), using an *in situ* blood-perfused mesenteric bed, did not observe an impairment of endothelial function. Thus, if the production of nitric oxide is deficient in the mesenteric bed of hypertensive rats, then the ability of L-NAME to potentiate vasoconstriction should be greater in SD and WKY versus SHR.

Methods

Age-matched (16–20 weeks old) male SD, WKY and SHR rats were anaesthetized by intraperitoneal (i.p.) injections of sodium pentobarbitone (65 mg kg^{-1}) and their systolic blood pressure measured via a cannular (PE 50) inserted into the femoral artery. The cannula, filled with heparinized saline (25 i.u. ml^{-1}), was connected to a Statham pressure transducer and the recordings monitored on a Gould physiopolygraph (model RS 3400). Immediately after blood pressure measurement, the abdominal cavity of individual rats was opened, the mesenteric artery was cannulated through an incision at its confluence with the dorsal aorta and then isolated as previously described by McGregor (1965). The mesenteric bed was flushed with heparinized PSS and subsequently transferred to a warmed chamber and perfused with PSS (maintained at 37°C and gassed with 95% O_2:5% CO_2) at a constant flow rate of 5 ml min^{-1} using a Microperplex peristaltic pump (LKB Bromma, model 2132). Changes in perfusion pressure were recorded via a Statham pressure transducer coupled to a Grass polygraph recorder (model 7E).

The PSS used had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5, glucose 11.1 and where applicable, 1% polyvinylpyrrolidone (PVP). The PSS routinely contained indomethacin (1 μM) and where KCl was used as the pressor agent, also contained 0.1 μM prazosin; the pH of the solution, after saturation with 95% O_2:5%CO_2 gas mixture, was 7.4. Tissues were allowed to equilibrate for 1 h before the start of the experiments. In studies where endothelial denudation was required, this was achieved by an infusion of distilled water for 5 min followed by equilibration in normal PSS for 1 h before challenging with drugs. Endothelial integrity was ascertained functionally with bolus injection of acetylcholine after raising the vascular tone with an infusion of 0.5–1 μM cirazoline.

Experimental protocol

Series 1 and 2 involved studies, as outlined below on SD rats. Series 3 involved a comparison between SHR and WKY with a protocol identical to that outlined for cirazoline and KCl at the perfusion rate of 5.0 ml min^{-1}, in series 1 and 2.

Series 1: The first series of experiments, using mesenteric vascular beds isolated from SD rats, was performed to determine the effects of changes in perfusion rate and infusion of L-NAME on: (a) the basal perfusion pressure, and (b) the vasoconstrictor responses to cirazoline in vascular beds perfused with PSS at 2.5, 5.0 and 10.0 ml min^{-1}. Arterial beds were usually set-up and equilibrated with PSS at the flow rate of 2.5 ml min^{-1}, this was followed by a bolus injection of the ED_{50} (determined from preliminary experiments) of cirazoline; subsequently, the flow rate was increased to 5.0 and 10.0 ml min^{-1} respectively, and, during perfusion at each flow rate, the test dose of cirazoline was injected and the changes in perfusion pressure measured. This procedure was performed in the absence of, and during infusion of L-NAME using either PSS or PSS + 1% PVP to adjust viscosity and maintain osmotic pressure. A separate study of the actions of L- and D-NAME on the dose-response curves of the α-adrenoceptor agonists was then conducted, using PSS and a constant perfusion rate of 5.0 ml min^{-1}.

Series 2: Experiments in this series, also conducted at the perfusion rate of 5.0 ml min^{-1}, were aimed at determining the mode of interactions of L- and D-NAME with KCl vasoconstrictor responses in freshly isolated, and in 48 h cold-stored (4–5°C), vascular beds which were either intact or denuded of endothelium. The protocol, as for cirazoline, involved the establishment of dose-response curves to the agonist in the absence of, and during infusion of a particular concentration of L-, or D-NAME. Loss of vasoconstrictor and vasodilator responses to bolus injections of veratridine and infusion of capsaicin respectively were used to assess the viability or otherwise of periarterial nerves to the mesenteric beds after cold-storage. Additional experiments were performed to assess the: (a) influence of dexamethasone on L-NAME-KCl interaction; and, (b) contributions of Na^+/K^+-ATPase to the action of L-NAME. In (a), the effect of L-NAME on KCl vasoconstriction was determined in PSS containing 0.5 μM dexamethasone and then compared over a 6 h period with

Table 1 Effects of perfusion rate and inclusion of 1% polyvinylpyrrolidone (PVP) on the basal perfusion pressure (BPP) and the potentiating action of 5 μM N^G-nitro-L-arginine methyl ester (L-NAME) (measured as potentiating factors, PFs) on cirazoline-induced pressor responses in mesenteric arterial beds of normotensive Sprague-Dawley (SD) rats

Experimental conditions		*BPP* (mmHg)	*PF* (ED_{50}control:ED_{50}L-NAME)
2.5 ml min^{-1}	PSS	8.8 ± 0.7 (5)	2.6 ± 0.3 (6)
	PSS + 1% PVP	*13.2 ± 0.4 (5)	*1.8 ± 0.4 (5)
5.0 ml min^{-1}	PSS	18.5 ± 1.4 (24)	2.5 ± 0.3 (6)
	PSS + 1% PVP	*23.6 ± 1.2 (6)	*1.7 ± 0.3 (5)
10.0 ml min^{-1}	PSS	30.2 ± 1.7 (5)	2.4 ± 0.2 (6)
	PSS + 1% PVP	*37.9 ± 2.1 (5)	*1.4 ± 0.2 (5)

Data represent the means ± s.e.mean, *n* values in parentheses. Statistically significant difference ($P < 0.05$) from the corresponding values obtained during perfusion with plain physiological salt solution (PSS).

corresponding responses obtained during perfusion with PSS without dexamethasone, and the time control. Experiments ascertaining the role of Na^+/K^+-ATPase were carried out in mesenteric arterial beds perfused with K^+-free PSS and involved testing the action of L-NAME against the vasoconstrictor effects of cirazoline, and, in the case of KCl, vasodilator effects after elevating the arterial tone with an infusion of cirazoline (0.5 μM).

Series 3: This essentially comprised experiments as outlined for the interaction of L-NAME with the α-adrenoceptor agonists or KCl in series 1 and 2 above but with SHR and WKY. All experiments in this series were performed in vascular beds perfused at a rate of 5.0 ml min^{-1}.

Drugs

The following were used: acetylcholine bromide, dexamethasone, indomethacin, N^G-nitro-L-arginine methyl ester, ouabain, capsaicin (8-methyl-*N*-vanillyl 6-nonenamide), tetrodotoxin, veratridine and polyvinylpyrrolidone (PVP) purchased from Sigma Chemical Co., MO, U.S.A.: N^G-nitro-D-arginine methyl ester was from Bachem Inc. (Torrance, CA, U.S.A.). Cirazoline and prazosin, as hydrochloride salts, were generous gifts from Synthélabo Laboratories, Paris, France, and Pfizer Inc., Arnprior, Ontario, Canada.

Statistical analysis

Data were analysed by analysis of variance and, for multiple comparison of results, Duncan's Multiple Range test was used to compare group means. In all cases, a probability of error of less than 0.05 was selected as the criterion for statistical significance. Potentiation factors (PF), defined as

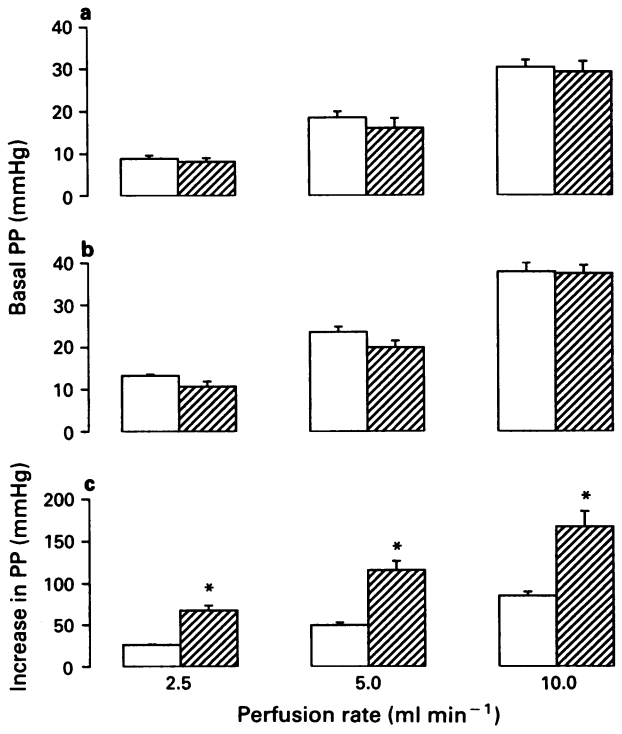

Figure 1 Differential effects of N^G-nitro-L-arginine methyl ester (L-NAME) on the basal perfusion pressure and cirazoline-induced vasoconstriction in isolated mesenteric arterial beds from SD rats perfused with either physiological salt solution (PSS, a and c) or PSS + 1% polyvinylpyrrolidone (PVP, b). Arterial beds were perfused at the rates indicated and in all panels the open and hatched columns represent the measured parameters in the absence, and in the presence of 100 μM (a and b) and 5 μM (c), L-NAME respectively. Data plotted are the means ± s.e.mean ($n = 6-8$ experiments).

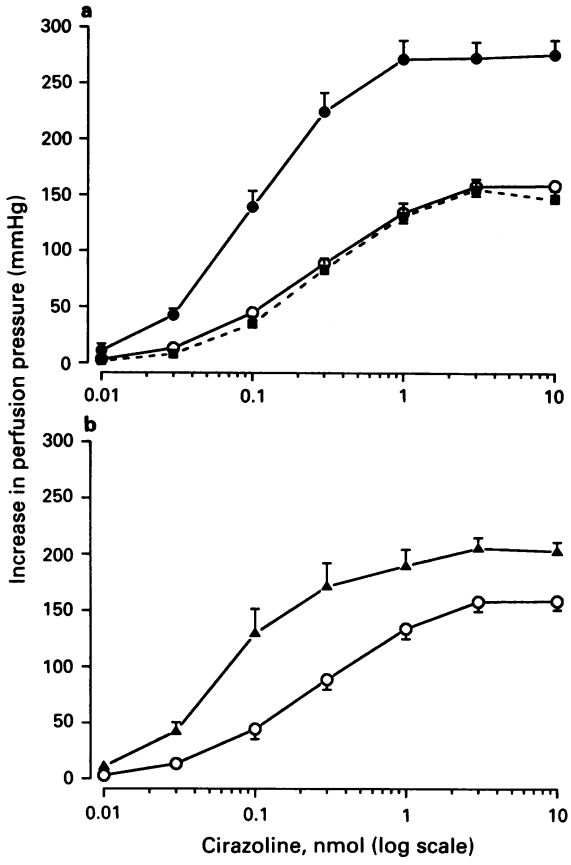

Figure 2 Potentiation by 5 μM N^G-nitro-L-arginine methyl ester (L-NAME, a) or endothelium denudation (b) of cirazoline-induced vasoconstrictor responses in mesenteric arterial beds isolated from Sprague Dawley rats. In (a), (○), (●) and (■) represent the responses obtained to cirazoline in the absence, and, in the presence of 5 μM L- and D-NAME respectively; while panel (b) compares cirazoline responses in endothelium-intact (○) and endothelium-denuded (▲) arterial beds. Each point on the graphs represents the mean ± s.e.mean, $n = 5-8$ experiments.

the ratios of ED_{50} in the absence, to that in the presence of L-NAME; ED_{50} denotes the doses of cirazoline or KCl that produced 50% of the maximal vasoconstrictor effect and were obtained from individual dose-response curves under each of the experimental conditions.

Results

Mesenteric basal perfusion pressure: influence of perfusion rate, PVP and L-NAME

The basal perfusion pressure of mesenteric arterial beds perfused with PSS increased significantly with increasing perfusion rates (Table 1) and the same trend was observed in arterial beds perfused with PSS + 1% PVP. However, the basal perfusion pressure values of tissues perfused with PSS + 1% PVP were signifcantly ($P < 0.05$) greater than the corresponding values in PSS (Table 1). Infusion of L-NAME in concentrations up to 100 μM did not alter the basal perfusion pressure at any of the perfusion rates, regardless of whether PVP was present (Figure 1). Conversely, the cirazoline-induced vasoconstrictor response at each of the perfusion rates was significantly potentiated by 5 μM L-NAME (see Figure 1). As can be seen in Table 1, the potentiation factors (defined in Methods section) were not significantly different at each of the perfusion rates, but were significantly ($P < 0.05$) greater in PSS versus PSS + 1% PVP; for this reason, subsequent experiments were performed in PSS.

Effects of L-NAME on mesenteric vasoconstrictor responses to cirazoline in normotensive Sprague Dawley rats

In normotensive Sprague Dawley rats, bolus injection of cirazoline (0.01–10.0 nmol) initiated dose-dependent pressor responses with ED_{50} values of 3.1 ± 0.5 ($n = 6$) and 1.8 ± 0.4 ($n = 5$) nmol in endothelium-intact and -denuded preparations respectively; the ED_{50} value is significantly ($P < 0.05$) lower in denuded than in intact preparations. D-NAME (50 μM), regardless of perfusion rate, was completely devoid of effect on the basal perfusion pressure but L-NAME (5 μM) caused a leftward shift of the dose-response curves to cirazoline with a marked increase in the maximal effect (Figure 2). However, in arterial bed preparations with intact endothelium but perfused with Ca^{2+}-free PSS, and in those denuded of endothelium by an infusion of distilled water for 5 min, L-NAME failed to potentiate cirazoline-induced vasoconstriction (Figure 3).

Effects of L-NAME on KCl-induced responses

Bolus injections of KCl (1–100 μmol) initiated pressor responses with ED_{50} values of 1.09 ± 0.10 ($n = 5$) and 1.11 ± 0.15 ($n = 5$) μmol in endothelium-intact and -denuded arterial preparations respectively, and, regardless of the presence or absence of endothelium, L-NAME potentiated the pressor responses to the same extent (Figure 4). Arterial beds cold-stored (4–5°C) for 2 days responded in a dose-dependent manner to bolus injections of KCl, but the magnitude of such responses were reduced as compared to similar effects in fresh tissues, and, unlike fresh preparations, responses were not potentiated by L-NAME. Furthermore, infusion of 0.1 μM capsaicin, which caused complete vasodilatation of fresh tissues was ineffective in cold-stored preparations; also, bolus injections of veratridine (1–30 nmol), unlike their effect in fresh preprations, failed to elicit vasoconstrictor action in cold-stored arterial beds (Figure 5).

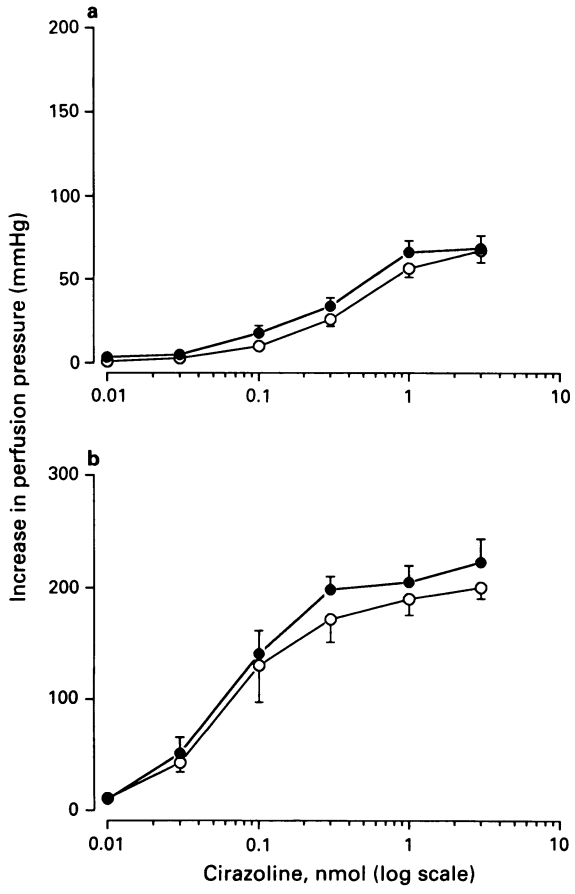

Figure 3 Lack of potentiation by N^G-nitro-L-arginine methyl ester (L-NAME) of cirazoline responses in isolated arterial beds with intact endothelium and perfused with Ca^{2+}-free PSS (a) or in endothelium-denuded arterial beds (b). In both panels, (○) and (●) represent the control and the responses in the presence of 5 μM L-NAME respectively. Each point on the graphs represents the mean ± s.e.mean, $n = 6$ experiments.

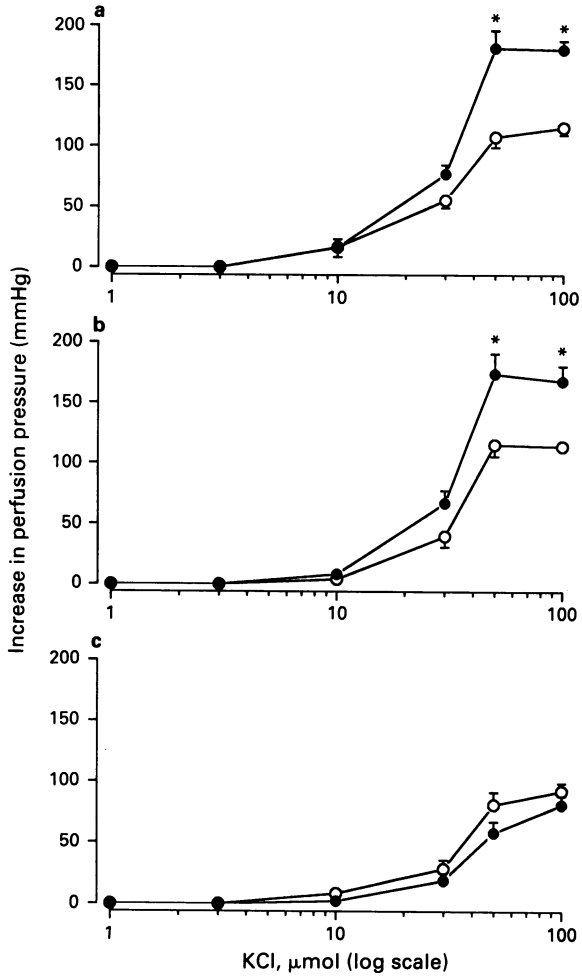

Figure 4 The effect of N^G-nitro-L-arginine methyl ester (L-NAME) on KCl dose-response curves in isolated perfused mesenteric arterial beds from SD rats. The data plotted in (a) and (b) were obtained, respectively, from endothelium-intact and denuded freshly isolated arterial beds, while in (c) data presented are from arterial beds denervated by cold storage for 48 h at 4–5°C. In all panels, (○) and (●) represent the responses in the absence and in the presence of 5 μM L-NAME, respectively. Each point on the graphs represents the mean ± s.e.mean ($n = 6$), and *denotes statistically significant difference ($P < 0.05$) from corresponding control.

In endothelium-denuded mesenteric arteries, the pressor responses obtained to bolus injections of 50 μmol KCl at 2, 4 and 6 hourly periods were not significantly different (107.5 ± 6.9, 115.3 ± 9.7 and 114.3 ± 7.1 respectively), and L-NAME (5 μM) potentiated the KCl effects respectively by 40.6 ± 4.3, 33.7 ± 3.1 and 37.9 ± 3.9% in the presence of dexamethasone. Potentiation of KCl-induced vasoconstriction by L-NAME in the absence of dexamethasone, over the same observation period was not significantly different from that in its presence. Mesenteric arterial beds, equilibrated with K^+-free PSS, constricted to cirazoline and such vasoconstrictor responses were potentiated by L-NAME (Figure 6); however, ouabain-sensitive KCl-induced vasodilator responses in arterial beds preconstricted with cirazoline were not inhibited by 1 mM L-NAME (Figure 7).

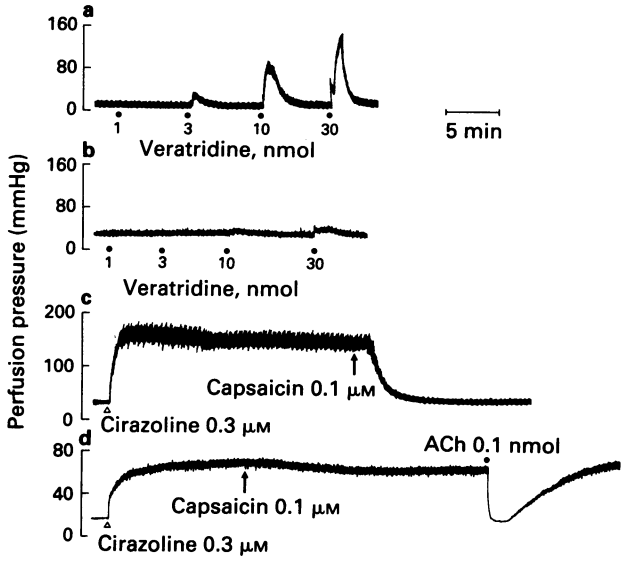

Figure 5 Representative tracings of the comparative effects of veratridine (a and b) and capsaicin (c and d) in freshly isolated (a and c respectively) versus 48 h cold-stored (4–5°C; b and d respectively) mesenteric arterial beds of Sprague Dawley rats. Veratridine doses (●), given by bolus injections, are in nmol; capsaicin (0.1 μM) was infused into cirazoline (0.3 μM)-preconstricted arterial beds and the arrows denote the start of the infusion.

Interactions of L-NAME with cirazoline and KCl in arterial beds isolated from WKY and SHR

WKY rats used in our study had mean systolic/diastolic blood pressures of 95.6 ± 1.8/51.5 ± 0.7 mmHg and a mean heart rate of 311.1 ± 16.3 beats min^{-1} ($n = 10$) while the mean blood pressures for the SHR were 174.6 ± 7.6/111.5 ± 4.5 mmHg and the heart rate was 372.3 ± 8.2 beats min^{-1} ($n = 13$). The systolic and diastolic blood pressures and the heart rates in SHR were significantly ($P < 0.05$) higher than the comparative values for the WKY.

As previously noted for SD rats, L-NAME failed to change the basal perfusion pressure in WKY and SHR, but, at 5 μM, it markedly reduced the ED_{50} values for cirazoline but not KCl, and increased the maximal pressor effects to both compounds in both WKY and SHR (Table 2). Although mesenteric arterial beds from the SHR were more sensitive to cirazoline than those from WKY (ED_{50}; 2.18 ± 0.13 versus 2.94 ± 0.21 nmol respectively), there were no apparent differences in the potentiation, expressed as potentiation factors (PFs), of pressor effects induced by L-NAME in the two groups of rats (Table 2).

Discussion

Arginine derivatives, such as N^G-nitro-L-arginine methyl ester, have been shown to inhibit NO synthesis and endothelium-dependent relaxation and have been used extensively in studies involving the role of NO synthesis in the regulation of vascular tone and blood pressure (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Dohi *et al.*, 1990; Moncada *et al.*, 1991). The present study, with isolated mesenteric arterial beds perfused with PSS, provides evidence that L-NAME, although lacking an effect on the basal tone, caused marked potentiation of α-adrenoceptor-mediated vasoconstriction in an endothelium-dependent manner. KCl-induced vasoconstrictor responses were also enhanced by L-NAME, but, in an endothelium- and time-independent, and dexamethasone-insensitive manner; such enhancement was completely absent in cold-denervated arterial beds. The L-NAME-mediated potentiation of

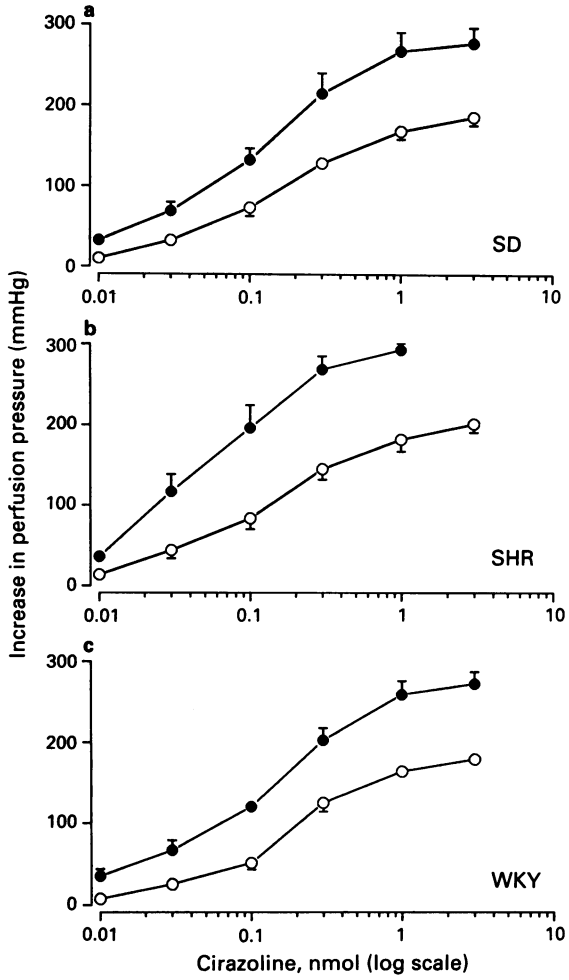

Figure 6 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) on dose-response curves to cirazoline in mesenteric arterial beds isolated from normotensive Sprague Dawley (SD, a), Wistar Kyoto (WKY, c) and spontaneously hypertensive (SHR, b) rats. In all panels, (○) represents the control responses, while (●) represents the responses during an infusion of 5 μM L-NAME. The arterial beds were perfused with K^+-free physiological salt solution, and the points on the graphs represent the means ± s.e.mean, $n = 5$.

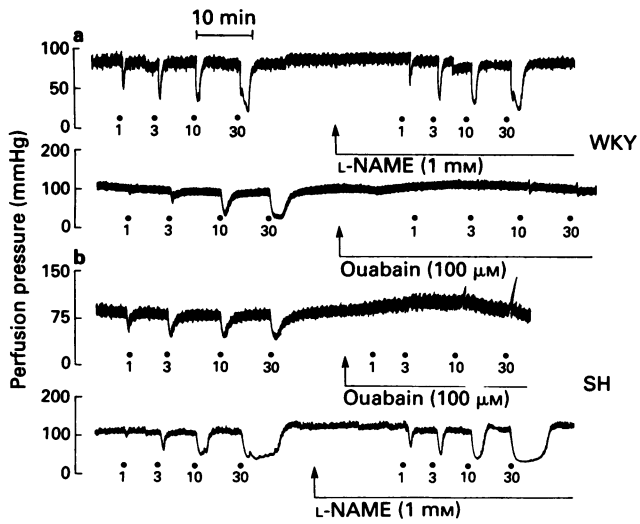

Figure 7 Effects of 1 mM N^G-nitro-L-arginine methyl ester (L-NAME) and 100 μM ouabain on KCl-induced vasodilator responses in mesenteric arterial beds perfused with K^+-free physiological salt solution and preconstricted with an infusion of 0.5 μM cirazoline. Arterial beds in (a) were isolated from normotensive Wistar Kyoto (WKY) rats, while those in (b) were from spontaneously hypertensive rats (SHR). Doses of KCl indicated by (●) are in μmol.

Table 2 Effects of N^G-nitro-L-arginine methyl ester (L-NAME, 5 μM) on cirazoline- and KCl-induced pressor responses in normotensive Sprague Dawley (SD), Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats

Agonists		*SD*	*WKY*	**SHR*
Cirazoline	PFs	2.95 ± 0.25	3.55 ± 0.50	3.51 ± 0.48
	E_{max} (% increase)	65.4 ± 7.1	69.4 ± 8.2	63.7 ± 9.4
KCl	PFs	1.09 ± 0.10	1.05 ± 0.07	1.23 ± 0.13
	E_{max} (% increase)	48.5 ± 9.4	57.1 ± 8.3	43.4 ± 7.4

Data represent the means ± s.e.mean ($n = 5-6$), and, *denotes that both the PFs (potentiation factors defined in Methods) and the % increases in the E_{max} obtained in arterial beds from SHR for both agonists did not differ significantly ($P > 0.05$) from those obtained for normotensive SD and WKY. KCl experiments were performed in the presence of 10 nM prazosin added to perfusion medium.

cirazoline and KCl, which occurred in arterial beds isolated from both normotensive rats and SHR, was neither mediated via an action of L-NAME on Na^+/K^+-ATPase, nor on the inducible smooth muscle NO synthase, but rather, on endothelial and neuronal NO synthase(s).

Administration of L-NMMA to conscious guinea-pigs (Aisaka *et al.*, 1989), and rabbits (Rees *et al.*, 1989) or to pithed rats (Tabrizchi & Triggle, 1992) and of L-NAME to conscious rats (Gardiner *et al.*, 1990), results in a marked hypertension that has been ascribed to the inhibition of the endogenous synthesis of NO. L-NMMA also causes coronary vasoconstriction in the rabbit isolated perfused heart (Amezcua *et al.*, 1989). In the present study no pressor response was observed during the infusion of L-NAME, and the inclusion of 1% PVP as a plasma protein replacement made no difference. These data suggest that NO may not be spontaneously released under the basal perfusion pressure conditions in the mesenteric vascular bed and/or that circulating vasoconstrictors, or other factors, may be required to trigger release. If the presence of a vasoconstrictor, or active tone, is required as a trigger for NO synthesis and release, conceivably the stimulus is Ca^{2+} entry via the receptor-operated calcium channels (ROCC)/cation channels present in the endothelial cell membrane, and the resultant increase in intracellular levels of Ca^{2+} ions stimulates the *de novo* synthesis of NO.

The endothelium has been postulated to serve as a pressure and a flow sensor (Rubanyi *et al.*, 1990); thus, both of these parameters are critical to the release of endothelium-derived vasoactive factors. However, an increase in the perfusion rate, but not pressure, has been demonstrated to increase the rate of NO release in porcine cultured aortic endothelial cells under basal and stimulated conditions (Kelm *et al.*, 1991). In the present study, L-NAME was still without effect despite the assumed increase in shear stress resulting from an increase in the perfusion rate from 2.5 to 10.0 ml min^{-1}. This finding supports an earlier conclusion that in the rat mesenteric bed endothelium derived NO, (EDNO) is less important than the endothelium-derived hyperpolarizing factor, or EDHF (Adeagbo & Malik, 1990; Adeagbo & Triggle, 1992; 1993). Furthermore, a recent report by Garland & McPherson (1992) also indicates that ACh-mediated hyperpolarization in the rat small mesenteric artery is not mediated by NO.

The present study has demonstrated a stereo-specific enhancement of cirazoline-induced vasoconstriction by nitro-arginine methyl ester. L-NAME but not D-NAME caused a leftward shift, accompanied by a significant increase in the maximal effect, of cirazoline dose-response curves. Since this L-NAME-induced potentiation was abolished in endothelium-denuded mesenteric arterial beds, it can be concluded that the action of L-NAME resulted from an inhibitory action on NO synthesis in endothelial cells. The endothelial NO synthase requires Ca^{2+} and NADPH as co-factors for the conversion of L-arginine to NO and L-citrulline (Myers *et al.*, 1989); thus, the failure of L-NAME to potentiate α-adrenoceptor-mediated pressor responses obtained during perfusion with Ca^{2+}-free PSS further supports the conclusion that endothelial NO synthase is the target of L-NAME action in the present study.

KCl-induced vasoconstrictor responses were also potentiated by L-, but not D-NAME. However, unlike for cirazoline, the ED_{50}s for KCl were not altered by L-NAME, but there were significant increases in the E_{max} in both endothelium-intact and denuded preparations. These results suggest that a component of the effect of L-NAME in mesenteric arterial beds might be independent of the endothelium. The extra-endothelial sites that may contribute to the potentiation induced by L-NAME, include: the inducible smooth muscle, and/or constitutive neuronal NO synthase(s), non-specific interference with the sodium pump, or a direct facilitation of smooth muscle contractile process.

Endotoxin contamination of the mesenteric vasculature may occur during separation of the vascular bed from the bowel and thus, it is conceivable that the inducible smooth muscle NO synthase might serve as an additional target for L-NAME action. If indeed the inducible smooth muscle NO synthase, as described in the rat aortic rings (Schini & Vanhoutte, 1991; Moritoki *et al.*, 1991), is responsible for the non-endothelium-dependent potentiation of KCl pressor responses by L-NAME, then inclusion of a glucocorticoid, such as dexamethasone, in the perfusion medium should counteract the effect of L-NAME. Glucocorticoids have been demonstrated to prevent the induction of smooth muscle NO synthase (Rees *et al.*, 1990). However, the addition of dexamethasone to the perfusing PSS failed to prevent the potentiation by L-NAME of KCl pressor activity and thus our data are incompatible with this hypothesis. This suggests that the mechanism of endothelial-independent potentiation of the KCl effect by L-NAME observed in the present study does not involve the inducible smooth muscle NO synthase. It is also evident from the present study, that the Na^+/K^+-ATPase is not involved in the mechanism of action of L-NAME since L-NAME in concentrations up to 1 mM was totally ineffective against the ouabain-sensitive vasodilatation produced by KCl following the infusion of K^+-depleted PSS, and furthermore, L-NAME still potentiated the cirazoline-induced pressor responses obtained in mesenteric beds perfused with K^+-depleted PSS.

The rat mesenteric arterial bed has been shown to have dense adrenergic (Furness & Marshall, 1974), peptidergic (Ganz *et al.*, 1986) and capsaicin-sensitive sensory (Wharton *et al.*, 1986; Manzini & Perretti, 1988) innervation. It is conceivable that the release of NO from any of these neuronal sources may contribute to the potentiating effects of L-NAME on KCl vasoconstriction. The neural NO synthase is a homologue of the endothelial enzyme, and shares not only co-factor requirements (Bredt & Snyder, 1990; Crossin, 1991), but, also, sensitivity to nitro-L-arginine; an IC_{50} of 0.05 μM has been reported (Lambert *et al.*, 1991). That L-NAME-induced enhancement of KCl vasoconstriction is mediated via inhibition of neuronal NO synthase is evident from the complete loss of potentiation in arterial beds denervated by cold storage (4–5°C) for 2 days. Cold storage is used extensively as a denervation procedure (Akbarali *et al.*, 1987; Jurkiewicz *et al.*, 1992), and the procedure also abol-

ished nerve-mediated vasoconstriction induced by veratridine, and, though less consistently, capsaicin-induced vasodilatation. Veratridine depolarizes nerve terminals via the activation of Na^+ channels (Kirpekar & Prat, 1979). High external K^+ concentrations have previously been demonstrated to stimulate the release of EDRF (presumably NO) in canine femoral arteries (Rubanyi & Vanhoutte, 1988) and to stimulate simultaneously NO release and increase in tissue cyclic GMP levels in cerebellar slices (Dickie *et al.*, 1990).

Another important aspect of the present study pertains to the comparison of L-NAME effects on cirazoline- and KCl-induced pressor activities of Sprague-Dawley, Wistar Kyoto and spontaneously hypertensive rats. The rationale for this comparison is based on the hypothesis that impairment of endothelial function in the hypertensive state reflects a reduced activity/level of the NO synthase, so that a reduced degree of potentiation of pressor activity should be observed. Our data have revealed that although the SHR were significantly more sensitive to cirazoline than SD or WKY rats, the action of L-NAME was qualitatively similar in the three strains of rat *viz* significantly lower ED_{50}s and greater E_{max} values for cirazoline in the presence of L-NAME. In addition, potentiation of pressor responses by L-NAME (measured as PFs) was statistically similar in all strains of rat used. Thus, our data indicates that there is no dysfunction of NO synthase activity in the mesenteric vessels of the SHR. An impairment of endothelium-dependent relaxation has been reported in both isolated thoracic aortic rings from genetically hypertensive rats (Konishi & Su, 1983; Winquist *et al.*, 1984; Lüscher & Vanhoutte, 1986) and small (DeMey & Gray, 1985; Mayhan *et al.*, 1987; Dohi *et al.*, 1990) blood vessels. It is however, worthy of note that an enhancement of endothelial function in hypertension has also been reported (Webb *et al.*, 1987), and also that EDRF release in bioassay experiments does not differ between hypertensive and normotensive control rats (Van de Voorde & Leusen, 1986). Lüscher, in a recent editorial (1990), has concluded that the apparent impairment of endothelial function in the SHR is a result of an increase in the release of a contracting factor(s) rather than decrease in a relaxing factor(s). In a recent study Tabrizchi & Triggle (1991) reported that bolus i.v. injections of L-NAME augmented the increase in blood pressure resulting from stimulation of the sympathetic nerves in the pithed rat, and that this increase was greater in SHR than in WKY. These authors concluded that the levels of L-arginine-derived NO may be higher in SHR and secondary to the elevated blood pressure of the SHR.

The striking disparity between the enhancement of pressor responses by L-NAME and that due to endothelial denudation brought about by an infusion of distilled water deserves comment. Significantly greater potentiation by L-NAME than endothelial denudation suggests: either (a) that distilled water damaged the underlying smooth muscle cells, or (b) the contribution of a non-cyclo-oxygenase, indomethacin-insensitive endothelium-derived contracting factor(s) which is only unmasked after inhibition of endothelial NO synthase, or, (c) the possibility of L-NAME exhibiting an inhibitory effect on both endothelial and smooth muscle NO synthases. Damage to the smooth muscle cells resulting from the distilled water treatment is an unlikely explanation since sodium nitroprusside-induced vasodilatation was, if anything, potentiated in distilled water-treated versus control, untreated, arterial beds. The enhancement of sodium nitroprusside-induced vasodilatation following endothelial denudation has been previously reported by Ralevic *et al.* (1991). Conceivably the inhibition of NO synthesis promotes the synthesis of endothelial-derived contracting factor(s). Furthermore, a cyclic GMP independent action of NO on Ca^{2+} homeostasis has been reported (Garg & Hassis, 1991) and thus L-NAME may prevent the NO-mediated decrease in cytosolic free Ca^{2+}.

In conclusion, the present study demonstrates that L-NAME potentiated α-adrenoceptor-and KCl-mediated pressor responses in perfused rat mesenteric arterial beds via an inhibitory action on endothelial, and neuronal NO synthase(s). This potentiation involved neither the dexamethasone-sensitive inducible smooth muscle NO synthase, nor Na^+/K^+-ATPase. Furthermore, since the effects of L-NAME were similar in normotensive SD and WKY compared to SHR, our study indicates that NO synthase activity is not impaired in the mesenteric bed of spontaneously hypertensive rats.

This research project was supported by a grant-in-aid (to C.R.T.) from the Alberta Heart and Stroke Foundation. R.T. was supported by Medical Research Council and Alberta Heritage Foundation for Medical Research Fellowships.

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(Received March 19, 1993
Revised June 17, 1993
Accepted August 26, 1993)

Br. J. Pharmacol. (1994), **111**, 21–28

Bradykinin-stimulated phosphoinositide metabolism in cultured canine tracheal smooth muscle cells

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1 Stimulation of bradykinin (BK) receptors coupled to phosphoinositide (PI) hydrolysis was investigated in canine cultured tracheal smooth muscle cells (TSMCs). BK, kallidin, and des-Arg^9-BK, stimulated [^{3}H]-inositol phosphates (IPs) accumulation in a dose-dependent manner with half-maximal responses (EC_{50}) at 20 ± 5, 13 ± 4, and 2.3 ± 0.7 nM, ($n = 5$), respectively.

2 D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, BK B_2 receptor antagonists, were equipotent in blocking the BK-induced IPs accumulation with $pK_B = 7.1$ and 7.3, respectively.

3 Short-term exposure of TSMCs to phorbol 12-myristate 13-acetate (PMA, 1 μM), attenuated BK-stimulated IPs accumulation. The concentrations of PMA that gave half-maximal and maximal inhibition of BK-induced IPs accumulation were 15 ± 4 nM and 1 μM, $n = 3$, respectively. The inhibitory effect of PMA on BK-induced response was reversed by staurosporine, a protein kinase C (PKC) inhibitor, suggesting that the inhibitory effect of PMA was mediated through the activation of PKC.

4 Prolonged incubation of TSMCs with PMA for 24 h, resulted in a recovery of receptor responsiveness which may be due to down-regulation of PKC. The inactive phorbol ester, 4α-phorbol 12, 13-didecanoate at 1 μM, did not inhibit this response.

5 The site of this inhibition was further investigated by examining the effect of PMA on AlF_4^--induced IPs accumulation in canine TSMCs. AlF_4^--stimulated IPs accumulation was inhibited by PMA treatment, suggesting that the G protein(s) can be directly activated by AlF_4^-, which is uncoupled from phospholipase C by PMA treatment.

6 Incubation of TSMCs in the absence of external Ca^{2+} or upon removal of Ca^{2+} by addition of EGTA, caused a decrease in IPs accumulation without changing the basal levels. Addition of Ca^{2+} (3–620 nM) to digitonin-permeabilized TSMCs stimulated IPs accumulation was obtained by inclusion of either guanosine 5′-O-(3-thiotriphosphate) (GTPγS) or BK. The combination of GTPγS and BK caused an additive effect on IPs accumulation.

7 Pretreatment of TSMCs with cholera toxin enhanced BK-stimulated IPs accumulation, whereas there was no effect with pertussis toxin.

8 These data suggest that BK-stimulated PI metabolism is mediated by the activation of BK B_2 receptors coupling to a G protein which is not blocked by cholera toxin or pertussis toxin treatment and dependent on external Ca^{2+}. The transduction mechanism of BK coupled to PI hydrolysis is sensitive to feedback regulation by PKC.

Keywords: Bradykinin; phorbol ester; protein kinase C; inositol phosphates; G protein; canine tracheal smooth muscle cells

Introduction

Bradykinin (BK) is a classic mediator of inflammatory diseases of the airways and may be implicated in allergic asthma (Christiansen *et al.*, 1987). In the airways, BK causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). Most of the biological actions of BK are mediated through at least two different receptors, termed BK B_1 and B_2 receptors, which have been pharmacologically characterized using different kinin analogues with agonist and antagonist properties (Regoli *et al.*, 1990). In many cell types, including the neuroblastoma-glioma hybrid NG108-15 (Osugi *et al.*, 1987), glioma C6-4-2 (Reiser *et al.*, 1990), astrocytoma 1321N1 cells (Helper *et al.*, 1987), and bovine tracheal smooth muscle cells (TSMCs) (Marsh & Hill, 1992), BK receptors activate phospholipase C (PLC) mediated phosphoinositide (PI) hydrolysis in the plasma membrane. The resultant increase in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) release Ca^{2+} from internal stores and activate protein kinase C (PKC), respectively (Nishizuka, 1988; Berridge & Irvine, 1989).

Binding and functional studies have provided evidence for subtypes of B_2 receptors in guinea-pig ileum and lung and in rat myometrium membranes and vas deferens (Manning *et al.*, 1986; Braas *et al.*, 1988; Plevin & Owen, 1988; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991). The mechanisms involved in smooth muscle contractile response are not completely understood particularly in the airways. One possible mechanism of BK-induced bovine smooth muscle contraction is an increase in PI hydrolysis mediated by B_2 receptors (Marsh & Hill, 1992). There are several lines of evidence that PLC is coupled to a variety of cell surface receptors via a guanine nucleotide binding protein (Sternweis & Smrcka, 1992). The stable analogue of GTP, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), has been shown to increase IPs accumulation in permeabilized tracheal smooth muscle of rabbit (Rosenberg *et al.*, 1991) and man (Murray *et al.*, 1989). In some studies, PI hydrolysis has been shown to be dependent on calcium, presumably due to the requirement of this ion for PLC activity (Fisher *et al.*, 1989; Eberhard & Holz, 1991). In other work, however, no dependence on calcium has been observed (Berridge & Irvine, 1989).

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Many studies have also suggested that BK B_2 receptors are coupled to G proteins in other cell types (Higashida *et al.*, 1986; Burch & Axelrod, 1987; Murayama & Ui, 1987). For example, BK-induced inositol phosphates (IPs) accumulation was found to be sensitive to guanine nucleotide analogues in NG108 and 3T3 cells (Higashida *et al.*, 1986; Murayama & Ui, 1987). It has been reported that many signal transduction processes which require GTP may not be affected by pertussis or cholera toxin (Martin *et al.*, 1985; Aub *et al.*, 1986). Indeed, in NG108 cells, pertussis toxin (PTX)-insensitive G_q protein(s) may account for kinin receptor coupling to PLC (Gutowski *et al.*, 1991).

Agonist-activation of PI hydrolysis also leads to the formation of DAG. The ability of phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) which mimics the effect of DAG, to activate PKC (Castagna *et al.*, 1982), has provided a useful tool to examine the role of the DAG pathway related to smooth muscle contraction. By using PMA it was shown that stimulation of PI hydrolysis induced by α_1-adrenoceptor activation on DDT_1MF_2 cells (Leeb-Lundberg *et al.*, 1985) was inhibited by PMA, probably through phosphorylation of the receptors by PKC. Because DAG is one of products of PI hydrolysis, it is believed that PKC may be involved in 'desensitization' or 'down-regulation' of the muscarinic receptors in astrocytoma cells and could exert a feedback regulation of PI breakdown (Orellana *et al.*, 1985).

The purpose of the present study was to investigate the effect of BK on IPs accumulation and its regulation in canine TSMCs, using PTX, cholera toxin (CTX), PKC activator, and AlF_4^-, an activator of the regulatory proteins. These results conclude that BK induces PLC-mediated PI hydrolysis through the activation of BK B_2 receptors in canine TSMCs and that this reaction is negatively regulated by PKC activation. Evidence is also provided that BK B_2 receptors may be coupled to PLC via a PTX-and CTX-insensitive Gq protein.

Methods

Animals

Mongrel dogs, 10–20 kg, of either sex were purchased from a local supplier and used throughout this study. Dogs were housed in our animal facilities under automatically controlled temperature and light cycle and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.v.) and the lungs were ventilated mechanically via an orotracheal tube. The tracheae were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods of Yang *et al.* (1991). The trachea was cut longitudinally through the cartilage rings and the smooth muscle was dissected. The muscle was minced and transferred to the dissociation medium containing 0.1% collagenase IV, 0.025% DNase I, 0.025% elastase IV, and antibiotics in physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, $CaCl_2$ 1.1, $NaHCO_3$ 20, NaH_2PO_4 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were gently agitated at 37°C in a rotary shaker for 1 h. The released cells were collected and the residual was again digested with fresh enzyme solution for an additional hour at 37°C. The released cells were washed twice with DMEM/F-12 medium. The cells, suspended in DMEM/F-12 containing 10% FBS, were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cell number was counted and the suspension diluted with DMEM/F-12 to 2×10^5 cells ml^{-1}. The cell suspension (1 ml/well) was plated onto 12-well culture plates. The medium was changed after 24 h and then every 3 days. After a 5-day culture, cells were shifted to DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were cultured in DMEM/F-12 containing 1% FBS supplemented with IGF-I (10 ng ml^{-1}) and insulin (1 μg ml^{-1}) for 12–14 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by indirect immunofluorescence using a monoclonal antibody of light chain myosin (Gown *et al.*, 1985). Over 95% of the cell preparation was composed of smooth muscle cells.

Accumulation of inositol phosphates

Effect of BK on the hydrolysis of PI was assayed by monitoring the accumulation of ^3H-labelled IPs as described by Berridge *et al.* (1983). Cultured TSMCs were incubated with 5 μCi ml^{-1} of *myo*-[2-^3H]-inositol at 37°C for 2 days. TSMCs were washed two times with PBS and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM): NaCl 117, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 2.4, glucose 1, HEPES 20 and LiCl 10 at 37°C for 30 min. After BK was added at the concentration indicated, incubation was continued for another 60 min in the presence of 10 μM phosphoramidon. Reactions were terminated by addition of 5% perchloric acid followed by sonication and centrifugation at 3,000 *g* for 15 min.

The percholic acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied for a column of AG1-X8, formate form, 100–200 mesh (Bio-Rad). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free *myo*-[^3H]-inositol and glycerophosphoinositol, respectively. Total IPs was eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^3H]-IPs was determined in a radiospectrometer (Beckamn LS5000TA, Fullerton, CA, USA).

Permeabilized cells

After the prelabelling period, TSMCs were washed twice with PBS and then permeabilized in potassium glutamate-EGTA-HEPES buffer (KGEH) containing (mM): K^+ glutamate 139, ATP 2, $MgCl_2$ 4, LiCl 10, EGTA 2, HEPES 20 (pH 7.4), and 10 μM digitonin, as described by Eberhard & Holz (1987). Cells were allowed to permeabilize for 5 min at 37°C. The permeabilizing buffer was discarded and cells were washed twice with KGEH buffer without digitonin. Incubations were continued for 15 min at 37°C. The required free Ca^{2+} concentrations ($[Ca^{2+}]_f$) were achieved by the addition of various amounts of $CaCl_2$ to the KGEH buffer containing 2 mM EGTA. The $[Ca^{2+}]_f$ was determined by the addition of 10 μM fura-2 (K^+-salt) using a SLM spectrofluorometer (Aminco SLM, Urbana, IL, U.S.A.). BK at the indicated concentration was added and incubation continued for 60 min. Reactions were terminated by the addition of 5% perchloric acid. The procedures for extraction, separation, and quantification of IPs were the same as those used for intact cells.

ADP-ribosylation

Cultured TSMCs were incubated with PTX (100 ng ml^{-1}) or CTX (10 μg ml^{-1}) at 37°C for 4 h. Each plate was rinsed with cold PBS and once with buffer A (mM) containing Tris-HCl 20, pH 7.5, NaCl 10, EGTA 1 and phenylmethylsulphonyl fluoride 1 (PMSF). Cells were scraped from the dish into buffer A and homogenized with a polytron (set 5 for 30 s). After removing the nuclei and unbroken cells by centrifugation at 400 *g* for 10 min, the supernatant was centrifuged at 10,000 *g* for 30 min. The final pellet was suspended in 25 mM Tris-HCl, pH 7.4 containing 2.5 mM $MgCl_2$. Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

ADP-ribosylation was performed as described (Katada & Ui, 1982) with minor modifications: 100 μg of total protein

from the membrane-rich fraction was diluted with an equal volume of ribosylation buffer (Tris-HCl 25 mM, pH 7.4, thymidine 10 mM, GTP 1 mM, $MgCl_2$ 2.5 mM, EDTA 1 mM), and 5 mM dithiothreitol (DTT). PTX was activated by incubation with 50 mM DTT in 25 mM Tris-HCl, pH 7.4 at 37°C for 1 h and added to a final concentration of 5 μg ml^{-1}. [^{32}P]-NAD^+ (5 μCi/sample) was added with cold NAD^+ to a final concentration of 10 μM and the reaction mixture was incubated at 37°C for 30 min. ADP ribosylation reaction was terminated by 5% trichloroacetic acid precipitation, and the samples were resolved by SDS-polyacrylamide (10%)-gel electrophoresis (Laemmli, 1970). Gels were stained with Commassie Brilliant Blue, dried, and autoradiographed. For CTX labelling, 5 μg CTX/sample was included in the ADP-ribosylation mixture.

Analysis of data

The EC_{50} of BK for stimulating IPs accumulation was estimated by Graph Pad Program (Graph Pad, San Diego, CA, U.S.A.). The dissociation constants (K_B) of BK antagonists were estimated by the method of Furchgott (1972), using their ability to antagonize BK-mediated IPs accumulation.

The data were expressed as the mean ± s.e.mean of at least four experiments with statistical comparisons based on a two-tailed Student's *t*-test at a $P<0.01$ level of significance.

Chemicals

DMEM/F-12 medium and FBS were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Insulin and IGF-I were from Boehringer Mannheim (GmbH, Germany). Fura-2 was from Molecular Probes Inc (Eugene, OR, U.S.A.). *Myo*-[^{3}H]-inositol (18 Ci $mmol^{-1}$) and [^{32}P]-NAD^+ (65 Ci $mmol^{-1}$) were from Amersham (Buckinghamshire, England). D-Arg[Hyp^3, D-Phe^7]-BK, D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, des-Arg^9-BK and kallidin were from Bachem California (Torrance, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.).

Results

Agonist-induced IPs accumulation

To study agonist-stimulated IPs accumulation, canine cultured TSMCs were labelled for 48 h with [^{3}H]-inositol, treated with agonist and extracted. The aqueous phase of cell extracts was used to determine the amount of [^{3}H]-IPs. Carbachol (100 μM), histamine (100 μM), 5-hydroxytryptamine (5-HT, 100 μM), and BK (1 μM), produced IPs accumulation of 9.1 ± 0.5, 2.6 ± 0.2, 10.5 ± 0.2, and 7.1 ± 0.3 fold, respectively, greater than the basal level (8600 ± 500 d.p.m./well; $P<0.001$, $n=3$). BK-induced IPs accumulation increased rapidly up to 10 min of incubation and reached a maximal value (52700 ± 2500 d.p.m./well, $n=3$) at 60 min in the presence of 10 mM LiCl. LiCl alone caused no significant accumulation of IPs within 60 min. The effects of BK, kallidin, and des-Arg^9-BK on IPs accumulation was dose-dependent over the range of 1 nM to 10 μM with EC_{50} values of 20 ± 5, 13 ± 4, and 2.3 ± 0.7 nM, ($n=5$), respectively (Figure 1). Among these agonists, des-Arg^9-BK at maximally effective concentration caused the smallest increase in IPs accumulation.

Effects of BK B_2 antagonists on BK-induced IPs accumulation

To determine which type of BK receptor is involved in the activation of PLC in canine TSMCs, we examined the effects of D-Arg[Hyp^3,D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, selective BK B_2 receptor antagonists, on BK-induced IPs accumulation. As shown in Figure 2, preincubation of TSMCs with these antagonists inhibited the BK-induced IPs accumulation. The concentration-effect relationship of BK was shifted to the right in a nearly parallel fashion, without changing the maximal response, upon addition of 5 μM D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8}, D-Phe^7]-BK. The EC_{50} values of BK were increased from 20 ± 5 nM to 2.1 ± 0.4 and 1.4 ± 0.3 μM, $n=4$, in the presence of D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, respectively. The dissociation constants (pK_B) were calculated from the dose-ratios and were 7.1 and 7.3 for D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, respectively. In contrast, the BK B_1 receptor antagonist, [D-Arg^9, Leu^8]-BK, did not change the BK-induced response (data not shown). These results indicated that the receptors mediating BK-induced IPs accumulation had similar pharmacological properties to BK B_2 receptors (Regoli *et al.*, 1990; Marsh & Hill, 1992).

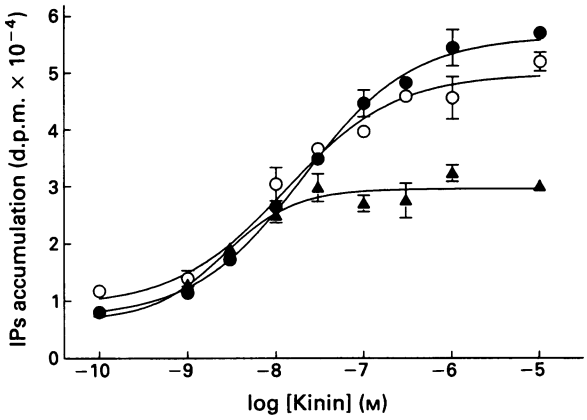

Figure 1 Dose-response curve for bradykinin (BK), kallidin-, and des-Arg^9-BK-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Agonist (1 nM–10 μM) was added and the reaction was stopped after 60 min incubation. [^{3}H]-IPs were determined as described under Methods. Each point represents the mean ± s.e.mean of five separate experiments determined in triplicate. (●) BK; (○) kallidin; (▲) des-Arg^9-BK.

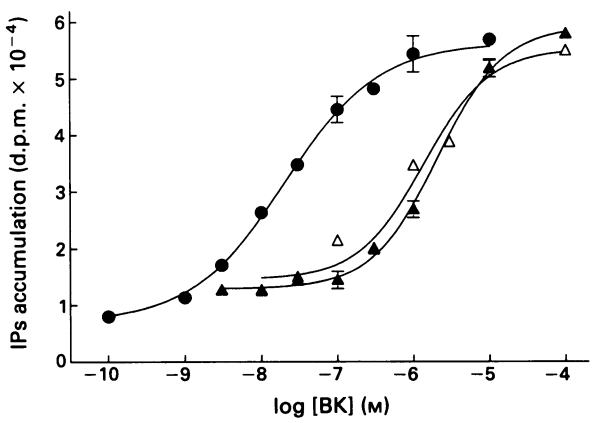

Figure 2 Inhibition of bradykinin (BK)-induced inositol phosphates (IPs) accumulation in TSMCs by D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK. [^{3}H]-IPs were determined as described under Methods. Each point represents the mean ± s.e.mean of four experiments determined in triplicate. BK-stimulated IPs accumulation was measured in the absence (●) and presence of D-Arg[Hyp^3,D-Phe^7]-BK (△, 5 μM) and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK (▲, 5 μM).

Effect of phorbol esters on BK-induced IPs accumulation

In order to determine whether PKC activation by phorbol esters causes an inhibition on BK-induced PI response, the concentration-response relationship for PMA inhibition of BK-stimulated IPs accumulation was examined in cultured TSMCs. As shown in Figure 3, half-maximal and maximal inhibition of PMA on BK-stimulated IPs accumulation occurred at 15 ± 4 nM and 1 μM, $n = 3$, respectively. PMA had no effect on the basal levels of IPs accumulation at any of the concentrations tested. Furthermore, [^{3}H]-inositol-labelled TSMCs were treated with PMA and 4α-PDD (1 μM) and then stimulated with 1 μM BK in the continuous presence of phorbol esters (Figure 4). Treatment of TSMCs with 1 μM PMA for 30 min led to an inhibition of BK-stimulated IPs accumulation by 48% ($P<0.001$, $n = 4$, as compared with the control). However, when TSMCs were treated with 1 μM PMA for 24 h, the extent of BK-stimulated IPs accumulation was greater than that of TSMCs of the control group ($P<0.01$, $n = 4$). The inactive phorbol ester, 4α-phorbol 12, 13-didecanoate (4α-PDD, 1 μM), did not block BK-induced IPs accumulation (Figure 4). When TSMCs were pretreated with staurosporine (1 μM), a potent PKC inhibitor, the inhibitory effect of PMA on BK-stimulated IPs accumulation was reversed (Figure 4).

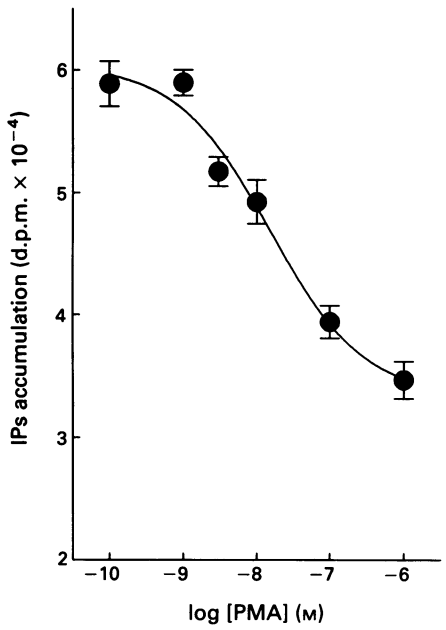

Figure 3 Concentration dependence of phorbol 12-myristate 13-acetate (PMA) inhibition of bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells prelabelled with [^{3}H]-inositol were washed with KHS, treated with various concentrations of PMA for 30 min and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of three separate experiments determined in triplicate. The basal level and BK-induced IPs accumulation were 11690 ± 1170 and 61290 ± 2790 d.p.m./well, respectively.

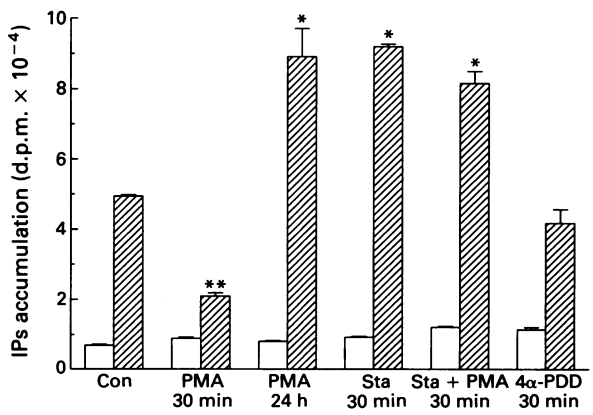

Figure 4 Effects of phorbol ester and staurosporine treatment on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. [^{3}H]-inositol-labelled TSMCs were pretreated with phorbol 12-myristate 13-acetate (PMA, 1 μM), staurosporine (Sta, 1 μM), staurosporine plus PMA, or 4α-phorbol 12,13-didecanoate (4α-PDD, 1 μM) for the indicated time and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of four separate experiments determined in triplicate. $*P<0.01$; $**P<0.001$, as compared with non-treated cells (Con) stimulated by BK. Open column, basal level; hatched column, stimulated by BK.

Effect of PTX and CTX on BK-induced IPs accumulation

To gain insight into the identity of the coupling G protein in the BK signal transduction mechanism, cultured TSMCs

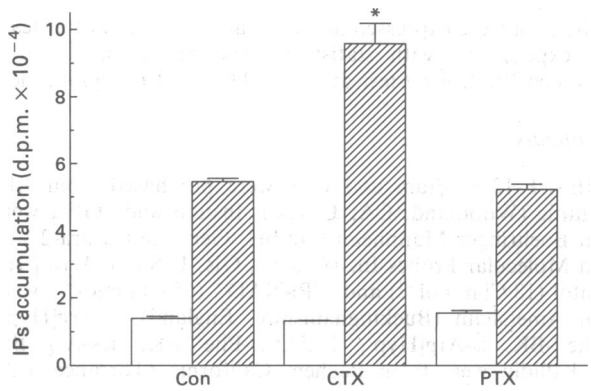

Figure 5 Effects of cholera toxin and pertussis toxin on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells were treated with either cholera toxin (CTX, 10 μg ml^{-1}) or pertussis toxin (PTX, 100 ng ml^{-1}) for 4 h and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results presented are the mean ± s.e.mean of five separate experiments determined in triplicate. $*P<0.001$, as compared with control (Con) cells. Open column, basal level; hatched column, stimulated by BK.

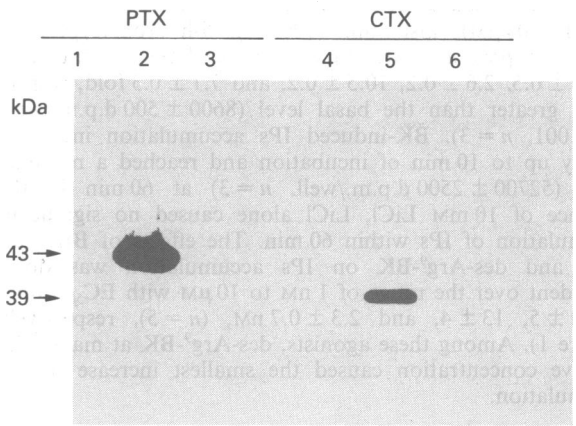

Figure 6 ADP-ribosylation of the G proteins. Cultured TSMCs were pretreated with either pertussis toxin (PTX, 100 ng ml^{-1}, lane 3), or cholera toxin (CTX, 10 μg ml^{-1}, lane 6) for 4 h. Control and toxin-treated samples were then assayed for ADP-ribosylation of the remaining available substrates in the presence of [^{32}P]-NAD$^+$ and PTX (Lanes 2–3) or CTX (Lanes 5–6) as described in Methods. Lane 1, control for PTX assay; Lane 4, control of CTX assay.

were preincubated with either CTX or PTX, and IPs accumulation in response to BK was examined. As shown in Figure 5, pretreatment of TSMCs with PTX (100 ng ml^{-1}, 4 h) had no effect on the basal or BK-stimulated IPs accumulation. In contrast, incubation of TSMCs with CTX (10 μg ml^{-1}, 4 h) enhanced BK-induced IPs accumulation by 75% ($P<0.001$, $n=5$, as compared with control).

ADP-ribosylation of G protein

To characterize the alteration of the α-subunit of G proteins by PTX and CTX, cultured TSMCs were incubated in the presence or absence of these toxins, and then assayed for ADP-ribosylation of any remaining unmodified G protein in the presence of [^{32}P]-NAD$^+$ (Figure 6). Control cultures of TSMCs showed a major substrate for PTX at 43 kDa, which was completely ribosylated under the experimental conditions used here. The major substrate for CTX was evident with a molecular weight of 41 kDa. Pretreatment of TSMCs with PTX or CTX for 4 h resulted in complete abolition of label incorporation into this substrate. These results confirmed that pretreatment of TSMCs with these two toxins is adequate to determine the roles of various G proteins involved in the BK signal transduction.

Effect of PMA on direct stimulation of G proteins

To determine the site of PMA treatment-related decrease in IPs accumulation induced by BK, AlF_4^- was used to stimulate directly G proteins to generate IPs. Figure 7 reveals that 1 μM BK or 10 μM AlF_4^- elicited essentially identical IPs accumulation in intact TSMCs. Pretreatment of TSMCs with 1 μM PMA for 30 min, inhibited the IPs accumulation induced by BK or AlF_4^- ($P<0.001$, $n=3$, as compared with non-treated cells). Parallel experiments were performed with digitonin-permeabilized TSMCs and GTPγS to activate G protein directly. IPs accumulation induced by GTPγS was also inhibited by PMA treatment (data not shown). These results suggest that the inhibitory effect of PMA on IPs accumulation may be due to activation of PKC and subsequently uncouple G proteins to PLC.

Dependence of BK-stimulated IPs accumulation on extracellular Ca^{2+}

To determine whether Ca^{2+} influx is required for the activation of PLC, BK-induced IPs accumulation was performed in Ca^{2+}-free KHS buffer. Results in Figure 8 illustrate the dependence of BK-induced IPs accumulation on Ca^{2+} influx. TSMCs preincubated in Ca^{2+}-free KHS or in Ca^{2+}-free KHS plus 0.5 mM EGTA for 5 min, led to an attenuation of the BK-induced response. Furthermore, BK-induced IPs accumulation was slightly affected by pretreatment with the Ca^{2+}-channel blockers diltiazem and verapamil at a concentration of 10 μM (Figure 9). However, Ni^{2+} (5 mM) significantly inhibited BK-induced IPs accumulation (Figure 9; $P<0.001$, $n=3$, as compared with control).

To assess more directly the role played by physiologically relevant Ca^{2+} concentrations in the regulation of the activity of PLC, [^{3}H]-inositol-labelled TSMCs were first permeabilized in digitonin-containing KGEH buffer and then exposed to Ca^{2+}-EGTA buffer, in which the $[Ca^{2+}]_f$ had been measured directly by the addition of fura-2 (free acid). IPs accumulation was measured after 60 min incubation. Elevation of $[Ca^{2+}]_f$ from 3 to 620 nM resulted in a 35% increase in IPs accumulation, with an EC_{50} of 163 ± 18 nM, $n=3$ (Figure 10). The addition of 50 μM GTPγS further potentiated this Ca^{2+}-dependent IPs accumulation with an EC_{50} for Ca^{2+} of about 25 ± 7 nM ($n=3$). The effect of BK was potentiated by the inclusion of 50 μM GTPγS, such that, under these conditions, the $[Ca^{2+}]_f$ required to elicit a half-maximal increase in IPs accumulation was reduced from 125 ± 15 nM to 3.9 ± 1.2 nM ($n=3$). When BK and GTPγS were added simultaneously, the stimulated accumulation of IPs was 313 ± 26% of control (n = 3), a value that compares favourably with the degree of stimulation obtained in intact cells. Therefore, GTPγS and BK together elicit an additive increase in IPs accumulation.

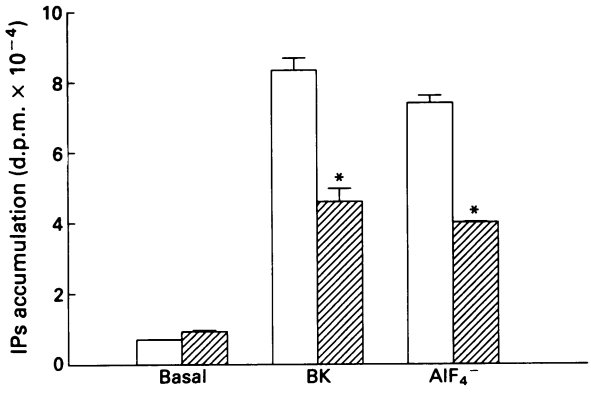

Figure 7 Effects of phorbol 12-myristate 13-acetate (PMA) on direct stimulation of G proteins coupling to phosphoinositide (PI) hydrolysis in intact TSMCs. Cells prelabelled with [^{3}H]-inositol were washed with KHS and incubated in the presence or absence of 1 μM PMA for 30 min. The cells were then exposed to 1 μM bradykinin (BK) or 10 μM AlF_4^- (10 μM $AlCl_3$ + 10 mM NaF) for 60 min. The accumulation of inositol phosphates (IPs) was determined as described under Methods. Values are expressed as the mean ± s.e.mean from three separate experiments determined in triplicate. *$P<0.001$, as compared with untreated cells stimulated by BK. Open column, control; hatched column, treated with PMA.

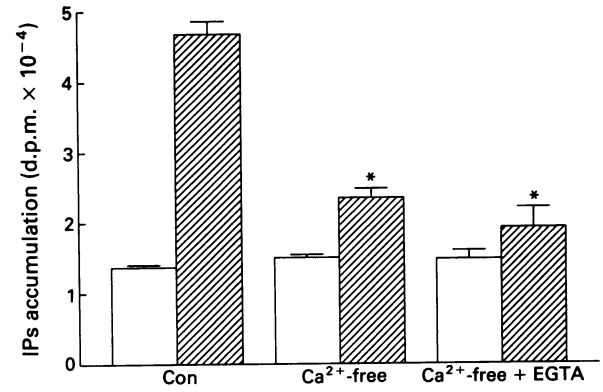

Figure 8 Calcium dependence of bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. Cells were prelabelled with [^{3}H]-inositol for 2 days. Labelled cells were incubated in either KHS, Ca^{2+}-free KHS or Ca^{2+}-free KHS plus 0.5 mM EGTA. Then BK (1 μM) was added and continuously incubated for 60 min. [^{3}H]-IPs were determined as described under Methods. Results are the mean ± s.e.mean of three separate experiments determined in triplicate. *$P<0.001$, as compared with that of cells induced by BK alone in KHS buffer (Con). Open column, basal level; hatched column, stimulated by BK.

Discussion

It is well established that receptor activation by neurotransmitters, growth factors, hormones, and light causes rapid PI hydrolysis (Rana & Hokin, 1990). In the present study, we have demonstrated that BK induces PI turnover in canine TSMCs by interacting with its receptors coupled to PLC via a G protein. BK-stimulated IPs accumulation is time- and dose-dependent, and regulated by $[Ca^{2+}]_f$ and GTPγS, a non-hydrolyzable analogue of GTP. BK-stimulated inositol

phospholipid-specific PLC may be regulated by PKC stimulation via the formation of DAG. Short-term treatment of TSMCs with PMA dramatically inhibits BK-stimulated IPs accumulation. One possible consequence for the activation of this arm of the second messenger pathway is the presence of a short inhibitory feedback loop in which DAG formation and PKC activation cause an attenuation of agonist-stimulated PLC activity. This mechanism has been proposed for the regulation of agonist-stimulated PLC in a number of cell types (Castagna *et al.*, 1982; Leeb-Lungberg *et al.*, 1985; Orellana *et al.*, 1985).

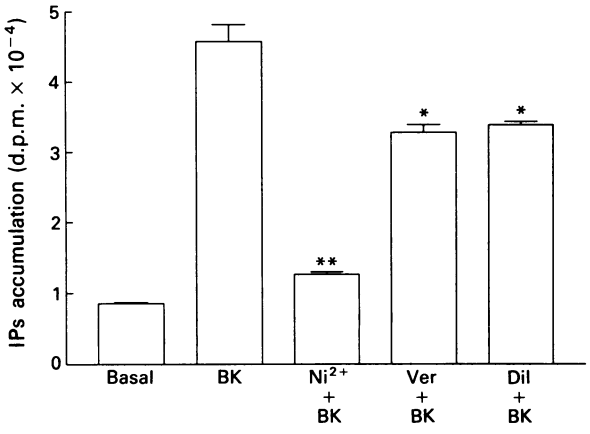

Figure 9 Effects of Ca^{2+}-channel blockers on bradykinin (BK)-stimulated [^{3}H]-inositol phosphates ([^{3}H]-IPs) accumulation in cultured TSMCs. [^{3}H]-inositol-labelled cells were preincubated with diltiazem (Dil, 10 μM), verapamil (Ver, 10 μM), or Ni^{2+} (5 mM) for 30 min and then exposed to BK (1 μM) for 60 min. [^{3}H]-IPs were determined as described under Methods. Results are the mean ± s.e.mean of three separate experiments determined in triplicate. $*P<0.01$; $**P<0.001$, as compared with control.

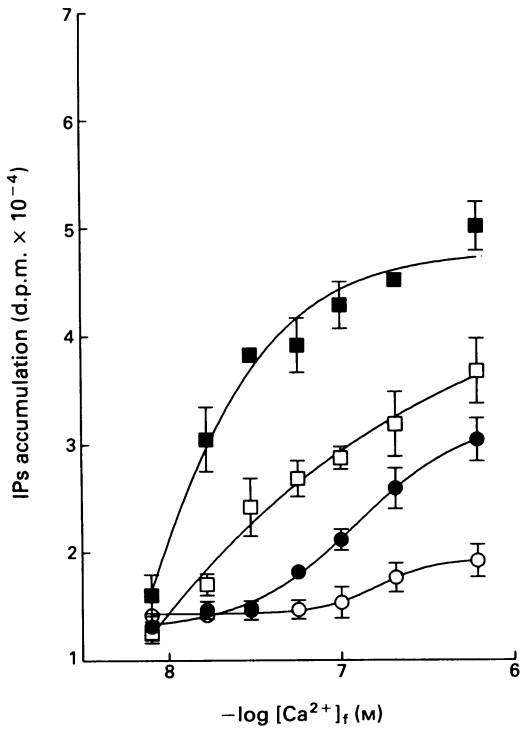

Figure 10 Increase in $[Ca^{2+}]_f$ causes the release of inositol phosphates (IPs) from digitonin-permeabilized TSMCs. [^{3}H]-inositol-prelabelled cells were permeabilized and incubated in KGEH buffer with added Ca^{2+} to achieve the required $[Ca^{2+}]_f$ (as measured by the addition of fura-2). Cells were incubated for 60 min in the absence (○) or presence of either bradykinin (BK, ●, 1 μM), guanosine 5'-O-(3-thiotriphosphate) (GTPγS, □, 50 μM), or BK plus GTPγS (■). The EC_{50} values for basal, BK, GTPγS, and BK plus GTPγS were 163, 125, 25, and 3.9 nM, respectively. Results are expressed as means ± s.e.mean from three separate experiments determined in triplicates. $[Ca^{2+}]_f$ values of 7.93, 16.61, 39.89, 56.35, 100.4, 207.3 and 616.8 nM were obtained at Ca^{2+}/EGTA molar ratios of 0.25, 0.38, 0.50, 0.63, 0.75, 0.88 and 1, respectively, with EGTA maintained at a concentration of 2 mM.

The discrepancy in the dose-response relationship for the effects of BK, kallidin, and des-Arg^9-BK on the IPs accumulation in canine TSMCs is consistent with those reported by others using different tissues and cell preparation (Regoli *et al.*, 1990). Our data obtained from the IPs accumulation induced by these agonists show that BK and kallidin produce a larger response than des-Arg^9-BK (Figure 1). The EC_{50} value of des-Arg^9-BK is very low, *circa* 2 nM. This may imply that there is a significant population of B_1 receptors in cultured TSMCs. It has been suggested that BK induces its effects through at least two types of receptors, which have been characterized as B_1 and B_2 receptors (Regoli *et al.*, 1990). BK and kallidin have high affinity for B_2 receptors and low affinity for the B_1 receptors (Regoli *et al.*, 1990). In contrast, des-Arg^9-BK has low affinity for B_2 receptors but high affinity for the B_1 receptors (Regoli *et al.*, 1990). Therefore, our findings may reflect the presence of both B_1 and B_2 receptors in canine TSMCs.

As an alternative approach to define the receptor subtypes, the determination of antagonist affinities can provide more accurate information than the use of agonist relative potencies. From the experiments, the effects of discriminating antagonists have been analysed at the level of the biochemical response to BK. The results obtained with D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK, differentiated the receptor subtype mediating IPs accumulation (Figure 2). The pK_B values for the B_2 antagonists D-Arg[Hyp^3, D-Phe^7]-BK and D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-BK were 7.1 and 7.3, respectively, consistent with the involvement of B_2 receptors in the IP response to BK. These results are in good agreement with those reported by others in the rabbit jugular vein (Regoli *et al.*, 1990) and in bovine TSMCs (Marsh & Hill, 1992).

Because PKC activation is associated with several cellular responses, phorbol ester-mediated inhibition of IP_3 formation might occur at one or more different sites. In a number of cell types, elevation of $[Ca^{2+}]_i$ by Ca^{2+}-mobilizing agonists known to act by receptor-mediated stimulation of PI turnover, has been shown to be inhibited by phorbol esters (Castagna *et al.*, 1982; Leeb-Lundberg *et al.*, 1985; Orellana *et al.*, 1985). It has been suggested that protein phosphorylation mediated by interaction of phorbol ester with PKC may be the mechanism by which PMA modulates hormone-sensitive PI metabolism. One proposed mechanism by which phorbol ester might attenuate a rise in IP_3 is due to its increasing degradation mediated by activation of a phosphomonoesterase specific for IP_3 (Watson & Lapetina, 1985; Connolly *et al.*, 1986). The activity of this cytosolic enzyme increases after phosphorylation by PKC which provides a mechanism for the inhibition of the agonist-induced rise in IP_3 concentration in platelets. We observed that PMA blocks both BK-stimulated IPs accumulation and also AlF_4^--mediated IPs formation in canine TSMCs (Figures 4 and 7). After prolonged treatment of TSMCs with PMA, BK induced a fast rise in IPs accumulation, which reaches a plateau greater than that of the control level. We therefore propose that DAG, which is formed during stimulation with BK, prevents any additional increase in IPs accumulation via PKC activation. Our results indicate that PMA acts through the activation of PKC with subsequent protein phosphorylation, since staurosporine, a potent PKC inhibitor, blocks the inhibitory effect of PMA.

BK-stimulated IPs accumulation is almost completely dependent on the presence of extracellular Ca^{2+} (Figure 8). This calcium dependence is similar to PI response to agonists in several types of cells (Fisher *et al.*, 1989; Eberhard & Holz,

1991; Yang & Chou, 1992). In the present study, TSMCs prelabelled with [^{3}H]-inositol for 2 days and then exposed to BK, caused a rapid release of IPs in the presence of external Ca^{2+} (1.8 mM). However, Ca^{2+}-free buffer or inclusion of 0.5 mM EGTA almost completely attenuates the BK-induced increase in IPs accumulation. Furthermore, the BK-induced effect is slightly affected by the presence of the Ca^{2+}-channel blockers, diltiazem and verapamil (Figure 9). However, the non-selective Ca^{2+}-channel blocker, Ni^{2+}, did inhibit the BK-stimulated response (Figure 9), indicating that Ca^{2+} influx may be required for the activation of PLC through an unidentified pathway.

Further indication for the requirement of Ca^{2+} for PI hydrolysis has been obtained from experiments with digitonin-permeabilized TSMCs (Figure 10). In the presence of BK and GTPγS, the extent of PI breakdown in permeabilized TSMCs appears to be the same as that obtained in intact cells stimulated by BK alone. Since GTPγS alone could also stimulate IPs accumulation in permeabilized cells, a guanine nucleotide binding protein might be involved in the transduction process (Evans *et al.*, 1985; Merritt *et al.*, 1986; Jones *et al.*, 1988). Although BK does enhance IPs accumulation without the addition of GTPγS, this ability presumably reflects the presence of residual endogenous guanine nucleotides within the permeabilized cells. However, the combination of BK and GTPγS has an additive effect on IPs accumulation. GTPγS may play a role in the breakdown of PI that reduces the requirement of $[Ca^{2+}]_f$ for PLC activity (Bradford & Rubin, 1986; Smith *et al.*, 1986). The availability of $[Ca^{2+}]_f$ is required for stimulation of PI hydrolysis. A half-maximal (EC_{50}) increase of IPs accumulation induced by BK alone occurs at a $[Ca^{2+}]_f$ greater than 125 nM, while in the presence of GTPγS, only 3.9 nM $[Ca^{2+}]_f$ is required for BK to induce a half-maximal increase of IPs accumulation. Therefore, guanine nucleotides appear to sensitize the transduction process such that BK effectively initiates PI hydrolysis at a $[Ca^{2+}]_f$ that is encountered in quiescent cells.

G proteins are involved in receptor coupling to PLC activity for many agonists (Sternweis & Smrcka, 1992). Although we have shown that the presence of an ADP-ribosylated protein of molecular weight at 43 and 41 kDa is demonstrated in PTX- and CTX-treated cells (Figure 6), respectively, our results demonstrate that BK stimulation of PLC activity is not sensitive to inhibition by these two toxins (Figure 5). This suggests that a coupling process occurs in these cells through a mechanism which is not mediated through a PTX- or CTX-sensitive G protein. Similar results have been reported for several cell types by others (Etscheid & Villereal, 1989; Galron *et al.*, 1990; Gutowski *et al.*, 1991). BK-stimulated IPs accumulation in TSMCs is enhanced by short-term (4 h) treatment with CTX (Figure 5). Signal transduction processes have been reported that require GTP but are not affected by toxins (Martin *et al.*, 1985; Aub *et al.*, 1986). Furthermore, it has been shown that treatment of cells with CTX or PTX enhances PI turnover in rat heart myocytes and human foreskin fibroblasts (Etscheid & Villereal, 1989; Galron *et al.*, 1990). The precise mechanism of action of these toxins is not known.

In conclusion, our results demonstrate that BK-stimulated IPs accumulation is mediated by the activation of PLC coupling to a G protein which is not blocked by CTX or PTX treatment and activated by external Ca^{2+}. Short-term PMA treatment results in a negative feedback regulation on agonist-induced IPs accumulation. Long-term PMA treatment might be associated with augmenting responses to agonists, perhaps due to down-regulation of PKC and the loss of its inhibitory function. These results suggest that physiological activation of PKC might serve as a modulator of cellular responses induced by IP_3. The site of PMA inhibition appears to be at a postreceptor level. Determination of how DAG and other second messengers modulate agonist-induced cellular responses is important in order to clarify the mechanisms underlying bronchial hyperreactivity of the airway in asthma.

This work was supported by grants CMRP-340 and 273 from Chang Gung Medical Research Foundation and NSC81-0412-B182-007 to C.M.Y., and CMRP-403 and NSC81-0420-B182-549 to S.F.L. from The National Science Council, Taiwan. The authors are greatly indebted to Dr Anthony Herp at Chang Gung Medical College for his critical reading of the manuscript and suggestions. Appreciation is also expressed to Dr Delon Wu for his encouragement.

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(Received June 28, 1993
Revised August 31, 1993
Accepted September 3, 1993)

Br. J. Pharmacol. (1994), **111**, 29–34

Contractile properties of synthetic cationic polypeptides in guinea-pig isolated trachea

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1 The synthetic polypeptides, poly-L-arginine, poly-L-lysine and poly-D-lysine contract guinea-pig isolated trachea in a concentration-dependent, epithelium-independent manner. Indomethacin augmented the contractile response to poly-L-arginine.

2 The contractile response to poly-L-arginine was not significantly inhibited by nicardipine, a selective L-type calcium channel blocker or by the histamine H_1-receptor antagonist, mepyramine nor significantly augmented by the neutral endopeptidase inhibitor, phosphoramidon.

3 The contractile response to poly-L-arginine was inhibited in a concentration-dependent manner by prior incubation of guinea-pig tracheal rings with a number of anionic polypeptides including, low molecular weight heparin, poly-L-aspartic acid and bovine serum albumin.

4 *In vitro* capsaicin-induced desensitization failed to attenuate the contractile response to poly-L-arginine, suggesting little, if any role for sensory neuropeptides in the functional response in the guinea-pig.

5 Synthetic polypeptides induce an epithelium-independent, charge-dependent contraction of guinea-pig isolated trachea.

Keywords: Cationic polypeptides; airway smooth muscle of guinea-pig; trachea; heparin; capsaicin; poly-L-arginine, epithelium

Introduction

A number of studies have demonstrated the presence of elevated numbers of eosinophils in bronchoalveolar lavage fluid 6 h following allergen challenge in asthmatics (De Monchey *et al.*, 1985). Similarly, elevated levels of eosinophils are observed in bronchoalveolar lavage fluid (Wardlaw *et al.*, 1988) and in bronchial biopsies (Jeffery *et al.*, 1989; Beasley *et al.*, 1989) from patients with mild asthma. Furthermore, eosinophil-derived granule products have also been demonstrated in bronchial biopsies by immunofluorescence (Filley *et al.*, 1982; Venge *et al.*, 1988) and electron transmission microscopy (Jeffery *et al.*, 1992). It has been suggested that eosinophil-derived cationic proteins including, major basic protein (MBP), a highly charged protein, might be implicated in the pathogenesis of asthma (Hamann *et al.*, 1991). Indeed, a number of studies have demonstrated that MBP can increase ion flux and prostaglandin synthesis in airway epithelium (Jacoby *et al.*, 1988), reduce ciliary motility (Hastie *et al.*, 1987), induce damage to airway epithelium (Motojima *et al.*, 1989), induce histamine release from human basophils and rat mast cells (O'Donnell *et al.*, 1983; Zheutlin *et al.*, 1984), mediate airway smooth muscle contraction (White *et al.*, 1990; Gundel *et al.*, 1991; Barker *et al.*, 1991), promote oedema (Needham *et al.*, 1988) and increase airways responsiveness to spasmogens (Brofman *et al.*, 1989; White *et al.*, 1990; Gundel *et al.*, 1991; Barker *et al.*, 1991; Uchida *et al.*, 1993).

Naturally occurring cationic polypeptides, including MBP, contain basic amino acids, namely arginine and lysine. Thus, synthetic polypeptides rich in basic amino acids might be used to model natural polypeptides. In this regard, a number of the biological activities attributed to MBP may be mimicked by synthetic polypeptides. Thus, synthetic polypeptides mediate the degranulation of human basophils (Foreman & Lichtenstein, 1980; Coleman *et al.*, 1981), rat and hamster mast cells (Leung & Pearce, 1984) but not human lung mast cells (Church *et al.*, 1982) and stimulate the release of arachidonic acid and prostaglandins from fibroblast (Shier *et al.*, 1984; Shier & Dubouidieu, 1986) and cultured aortic endothelial cells (Needham *et al.*, 1988). Furthermore, cationic polypeptides can increase the permeability of airway epithelium (Herbert *et al.*, 1991; Yu *et al.*, 1992; Coyle *et al.*, 1992), promote pulmonary (Vehaskari *et al.*, 1984) and dermal oedema (Needham *et al.*, 1988; Antunes *et al.*, 1990) and induce airways hyperresponsiveness to spasmogens both *in vitro* (Coyle *et al.*, 1992) and *in vivo* (Coyle *et al.*, 1993; Uchida *et al.*, 1993). In the present study we have investigated the role of synthetic polypeptides for their ability to contract airway smooth muscle *in vitro*.

Methods

Tissue preparation

Albino guinea-pigs (300–500 g) were killed by cervical dislocation and the trachea removed and placed in cold (4°C) Krebs-Henseleit solution, aerated with 95% O_2 and 5% CO_2. Since it has previously been shown that the epithelium can influence guinea-pig tracheal smooth muscle sensitivity to spasmogens (Goldie *et al.*, 1986), the epithelium was removed from all preparations with a wooden probe. To confirm the removal of the epithelium, paraffin embedded tracheal sections (8 μm) were stained with haematoxylin-eosin and examined at the light microscopic level.

Tracheal rings (2 mm) were suspended in dimethyldichlorosilane (40%)-treated 1 ml organ baths under an optimal tension of 1 g in Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 at 37°C. Tissues were allowed to equilibrate for 30 min and changes in Krebs-Henseleit solution were made every 10 min. Methacholine (10 μM) was added to the bath and after the contractile response had reached plateau, the tissues were washed 5 times over a 15 min period and allowed to equilibrate for a further 30 min. This procedure was repeated a second time.

Experimental protocol

Cumulative concentration-effect curves of poly-L-arginine (mol. wt. 11600; 30–1000 μg ml^{-1}) were investigated in epi-

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thelium-intact or epithelium-denuded guinea-pig tracheal preparations in the absence or presence of indomethacin (5 μM). In other experiments, functional responses to the synthetic polypeptide, poly-L-lysine and poly-D-lysine (mol. wt. 25000; 30–1000 μg ml^{-1}) were also assessed in epithelium-denuded, indomethacin-pretreated guinea-pig tracheal preparations.

The nature of the poly-L-arginine-induced contraction was investigated in other experiments. Thus, poly-L-arginine cumulative concentration-effect curves were performed in epithelium-denuded, indomethacin-treated guinea-pig tracheal rings in the absence or presence of the histamine H_1-receptor antagonist, mepyramine (10 μM; 30 min preincubation) and the L-type calcium channel blocker, nicardipine (1 μM; 30 min). Histamine concentration-effect curves were performed in the absence or presence of mepyramine (10 μM). The effect of nicardipine (1 μM) on the response to potassium ions and methacholine was also investigated. In further experiments, cumulative concentration-effect curves to poly-L-arginine were performed in the absence or presence of the neutral endopeptidase inhibitor phosphoramidon (10 μM; 30 min) in indomethacin-treated epithelium-intact and epithelium-denuded guinea-pig tracheal preparations.

In other experiments, the response to poly-L-arginine was evaluated in epithelium-denuded, indomethacin-treated guinea-pig tracheal rings in vehicle or capsaicin-desensitized preparations. Tracheal rings were incubated with capsaicin (10 μM) or vehicle control (1% ethanol) for 15 min, then washed every 5 min over a 15 min period and allowed to equilibrate for a further 15 min. A consecutive administration of capsaicin (10 μM) or vehicle was performed to demonstrate capsaicin-induced desensitization. Tissues were then washed 5 times over a 15 min period then allowed to equilibrate for a further 15 min. The contractile response to poly-L-arginine was then reassessed in capsaicin- or vehicle-treated tracheal preparations.

In further experiments, the contractile response to poly-L-arginine (1000 μg ml^{-1}) was investigated following 30 min incubation with a number of anionic polypeptides including, low molecular weight heparin (10–1000 units ml^{-1}), poly-l-aspartic acid (10–1000 μg ml^{-1}) and bovine serum albumin (100–1000 μg ml^{-1}) in epithelium-denuded, indomethacin-treated, guinea-pig tracheal preparations.

Analysis of results

The contractile agonist potency (EC_{25}) or (EC_{50}), is expressed as the geometric mean together with 95% confidence limits. The contractile response to agonists is expressed in terms of the maximal contractile response to methacholine (100 μM). Data were analysed by analysis of variance and Dunnett's *t* test used to test differences in control and treatment means (Wallenstein *et al.*, 1980). In other cases, non-paired Student's *t* test was used. Data were considered significant if $P<0.05$.

Drugs

The following were used: indomethacin, methacholine hydrochloride, mepyramine, nicardipine hydrochloride, poly-L-arginine (mol. wt. 11600), poly-L and poly-D-lysine (mol. wt. 25000), poly-L-aspartic acid (mol. wt. 14000), bovine serum albumin, capsaicin (Sigma); heparin sodium (Delta West). Except where otherwise stated all drugs were dissolved in Krebs Henseleit solution. The composition of the Krebs-Henseleit solution was (mM): NaCl 117.6, KCl 5.4, $MgSO_4$. $7H_2O$ 0.57, $KH_2.PO_4$ 1.03, $NaHCO_3$ 25.0, glucose 11.1 and $CaCl_2.2H_2O$ 2.5. Stock concentrations (0.01 M) of indomethacin, capsaicin and nicardipine were made up in 0.5% Na_2CO_3, 100% ethanol and distilled water respectively. The appropriate dilution was then made in Krebs-Henseleit solution.

Results

Cationic polypeptides

Poly-L-arginine (mol. wt. 11600; 30–100 μg ml^{-1}) caused a concentration-dependent contraction in epithelium-intact and epithelium-denuded preparations in the absence or presence of indomethacin (Figure 1). Analysis of the data represented in Figure 1 revealed a significant interaction between the factors indomethacin and the different concentrations of poly-L-arginine tested ($P<0.001$). Further analysis revealed that the potentiating effect of indomethacin was significant at 300 μg ml^{-1} ($P<0.001$) and 1000 μg ml^{-1} ($P<0.001$) poly-L-arginine. In contrast, removal of the epithelium failed to alter significantly the response to poly-L-arginine ($P>0.05$).

Both poly-L-lysine (EC_{25}; 95% confidence limits: 522 μg ml^{-1} (208–1312), $n=5$) and poly-D-lysine (619 μg ml^{-1} (185–2075), $n=5$) contracted guinea-pig tracheal preparations with equal potency ($P>0.05$; Figure 2).

Nicardipine (1 μM) which blocks L-type calcium channels failed to alter significantly airway smooth muscle sensitivity (Figure 3a; EC_{25}, 95% confidence limits: control 181 μg ml^{-1} (122–267); nicardipine-treated: 340 μg ml^{-1} (159–728), $n=5$, $P>0.05$) or E_{max} (control: 53.6 ± 2.8% methacholine E_{max}; nicardipine-treated; 49.4 ± 5.4%, $n=5$, $P>0.05$) to poly-L-arginine. In contrast, nicardipine (1 μM) significantly attenuated the contractile response to exogenously administered potassium ions (Figure 3b). Furthermore, the contractile potency (EC_{50}) to methacholine was also partially attenuated in nicardipine-treated preparations (control: 95 nM (52–174), $n=3$; nicardipine-treated: 155 nM (104–230), $n=3$, $P<0.05$).

The histamine H_1-receptor antagonist, mepyramine, failed to reduce significantly the contractile potency (EC_{25}, 95% confidence limits; control 372 μg ml^{-1} (170–813), $n=8$, vs mepyramine-treated; 389 μg ml^{-1} (204–741), $n=8$, $P>0.05$)

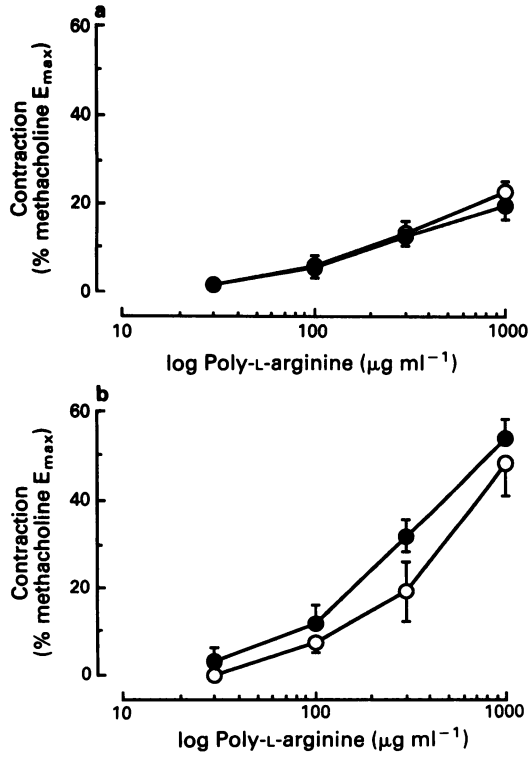

Figure 1 Concentration-dependent contraction of guinea-pig tracheal preparations to poly-L-arginine (mol. wt. 11600) in the presence (○) or absence (●) of epithelium in the absence (a) or presence (b) of indomethacin (5 μM). Each point represents the mean ± s.e.mean of 5 values.

or to alter the contractile response to poly-L-arginine (1 mg ml^{-1}) (% methacholine E_{max}: control; 40 ± 6%, $n = 8$ vs mepyramine-treated; 37 ± 6%, $n = 8$, $P > 0.05$). In contrast, the contractile potency to histamine (EC_{50}, 95% confidence limits; control: 1.04 μM (0.87–1.23), $n = 3$) was significantly reduced in mepyramine-treated preparations (3.6 mM (0.77–16.9) $n = 3$, $P < 0.001$ cf. control).

Role of sensory neuropeptides

The role of sensory neuropeptides on the contractile response to poly-L-arginine was assessed indirectly in preparations acutely desensitized to capsaicin. The effect of prior exposure of tissue to capsaicin (10 μM) on the subsequent response to poly-L-arginine is shown in Figure 4. Capsaicin (10 μM) contracted guinea-pig tracheal tissue to 55 ± 4% methacholine E_{max} ($n = 6$). The tissue failed to respond to capsaicin when administered a second time. In contrast, capsaicin-treatment failed to reduce significantly guinea-pig tracheal smooth muscle sensitivity (EC_{25}, 95% confidence limits: vehicle; control: 257 μg ml^{-1} (145–456); capsaicin-treated; 459 μg ml^{-1} (286–736), $n = 6$, $P > 0.05$) or E_{max} (vehicle control: 54.8 ± 4.5%; capsaicin-treated; 43.9 ± 6.1%, $n = 6$, $P > 0.05$) to poly-L-arginine.

The neutral endopeptidase inhibitor, phosphoramidon (10 μM) failed to potentiate significantly contractile responses to

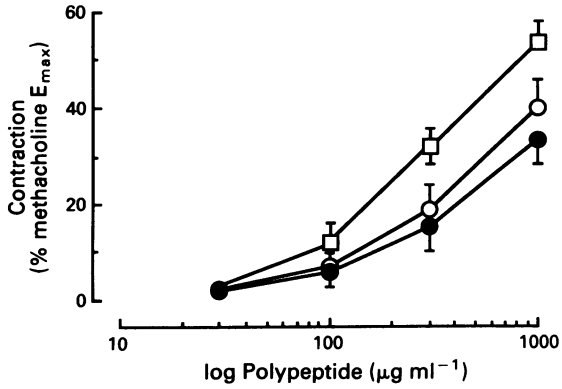

Figure 2 Concentration-dependent contraction of epithelium-denuded guinea-pig tracheal preparations to poly-L-lysine (mol. wt. 25000; ○) and poly-D-lysine (mol. wt. 25000; ●) in the presence of indomethacin (5 μM). Each point represents the mean ± s.e.mean of 7 values. Concentration-response curve to poly-L-arginine (mol. wt. 11600) is shown for comparison (□).

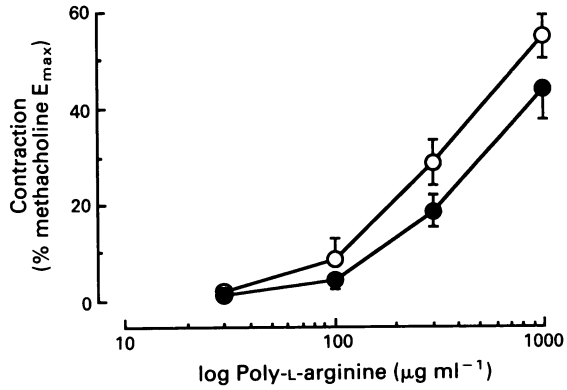

Figure 4 Concentration-dependent contraction of epithelium-denuded guinea-pig tracheal preparations to poly-L-arginine (mol. wt. 11600) following vehicle-treatment (○) or capsaicin-induced desensitization (●). Each point represents the mean ± s.e.mean of 5 values. Indomethacin (5 μM) was present.

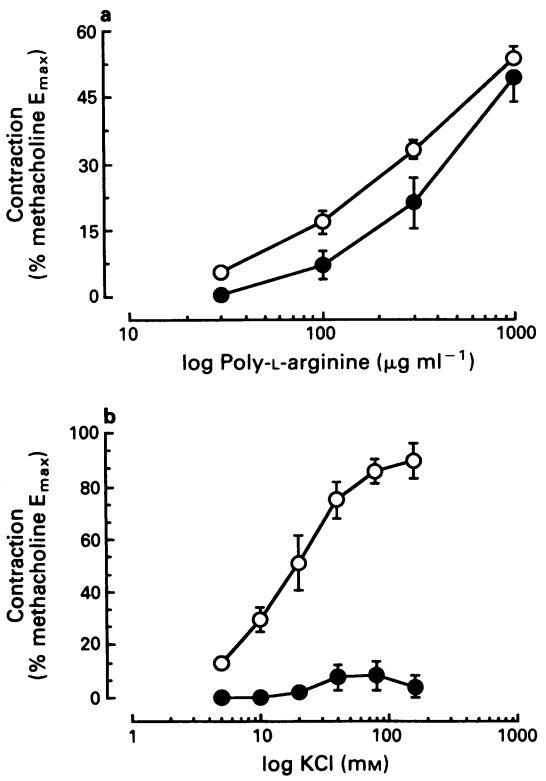

Figure 3 Concentration-dependent contraction of epithelium-denuded guinea-pig tracheal preparations to (a) poly-L-arginine (mol. wt. 11600) and (b) potassium ions in the absence (○) or presence (●) of nicardipine (1 μM). Each point represents the mean ± s.e.mean of 5 values. Indomethacin (5 μM) was present.

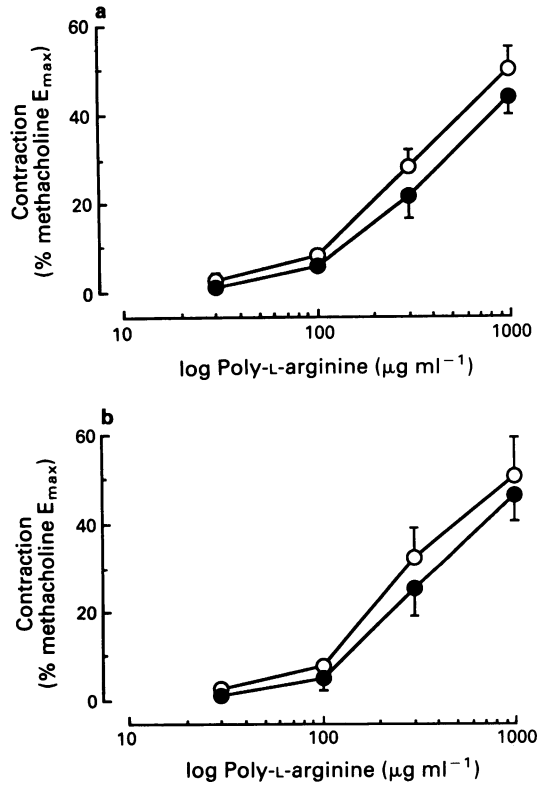

Figure 5 Concentration-dependent contraction of (a) epithelium-intact and (b) epithelium-denuded, guinea-pig tracheal preparations to poly-L-arginine (mol. wt. 11600) in the absence (○) or presence (●) of the neutral endopeptidase inhibitor, phosphoramidon (10 μM). Each point represents the mean ± s.e.mean of 5 values. Indomethacin (5 μM) was present.

poly-L-arginine in epithelium-intact (EC_{25}, 95% confidence limits; control: 324 $\mu g\ ml^{-1}$ (218–481), $n = 5$ vs phophoramidon-treated: 283 $\mu g\ ml^{-1}$ (193–415), $n = 5$, $P > 0.05$) or denuded preparations (EC_{25}, 95% confidence limits; control: 330 $\mu g\ ml^{-1}$ (184–590), $n = 5$ vs phosphoramidon-treated: 262 $\mu g\ ml^{-1}$ (204–336), $n = 5$, $P > 0.05$) treated with indomethacin ($P > 0.05$, Figure 5).

Effect of anionic polypeptides

The anionic polypeptides heparin, poly-L-aspartic acid and bovine serum albumin induced small contractile responses of $17 \pm 5\%$, $17 \pm 4\%$ and $12 \pm 6\%$ methacholine E_{max}, respectively ($n = 5$), at the maximum concentrations tested. In most cases the contractile response had returned to baseline after 30 min. The anionic polypeptides heparin (Figure 6a), poly-L-aspartic acid (Figure 6b) and bovine serum albumin (Figure 6c) inhibited poly-L-arginine (1 $mg\ ml^{-1}$)-induced contraction of guinea-pig tracheal preparations in a concentration-dependent manner. This inhibitory effect was a consequence of the physical binding of the anionic polypeptides to poly-L-arginine as demonstrated by an increase in the turbidity of the Krebs-Henseleit solution in the bath. The inhibitory potency was calculated for these anionic polypeptides yielding IC_{50} values for heparin, poly-L-aspartic acid and bovine serum albumin of 96.4 u ml^{-1}(74.0–126.0), 0.3 $mg\ ml^{-1}$ (0.1–0.9) and 5.8 $mg\ ml^{-1}$ (3.3–10.3), respectively ($n = 5$).

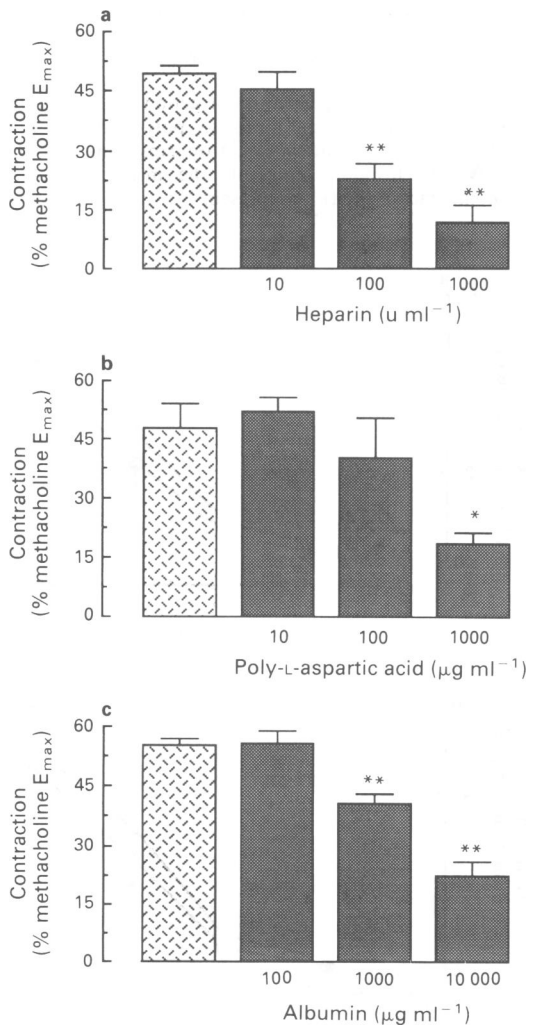

Figure 6 Histogram representing poly-L-arginine (1000 $\mu g\ ml^{-1}$)-induced contraction of epithelium-denuded guinea-pig tracheal preparations in the absence (stippled column) or presence (shaded column) of (a) heparin, (b) poly-L-aspartic acid and (c) bovine serum albumin. Vertical lines represent s.e.mean of 5 observations. Indomethacin (5 μM) was present. $^*P < 0.05$, $^{**}P < 0.01$ cf. control (Dunnett's t test).

Discussion

The results from this study confirm that synthetic polypeptides cause contraction of guinea-pig isolated tracheal preparations. The mechanism of action is not clear but appears to be epithelium-independent, not to be mediated by the activation of airway smooth muscle histamine H_1-receptors, or secondary to the release of sensory neuropeptides and does not appear to involve the opening of dihydropyridine-sensitive calcium channels. However, the cationic nature of the synthetic polypeptides is apparently important for mediating airway smooth muscle contractions.

The contractile response mediated by poly-L-arginine was unaffected by epithelium removal but augmented by cyclo-oxygenase inhibition, most probably as a consequence of the inhibition of the basal release of prostanoids by guinea-pig tracheal preparations (Braunstein *et al.*, 1988; Hay *et al.*, 1988). Although the contractile response to the cationic polypeptides was independent of the release of prostanoids, other activities including, increased permeability of the airway epithelium (Yu *et al.*, 1992) and dermal oedema (Needham *et al.*, 1988) have been shown to be dependent on cyclo-oxygenase products.

The finding that both the laevo- and dextro-isomers of polylysine induced concentration-dependent contraction, confirm the importance of cationic charge in mediating airway smooth muscle contraction. The activity of poly-L-lysine as a spasmogen in guinea-pig isolated trachea appeared to be less than that of poly-L-arginine. This has also been observed for histamine release (Foreman & Lichtenstein, 1980) and dermal oedema (Antunes *et al.*, 1990). The difference in the activities of the two polypeptides might be a consequence of the greater hydrophobicity displayed by poly-L-arginine than by poly-L-lysine (Ichimura & Zama, 1977; Ichimura *et al.*, 1978). Furthermore, the importance of charge to the response to synthetic polypeptides is reflected in the ability of various anionic polypeptides to inhibit the contractile response to poly-L-arginine. Both low molecular weight heparin and poly-L-aspartic acid inhibited poly-L-arginine-induced contraction. This was a consequence of the neutralisation of the cationic charge by the anionic polypeptides which was reflected by increases in the turbidity of the Krebs-Henseleit solution. Poly-L-aspartic acid was approximately 20 times more potent than bovine serum albumin as an inhibitor of poly-L-arginine-induced contraction, probably because of the greater anionic charge of poly-L-aspartic acid. Other studies have demonstrated that heparin is a potent inhibitor of cationic polypeptide-induced oedema (Chang *et al.*, 1987; Needham *et al.*, 1988; Antunes *et al.*, 1990) and airways hyperresponsiveness (Coyle *et al.*, 1992; 1993). Furthermore, heparin was significantly more effective than bovine serum albumin as an inhibitor of polypeptide-induced pulmonary oedema (Chang *et al.*, 1987).

Synthetic cationic polypeptides have been used as tools to study the biological properties of various naturally occurring cationic polypeptides such as MBP. This eosinophil-derived peptide contains 17 arginine residues (Wasmoen *et al.*, 1989) but the role of charge in the biological activity of MBP is not clear. Other naturally occurring polypeptides including, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) did not mimic the ability of MBP to induce airways hyperresponsiveness (Gundel *et al.*, 1991). Similarly, MBP is more effective than ECP and EPO in mediating toxicity toward the epithelium (Motojima *et al.*, 1989), MBP but not ECP and eosinophil-derived neurotoxin mediate the release of histamine from human basophils (Zheutlin *et al.*, 1984) and cationic polypeptides of a similar molecular weight and

charge to MBP failed to mimic the response to MBP (Hastie *et al.*, 1987; Jacoby *et al.*, 1988). Furthermore, denatured MBP, which presumably retains its charged nature, does not contract guinea-pig trachea *in situ* (White *et al.*, 1990) and is significantly less active than native MBP in releasing histamine from human basophils (O'Donnell *et al.*, 1983; Zheutlin *et al.*, 1984). These studies indicate that factors other than charge, such as molecular conformation, might be important in determining biological activity. Alternatively, it has been proposed that native MBP which is prone to aggregate at neutral pH, is likely to possess greater charge than the reduced and alkylated form of MBP (Zheutlin *et al.*, 1984). Furthermore, synthetic cationic polypeptides also increase airways responsiveness to spasmogens *in vivo* (Coyle *et al.*, 1993; Uchida *et al.*, 1993). Nevertheless, there is a qualitative difference between the contractile response elicited by MBP and poly-L-arginine, namely that MBP-induced contraction of guinea-pig trachea is epithelium-dependent, with no response observed following epithelium removal (White *et al.*, 1990). In contrast, epithelium removal does not inhibit the response to synthetic cationic polypeptides (this study). Thus, the contractile properties of synthetic polypeptides do not appear to mimic closely MBP, although they are all inactivated by anionic polypeptides. Thus, poly-L-glutamic acid inhibits MBP-induced bronchospasm (Barker *et al.*, 1991) and bronchial hyperresponsiveness (Gundel *et al.*, 1991), while heparin has been shown to inhibit MBP-induced airway epithelium damage (Motojima *et al.*, 1989). Cationic charge is important for the biological inactivation of cationic polypeptides and in this regard, synthetic polypeptides provide a good model of naturally occurring polypeptides.

The mechanism of action of cationic polypeptide-induced airway smooth muscle contraction is not clear, but a direct action on airway smooth muscle and/or on neuronal pathways cannot be excluded. Synthetic cationic polypeptides appear to increase cell membrane permeability as a consequence of binding to negatively charged residues on the surface of the membrane (Larsen, 1967), whereas ECP but not MBP form transmembrane pores in plasma bilayers (Young *et al.*, 1986). At higher concentrations, MBP increases the permeability of the bilayers as a result of physical damage to their structural integrity (Young *et al.*, 1986). It is thought that the charged nature of MBP facilitates binding to the cell surface, whereupon the hydrophobic regions of the molecule penetrate the lipid bilayer (Wasmoen *et al.*, 1989) and/or promote clustering of negatively charged components of the cell membrane (Abu-Ghazaleh *et al.*, 1992), thereby increasing cell membrane permeability. Whether synthetic cationic polypeptides and MBP increase cell membrane permeability via the same mechanism is not clear.

The contractile response to poly-L-arginine was not a consequence of the opening of voltage-gated calcium channels. Whether, poly-L-arginine might induce contraction as a result of a non-specific increase in cell permeability to calcium is unknown. Furthermore, neither sensory neuropeptides nor mast cell-derived histamine are responsible for mediating the contractile response to poly-L-arginine.

This research was supported by grants from the National Health and Medical Research Council of Australia and from the Clive and Vera Ramaciotti Foundation of Australia.

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(Received May 17, 1993
Revised August 5, 1993
Accepted September 3, 1993)

Br. J. Pharmacol. (1994), **111**, 35–41

Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats

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1 Streptozotocin-induced diabetic rats (Wistar) were implanted with sustained release insulin pellets (release rate = 4 u day^{-1}) or with placebo pellets (palmitic acid) from the onset of glycosuria.

2 Noradrenaline sensitivity, endothelium-dependent relaxation to acetylcholine and endothelium-independent relaxation to sodium nitroprusside were assessed in mesenteric resistance arteries from the insulin-treated (IT) diabetic animals and compared to placebo-implanted (PI) diabetics and age-matched controls.

3 Arteries from PI-diabetic rats (8–10 weeks) demonstrated an enhanced maximal response to noradrenaline compared to controls, which was not prevented by insulin treatment (control 2.65 ± 0.17 mN mm^{-1}, $n = 18$ arteries *versus* PI-diabetic 3.73 ± 0.40 mN mm^{-1}, $n = 5$, $P < 0.05$; control *versus* IT-diabetic 4.02 ± 0.19 mN mm^{-1}, $n = 22$, $P < 0.001$). Sensitivity to noradrenaline was similar between the three groups.

4 In the presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), IT and PI arteries were more sensitive to noradrenaline than control arteries (pEC_{50}: control 5.75 ± 0.08, $n = 17$, *versus* PI-diabetic 6.14 ± 0.09, $n = 8$, $P < 0.05$; control *versus* IT-diabetic 6.38 ± 0.08, $n = 20$, $P < 0.001$).

5 The maximum contractile response to depolarizing 125 mM K^+ was significantly enhanced in IT-diabetic arteries but not PI-diabetic when compared to control arteries (maximum response: control 3.74 ± 0.15 mN mm^{-1}, $n = 18$, *versus* PI-diabetic 3.61 ± 0.19 mN mm^{-1}, $n = 11$, NS; control *versus* IT-diabetic 4.66 ± 0.18 mN mm^{-1}, $n = 22$, $P < 0.001$).

6 Endothelium-dependent relaxation to acetylcholine was profoundly impaired in the PI-diabetic arteries, but in the IT-diabetic arteries was not significantly different from controls (pEC_{50}: control 7.64 ± 0.19, $n = 17$, *versus* PI-diabetic 6.07 ± 0.12, $n = 8$, $P < 0.001$; control *versus* IT-diabetic 7.36 ± 0.09, $n = 22$, NS).

7 Endothelium-independent relaxation to sodium nitroprusside was slightly but significantly impaired in the PI-diabetic arteries, but was not significantly different in the IT-diabetic arteries compared to controls (pEC_{50}: control 7.78 ± 0.10, $n = 13$, *versus* PI-diabetic 7.31 ± 0.13, $n = 13$, $P < 0.05$; control, *versus* IT-diabetic 7.64 ± 0.09, $n = 16$, NS).

Keywords: Mesenteric resistance arteries; vascular endothelium; vascular smooth muscle; chemically induced diabetes in rats; streptozotocin: insulin treatment

Introduction

Abnormal function of the vascular endothelium has been implicated as one of the underlying mechanisms of microvascular disease in diabetes. Recent studies in man have identified impaired endothelium-dependent relaxation to acetylcholine in the smooth muscle of the corpora cavernosa of impotent diabetic men (De Tejada *et al.*, 1989), in isolated resistance arteries from gluteal subcutaneous fat from type I (insulin-dependent) diabetic subjects (McNally *et al.*, 1992), and in the forearm of subjects with type II (non-insulin dependent) diabetes mellitus (McViegh *et al.*, 1992). Elliot *et al.* (1992), have also reported altered basal and stimulated nitric oxide (NO) synthesis to carbachol in the forearm of microalbuminuric type I diabetic patients. There is also some evidence to suggest that the sensitivity of the underlying smooth muscle to exogenously derived nitric oxide may be altered in human diabetes (McViegh *et al.*, 1992; Calver *et al.*, 1992). Endothelium-dependent and -independent defects could, therefore, account for impaired NO mediated relaxation. In a recent study by Smits *et al.* (1993), however, forearm blood flow responses to both endothelium-dependent and -independent relaxation were found to be normal.

Extensive functional studies in conduit arteries from animals with chemically-induced diabetes comprise a largely confusing literature. Several studies, however, describe enhanced reactivity to noradrenaline (MacLeod & McNeill, 1985; Harris & MacLeod, 1988; Pieper & Gross, 1988; White & Carrier, 1988; Cohen *et al.*, 1990) and impaired endothelium-dependent relaxation (Oyama *et al.*, 1986; Pieper & Gross, 1988; Durante *et al.*, 1988; Kamata *et al.*, 1989; Tanz *et al.*, 1989; Mayhan, 1989; Abiru *et al.*, 1990; Tesfamariam *et al.*, 1990; Mayhan *et al.*, 1991; Cameron & Cotter, 1992). We have previously demonstrated in streptozotocin-induced diabetes in rats, that both sensitivity to noradrenaline and endothelium-dependent relaxation to acetylcholine are abnormal in isolated mesenteric resistance arteries (Taylor *et al.*, 1992) and in the whole perfused mesenteric circulation (Taylor *et al.*, 1994 following paper). The first of these studies (Taylor *et al.*, 1992) also suggested the observed abnormalities can be explained, at least in part, by a deficit in the production of NO in the endothelial cells of the resistance vasculature.

In animal models of diabetes, insulin treatment has been shown to reverse the decreased responsiveness to endothelin-1 in aortic rings (Hodgson & King, 1992), to prevent impaired endothelium-dependent relaxation to histamine in aortic rings

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(Tanz *et al.*, 1989) and to prevent both contraction and relaxation defects (Takiguchi *et al.*, 1989) in the rat perfused mesentery. No previous study has determined the effect of insulin treatment on abnormalities of isolated resistance arteries. In the present study, therefore, we aimed to investigate the effect of sustained release insulin replacement on resistance vascular function in rats with streptozotocin-induced diabetes.

Methods

Induction of experimental diabetes

Diabetes was induced in female Wistar rats by intraperitoneal injection of streptozotocin (45 mg kg^{-1}) dissolved in citrate buffer. Onset of diabetes was confirmed by the presence of glycosuria 48 h after injection. Blood samples for the measurement of plasma glucose (glucose oxidase method: YSI model 23 AM Glucose Analyser, Yellow Springs, OH, U.S.A.) and rat and bovine plasma insulin concentrations (RIA, Sönksen, 1976) were obtained by cardiac puncture after cervical dislocation. Control rats were housed separately from the diabetic animals and all animals were given free access to food and water.

Insulin treatment

Following the onset of glycosuria, 48 h after streptozotocin administration, diabetic animals were treated with insulin with sustained release insulin pellets, containing bovine insulin (17%) and palmitic acid (83%) as an excipient (Wang, 1991). Slow surface erosion of the implants *in vivo* gradually releases the entrapped insulin, equivalent to a dose of 4 units day^{-1}. The insulin pellets (2 mm × 7 mm) were sterilised in 10% betadine solution before being implanted subcutaneously between the scapulae using a specially designed (12 gauge) trocar and stylet. This procedure was carried out under a short acting anaesthetic (Hypnorm, 0.5 ml kg^{-1} bodyweight, i.m.) requiring no suturing or clips for closure; 48 h after implantation of the insulin pellets, and in all subsequent routine tests, there was no evidence of glycosuria or ketonuria using reagent strips for urinalysis (Labstix). As controls for the implantation of the insulin, diabetic animals were implanted with placebo pellets containing only palmitic acid which were otherwise identical to the insulin pellets.

After 8–10 weeks animals were killed by cervical dislocation and a blood sample was taken by cardiac puncture for the plasma insulin assays and the determination of plasma glucose concentrations.

Assessment of vascular function

The mesentery was removed and placed in physiological salt solution (PSS). The solution consisted of (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 5.5, KH_2PO_4 1.18, $MgSO_47H_2O$ 1.17, KCl 4.7, $CaCl_2$ 2.5, ethylenediamine-tetra-acetic acid disodium salt (EDTA) 0.026. In order to obtain arteries of approximately equal diameter in control and experimental animals, the third branch mesenteric arteries were routinely dissected from control and insulin-treated (IT)-diabetic rats and the fourth order mesenteric arteries dissected from the placebo-implanted (PI)-diabetic animals (mean internal diameter ± s.e.mean: control 286 ± 9 μm, $n = 18$: IT-diabetic 316 ± 12 μm, $n = 22$: PI-diabetic 293 ± 12 μm, $n = 13$, P not significant). The arteries were dissected free of connective tissue under a light microscope and mounted as ring preparations on a small vessel myograph (Mulvany & Halpern, 1977) capable of measuring isometric tension. Arteries were bathed in PSS at 37°C and bubbled with 5% CO_2/95% O_2 and their passive tension and internal circumference were determined. The arteries were then set to an internal circumference equivalent to 90% of that which they would experience when relaxed *in situ* under a transmural pressure of 100 mmHg (the maximum active tension for the minimum resting tension is developed at approximately this circumference; Mulvany & Halpern, 1977). Arteries were then subjected to a routine run-up procedure in which they were contracted for 2 min every 10 min on four occasions according to the following protocol. The first and fourth contractions were produced with 5 μM noradrenaline in 125 mM potassium solution (KPSS, equimolar substitution of KCl for NaCl in PSS). The second was with 5 μM noradrenaline in PSS and the third with KPSS. Arteries failing to produce a maximum active tension equivalent to a pressure of 100 mmHg on the final contraction were rejected.

All experiments were conducted in the presence of the cyclo-oxygenase inhibitor indomethacin; as in a previous study of endothelial function of STZ-diabetic rats (Taylor *et al.*, 1992) we found no evidence for abnormalities of indomethacin-sensitive components of relaxation. Following the routine run up procedure, the vessels were incubated for 10 min in indomethacin (10 μM) and their responses to increasing concentrations of noradrenaline then determined at 3 min intervals (0.1 mM–10.0 mM cumulative addition). The bath was then washed three times with PSS and a further 15 min washout period allowed before the arteries were submaximally contracted for 3 min with the concentration of noradrenaline required to produce approximately 80% of the maximum response. Relaxation to acetylcholine was subsequently assessed by adding increasing concentrations of acetylcholine at 2 min intervals (final bath concentrations 1 nM–10 μM). After a washout period of 5 min, the NO synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester 10^{-4} M) was added to the bath and 10 min later a cumulative concentration-response to noradrenaline repeated. Finally, and in the continued presence of L-NAME, arteries were precontracted with noradrenaline as described, and after 3 min were subjected to increasing concentrations of the endothelium-independent nitrovasodilator sodium nitroprusside (SNP), at 2 min intervals (0.01–10 μM).

Drugs

Chemicals used in this investigation were noradrenaline (Winthrop Laboratories): acetylcholine, indomethacin, sodium nitroprusside, L-NAME, (all from Sigma); streptozotocin (gift from Dr MacLeod, Upjohn Co. Kalamazoo, U.S.A.) and Hypnorm (Jannsen Pharmaceutics). Chemicals were prepared as stock solutions solubilized in PSS, except indomethacin which was prepared as a 1 mM stock solution in phosphate buffer consisting of (mM): KH_2PO_4 20, $NaH_2PO_4.2H_2O$ 120, pH balanced to 7.8. All concentrations are expressed as the final molar concentrations in the organ bath. Sustained release insulin implants and placebo implants (Linplant) from Mollegaard, Denmark.

Statistical analysis

All values are given as the mean ± s.e.mean. Tension is given as active wall tension (mN mm^{-1} artery length) ± s.e.mean and the relaxation responses to acetylcholine as a percentage of the initial precontraction to noradrenaline. The negative log of the concentration of a drug required to affect 50% of the maximum response (pEC_{50}) was calculated as the mean ± s.e.mean of the individual pEC_{50}s using non linear regression, and the sigmoid equation of the curve fitting programme 'GraphPad' (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical comparisons of the pEC_{50} values and maximum responses between the groups were achieved by one way analysis of variance with the Bonferonni correction of P values for multiple comparisons. Paired two-tailed t tests were employed in the comparisons of sequential concentration-effect curves in the presence and absence of inhibitors. Significance was assumed if $P < 0.05$.

Results

The glucose concentration of the plasma obtained from PI-diabetic animals at the time of study (8–10 weeks after induction of diabetes) was 44.28 ± 4.10 mM ($n = 5$) whereas the plasma glucose of the insulin-treated diabetic animals was not significantly different from that of controls (8.72 ± 1.81 mM, $n = 11$, *versus* 6.31 ± 0.44 mM, $n = 14$). Bovine insulin concentrations in the implanted animals were 46.68 ± 11.11 mu l^{-1} ($n = 11$) and below the detectable limit in the control and placebo-treated animals. Endogenous levels of insulin in the control animals were 19.64 ± 6.25 mu l^{-1} ($n = 14$), lower than that of bovine insulin in the treated animals ($P < 0.05$). At the time of implantation, IT-diabetic animals weighed 222 ± 2 g, and following insulin treatment weighed 302 ± 4 g, a significant weight gain ($P < 0.0001$), and greater than the normal weight for age matched animals (250–270 g). In contrast PI-diabetics did not show any significant weight gain, being 240 ± 8 g before, and 249 ± 4 g after treatment (P not significant).

Contractile responses to noradrenaline

The exposure of arteries to noradrenaline led to a concentration-dependent rise in tension in all experimental groups. There was no significant difference in the sensitivity between the three groups (pEC_{50}: control 5.64 ± 0.07, $n = 18$; PI-diabetic 5.79 ± 0.12, $n = 5$; IT-diabetic 5.78 ± 0.05, $n = 22$, P not significant). However, the PI-diabetic arteries demonstrated an enhanced maximum contractile response to noradrenaline compared to controls (maximum response; PI-diabetic 3.73 ± 0.40 mN mm^{-1}, $n = 5$, *versus* control 2.65 ± 0.15 mN mm^{-1}, $n = 18$, $P < 0.001$). Insulin treatment did not prevent this enhanced contractile response to noradrenaline (maximum response: IT-diabetic 4.02 ± 0.19 mN mm^{-1}, $n = 22$, *versus* control 2.65 ± 0.17 mN mm^{-1}, $n = 18$, $P < 0.001$) Figure 1.

The addition of L-NAME in control arteries led to a highly significant increase in the maximum response to noradrenaline from 2.65 ± 0.17 mN mm^{-1} to 3.56 ± 0.16 mN mm^{-1}, $n = 17$, $P < 0.001$, although there was no significant increase in sensitivity (pEC_{50}: from 5.64 ± 0.07, to 5.75 ± 0.08, $n = 17$, P not significant). In the PI-diabetic arteries the addition of L-NAME did not affect the maximum response to noradrenaline (from 3.73 ± 0.40 mN mm^{-1} to 4.01 ± 0.18 mN mm^{-1}, $n = 5$, P not significant) but led to a significant increase in sensitivity (pEC_{50}: from 5.79 ± 0.12 to 6.12 ± 0.13, $n = 5$, $P < 0.005$). The PI-diabetic arteries in the presence of L-NAME demonstrated enhanced sensitivity to noradrenaline compared to control arteries in the presence of L-NAME (pEC_{50}: control 5.75 ± 0.08, $n = 17$ *versus* PI-diabetic 6.14 ± 0.09, $n = 8$, $P < 0.05$). In the arteries from the IT-diabetic rats the addition of L-NAME caused both an increase in maximum response (from 4.02 ± 0.19 mN mm^{-1} to 4.65 ± 0.20 mN mm^{-1}, $n = 20$, $P < 0.005$) and a substantial increase in sensitivity to noradrenaline (pEC_{50}: from 5.80 ± 0.05, to 6.38 ± 0.08, $n = 20$, $P < 0.001$). In the presence of L-NAME the IT-diabetic arteries demonstrated greater sensitivity to noradrenaline than control arteries (pEC_{50}: 6.38 ± 0.08, $n = 20$, *versus* 5.75 ± 0.08, $n = 17$, $P < 0.001$). No significant difference was observed between the maximum responses of the PI-diabetic arteries and that of the control arteries (maximum response: 4.01 ± 0.18 mN mm^{-1}, $n = 8$ *versus* 3.56 ± 0.16 mN mm^{-1}, $n = 17$, P not significant) or

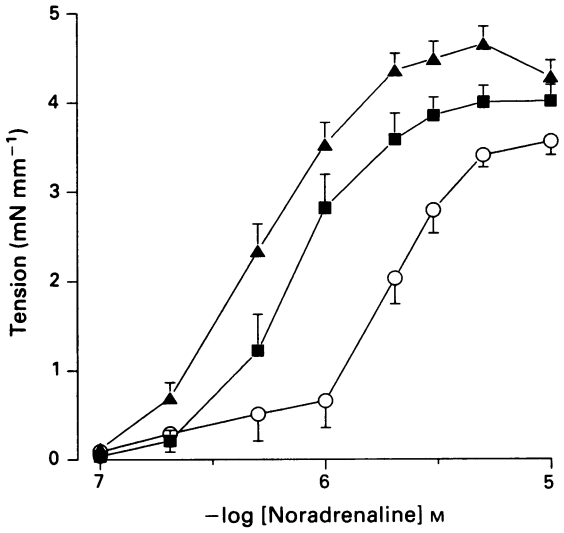

Figure 2 Noradrenaline concentration-effect curves, in the presence of indomethacin (10 μM) and the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (10^{-4} M), in isolated mesenteric resistance arteries from control (○, $n = 17$) placebo-implanted-diabetic (■, $n = 8$) and insulin-treated-diabetic rats (▲, $n = 20$).

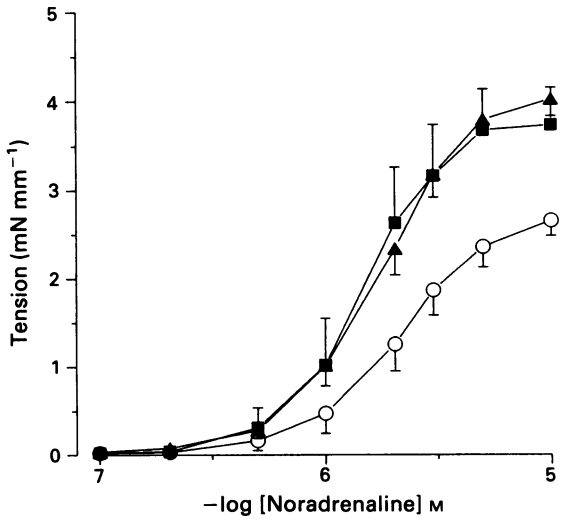

Figure 1 Noradrenaline concentration-effect curves, in the presence of indomethacin (10 μM), in isolated resistance mesenteric arteries from control (○, $n = 18$), placebo-implanted-diabetic (■, $n = 5$) and insulin-treated-diabetic rats (▲, $n = 22$).

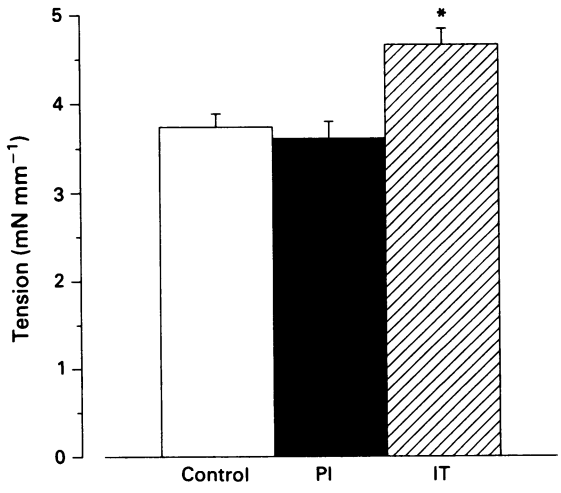

Figure 3 Maximal contraction to 125 mM depolarizing potassium (KPSS) in isolated mesenteric resistance arteries from control ($n = 18$), placebo-implanted (PI)-diabetic ($n = 11$) and insulin-treated (IT)-diabetic rats ($n = 22$).

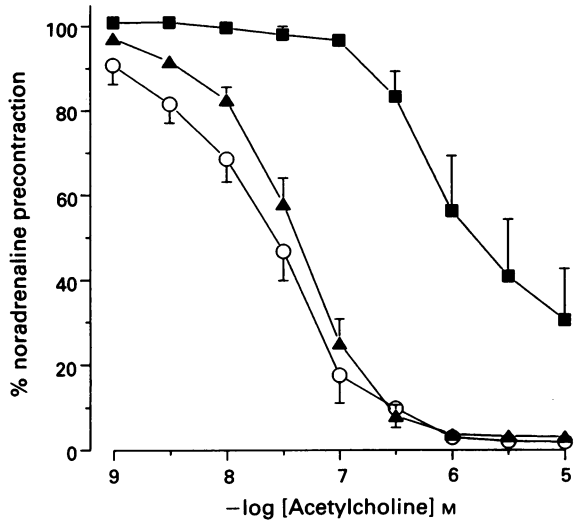

Figure 4 Acetylcholine concentration-effect curves, in the presence of indomethacin, in isolated mesenteric resistance arteries from control (○, $n = 17$), placebo-implanted-diabetic (■, $n = 8$), and insulin-treated-diabetic rats (▲, $n = 22$).

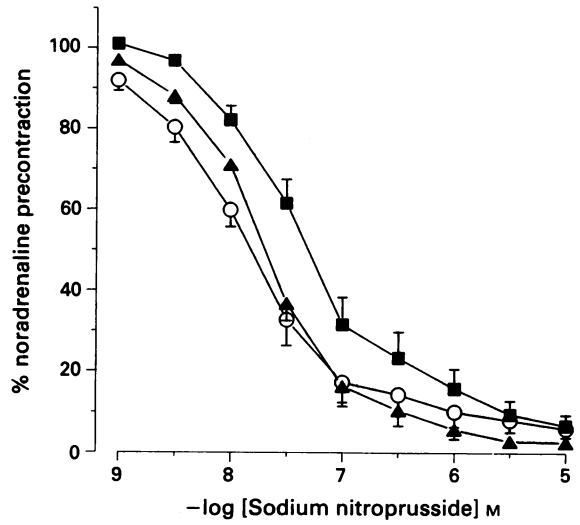

Figure 5 Endothelium-independent relaxation responses to sodium nitroprusside in isolated mesenteric resistance arteries from control (○, $n = 13$) placebo-implanted-diabetic (■, $n = 13$) and insulin-treated-diabetic rats (▲, $n = 16$) in the presence of indomethacin and N^G-nitro-L-arginine methyl ester (10^{-4} M).

between the PI-diabetic arteries and the IT-diabetic arteries (maximum response: 4.01 ± 0.18 mN mm^{-1}. $n = 8$, *versus* 4.65 ± 0.20 mN mm^{-1}, $n = 20$, P not significant). However, in the IT-diabetic arteries the maximum response to noradenaline in the presence of L-NAME was significantly raised above the controls (maximum response: 4.65 ± 0.20 mN mm^{-1}, $n = 20$, *versus* 3.56 ± 0.16 mN m^{-1}, $n = 17$, $P < 0.001$) Figure 2.

Arteries from the IT-diabetic animals demonstrated an enhanced response to 125 mM potassium (KPSS) relative to controls (4.66 ± 0.18 mN mm^{-1}, $n = 22$, *versus* 3.74 ± 0.15 mN mm^{-1}, $n = 18$, $P < 0.001$). Those from PI-diabetic animals were not significantly different from the controls (3.61 ± 0.19 mN mm^{-1}, $n = 11$) Figure 3.

Endothelium-dependent relaxation

The cumulative addition of acetylcholine, in half log molar increments, led to a concentration-dependent relaxation of arteries precontracted submaximally with noradrenaline, in all three experimental groups. Arteries from the PI-diabetic animals demonstrated profoundly reduced relaxation to acetylcholine compared to the control group (pEC_{50}: PI-diabetic 6.07 ± 0.12, $n = 8$, *versus* control 7.64 ± 0.19, $n = 17$, $P < 0.001$). Maximum relaxation was also significantly reduced in the PI-diabetic arteries compared to controls (maximum relaxation: PI-diabetic 69.5 ± 12.1, $n = 8$, *versus* control 98.5 ± 0.4, $n = 17$, $P < 0.001$). In the IT-diabetic arteries there was, however, no obvious impairment of acetylcholine-induced relaxation, compared with controls (pEC_{50}: IT-diabetic 7.36 ± 0.09, $n = 22$, *versus* control 7.64 ± 0.19, $n = 17$, P not significant. Maximum relaxation: IT-diabetic $93.8 \pm 0.6\%$, $n = 22$, *versus* control 98.5 ± 0.4, $n = 17$, P not significant) Figure 4.

Endothelium-independent relaxation

Concentration-dependent relaxation to increasing concentrations of sodium nitroprusside was observed in arteries from all three groups. PI-diabetic arteries did, however, show slight attenuation of the relaxation response compared to the control group (pEC_{50}: PI-diabetic 7.31 ± 0.13, $n = 13$, *versus* control 7.78 ± 0.10, $n = 13$, $P < 0.05$). This attenuation of the response was not observed in the insulin-treated group in which the response was not significantly different from that of the control group (pEC_{50}: IT-diabetic 7.64 ± 0.09, $n = 16$, *versus* control 7.78 ± 0.10, $n = 13$, P not significant) Figure 5.

Discussion

The results of this study, although in a different strain of rat, are in agreement with our previous observations in the STZ-diabetic rat model (Taylor *et al.*, 1992) in which we demonstrated impaired endothelium-dependent relaxation to acetylcholine. In addition we have demonstrated that insulin treatment prevents the development of impaired endothelium-dependent relaxation in isolated resistance arteries from the mesenteric circulation of these animals. This is in agreement with two previous studies in streptozotocin-diabetic rats; Takiguchi *et al.* (1989) have demonstrated that insulin treatment improves impaired acetylcholine relaxation in the isolated perfused mesentery and Tanz *et al.* (1989) have shown that insulin treatment prevents impaired histamine relaxation in 12 week streptozotocin-diabetic rat aorta. Our previous study suggested that the impaired endothelium-dependent relaxation resulted from reduced NO release and insulin therapy may, therefore, prevent this deficit. This is suggested by the experiments using the NO synthase inhibitor L-NAME which indicated greater noradrenaline-stimulated NO release in the arteries from the treated compared to untreated diabetic rats. Reduced NO release in diabetes is consistent with reports of impaired endothelium-dependent relaxation in human isolated resistance arteries in type I diabetes (McNally *et al.*, 1992) and in a study of forearm blood flow in type II diabetes (McVeigh *et al.*, 1992). In addition, other studies in type I diabetes have reported an impaired basal release of NO in the forearm circulation (Calver *et al.*, 1992; Elliot *et al.*, 1992). However, not all have found evidence of impaired NO production (Halkin *et al.*, 1991; Smits *et al.*, 1993). The differences could reflect glycaemic control: better controlled patients may be analogous to the sustained release, insulin-treated rats, whereas, poor control could be associated with poor endothelial function.

Insulin therapy results not only in the normalisation of serum glucose, but also many of the attendant sequelae of diabetes. Reversal of hyperglycaemia is, however, the most likely factor which prevents endothelial dysfunction. Vascular endothelial cells are particularly susceptible targets as glucose transport is independent of insulin (Green & Jaspan, 1990) and the cells are, therefore, at risk of excessive glucose entry when ambient concentrations are high. Activation of the polyol pathway by glucose has been cited as a possible mechanism contributing to reduced nitric oxide production (Cameron & Cotter, 1992). However, in our recent study in which STZ-diabetic rats were treated with the aldose reductase inhibitor,

ponalrestat, no reversal of the endothelial dysfunction in the mesenteric circulation was apparent (Taylor *et al.*, 1994). Other mechanisms by which hyperglycaemia may directly affect endothelial function include the formation of advanced glycosylation end products (AGEs) which can effectively quench nitric oxide, so reducing relaxation (Brownlee *et al.*, 1988; Bucala *et al.*, 1991; Makita *et al.*, 1991; Hogan *et al.*, 1992). Glucose metabolites such as glucose-6-phosphate (G6P) may also lead to intracellular glycosylation and quenching of NO, and may cause extensive damage of intracellular constituents by the generation of free radicals (Molinatti *et al.*, 1990). The breakdown of nitric oxide by free radicals, generated by hyperglycaemia-induced cyclo-oxygenase activity may also contribute to impaired endothelium-dependent relaxation (Tesfamariam & Cohen, 1992). Also a decrease in antioxidant activity, including the reported reductions in glutathione through stimulation of the polyol pathway (Dvornik, 1987), and associated superoxide dismutase activity (Tagami *et al.*, 1992), would lead to a reduced scavenging of free radicals. This has been shown to be prevented in aortic endothelial cells of alloxan diabetic rabbits by treatment with insulin (Tagami *et al.*, 1992).

Insulin also reverses hyperlipidaemia in man and in animal models of diabetes. It is highly relevant, therefore, that hypercholesterolaemia (Creager *et al.*, 1990; Chowienczyk *et al.*, 1992) and atherosclerosis (Zeiher *et al.*, 1991) in man are associated with impaired endothelium-dependent relaxation. Moreover, oxidised low density lipoproteins (LDLs) inhibit relaxations mediated by NO (Jacobs *et al.*, 1990), and LDLs are elevated in diabetes (Sosenko *et al.*, 1980). Reversal of hyperlipidaemia may, therefore, provide an alternative mechanism for insulin reversal of endothelial dysfunction. Some lipids, notably 3-omega fatty acids, stimulate NO production and it is of interest that McVeigh *et al.* (1993) have recently reported dietary fish oil supplementation reverse to abnormal endothelium-dependent relaxation in the forearm of patients with type II diabetes.

Mesenteric resistance arteries from the PI-diabetic rats demonstrated an enhanced maximum response to noradrenaline, but no change in sensitivity, in contrast to our previous study in which we observed both were increased (Taylor *et al.*, 1992). The difference could reflect the strain of rat used or the shorter duration of diabetes (5–6 weeks) in the earlier study. The increased contractility to noradrenaline was unlikely to be the result of enhanced constrictor prostanoid production as suggested by some (Agrawal & McNeill, 1987) as the experiments were carried out in the presence of indomethacin. In our earlier study (Taylor *et al.*, 1992) in rats with shorter duration of diabetes, we attributed enhanced noradrenaline reactivity to reduced release of NO since the addition of L-NAME to the arteries resulted in identical tension development in control and diabetic arteries when contracted with noradrenaline. In the present study in animals with untreated diabetes of longer duration (8–10 weeks), the diabetic arteries continued to display an enhanced response to noradrenaline in the presence of L-NAME when compared to controls. This suggests a second, duration-dependent mechanism and might be associated with autonomic neuropathy. Autonomic neuropathy develops several weeks after STZ injection resulting in reduced endogenous noradrenaline release (Sato *et al.*, 1989), and could lead to upregulation of adrenoceptors and increased sensitivity to exogenous noradrenaline. Cameron & Cotter (1992), Scarborough & Carrier (1983) and Wong & Tzeng (1992), have all implicated an enhanced α_2-adrenoceptor mediated component of noradrenaline contraction in animal models of diabetes. Enhanced pressor responses to exogenous noradrenaline have also been documented in insulin-dependent, diabetic patients (Moorhouse *et al.*, 1966; Christlieb *et al.*, 1976; Eichler *et al.*, 1992).

The increase in reactivity to noradrenaline in the diabetic rats was not prevented by sustained release insulin treatment despite normalisation of plasma glucose. As these treated animals would not have had diabetes for a sufficient duration to develop neuropathy this is unlikely to be a reflection of irreversible autonomic neuropathy. This would suggest that insulin itself may be affecting contractility in the treated group. Indeed serum levels of exogenous bovine insulin in the treated diabetics were moderately raised compared to those of endogenous insulin in the non-diabetic controls. The weight gain following insulin treatment is also indicative of a degree of hyperinsulinaemia. The observation that the contractile response to 125 mM potassium (KPSS) was elevated in the insulin-treated diabetic animals but not in the placebo-implanted group could suggest a chronic effect of raised exogenous insulin on the contractile mechanism. This is consistent with the data of Pfaffman *et al.* (1982) in which there was a tendency for K^+-induced contractions (70 mM) in insulin-treated diabetic aortas to develop greater tension than controls. Insulin could increase contractile responses through the promotion of cell growth and proliferation of vascular smooth muscle (Stolar, 1988; Banskota *et al.*, 1989).

Most studies investigating the effects of insulin on vascular smooth muscle function have been acute studies *in vitro* and are, therefore, likely to be of little relevance to the abnormalities of noradrenaline-induced contraction seen in diabetic and insulin-treated animals in this *in vitro* preparation in the absence of insulin. Indeed acute and chronic responses to insulin may be quite different (Gans & Donker, 1991). Acutely, insulin, usually at supraphysiological concentration, causes vasodilatation in animals (Liang *et al.*, 1982; Yagi *et al.*, 1989; Standley *et al.*, 1991; Wambach & Liu, 1991) and in a man (Scott *et al.*, 1988), but there is also evidence that insulin may act synergistically with noradrenaline to enhance constriction (Gans *et al.*, 1990; Townsend *et al.*, 1992).

Relaxation to SNP was normal in the IT-diabetic rats but significantly reduced in the PI-diabetic rats. This would be expected if AGEs were present in the untreated animals, as quenching of NO would lead to reduced efficacy of SNP since this compound generates free NO in aqueous solution (Ignarro *et al.*, 1980). In our earlier study we reported a normal response to SNP in diabetic animals of 6 weeks duration; the difference between that and the present study may reflect AGE production associated with the longer duration of diabetes. Bucala *et al.* (1991), also found impaired acetylcholine and nitroglycerin vasodilator responses *in vivo* in STZ rats of 2 month duration which were associated with AGE formation in the artery wall. They found that short term insulin treatment did not reverse this defect and suggested that it was due to the irreversible effect of these NO quenching compounds. It would be of interest to determine, in this preparation, therefore, whether insulin treatment reverses rather than prevents the endothelium-dependent and -independent relaxation defects after prolonged diabetes.

In conclusion, sustained release insulin treatment from the onset of streptozotocin-induced diabetes prevents the development of impaired endothelium-dependent and endothelium-independent relaxation in rat mesenteric resistance arteries. The most likely mechanisms, would appear to be the preservation of endothelial NO production, but the prolongation of the half-life of NO through reduced free radical, superoxide and AGE formation may also play a role. In contrast insulin-treatment does not prevent the enhanced contractility to noradrenaline associated with streptozotocin-induced diabetes. This underlying enhancement of contractility to noradrenaline in insulin-treated experimental diabetes could contribute to increased peripheral vascular resistance and to the development of diabetic hypertension.

This work was funded through a collaborative PhD studentship between the Medical Research Council and ZENECA Pharmaceuticals. We are grateful to Dr Richard Jones for thoughtful discussion and suggestions.

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(Received April 23, 1993
Revised August 13, 1993
Accepted September 3, 1993)

Br. J. Pharmacol. (1994), **111**, 42–48

Endothelial function in the isolated perfused mesentery and aortae of rats with streptozotocin-induced diabetes: effect of treatment with the aldose reductase inhibitor, ponalrestat

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1 Noradrenaline sensitivity and relaxation to acetylcholine were investigated in the isolated perfused mesentery and in aortic rings of control and streptozotocin (STZ)-induced (50 mg kg^{-1}) diabetic Charles River rats.

2 In addition, noradrenaline sensitivity and acetylcholine relaxation were similarly assessed in streptozotocin-induced diabetic rats treated from the time of onset of diabetes with the aldose reductase inhibitor, ponalrestat (100 mg kg^{-1} day^{-1}).

3 The untreated diabetic rats (2–10 weeks after injection of STZ) demonstrated enhanced vascular sensitivity to noradrenaline in the perfused mesenteric arterial tree, compared with age matched controls (pEC_{50} [− log concentration (M)]: diabetic 5.62 ± 0.09, $n = 18$, *versus* control 5.23 ± 0.07, $n = 16$, $P < 0.01$).

4 Acetylcholine-induced relaxation was significantly impaired in the perfused mesentery of the diabetic animals compared to controls (pED_{50} [− log dose (mol)]: diabetic 9.87 ± 0.10, $n = 20$, *versus* controls, 10.29 ± 0.09, $n = 20$, $P < 0.05$).

5 In contrast, the aortic ring preparations demonstrated no significant functional differences between the diabetic and control groups in response to either noradrenaline (pEC_{50}: diabetic 7.66 ± 0.08, $n = 15$, *versus* controls 7.55 ± 0.06, $n = 15$, NS), or acetylcholine (pEC_{50}: diabetics 7.30 ± 0.06, $n = 15$, *versus* controls 7.40 ± 0.09, $n = 15$, NS).

6 Treatment with the aldose reductase inhibitor, ponalrestat, did not affect the increased vascular reactivity to noradrenaline, or impaired relaxation to acetylcholine in the perfused mesentery.

Keywords: Mesenteric resistance arteries of rat; rat aorta; vascular endothelium; vascular smooth muscle; chemically induced diabetes; streptozotocin; aldose reductase inhibitors; endothelium derived relaxing factor

Introduction

Abnormal function of the vascular endothelium is now identified with a growing number of conditions associated with arterial disease (Creager *et al.*, 1990; Zeiher *et al.*, 1991; Chowienczyk *et al.*, 1992), including diabetes (Hsueh & Anderson, 1992). There is evidence that impotence in diabetic men may be explained by impairment in the endothelium-dependent relaxation of the smooth muscle of the corpora cavernosa (de Tejada *et al.*, 1989). Other indicators include the demonstration of abnormal relaxation to acetycholine in the forearm of subjects with non-insulin dependent (type II) diabetes mellitus (NIDDM) (McVeigh *et al.*, 1992), and reduced basal and stimulated nitric oxide (NO) synthesis in patients with insulin-dependent diabetes associated with microalbuminuria (Elliot *et al.*, 1992). In a preliminary report, abnormal endothelium-dependent relaxation to acetylcholine has also been described in resistance arteries from the subcutaneous fat of diabetic subjects without proteinuria (McNally *et al.*, 1992). There is also some evidence that reduced responsiveness of the underlying vascular smooth muscle to NO may contribute to poor endothelial-dependent relaxation (McVeigh *et al.*, 1992; Calver *et al.*, 1992). Indirect evidence for endothelial dysfunction in diabetes includes the observation that the marker of endothelial damage, Von Willebrand's Factor, is elevated in the serum of patients with NIDDM who develop diabetic nephropathy (Stehouwer *et al.*, 1991), and is strongly associated with the development of increased urinary albumin excretion (Stehouwer *et al.*, 1992).

In animal models of diabetes there is histological evidence (Arbogast *et al.*, 1984) that vascular endothelium is abnormal. Functional studies in conduit arteries from animals with chemically induced diabetes have not been uniform in their findings, but impaired endothelium-dependent relaxation has been described (Oyama *et al.*, 1986; Pieper & Gross, 1988; Durante *et al.*, 1988; Kamata *et al.*, 1989; Tanz *et al.*, 1989; Mayhan, 1989; Abiru *et al.*, 1990; Tesfamariam *et al.*, 1990; Mayhan *et al.*, 1991; Cameron & Cotter, 1992a), together with increased vascular reactivity to noradrenaline (MacLeod & McNeill, 1985; Harris & MacLeod, 1988; Piper & Gross, 1988; White & Carrier, 1988; Cohen *et al.*, 1990). We have recently reported that resistance arteries from the mesenteric circulation of streptozotocin (STZ)-induced diabetic rats also display grossly impaired endothelium-dependent relaxation to acetylcholine, and an enhanced sensitivity to noradrenaline (Taylor *et al.*, 1992). Using specific inhibitors of NO synthase we demonstrated that both these abnormalities were at least in part due to the reduced production/release of endothelium-dependent relaxing factor.

Amongst the mechanisms that have been proposed to account for diabetic vascular dysfunction, underlying hyperglycaemia is being seen as increasingly central to its aetiology. Several explanations have been put forward which might associate hyperglycaemia with reduced endothelium-dependent relaxation. These include a role for the glucose-stimulated increase in polyol pathway activity and the redox changes associated with increased sorbitol flux (Williamson *et al.*, 1990; Cameron & Cotter, 1992a). Increased sorbitol levels have been identified in aortic endothelium of diabetic rats (Brolin & Naeser, 1988) and could lead to endothelial dysfunction in a number of ways. An increase in sorbitol could compromise endothelial function by a purely osmotic effect (Green & Jaspan, 1990). Increased activity of the polyol

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pathway is also associated with increased utilization of NADPH, which may lead to reduced availability of cellular NADPH, an essential cofactor of NO synthase (Cameron & Cotter, 1992a). Increased polyol pathway activation will also lead to a reduction in glutathione (Dvornik *et al.*, 1987), which in turn may reduce superoxide dismutase activity (Loven *et al.*, 1986). Thus there may be reduced production of NO, together with increased degradation by free radicals. Increased sorbitol flux is also associated with myoinositol depletion which in turn is responsible for a reduction in Na/K ATPase activity (Simmons & Winegrad, 1989). Inhibition of Na/K ATPase can lead to reduced endothelium-dependent relaxation in resistance arteries (Wolfson & Poston, 1991) and could contribute, therefore, to endothelial dysfunction in diabetes.

The rate limiting enzyme in the polyol pathway, aldose reductase, catalyzes the conversion of glucose to sorbitol, which is followed by the conversion of sorbitol to fructose by sorbitol dehydrogenase. There has been considerable interest, therefore, in the possible use of aldose reductase inhibitors (ARI) as a potential means of reversal of the nervous and vascular defects of diabetes. Clinical trials of aldose reductase inhibitors have shown some promise (Sima *et al.*, 1988; Boulton *et al.*, 1990), but have not proven as beneficial as animal studies had intimated (Green & Jaspan, 1990). It has been suggested that the lack of success of ponalrestat, in particular, was due to the inadequate inhibition of aldose reductase achieved with the dose prescribed in clinical trials (Cameron & Cotter, 1992b).

Two recent studies, in conduit arteries, have shown that treatment with an aldose reductase inhibitor (ARI) prevents the development of impaired endothelium-dependent relaxation in STZ-diabetic rat aorta (Cameron & Cotter, 1992a) and alloxan-diabetic rabbit aorta (Tesfamariam *et al.*, 1993). Until now, no study has looked at the effect of ARI treatment on diabetic resistance vascular function. In this study we have investigated the effect of treatment of diabetic rats with ponalrestat, which has shown only very modest benefit in clinical trials (Sima *et al.*, 1988). However, at higher doses, it has recently been shown to prevent nerve conduction velocity deficits in STZ-diabetic rats, which has been ascribed to the improvement of nerve blood flow (Cameron & Cotter, 1992b). In addition, a preliminary report by Chess-Williams & Otter (1993) has also shown that ponalrestat (25 mg kg^{-1} day^{-1}) prevented endothelial dysfunction in diabetic rat aorta.

In order to ascertain whether a different model of resistance artery function showed the same defect as that we have previously described in isolated mesenteric resistance arteries, we have chosen to use the isolated perfused mesentery (a preparation based on the method of McGregor, 1965) in this study. By way of comparison, we have also investigated constrictor responses and endothelium-dependent relaxation in aortic ring preparations taken from the same animals.

Methods

Animals

Female adult Charles River rats (ZENECA, Alderly Park) were used in this study (220–250 g). Animals were housed separately according to their experimental groups and maintained in a temperature- and light-controlled environment. Food and water were given *ad libitum*.

Induction of experimental diabetes

Diabetes was induced in Charles River rats ($n = 40$) by i.p. injection of streptozotocin, STZ, (50 mg kg^{-1}, ZENECA Pharmaceuticals). Half of the diabetic group were treated daily with the aldose reductase inhibitor, ponalrestat (intraoral administration via a ball-ended stainless steel dosing cannula, 100 mg kg^{-1} day^{-1}, a dose shown to achieve 95% inhibition of sorbitol accumulation in sciatic nerve tissue of STZ-diabetic rats, ZENECA Pharmaceuticals). Experiments began 2–10 weeks after induction of diabetes with experiments being run in parallel, one rat from each experimental group. All elements of the protocol were carried out on each animal. Blood samples were obtained by cardiac puncture at the time of exsanguination of the animals. Plasma glucose levels were then assayed spectrophotomically (glucose oxidase analyser).

Isolated perfused mesentery preparation

Vascular reactivity to noradrenaline and the dilator response to acetylcholine were assessed in the isolated perfused mesentery of rats from each of the three groups. The rats were anaesthetized by means of i.p. injection of 'Intraval' (thiopentone, 150 mg kg^{-1}) and cannulated at the superior (anterior) mesenteric artery. The animals were then killed by exsanguination and the mesentery isolated under 'constant flow' perfusion with Krebs solution using a peristaltic pump (6.5 ml min^{-1}, McGregor, 1965). The Krebs buffer was of the following composition (mM): NaCl 120, $NaHCO_3$ 25, D-glucose 5.5, KH_2PO_4 1.2, $MgSO_47H_2O$ 1.2, KCl 4.7, $CaCl_2$ 2.5, ethylenediamine-tetra-acetic acid disodium salt (EDTA) 0.026, maintained at 37°C and bubbled with 95% O_2/5% CO_2. Changes in the resistance across the mesenteric arterial tree were measured by monitoring back pressure with a pressure transducer in series between the pump and the preparation. Preparations were allowed to equilibrate for 40 min. Noradrenaline concentration-effect curves were constructed by administration of the drug at a known concentration within the perfusate for 10 min intervals (10^{-7}–10^{-4} M). Following a 20 min washout period, the preparation was precontracted with a supramaximal concentration of noradrenaline. Acetylcholine concentration-effect curves were constructed on top of the noradrenaline contraction through a series of bolus injections, at 2 min intervals, via a self sealing rubber septum (10^{-13}–10^{-8} No of moles). Bolus injections were restricted to small volumes to avoid artifacts (<4 µl).

Aortic ring preparations

A section of the thoracic aorta between the aortic arch and the diaphragm was dissected out from the same animals following exsanguination. These were mounted as ring preparations and normalized for the measurement of isometric tension in 20 ml organ baths containing Krebs buffer (maintained at 37°C and bubbled with 95% O_2/5% CO_2 throughout). After a standard equilibration time of 40 min under a resting tension of 1 g, during which the Krebs buffer solution was changed every 20 min, cumulative concentration-effect curves to noradrenaline were constructed by addition of small volumes of stock solution to the organ bath (10^{-9}–3×10^{-5} M). Contractile responses were expressed as a percentage of the maximum response. Following washout, a 20 min recovery period was allowed before vessels were submaximally precontracted with the required noradrenaline dose to achieve about 80% of the maximum response. When the noradrenaline-induced preincubation reached a plateau level, acetylcholine was added cumulatively as small volumes of stock solution in half log molar increments at 2 min intervals (10^{-9}–3×10^{-5} M). Relaxation was expressed as a percentage of the precontraction tension.

Materials

Chemicals used in the study included the following: noradrenaline (Sigma); acetylcholine (Sigma); streptozotocin (ZENECA Pharmaceuticals). Chemicals were prepared as stock solutions solubilized in Krebs, and made daily. Noradrenaline was dissolved with an equal weight of ascorbic acid to prevent oxidation. All concentrations are expressed as

final molar concentrations in the organ bath, except the bolus injection of acetylcholine in the perfused mesentery, which is expressed in terms of the number of moles injected.

Statistical analysis

All values are given as the mean ± s.e.mean. In both isolated perfused mesentery and aorta, noradrenaline-induced tension is expressed as a percentage of the maximum contractile response and the relaxation to acetylcholine as a percentage of the noradrenaline-induced precontraction. In the perfused mesentery preparations in which acetylcholine was administered as bolus injections, sensitivity to the agonist is expressed in terms of a pED_{50} defined as the negative log of the effective dose (No. of moles) required to produce half the maximum effect. In the aortic ring preparations in which the concentration of the agonist in the bath is known, the negative log of the effective concentration (M) of the drug required to produce 50% of the maximum effect (pEC_{50}) was employed. In either case, the index of sensitivity was calculated from each concentration-effect curve by fitting the data to the sigmoid equation of the curve fitting programme 'GraphPad' (GraphPad Software Inc., San Diego, CA, U.S.A.). One way analysis of variance and independent Student's t tests were employed in the statistical comparison of the pEC_{50} or pED_{50} values between groups. The Bonferroni correction was employed for multiple comparisons and significance was assumed if $P<0.05$ ('Instat' by GraphPad, San Diego, CA, U.S.A.). NS = not significant. Values are given ± s.e.mean.

Results

Animals injected with STZ experienced significant weight loss compared to age matched controls (mean weight: untreated diabetic 262.1 ± 5.78 g, $n = 21$, *versus* control 290.0 ± 7.12 g, $n = 22$, $P<0.01$). Treatment with the aldose reductase inhibitor (ARI) had no significant effect on weight loss in the diabetic animals (mean weight: ARI-treated diabetic 263.6 ± 4.22 g; $n = 22$; *versus* untreated diabetic 262.1 ± 5.78 g, $n = 21$, NS). Plasma glucose levels were significantly raised in the diabetic animals compared to controls (plasma glucose concentration: untreated diabetic 22.06 ± 3.09 mM, $n = 21$, *versus* control 4.15 ± 0.31 mM, $n = 22$, $P<0.001$). No difference was observed in the plasma glucose concentrations between ARI-treated and untreated diabetic animals (plasma glucose concentration: untreated diabetic 22.1 ± 3.1 mM, $n = 21$, *versus* ARI-treated diabetic 26.8 ± 2.8 mM, $n = 22$, NS).

Noradrenaline reactivity

Cumulative increases in the noradrenaline concentration of the perfusate led to a concentration-dependent increase in the perfusion pressure. The mesentery from the untreated diabetic animals displayed enhanced noradrenaline-sensitivity compared to age-matched controls (pEC_{50}: untreated diabetic 5.62 ± 0.09, $n = 18$, *versus* control 5.23 ± 0.07, $n = 16$, $P<0.01$). There was no significant difference in the response to noradrenaline between ARI-treated diabetic animals and untreated diabetic animals (pEC_{50}: ARI-treated diabetic 5.69 ± 0.07, $n = 18$, *versus* untreated diabetic 5.62 ± 0.09, $n = 18$, NS) (Figure 1). No significant correlation was observed between the pEC_{50} and duration of diabetes, in either group of diabetic animals.

Aortic ring preparations taken from the same untreated diabetic animals showed no significant change in noradrenaline sensitivity compared to those from control animals (pEC_{50}: untreated diabetic 7.66 ± 0.08, $n = 15$, *versus* control 7.55 ± 0.06, $n = 15$, NS). Moreover, ARI treatment had no significant effect on noradrenaline sensitivity in diabetic rat aortae (pEC_{50}: ARI-treated diabetic 7.71 ± 0.17, $n = 15$, *versus* untreated diabetic 7.66 ± 0.08, $n = 15$, NS) (Figure 2).

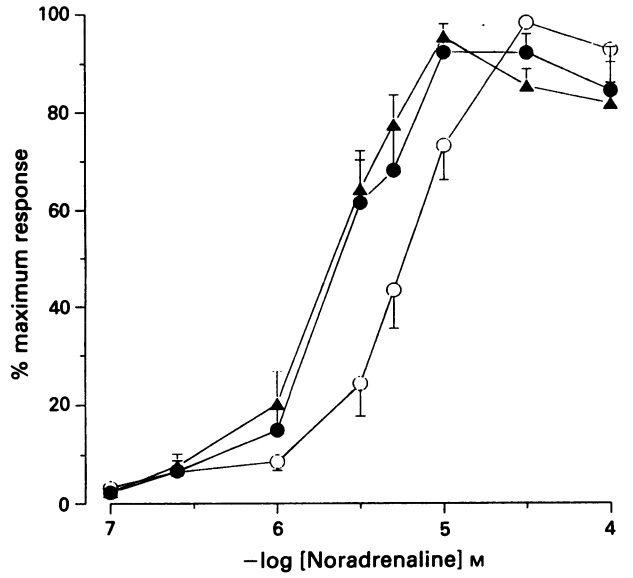

Figure 1 Noradrenaline concentration-effect curves for perfused mesentery preparations from control (○, $n = 16$), untreated diabetic (●, $n = 18$) and aldose reductase inhibitor (ARI) treated-diabetic rats (▲, $n = 18$).

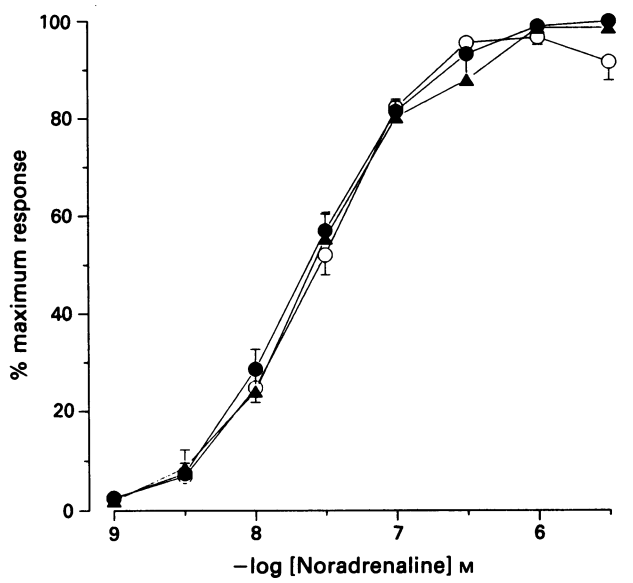

Figure 2 Noradrenaline concentration-effect curves for thoracic aortic ring preparations in control (○, $n = 15$), untreated diabetic (●, $n = 15$) and aldose reductase inhibitor (ARI) treated-diabetic rats (▲, $n = 15$).

Endothelium-dependent relaxation

In the perfused mesentery preparations, precontracted with noradrenaline, concentration-dependent decreases in perfusion pressure were observed in response to the bolus injections of increasing acetylcholine concentrations. Untreated diabetic rats demonstrated a significantly blunted dilator response to acetylcholine (pED_{50} [− log dose (No moles)]: diabetic 9.87 ± 0.10, $n = 20$, *versus* control 10.29 ± 0.09, $n = 20$, $P<0.05$). The ARI-treated diabetic group demonstrated no significant difference in sensitivity to acetylcholine

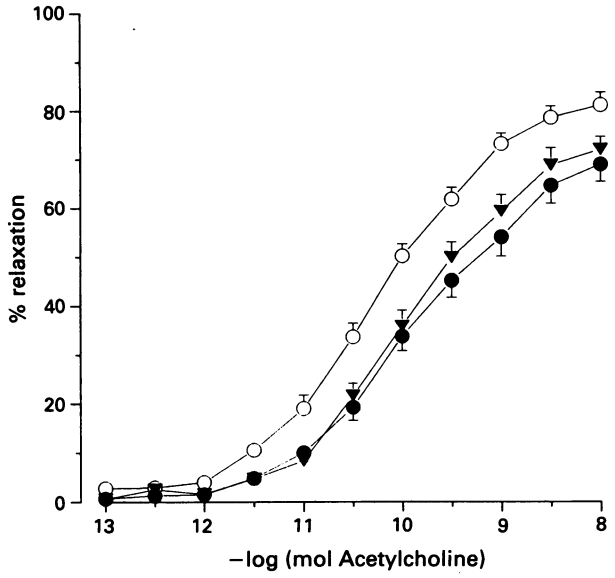

Figure 3 Acetylcholine-induced relaxation of isolated perfused mesenteric preparation after precontraction to noradrenaline in control (○, $n = 20$), untreated diabetic (●, $n = 20$) and aldose reductase inhibitor (ARI) treated-diabetic rats (▲, $n = 18$).

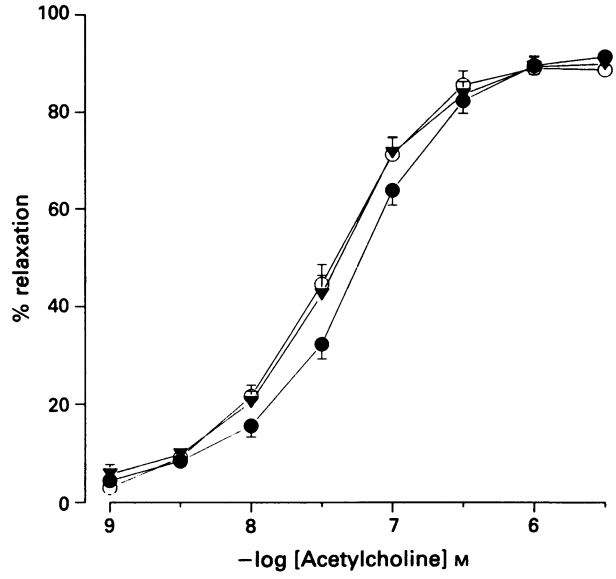

Figure 4 Acetylcholine-induced relaxation after precontraction to noradrenaline in thoracic aortic ring preparations from control (○, $n = 15$), untreated diabetic (●, $n = 15$) and aldose reductase inhibitor (ARI) treated-diabetic rats (▲, $n = 15$).

compared to the untreated diabetic group (pED_{50} [− log dose (No moles)]: ARI-treated diabetic 9.90 ± 0.13, $n = 18$, *versus* untreated diabetic 9.87 ± 0.10, $n = 20$, NS) (Figure 3). No significant correlation was observed between the pED_{50} and the duration of diabetes.

In contrast, endothelium-dependent relaxation to acetylcholine in the aortic ring preparations was not significantly affected in the diabetic rats (pEC_{50}: untreated diabetic 7.30 ± 0.06, $n = 15$, *versus* control 7.40 ± 0.09, $n = 15$, NS). In addition, ARI-treatment had no significant effect on acetylcholine sensitivity in the aortae from diabetic rats (pEC_{50} ARI-treated diabetic 7.40 ± 0.06, *versus* 7.30 ± 0.06, $n = 15$ for the untreated diabetic, NS) (Figure 4).

Discussion

The results of this study in the perfused mesentery of rats with STZ-diabetes agree with our previous findings in isolated mesenteric resistance arteries (Taylor *et al.*, 1992) in which we have observed enhanced sensitivity to noradrenaline and impaired endothelium-dependent relaxation to acetylcholine. It is unlikely that these defects are the result of a localized toxic effect of STZ *per se* as similar defects have been demonstrated in alternative models of diabetes, both chemically (Abiru *et al.*, 1990) and genetically-induced (Meraji *et al.*, 1987; Durante *et al.*, 1988; Kappagoda *et al.*, 1989). We previously reported that the observed abnormalities were due, at least in part, to the reduced production and release of nitric oxide. This has been confirmed in a recent study by Lawrence & Brain (1992) which has shown a deficit in the local production of nitric oxide in the cutaneous microvasculature of rats with STZ-induced diabetes.

The absence of these functional defects in the aortae from the same diabetic animals suggests that the resistance vasculature, with its direct relevance to the local control of blood flow, may be more sensitive to the damage associated with the metabolic sequelae of diabetes. This might suggest an underlying difference in the endothelial cell layer between resistance arteries and conduit arteries. Endothelial cells from small vessels do show different functional characteristics from those of larger vessels, for example, glucose may stimulate endothelial cell replication in retinal capillaries but is inhibitory in larger arteries and veins (Porta *et al.*, 1987). However, many previous studies in diabetic animals have shown defects of contractile and endothelial function in conduit arteries. The conflicting results could be explained by the later onset of the abnormalities in the conduit arteries compared to the resistance vasculature. Indeed, in a recent study investigating the time of onset of vascular abnormalities in diabetes, Wong & Tzeng (1992), only observed an enhanced contractile response to noradrenaline in rat aorta 12 weeks after induction of diabetes.

The enhanced sensitivity to noradrenaline seen in this and our previous study in diabetic rats (Taylor *et al.*, 1992) has been documented in several investigations in conduit arteries (MacLeod & McNeill, 1985; Harris & MacLeod, 1988; Pieper & Gross, 1988; White & Carrier, 1988; Cohen *et al.*, 1990) but not all (Head *et al.*, 1987; Gebremedhin *et al.*, 1989; Mulhern & Docherty, 1989). Proposed mechanisms include an increased α_2-adrenoceptor component of noradrenaline-induced contraction (Scarborough & Carrier, 1983; Wong & Tzeng, 1992) increased constrictor prostanoid production (Agrawal & McNeill, 1987), and reduced EDRF production (Taylor *et al.*, 1992).

The observation of impaired endothelium-dependent relaxation to acetylcholine in this and in our previous investigation (Taylor *et al.*, 1992) agrees with the majority of studies in conduit arteries of rats with experimentally-induced diabetes (Oyama *et al.*, 1986; Pieper & Gross, 1988; Durante *et al.*, 1988; Tanz *et al.*, 1989; Kamata *et al.*, 1989; Cameron & Cotter, 1992a), but several have found no impairment (White & Carrier, 1986; Wakabayashi *et al.*, 1987; Head *et al.*, 1987; Gebremedhin *et al.*, 1988; Mulhern & Docherty, 1989; Kappagoda *et al.*, 1989). The explanation for these discrepant findings is not obvious, but variations in the duration and severity of diabetes and species differences, could be contributory. In addition, the level of antioxidants in the diet of the animals may play a role. Tesfamariam & Cohen (1992), have found that the isolated aortae of rabbits which were pre-fed with an antioxidant (probucol) did not develop abnormal endothelium-dependent relaxation in response to elevated glucose, suggesting that dietary antioxidants may protect against hyperglycaemia-induced oxidative stress.

The primary aim of this study was to determine whether the administration of the aldose reductase inhibitor, ponalrestat, would prevent the development of endothelial dysfunction in rats with STZ-diabetes. As detailed in the

introduction, stimulation of the polyol pathway may lead, theoretically, to endothelial dysfunction through a number of effector pathways. Despite treatment from the onset of diabetes, there was no significant improvement in acetylcholine relaxation or noradrenaline sensitivity in the treated animals compared with those with untreated diabetes over an equivalent period. These results are in contrast with an *in vitro* study in rat aorta by Cameron & Cotter (1992a), in which treatment with a novel aldose reductase inhibitor, (4-amino-2,6-dimethylphenyl-sulphonyl) nitromethane (21 mg kg^{-1} day^{-1}) over 3 months of STZ-diabetes, completely prevented the development of abnormal endothelium-dependent relaxation to acetylcholine and the calcium ionophore A23187. In the untreated animals, the ratio of maximum contractions to noradrenaline and phenylephrine was enhanced suggesting an increase in α_2-adrenoceptor-mediated responses. This was partially corrected by treatment with the aldose reductase inhibitor. More recently, Tesfamariam *et al.* (1993) have also found that treatment of diabetic rabbits with a structurally similar aldose reductase inhibitor as that used in this study, zopolrestat (150 mg kg^{-1}), prevented the development of abnormal endothelium-dependent relaxation in aorta, although at a lower dose (50 mg kg^{-1}), zopolrestat did not prevent endothelial-dysfunction. Interestingly, nitric oxide production and vasoconstrictor prostanoid levels were unchanged by the drug (Tesfamariam *et al.*, 1993). The same group have also found that two structurally unrelated aldose reductase inhibitors (sorbinil and zopolrestat) prevented glucose-induced endothelial dysfunction *in vitro* in normal rabbit aorta (Tesfamariam *et al.*, 1992). Therefore, there appears to be a discrepancy between the effect of aldose reductase inhibition on endothelial function in resistance size arteries and conduit arteries in animal models of diabetes. It is unlikely that the duration of treatment provides an explanation as the rats in Cameron & Cotter's study (1992a) were treated over a similar period. Although aldose reductase activity is well described in the endothelium of aorta (Brolin & Naeser, 1988), it is not known whether it is expressed in the endothelial cells of the resistance arteries. Therefore, it is possible that the inhibitor is ineffective due to the absence of the enzyme. Alternatively, although we consider it unlikely, the dose of ponalrestat used in this study may be ineffective in achieving sufficient inhibition of aldose reductase in the mesenteric arteries. Nonetheless, we can conclude, at least in the circulation studied, that small arteries which contribute to peripheral vascular resistance maintain abnormal endothelial function despite the high dose of aldose reductase inhibitor.

If, as suggested above, the aldose reductase pathway is unimportant in the aetiology of endothelial dysfunction in the resistance vasculature of this animal model of diabetes, then alternative explanations must be sought. A number have been suggested including non-enzymatic glycosylation of proteins (Brownlee *et al.*, 1988; Bucala *et al.*, 1991; Hogan *et al.*, 1992), activation of protein kinase C (Lee *et al.*, 1989; Tesfamariam *et al.*, 1991), increased production of a constrictor prostanoid (Tesfamariam *et al.*, 1990; Tesfamariam & Cohen, 1992) and an increase in free radical production (Pieper *et al.*, 1992; Tesfamariam & Cohen, 1992). A number of recent studies have suggested a particularly important role for glycosylated proteins and increased free radical production, both of which can lead to the effective quenching of nitric oxide (Bucala *et al.*, 1991; Corbett *et al.*, 1992; Pieper *et al.*, 1992). These findings are consistent with the reduced relaxation to nitrovasodilators which we have observed in the diabetic rat mesenteric circulation after prolonged diabetes (Taylor *et al.*, 1994), but not in the short term (Taylor *et al.*, 1992). There is also evidence of reduced nitrovasodilator relaxation in the forearm circulation of diabetic patients (Calver *et al.*, 1992; McVeigh *et al.*, 1992).

In conclusion, the results of the present study are consistent with our previous observations of endothelial dysfunction in the mesenteric circulation in STZ-diabetic rats. The lack of effect of treatment with an aldose reductase inhibitor implies that the polyol pathway does not appear to play a predominant role in endothelial dysfunction in this circulation.

This work was funded as part of a collaborative Ph.D. studentship between the Medical Research Council and ZENECA Pharmaceuticals Limited.

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(Received April 23, 1993
Revised August 13, 1993
Accepted September 3, 1993)

Br. J. Pharmacol. (1994), **111**, 49–56

Comparison of the haemodynamic profiles of elgodipine and nicardipine in the anaesthetized dog

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1 The haemodynamic profile of elgodipine (1–30 $\mu g\,kg^{-1}$, i.v.), a new dihydropyridine calcium antagonist, has been compared directly with that of nicardipine (1–30 $\mu g\,kg^{-1}$, i.v.) in chloralose-anaesthetized dogs.

2 Nicardipine produced dose-related systemic, pulmonary and coronary vasodilatation accompanied by reflex tachycardia, inotropy and increases in cardiac output and myocardial oxygen consumption (MVO_2). Elgodipine had similar vasodilator and hypotensive properties to nicardipine but produced less reflex inotropy, little or no reflex tachycardia and did not increase MVO_2.

3 Both calcium antagonists were retested in a separate group of anaesthetized dogs pretreated with propranolol (1 $mg\,kg^{-1}$, i.v.) and atropine (0.3 $mg\,kg^{-1}$, i.v.) to abolish reflex autonomic tone to the heart and thus reveal the direct cardiac effects of each compound. Under these conditions both elgodipine and nicardipine decreased heart rate and cardiac contractility and slowed atrio-ventricular conduction. Elgodipine was approximately ten times more potent than nicardipine as a decelerator agent and slightly more potent in depressing cardiac contractility and increasing PR interval duration. Elgodipine, unlike nicardipine, slightly reduced the QTc interval of the electrocardiogram. Therefore, the potent decelerator effect of elgodipine, which was present throughout the dose-range, appears to be largely responsible for the suppression of reflex tachycardia observed when the baroreflex is functional.

4 Elgodipine is a potent systemic and coronary vasodilator with more marked direct cardiac effects than nicardipine, particularly with respect to slowing of heart rate. The ability of elgodipine to increase coronary blood flow without significant reflex tachycardia or increases in MVO_2 suggests that this compound will have a more favourable effect on myocardial oxygen supply/demand balance than nicardipine. The haemodynamic profile of elgodipine may be suitable for the treatment of angina.

Keywords: Elgodipine; nicardipine; dihydropyridine calcium antagonists; reflex tachycardia; vascular/cardiac selectivity; anaesthetized dog

Introduction

The success of the prototype calcium antagonists, nifedipine, verapamil and diltiazem in the treatment of a range of cardiovascular disorders has generated much interest in this mechanistic approach and has resulted in the development of a number of newer agents from the same drug class. This research for optimised calcium antagonists has focussed largely on variations in tissue selectivity profile and pharmacokinetic properties. Elgodipine is a recent example of the dihydropyridine structural family of calcium antagonists (Tamargo *et al.*, 1991) which demonstrates potent coronary dilator properties in the anaesthetized pig without significant depression of cardiac contractility (Sassen *et al.*, 1990). An acccount of the beneficial effects of elgodipine in patients with coronary artery disease has recently been published (Suryapranata *et al.*, 1992).

We have performed a detailed haemodynamic study of elgodipine in the anaesthetized dog. In order to strengthen the interpretation of the data obtained with elgodipine we have made direct comparison (in the same animals) with nicardipine, an established and clinically proven dihydropyridine calcium antagonist. Our objectives were, firstly, to identify similarities and differences between the two agents, and secondly, to evaluate the extent to which the haemodynamic profile of elgodipine appears appropriate for the treatment of angina or congestive heart failure. Angina is a traditional market for calcium antagonists and their efficacy, based on coronary vasodilator properties, is well accepted. However, occasionally, when used as monotherapy, the dihydropyridine calcium antagonists have been reported to cause paradoxical pro-ischaemic responses in patients with coronary artery disease (Lambert *et al.*, 1985; HINT Research Group, 1986; Gheorghiade *et al.*, 1989). The reasons for such adverse responses are still the subject of discussion, but possible explanations include excessive hypotension causing simultaneous coronary hypoperfusion and reflex tachycardia (which increases oxygen demand), or the phenomenon of coronary steal (Waters, 1991). In contrast, calcium antagonists are not yet established in the treatment of congestive heart failure, largely due to concerns about their potential for cardiac depression as a result of direct negative inotropic properties (Packer, 1989). Hence the interest in agents which appear to be highly vascular selective.

In performing this study we have chosen a chloralose-anaesthetized dog model (Humphries & O'Connor, 1988) which allows the calcium antagonists to be evaluated under two different experimental conditions. Firstly, in 'normal' anaesthetized animals in which baroreceptor reflexes are still functional, such that systemic vasodilatation results in reflex cardiac stimulation (inotropy and chronotropy) as has previously been demonstrated to occur in anaesthetized dogs maintained on chloralose (O'Connor *et al.*, 1982; Humphries & O'Connor, 1988). Subsequently, to test the effects of elgodipine and nicardipine in a second group of otherwise similar animals in which efferent vagal and sympathetic cardiac tone were abolished by pharmacological intervention. This latter series of experiments enables an evaluation of the direct cardiac properties of each compound, uncomplicated by reflex changes in cardiac function. The extrapolation to man of haemodynamic data obtained in anaesthetized animals requires caution. By including a reference agent of known clinical profile (nicardipine) and by controlling the level of function of cardiac reflexes, which in anaesthetized

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animals can vary considerably according to choice of anaesthetic and species, we have attempted to design a study which avoids some of the obvious pitfalls.

Methods

Surgical preparation

Twelve beagle dogs of either sex (13–17 kg) were anaesthetized initially with sodium thiopentone (20 mg kg^{-1}, i.v.) a short-acting barbiturate and anaesthesia maintained throughout the experiment with α-chloralose (80 mg kg^{-1}, i.v. + 20–30 mg kg^{-1} h^{-1}, i.v.). After tracheal intubation, the animals were artificially ventilated (Braun respiratory pump) with ambient air enriched with oxygen such as to maintain blood gas parameters (P_{O_2}, P_{CO_2}, pH) within normal limits.

A catheter was introduced into the right brachial artery and advanced into the thoracic aorta for measurement of arterial blood pressure. The anaesthetic infusion and administration of drugs was effected via catheters placed in left brachial and right femoral veins, respectively. A left thoracotomy was performed at the level of the fourth intercostal space and electromagnetic flow probes positioned around the ascending aorta and the circumflex coronary artery to measure aortic blood flow (cardiac output) and coronary blood flow, respectively. A catheter was placed in the pulmonary artery to measure pulmonary arterial pressure and another positioned in the left atrium to measure left atrial pressure. Left ventricular pressure was measured with a transducer-tipped catheter (Millar Instruments) introduced into the left ventricle via the right femoral artery. In order to preserve baroreceptor function, no catheterization was performed in the carotid vascular beds. The electrocardiogram (ECG, Lead II) was recorded continuously using subcutaneous needle electrodes.

Parameters measured

Aortic, pulmonary and left atrial pressures were measured with Gould Statham transducers connected to Hellige preamplifiers. The first derivative of left ventricular pressure (dP/dt) was obtained using a differentiator and dP/dt_{max} was used as the index of myocardial contractility. Aortic and coronary blood flows were recorded continuously with electromagnetic flowmeters (Carolina Medical Electronics) and the corresponding total peripheral and coronary vascular resistances were calculated by dividing mean arterial pressure by mean blood flow. Pulmonary vascular resistance was calculated by dividing mean pulmonary arterial pressure (following subtraction of mean left atrial pressure) by cardiac output. An indirect index of myocardial oxygen consumption (MVO_2) was calculated according to the formula of Rooke & Feigl (1982) shown below:

$$MVO_2 = 4.08 \times 10^{-4}(SBP \times HR) + 3.25 \times 10^{-4} \frac{(0.8SBP + 0.2DBP) \times HR \times SV}{BW} + 1.43$$

where SBP = systolic blood pressure (mmHg), HR = heart rate (beats min^{-1}), DBP = diastolic blood pressure (mmHg), SV = stroke volume (ml $beats^{-1}$) and BW = body weight (kg).

Left ventricular end diastolic pressure (LVEDP) was measured with a Millar transducer and heart rate measured from the ECG signal as were PR interval (an index of atrio-ventricular conduction time) and QTc interval (an index of ventricular repolarization time) the latter being corrected for changes in heart rate by use of the formula of Bazett (1920).

Experimental protocol

Animals were randomised in two experimental groups. In the first group ($n = 6$), the effects of elgodipine and nicardipine were investigated without any pretreatment. In the second group ($n = 6$), in order to study the effects of the compounds in the absence of cardiac reflexes, dogs were pretreated with propranolol (1 mg kg^{-1}, i.v.) and methylatropine (0.3 mg kg^{-1}, i.v.) to block cardiac sympathetic and vagal tones, respectively.

Each animal received bolus i.v. doses (1, 3, 10, 30 μg kg^{-1}) of elgodipine (E_1, E_3, E_{10}, E_{30}) and nicardipine (N_1, N_3, N_{10}, N_{30}). In each experimental group the order of administration of bolus doses was: E_1, N_1, E_3, N_3, E_{10}, N_{10}, E_{30}, N_{30} for 3 dogs, and N_1, E_1, N_3, E_3, N_{10}, E_{10}, N_{30}, E_{30} for the other 3 dogs. Recovery of basal cardiovascular parameters was awaited prior to administration of the next dose. Approximate recovery times necessary were: 15 min for 1 μg kg^{-1}, 20 min for 3 μg kg^{-1}, 40 min for 10 μg kg^{-1} and 60 min for 30 μg kg^{-1}. Analysis of the effects of the last dose given were followed only for 15 min, sufficient to define maximum effects.

Analysis of results

Cardiovascular parameters were measured just before (predose) and 1, 3, 7 and 15 min after each bolus administration and the percentage changes from corresponding pre-drug values were calculated. Results are expressed as means ± s.e.mean.

The effects of equal doses of elgodipine and nicardipine were compared at each of the dose levels tested. To identify significant differences between these two drugs, statistical analysis was performed on the individual absolute values using a three-way analysis of variance. In addition, to evaluate the effects of individual treatments, comparisons between values obtained at baseline (pre-dose) and at each subsequent measurement period were performed using a Dunnett test. For all cases the threshold for significance was fixed at $P < 0.05$.

Drugs

Elgodipine (IQB) and nicardipine (Sandoz Pharma) were dissolved in an isotonic solution of glucose (5%). Atropine methyl nitrate (Merck) and (±)-propranolol hydrochloride (Erypharma) were dissolved in saline (0.9%). Other drugs used were α-chloralose (Prolabo) and sodium thiopentone (Rhone Merieux).

Results

Tables 1 and 2 contain the basal values of cardiovascular parameters in the two experimental groups i.e. under control conditions and following cardiac autonomic blockade, prior to each drug dose. No significant differences were found (ANOVA) between the pre-dose values prior to elgodipine and the pre-dose values prior to nicardipine for each dose level compared (e.g. E_{30} and N_{30}).

In general the group of animals pretreated with propranolol and atropine demonstrated a lower heart rate, higher LVEDP, reduced cardiac contractility, reduced cardiac output, slower AV conduction and higher total peripheral resistance compared with the untreated group of animals.

The vehicle used for administration of the calcium antagonists (5 ml of isotonic glucose, i.v.) produced no significant modification of cardiovascular parameters in these groups of animals.

Table 1 Chloralose-anaesthetized dogs; basal values of cardiovascular parameters in the control group before each bolus dose

Parameters	*Units*	*Elgodipine* 1 μg kg^{-1}	*Nicardipine* 1 μg kg^{-1}	*Elgodipine* 3 μg kg^{-1}	*Nicardipine* 3 μg kg^{-1}	*Elgodipine* 10 μg kg^{-1}	*Nicardipine* 10 μg kg^{-1}	*Elgodipine* 30 μg kg^{-1}	*Nicardipine* 30 μg kg^{-1}
Heart rate	beats min^{-1}	140 ± 6	136 ± 5	138 ± 5	136 ± 5	137 ± 7	136 ± 6	139 ± 7	132 ± 6
Systolic blood pressure	mmHg	126 ± 6	127 ± 6	125 ± 5	125 ± 5	121 ± 5	124 ± 5	122 ± 4	122 ± 5
Diastolic blood pressure	mmHg	89 ± 4	90 ± 5	88 ± 4	87 ± 4	84 ± 3	85 ± 4	82 ± 4	81 ± 3
Mean blood pressure	mmHg	102 ± 5	102 ± 5	101 ± 4	100 ± 4	96 ± 4	98 ± 4	95 ± 4	95 ± 3
Pulmonary arterial pressure	mmHg	32 ± 2	32 ± 2	32 ± 1	32 ± 2	31 ± 1	31 ± 2	30 ± 2	29 ± 1
Left ventricular end diastolic pressure	mmHg	9.5 ± 0.7	9.6 ± 0.5	9.2 ± 0.6	9.2 ± 0.5	8.6 ± 0.6	8.7 ± 0.5	7.9 ± 0.6	7.8 ± 0.5
Left ventricular dP/dt_{max}	mmHg s^{-1}	2333 ± 245	2354 ± 255	2354 ± 251	2354 ± 273	2312 ± 260	2363 ± 262	2383 ± 244	2408 ± 223
Aortic blood flow	l min^{-1}	1.72 ± 0.17	1.70 ± 0.17	1.72 ± 0.14	1.73 ± 0.16	1.73 ± 0.12	1.70 ± 0.13	1.68 ± 0.10	1.70 ± 0.11
Coronary blood flow	ml min^{-1}	29.7 ± 5.8	30.0 ± 5.5	31.3 ± 5.4	32.0 ± 5.8	30.3 ± 5.0	31.7 ± 5.1	31.3 ± 4.9	30.0 ± 4.7
Stroke volume	ml beat^{-1}	12.5 ± 1.6	12.7 ± 1.5	12.5 ± 1.2	12.8 ± 1.4	12.8 ± 1.0	12.6 ± 1.1	12.2 ± 0.9	12.9 ± 1.0
Total peripheral resistance	mmHg l^{-1} min	61 ± 5	63 ± 6	60 ± 4	60 ± 5	57 ± 4	59 ± 4	57 ± 3	56 ± 2
Pulmonary vascular resistance	mmHg l^{-1} min	13.7 ± 1.2	13.8 ± 1.3	13.3 ± 1.1	13.3 ± 1.3	12.5 ± 0.6	13.0 ± 0.6	12.3 ± 0.8	11.6 ± 0.8
Coronary vascular resistance	mmHg ml^{-1} min	4.07 ± 0.70	3.95 ± 0.64	3.68 ± 0.59	3.62 ± 0.59	3.59 ± 0.54	3.51 ± 0.54	3.40 ± 0.51	3.49 ± 0.44
Myocardial oxygen consumption index	10^{-2} ml O_2 min^{-1} g^{-1}	13.0 ± 0.8	12.7 ± 0.7	12.8 ± 0.8	12.7 ± 0.7	12.4 ± 0.8	12.5 ± 0.8	12.4 ± 0.8	12.1 ± 0.7
PR interval duration	ms	79 ± 3	80 ± 3	80 ± 3	80 ± 3	81 ± 3	79 ± 3	79 ± 3	79 ± 3
Corrected QT interval duration	ms	316 ± 14	312 ± 15	309 ± 13	309 ± 13	310 ± 12	306 ± 12	310 ± 12	302 ± 12

Values are means ± s.e.mean ($n = 6$).

Table 2 Chloralose-anaesthetized dogs; basal values of cardiovascular parameters in the group of animals pretreated with propranolol and atropine before each bolus dose

Parameters	*Units*	*Elgodipine* 1 μg kg^{-1}	*Nicardipine* 1 μg kg^{-1}	*Elgodipine* 3 μg kg^{-1}	*Nicardipine* 3 μg kg^{-1}	*Elgodipine* 10 μg kg^{-1}	*Nicardipine* 10 μg kg^{-1}	*Elgodipine* 30 μg kg^{-1}	*Nicardipine* 30 μg kg^{-1}
Heart rate	beats min^{-1}	127 ± 7	125 ± 7	125 ± 7	125 ± 7	125 ± 7	123 ± 6	122 ± 7	121 ± 6
Systolic blood pressure	mmHg	127 ± 9	128 ± 9	125 ± 9	124 ± 8	122 ± 7	123 ± 6	116 ± 5	119 ± 6
Diastolic blood pressure	mmHg	89 ± 7	90 ± 6	85 ± 6	85 ± 5	81 ± 4	81 ± 4	72 ± 3	76 ± 3
Mean blood pressure	mmHg	102 ± 7	103 ± 7	99 ± 7	98 ± 6	94 ± 5	95 ± 5	86 ± 3	91 ± 4
Pulmonary arterial pressure	mmHg	27 ± 2	27 ± 2	26 ± 2	26 ± 2	26 ± 2	26 ± 2	24 ± 1	25 ± 2
Left ventricular end diastolic pressure	mmHg	11.0 ± 1.0	11.2 ± 0.8	10.6 ± 0.8	11.0 ± 0.8	10.2 ± 0.8	10.1 ± 0.7	9.4 ± 0.7	9.7 ± 0.8
Left ventricular dP/dt_{max}	mmHg s^{-1}	1833 ± 133	1908 ± 142	1883 ± 151	1867 ± 151	1912 ± 134	1925 ± 109	1863 ± 134	1908 ± 110
Aortic blood flow	l min^{-1}	1.22 ± 0.10	1.23 ± 0.08	1.25 ± 0.08	1.23 ± 0.08	1.25 ± 0.06	1.30 ± 0.07	1.27 ± 0.07	1.28 ± 0.06
Coronary blood flow	ml min^{-1}	26.7 ± 4.5	27.3 ± 4.3	28.0 ± 4.6	28.7 ± 4.3	28.0 ± 3.3	30.0 ± 4.3	27.7 ± 2.8	28.3 ± 3.2
Stroke volume	ml beat^{-1}	9.7 ± 0.9	10.0 ± 0.8	10.1 ± 0.8	10.0 ± 0.8	10.2 ± 0.8	10.8 ± 0.9	10.7 ± 1.2	10.8 ± 1.1
Total peripheral resistance	mmHg l^{-1} min	88 ± 14	86 ± 11	81 ± 9	81 ± 9	77 ± 6	75 ± 6	70 ± 5	72 ± 5
Pulmonary vascular resistance	mmHg l^{-1} min	14.0 ± 2.6	13.3 ± 2.6	13.0 ± 2.3	13.1 ± 2.3	13.2 ± 1.9	12.6 ± 2.1	11.5 ± 0.9	12.1 ± 1.4
Coronary vascular resistance	mmHg ml^{-1} min	4.25 ± 0.69	4.08 ± 0.56	3.89 ± 0.58	3.65 ± 0.40	3.60 ± 0.44	3.42 ± 0.39	3.30 ± 0.36	3.40 ± 0.39
Myocardial oxygen consumption index	10^{-2} ml O_2 min^{-1} g^{-1}	11.3 ± 0.9	11.4 ± 0.9	11.1 ± 0.9	11.0 ± 0.9	10.8 ± 0.7	11.0 ± 0.7	10.3 ± 0.6	10.5 ± 0.6
PR interval duration	ms	92 ± 3	91 ± 3	91 ± 3	90 ± 3	90 ± 3	90 ± 3	89 ± 3	90 ± 3
Corrected QT interval duration	ms	324 ± 9	326 ± 7	325 ± 8	324 ± 7	318 ± 7	320 ± 7	316 ± 6	318 ± 6

Values are means ± s.e.mean ($n = 6$).

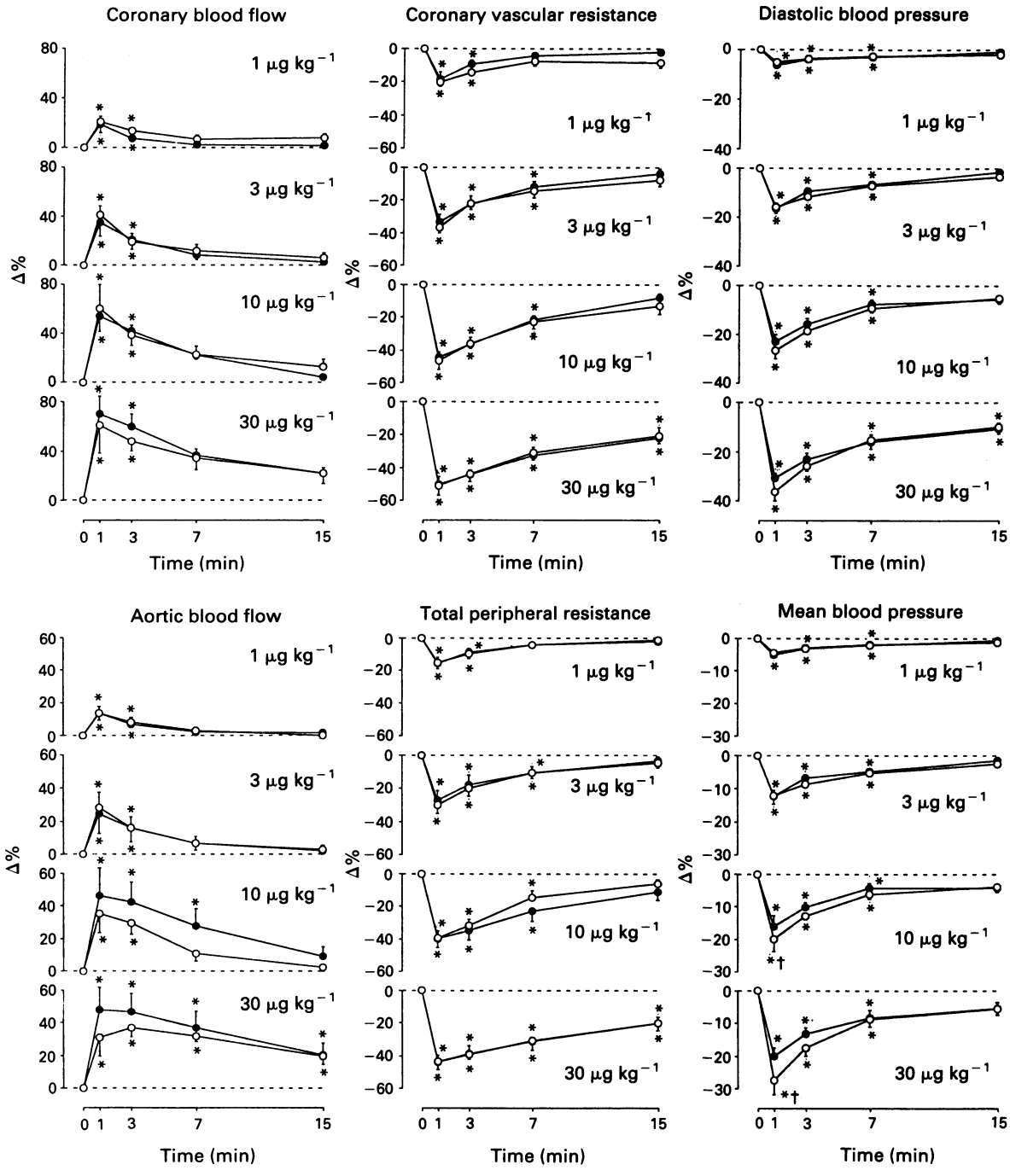

Figure 1 Comparison of the haemodynamic profiles of elgodipine (○) and nicardipine (●) in the control group of anaesthetized dogs, i.e. those having functional baroreflexes. Effects of a series of i.v. bolus doses of each compound are shown against coronary blood flow, coronary vascular resistance, diastolic blood pressure, aortic blood flow, total peripheral resistance and mean blood pressure. Points on the graphs represent mean % changes from pre-dose values ± s.e.mean ($n = 6$), measured 1, 3, 7 and 15 min after dosing. $^*P < 0.05$ versus predosing value; $\dagger P < 0.05$ elgodipine versus nicardipine.

Effects of elgodipine and nicardipine in non-pretreated animals

Figures 1 and 2 show a comparison of the effects produced by the different doses of elgodipine and nicardipine on twelve selected parameters in the control group of animals.

The systemic and coronary vasodilator effects of elgodipine and nicardipine were virtually identical. Over the dose range 1–30 μg kg^{-1}, i.v., both compounds produced quantitatively-similar, dose-related falls in coronary vascular resistance, total peripheral resistance and diastolic blood pressure accompanied by increases in coronary flow. Nicardipine, as a consequence of reflex cardiac stimulation, caused a slightly greater rise in cardiac output than elgodipine with correspondingly smaller reductions in systolic (not shown) and mean blood pressures. In general, the vasodilator effects of both compounds were rapid in onset, peaking after 1 min and recovering gradually thereafter with full recovery requiring 10–40 min depending upon the dose. Pulmonary vascular resistance was also reduced to the same extent by both drugs although the magnitude of the fall was smaller than that observed in systemic or coronary vascular beds (−26 ± 4% after elgodipine 30 μg kg^{-1}, −24 ± 5% after nicardipine 30 μg kg^{-1}). Pulmonary arterial pressure rose to a greater

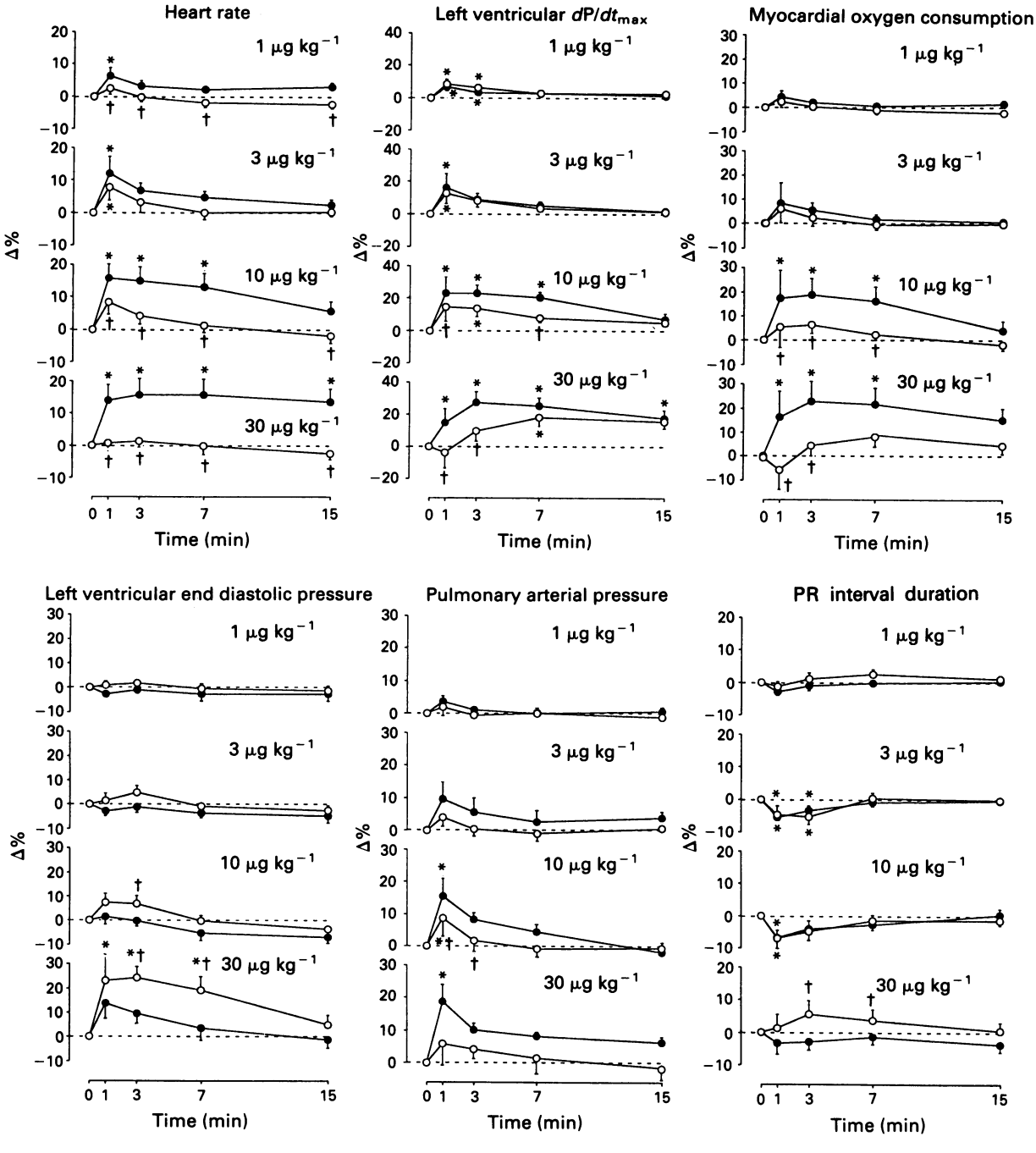

Figure 2 Comparison of the haemodynamic profiles of elgodipine (○) and nicardipine (●) in the control group of anaesthetized dogs, i.e. those having functional baroreflexes. Effects of a series of i.v. bolus doses of each compound are shown against heart rate, left ventricular dP/dt_{max}, myocardial oxygen consumption, left ventricular end diastolic pressure, pulmonary arterial pressure and PR interval duration. Points on the graphs represent mean % changes from pre-dose values ± s.e.mean ($n = 6$), measured 1, 3, 7 and 15 min after dosing. *$P < 0.05$ versus predosing value; †$P < 0.05$ elgodipine versus nicardipine.

extent following nicardipine than following elgodipine (Figure 2).

The vasodepressor effects of nicardipine were accompanied by dose-related increases in cardiac contractility (dP/dt_{max} + 27 ± 7% at 30 μg kg^{-1}) and heart rate (+16 ± 5% at 30 μg kg^{-1}) which were statistically significant at all doses studied. Elgodipine, despite causing similar or greater falls in blood pressure, did not significantly increase heart rate except after 3 μg kg^{-1} (+8 ± 4%). Nicardipine-induced changes in heart rate were greater than those associated with elgodipine at all doses (difference reached statistical significance after 1, 10 and 30 μg kg^{-1}). This contrast between the two compounds is particularly marked at 30 μg kg^{-1} (Figure 2). While nicardipine produced a substantial reflex tachycardia which was well sustained throughout the period during which blood pressure was lowered, heart rate was unchanged following elgodipine. Elgodipine produced significant dose-related increases in dP/dt_{max} which were less marked than those observed after nicardipine (significantly different at the two highest doses). As illustrated by the decrease in PR interval duration, both compounds accelerated AV nodal conduction at 3 and 10 μg kg^{-1} but not at the highest dose tested where elgodipine, in fact, showed a tendency to increase PR interval duration. Nicardipine caused dose-related increases in MVO_2 (+23 ± 9% after 30 μg kg^{-1}) whereas elgodipine did not significantly affect this parameter. This

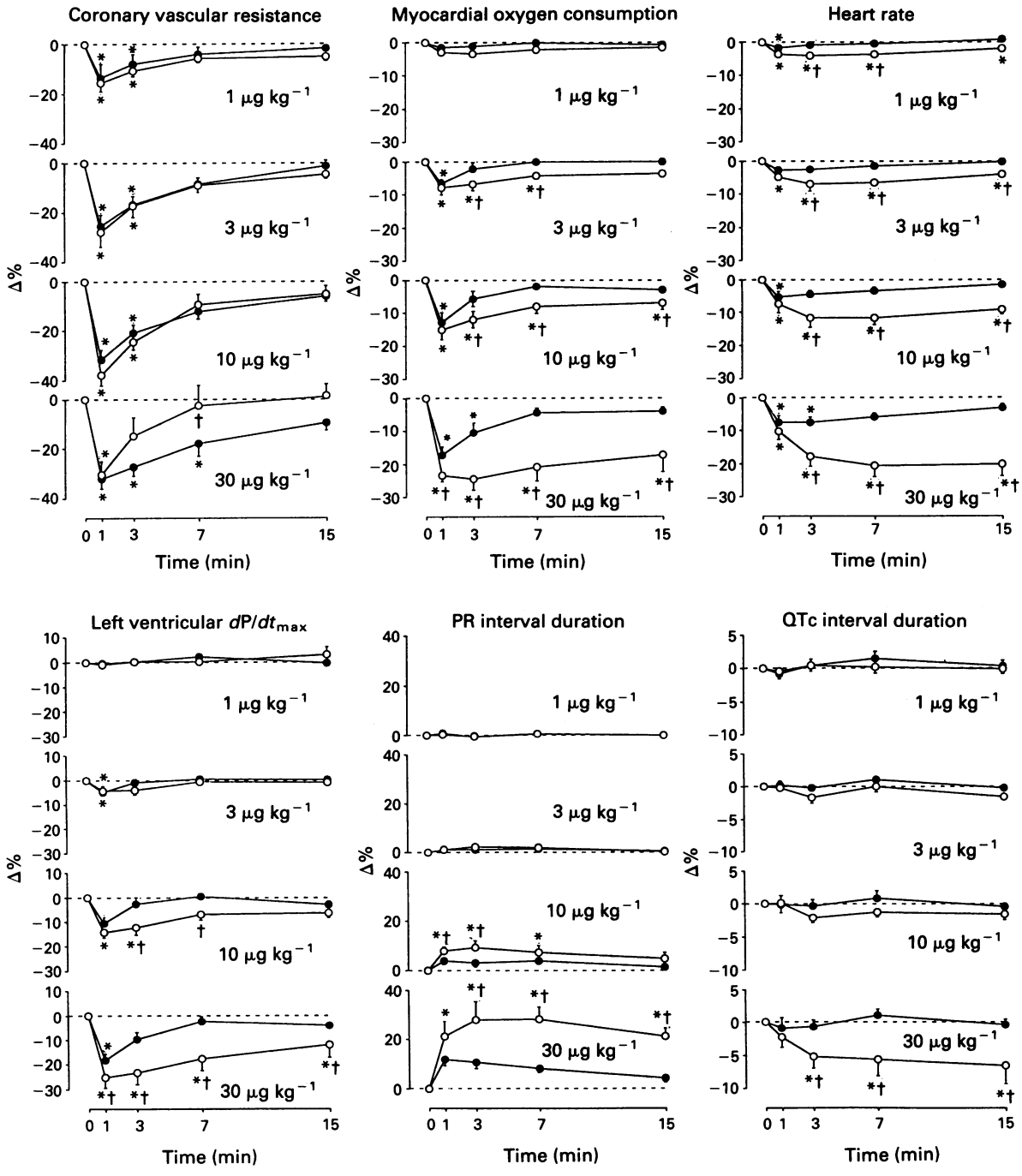

Figure 3 Comparison of the haemodynamic profiles of elgodipine (○) and nicardipine (●) in anaesthetized dogs pretreated with propranolol and atropine to block cardiac autonomic tone. Effects of a series of i.v. bolus doses of each compound are shown on selected parameters, coronary vascular resistance, myocardial oxygen consumpton, heart rate, left ventricular dP/dt_{max}, PR interval duration and QTc interval duration. Points on the graphs represent mean % changes from pre-dose values ± s.e.mean ($n = 6$), measured 1, 3, 7 and 15 min after dosing. *$P < 0.05$ versus predosing value; †$P < 0.05$ elgodipine versus nicardipine.

difference between the two compounds was statistically significant at 10 and 30 μg kg^{-1}. Neither drug affected LVEDP at low doses although elgodipine significantly increased this parameter at the highest dose.

Effects of elgodipine and nicardipine in animals pretreated with atropine and propranolol

Drug effects on selected parameters following elimination of cardiac reflex tone using the combination of propranolol and atropine are shown in Figure 3. Cardiac parameters are displayed preferentially since analysis of the direct cardiac effects of the two products was the objective of this part of the study.

After cardiac autonomic blockade the coronary (Figure 3) and systemic vasodilator properties of elgodipine and nicardipine were quantitatively similar but generally less prominent than observed in the absence of atropine and propranolol. Maximum reduction in total peripheral resistance following the highest doses of elgodipine and nicardipine were −26 ± 7% and −29 ± 6% in animals pretreated with propranolol and atropine, and −43 ± 4% and −44 ± 4% in non-pretreated animals, respectively. However, marked differences between the compounds were apparent on heart rate (Figure

3). Elgodipine 1–30 $\mu g\,kg^{-1}$ produced dose-related bradycardia, significant at all doses ($-21 \pm 3\%$ at 30 $\mu g\,kg^{-1}$) whereas nicardipine caused mild reductions in heart rate only at the two highest doses ($-8 \pm 2\%$ at 30 $\mu g\,kg^{-1}$). Comparison of the dose-response curves indicates that elgodipine was approximately ten times more potent than nicardipine in reducing heart rate. At 10 and 30 $\mu g\,kg^{-1}$, the bradycardia induced by elgodipine was slowly developing and prolonged compared to that induced by nicardipine. Both compounds caused dose-related inhibition of cardiac contractility and slowing of AV nodal conduction starting at 3 $\mu g\,kg^{-1}$ (Figure 3). For both parameters the effects of elgodipine were more pronounced than those of nicardipine, this difference being significant at the higher doses. Unlike nicardipine, elgodipine (30 $\mu g\,kg^{-1}$) resulted in a small but statistically significant abbreviation of the QTc interval ($-7 \pm 3\%$) of the ECG. In the presence of atropine and propranolol, both elgodipine and nicardipine dose-dependently decreased MVO_2, but the effects of elgodipine were longer lasting than those of nicardipine.

It is interesting to note that the difference in heart rate response between the two compounds observed when reflexes were functional was already maximal 1 min after dosing, whereas, following atropine and propranolol the greater inhibitory effect of elgodipine manifests itself progressively, becoming maximal 7 min after dosing. At 1 min the compounds showed similar decelerator properties.

Discussion

As vasodilators in our model, elgodipine and nicardipine appeared extremely similar and well-matched. Both produced dose-related peripheral and coronary vasodilatation over the range studied (1–30 $\mu g\,kg^{-1}$, i.v.) with a potency which was essentially equivalent. In addition, their vasodilator responses had similar kinetic characteristics and durations. A more detailed analysis of the data illustrates that both calcium antagonists showed a very slight selectivity for the coronary bed (e.g. elgodipine 3 $\mu g\,kg^{-1}$ produced $37 \pm 3\%$ reduction in coronary vascular resistance and $30 \pm 5\%$ reduction in total peripheral resistance). Since the coronary vascular bed autoregulates in response to changes in myocardial oxygen demand part of the coronary vasodilatation observed after nicardipine may have been due to the increase in MVO_2. This phenomenon is unlikely to contribute to elgodipine-induced coronary vasodilatation since elgodipine, unlike nicardipine, did not significantly elevate MVO_2 under control conditions. Despite similar pulmonary vasodilator effects, nicardipine increased pulmonary arterial pressure to a greater extent than elgodipine. This effect may be related to the reflex increase in cardiac output which tended to be more pronounced with nicardipine. Neither compound increased pulmonary artery pressure in dogs pretreated with atropine and propranolol (data not shown).

The equivalence of nicardipine and elgodipine as vasodilators in the control group contrasted with the dissimilarity of their cardiac profiles. Systemic vasodilatation and hypotension caused by nicardipine were accompanied at all doses by cardiac stimulation of reflex origin (tachycardia and inotropy). In these and other respects the profile of nicardipine was very similar to that previously reported for nifedipine in the same model (Humphries & O'Connor, 1988). Maintenance of anaesthesia with chloralose favours the manifestation of reflex cardiac effects since chloralose impairs baroreflex function to a lesser extent than other types of anaesthetic (Chenoweth & Van Dyke, 1969). Elgodipine also produced marked falls in diastolic blood pressure ($-36 \pm 4\%$ after 30 $\mu g\,kg^{-1}$, i.v.) but in contrast to nicardipine, these were associated with little or no reflex tachycardia. However, elgodipine did elicit significant reflex positive inotropic effects at all doses although these were smaller than produced by nicardipine. The studies performed in the presence of atropine and propranolol reveal that elgodipine has an important intrinsic inhibitory action on sinus rate. In this respect it was approximately ten times more potent than nicardipine despite having equivalent vasodilator potency. Suppression of reflex tachycardia by elgodipine is therefore largely due to this direct decelerator action of long duration which was present throughout the vasodilator dose-range.

Analysis of the temporal changes in heart rate and blood pressure suggests that, at least following 30 $\mu g\,kg^{-1}$, i.v., elgodipine, an additional property (over and above the direct decelerator action) may contribute to suppression of reflex tachycardia in the control group of animals. In this group of animals with functional reflexes, 1 min after dosing heart rate was unchanged following elgodipine administration whereas nicardipine-induced reflex tachycardia was maximal. However, at this time point in animals pretreated with atropine and propranolol, the direct decelerator effect of elgodipine was sub-maximal and not different from that of nicardipine. This suggests that elgodipine also possesses an anti-accelerator property of neurogenic origin which comes into operation rapidly to reduce reflex elevations of heart rate. One possible explanation would be an inhibitory effect of elgodipine on baroreceptor function. Changes in baroreceptor sensitivity have been reported following administration of other calcium antagonists (Heesch *et al.*, 1983; Warltier *et al.*, 1984).

The cardiovascular profile of elgodipine and, in particular, its ability to cause systemic and coronary vasodilatation without significant reflex tachycardia, distinguishes it from most other dihydropyridines which would tend to demonstrate a nicardipine-like profile in this model. However, FPL 62129 (Humphries & O'Connor, 1988) and MDL 72567 (Difrancesco *et al.*, 1986) are dihydropyridines which have also been reported to lower blood pressure acutely without reflex tachycardia in a fashion similar to elgodipine. In terms of overall profile, elgodipine appears to be positioned midway between, at one extreme, the vascular-selective dihydropyridines and, at the other, calcium antagonists of the verapamil and diltiazem types which show much more prominent direct cardiac effects, particularly on sinus and AV nodes (Taira, 1987). Presumably the mechanism responsible for the direct decelerator effect of elgodipine involves block of L-type calcium channels which play an important role in impulse generation and conduction in nodal tissues. In addition to blocking L-type calcium channels, elgodipine has been shown to possess antagonistic activity at T-type calcium channels in rat portal vein (J. Mironneau, personal communication). A similar T-type calcium channel exists in cardiac tissue where it is thought to have a role in pacemaker function (Hagiwara *et al.*, 1988). Therefore the possible involvement of T-channel block in the potent direct decelerator action of elgodipine cannot be excluded.

Haemodynamic studies with elgodipine in the anaesthetized pig (Sassen *et al.*, 1990) and in the conscious pig following myocardial infarction (van Woerkens *et al.*, 1991) have shown that elgodipine-induced vasodilatation is not associated with significant depression of cardiac contractility, leading to the suggestion that elgodipine would be suitable for use in congestive heart failure. However, our data indicate that elgodipine demonstrates only a moderate degree of vascular selectivity (i.e. vasodilator potency compared with depression of cardiac contractility) and is less vascular-selective than nicardipine. In our study the two compounds were equipotent as vasodilators while elgodipine produced greater inhibition of dP/dt_{max} than nicardipine when cardiac autonomic tone was eliminated. Similarly, reflex increases in dP/dt_{max} were smaller following elgodipine than with nicardipine. Clearly, there are many differences between the present study and those performed in the pig by other investigators (e.g. species, methodology, anaesthesia), however our comparison of nicardipine and elgodipine in the same animals remains a key element for interpretation of the data. Our study suggests that elgodipine does not possess marked vascular selectivity

and, under conditions when reflex cardiac activation is impaired (as commonly occurs in heart failure), manifests a negative inotropic effect at doses of $3\,\mu g\,kg^{-1}$, i.v. and greater. Therefore, it may not offer advantages over nicardipine in the treatment of heart failure.

When viewed in the context of angina the haemodynamic profile of elgodipine appears more attractive. Reflex tachycardia is an undesirable consequence of acute vasodilatation which may worsen cardiac ischaemia, either as a result of increased myocardial oxygen demand, or by reducing diastolic filling time thus impairing coronary perfusion (Boudoulas *et al.*, 1979). Unlike nicardipine which, when reflexes were functional, produced dose-related increases in MVO_2, elgodipine did not change this parameter significantly. Hence in our model, elgodipine shows potent coronary vasodilatation unaccompanied by increases in MVO_2, suggesting that it will have a favourable effect on myocardial oxygen supply/demand balance, a profile appropriate for the treatment of angina. To illustrate the possible clinical significance of this difference in profile between elgodipine and nicardipine, the latter drug has been reported to show pro-ischaemic properties (associated with tachycardia) in coronary artery disease patients (Lambert *et al.*, 1985; Gheorghiade *et al.*, 1989). However, whether elgodipine offers clinically-significant advantages over other dihydropyridines remains to be established. Reflex tachycardia to nifedipine is largely an acute phenomenon tending to diminish on chronic treatment and may be countered by co-administration of a β-adrenoceptor blocker, a regime which is acceptable in angina patients.

The effects of elgodipine on ECG are of interest in the light of a report (Bellissant *et al.*, 1991) that the compound, rather unexpectedly given the absence of changes in arterial pressure and heart rate, accelerated AV conduction and ventricular repolarization in human volunteers. In the anaesthetized dog, elgodipine produced changes in PR interval which were clearly the composite of reflex acceleration of AV nodal conduction and, at high doses, the direct conduction slowing property of the compound. In this respect the profile of elgodipine was as predicted, qualitatively similar to nicardipine and very like that of FPL 62129 (Humphries & O'Connor, 1988). However unlike nicardipine, elgodipine, at the highest dose tested, produced a small but statistically significant decrease in QTc interval, indicative of accelerated ventricular repolarization. Since this occurred in the presence of atropine and propranolol it represents a direct cardiac effect of the compound. The mechanism and the significance of this observation remain to be established.

In summary, elgodipine has systemic and coronary vasodilator properties similar to those of nicardipine but more important direct inhibitory cardiac effects, particularly with respect to the sinus node. Unlike nicardipine its hypotensive effects in chloralose-anaesthetized dog are not accompanied by reflex tachycardia or increases in MVO_2.

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(Received August 17, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 57–64

Effects of metabolic blockers on Ca^{2+}-dependent currents in cultured sensory neurones from neonatal rats

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1 The whole cell variant of the patch clamp technique was used to record high voltage-activated Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents from cultured neonatal rat dorsal root ganglion neurones. The aim of the project was to use these currents as physiological indices of intracellular Ca^{2+} regulation under control conditions and in the presence of metabolic inhibitors.

2 Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 μM) and sodium cyanide (1 μM) inhibited Ca^{2+} currents within 20 s, even when ATP was present in the patch pipette solution, suggesting a direct action on Ca^{2+} channels. These metabolic inhibitors did not affect Ca^{2+} current 'run down' or inactivation kinetics.

3 Cultured neonatal dorsal root ganglion neurones of the rat were relatively insensitive to the removal of glucose and ATP from the recording solutions for up to 3 h. These data suggest that the Ca^{2+} homeostatic mechanisms in these cells are highly resistant to metabolic insult.

4 However 2-deoxy-D-glucose (5 mM) in the extracellular recording medium with no ATP or glucose present did prolong the deactivation time of Ca^{2+}-activated Cl^- tail currents and increase the total charge flow following activation of a 500 ms voltage-activated Ca^{2+} current. This effect was prevented by inclusion of D-fructose 1,6-diphosphate (500 μM) in the patch pipette solution.

5 We conclude that some agents used to induce chemical hypoxia, such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and sodium cyanide, may interact directly with voltage-activated Ca^{2+} channels and are therefore not appropriate for use in studying disturbed neuronal Ca^{2+} homeostasis. However, the use of 2-deoxy-D-glucose in the absence of glucose and ATP does represent a model of disturbed Ca^{2+} homeostasis in cultured dorsal root ganglion neurones. In this study we have combined the whole cell recording technique with cultured neurones under conditions which produce a degree of metabolic stress as reflected by prolonged Ca^{2+}-activated Cl^- tail currents. The reduced efficiency of handling of intracellular Ca^{2+} loads may be an important factor contributing to the onset of neuronal damage during hypoxia and ischaemia.

Keywords: Cultured neurones; Ca^{2+} currents; Ca^{2+}-activated chloride currents; intracellular Ca^{2+} homeostasis; metabolic inhibition; cyanide; 2-deoxyglucose; fructose 1,6-diphosphate

Introduction

The damaging effects of hypoxia and ischaemia on certain neuronal populations appears to be the result of a failure of intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis, with the subsequent loss of control of numerous essential intracellular Ca^{2+}-dependent processes (Siesjö, 1981; Choi, 1988).

The maintenance of appropriately low free $[Ca^{2+}]_i$ is achieved by complex intracellular buffering mechanisms sited in the mitochondria, the endoplasmic reticulum and via Ca^{2+} binding proteins (Miller, 1991). Nevertheless, ultimately Ca^{2+} ions must be extruded across the plasma membrane to the external environment and this is achieved by several mechanisms including the Na^{2+}-Ca^{2+} exchanger (Blaustein, 1988) and the membrane bound Ca^{2+}-ATPase (McBurney & Neering, 1987; Benham *et al.*, 1992). These systems require ATP either directly, as in the case of the Ca^{2+}-ATPase, or indirectly due to the dependence on ATP of the Na^+-K^+ exchange required for normal functioning of the Na^+-Ca^{2+} exchange process and possibly for phosphorylation of the Na^+-Ca^{2+} exchange protein itself (DiPolo & Beaugé, 1983; 1987; Lagnado & McNaughton, 1990). An adequate supply of ATP is therefore essential for the maintenance of Ca^{2+} homeostasis in neurones and any impairment of this supply as occurs during hypoxia or ischaemia may lead to potentially harmful rises in $[Ca^{2+}]_i$. Maintained depolarization with prolonged activity of voltage-activated Ca^{2+} channels and activation of glutamate receptors, particularly the N-methyl-D-aspartate receptor ion channel complex, result in Ca^{2+} loads which pose a challenge to compromised Ca^{2+} homeostatic mechanisms (Meldrum *et al.*, 1985; Choi, 1988).

The whole cell recording configuration of the patch clamp technique is a powerful tool for the investigation of neuronal Ca^{2+} currents and Ca^{2+}-activated currents. Measurements of both Ca^{2+} induced inactivation of high voltage-activated Ca^{2+} currents (Chad & Eckert, 1986) and Ca^{2+}-activated Cl^- tail currents ($I_{Cl(Ca)}$) (Owen *et al.*, 1984; Mayer, 1985; Bader *et al.*, 1987; Currie & Scott, 1992) act as physiological indices of $[Ca^{2+}]_i$ close to the cell plasma membrane. We have studied the effects of various metabolic insults on these currents used as indicators of the efficiency of neuronal Ca^{2+} homeostasis. Previously we have shown that the lipid metabolite palmitoyl-DL-carnitine slows the rate of decay of $I_{Cl(Ca)}$, reflecting a change in the efficiency of cellular Ca^{2+} homeostasis (Stapleton *et al.*, 1992) and the possible involvement of caffeine-sensitive intracellular Ca^{2+} stores. A simple *in vitro* model of neuronal ischaemia/hypoxia, suitable for application to the whole cell configuration of the patch clamp technique would allow greater understanding of the processes involved in the onset of neuronal damage produced by such insults and may prove useful as an 'assay' system for the evaluation of agents with potential neuroprotective properties.

We have taken two approaches to simulate metabolic stress in our system: (1) the inhibition of oxidative phosphorylation by use of cyanide and the proton ionophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)

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(McLaughlin & Dilger, 1980); (2) the abolition of glucose uptake and metabolism with 2-deoxy-D-glucose (2-DG) and by removing ATP and glucose from the experimental recording conditions.

Methods

Cell culture

Primary cultures of dorsal root ganglion (DRG) neurones were prepared from 2 day old Wistar rats. DRG neurones were enzymatically and mechanically dissociated, plated on laminin-polyornithine coated cover-slips and maintained in culture for 2–3 weeks at 37°C in humidified air with 5% CO_2. F14 culture medium (Imperial Laboratories) supplemented with 10% horse serum (Gibco) was used with added penicillin (5000 i.u. ml^{-1}), streptomycin (5000 $\mu g\ ml^{-1}$), $NaHCO_3$ (14 mM) and nerve growth factor (20 ng ml^{-1}, Sigma). Cell cultures were refed with F14/horse serum mixture containing nerve growth factor (10 ng ml^{-1}) every 7 days.

Electrophysiology

The whole cell variant of the patch clamp technique was used (Hamill *et al.*, 1981). DRG neurones were voltage clamped by use of an Axoclamp 2A switching amplifier operated at a sampling rate of 28–35 kHz, with low resistance (3–7 MΩ) micropipettes. A pulse generator (Digitimer D4030) provided the timing of voltage command protocols. All experiments were performed at room temperature (approx. 23°C).

Standard recording medium contained (in mmol l^{-1}): choline chloride 130, KCl 3.0, $MgCl_2$ 0.6, $NaHCO_3$ 1.0, HEPES 10.0, tetraethylammonium bromide 25.0, D-glucose 4.0, $CaCl_2$ 2.0 and tetrodotoxin 0.0025. The pH was adjusted to 7.4 with NaOH and the osmolarity to 320 mOsmol l^{-1} with sucrose. The patch pipette solution contained (in mmol l^{-1}): CsCl 140, EGTA 1.1, $MgCl_2$ 2.0, $CaCl_2$ 0.1, ATP 2.0, HEPES 10.0, the pH was corrected to 7.2 with Tris (1 mM) and the osmolarity to 310 mOsmol l^{-1} again with sucrose. The estimated free Ca^{2+} concentration in the patch pipette solution was 16 nM. A modest amount of EGTA (1.1 mM) was used to buffer the Ca^{2+} in the patch pipette solution; this was present to ensure stability of Ca^{2+} currents and Ca^{2+}-activated currents under control conditions. The concentration of EGTA used was not sufficient to inhibit Ca^{2+}-activated Cl^- tail currents or currents activated by release of Ca^{2+} from intracellular stores. For several experiments both glucose and ATP were excluded from the recording and patch solutions respectively. In these cases the osmolarity was made up to the appropriate value with mannitol.

Drugs, dissolved in recording medium and after adjustment of pH, were applied extracellularly by low pressure ejection via a micropipette (tip diameter approximately 10 μm) positioned within 100 μm of the cell being recorded. Alternatively, DRG neurones were bathed in the recording medium containing the drug or incubated in culture medium containing the drug for varying periods prior to an experiment. Intracellular drug application was performed by including the drug in the patch pipette solution after suitable adjustment of the pH.

FCCP and sodium cyanide were obtained from Sigma; fructose 1,6-diphosphate (F1,6-DP) and 2DG from ICN Biochemicals.

Analysis

Net Ca^{2+} currents were evaluated following scaled linear subtraction of leakage and capacitative currents. Ca^{2+}-activated currents ($I_{Cl(Ca)}$) were assessed by measurement of the current amplitude 20 ms after the end of the voltage step command and by the time to reach 63% decay of the maximal inward current amplitude ($t_{63\%}$). An estimation of the total charge flow (Q) for each tail current was also made by measurement of the area under the tail current decay curve using an integration programme for tail currents of less than 4 s while for longer tail currents the traces were weighed. Since there was a wide distribution of tail current total charge flow these values have also been normalised (Q%) to give values as percentages of the response to the first voltage step command in each series.

All data are given as mean ± standard error of the mean (s.e.mean) and statistical significance has been assessed by a two-tailed Student's *t* test.

Results

Inhibition of oxidative phosphorylation

FCCP Extracellular application of the proton ionophore FCCP (5 μM) for 3 to 5 min by low pressure ejection caused a reduction in the steady state peak Ca^{2+} current amplitude (I_{max}) and the amplitude of the current measured at the end of a 100 ms voltage step command (I_{end}) when DRG neurones were activated from a holding potential (V_h) of −90 mV by voltage step commands to a clamp potential (V_c) of 0 mV (Table 1). This represents a 47% and 50% reduction in I_{max} and I_{end} respectively. The degree of inactivation of the currents was unaffected by FCCP (5 μM), with 54% inactivation of control currents and 51% inactivation occurring in the presence of FCCP, measured during a 100 ms voltage step command ($n = 8$). In five cells, incomplete recovery was seen 5 to 10 min after removal of the pipette containing FCCP (Table 1). The I_{max} recovered to 66% of its original value while I_{end} recovered to 81% of its original value.

Brief application (10–20 s) of FCCP (5 μM) reduced both I_{max} and I_{end} to the same extent as when applied until a steady state value had been reached as described above, I_{max} being reduced by 43% and I_{end} by 49% (Table 1). The recovery in all three cells was to 77% and 70% of the original value for I_{max} and I_{end} respectively. Hence the reduction in current amplitude occurs rapidly with no change in the degree of inactivation.

Low-voltage-activated Ca^{2+} currents, produced by stepping to a V_c of −30 mV, were not specifically studied in this investigation but on the occasions when they were seen, the

Table 1 Action of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 5 μM) on voltage-activated Ca^{2+} currents

Conditions	(n)	I_{max} (nA)	I_{end} (nA)
Control	8	−2.39 ± 0.27	−1.31 ± 0.23
3–5 min application	8	−1.27 ± 0.36**	−0.65 ± 0.26*
recovery	5	−1.59 ± 0.45	−1.06 ± 0.33
Control	3	−1.97 ± 0.24	−0.91 ± 0.16
10–20 s application	3	−1.14 ± 0.35	−0.46 ± 0.06

I_{max} and I_{end} were the Ca^{2+} current amplitude measured at the peak and end of 100 ms voltage step commands to 0 mV respectively. **$P < 0.005$; *$P < 0.02$ when compared with control values.

low voltage-activated component was also inhibited (Figure 1).

The current-voltage relationship for a cell under control conditions and after application of FCCP (5 μM) is shown in Figure 1. There was no shift in the voltage-dependence of activation and no change in the null potential (the potential at which there is no net current flow through the membrane).

To ensure that the effect seen with FCCP was not due to an increase in Ca^{2+} mediated inactivation we increased the EGTA concentration of the patch pipette solution to 20 mM. Under these conditions FCCP (5 μM) applied for 3 min inhibited I_{max} and I_{end} by 32% and 41% respectively ($n = 7$, $P < 0.02$). Additionally no FCCP-induced currents were seen during any of the experiments. These findings suggest that the major action of FCCP in reducing I_{Ca} is not by a Ca^{2+}-induced Ca^{2+} release mechanism.

In three cells to which FCCP (5 μM) was applied, Ca^{2+}-activated Cl^- tail currents were seen. The mean amplitude of the $I_{Cl(Ca)}$ measured 20 ms after the 100 ms voltage step command was -1.76 ± 0.54 nA under control conditions and -0.61 ± 0.16 nA after application of FCCP (5 μM) ($n = 3$). FCCP had no significant action on the decay of $I_{Cl(Ca)}$. However, this action was associated with a reduction in mean I_{max} of 58% and therefore much less Ca^{2+} was available to activate $I_{Cl(Ca)}$.

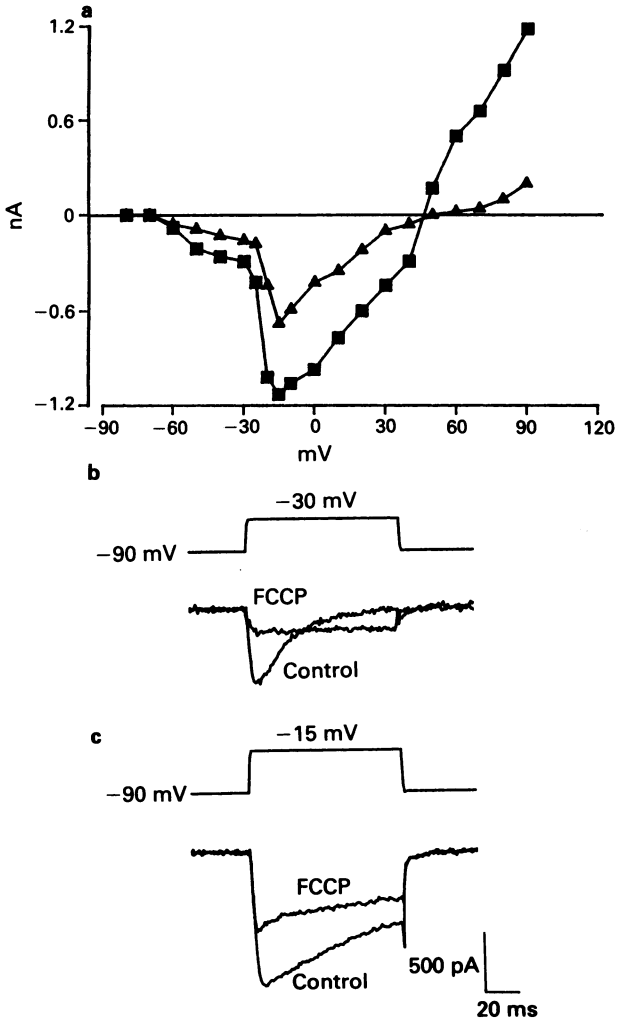

Figure 1 The actions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the peak voltage activated Ca^{2+} currents. (a) The current-voltage relationship for a neurone recorded under control conditions (■) and after 3 to 5 min application of 5 μM FCCP (▲). The maximum peak inward current is plotted after leak subtraction for each voltage step. Note that FCCP reduces Ca^{2+} channel currents over a wide voltage range without producing any shift in voltage dependence. Inset traces show, (b) low voltage-activated, T-type current and (c) high voltage-activated current activated from a holding potential of −90 mV by step depolarizations to −30 mV and −15 mV respectively. FCCP appears to affect the inactivation properties of the low threshold current as well as reduce the peak amplitude of high threshold Ca^{2+} currents.

In three cells the steady state inactivation kinetics were studied for both I_{max} and I_{end}. The potentials at which 50% steady-state inactivation occurred ($V_{0.5}$) for I_{max} and I_{end} after application of FCCP (5 μM) were not significantly different from values obtained under control conditions.

Similar results to those described above were obtained in three cells in which the concentration of FCCP used was 10 μM. I_{max} was reduced from -2.85 ± 0.47 nA to -1.47 ± 0.51 nA and I_{end} was reduced from -1.69 ± 0.44 nA to -1.05 ± 0.54 nA (48% reduction in I_{max} and 38% reduction in I_{end} with 65% and 81% recovery at 5 min respectively, $n = 3$).

More prolonged exposure to FCCP (5 μM) was studied by pretreating the cells with FCCP in the recording medium for 10 or 30 min or by incubation in the culture medium for 18 h. The cells were washed prior to an experiment and recording of Ca^{2+} currents made in normal recording medium in the absence of FCCP. After 10 or 30 min pretreatment I_{max} was measured every minute for 5 min and at 3 min intervals thereafter. There was no significant change in I_{max} nor in the degree of inactivation over the course of 8 min recording (Figure 2).

Eighteen hours incubation of cells with FCCP (5 μM) in the culture medium again did not give rise to any significant change in I_{max} nor in the degree of inactivation when currents were activated using the same protocol as above (inactivation of 44% and 45% at 1 and 5 min respectively, $n = 7$; Figure 2). However, after 18 h in culture with FCCP (5 μM) four out of seven cells were unable to tolerate the patch clamp conditions for more than 5 min. In the remaining three cells 'run-down' of the current over the course of 15 min recording occurred at a similar rate to that which would be expected under control conditions.

Cyanide The effects of sodium cyanide (1 μM) were studied in DRG neurones by both intra- and extracellular application.

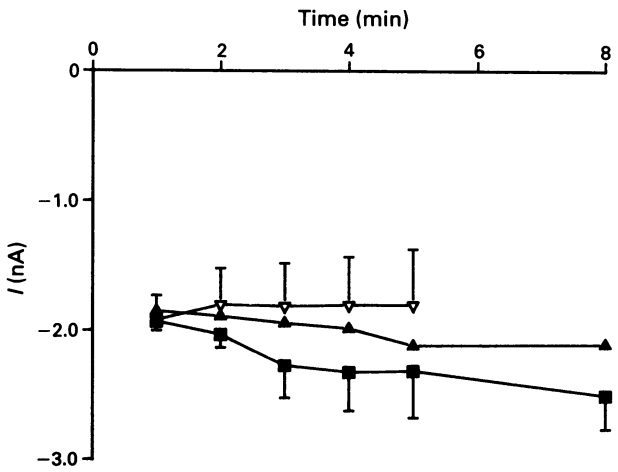

Figure 2 The effects of prolonged application of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on peak high voltage-activated Ca^{2+} currents. (a) Graph of time plotted against mean peak Ca^{2+} current recorded from cells pretreated with 5 μM FCCP for 10 min (■, $n = 3$), 30 min (▲, $n = 3$) and 18 h (▽, $n = 7$). Surprisingly, long term treatment with FCCP has no significant effect on Ca^{2+} currents recorded from cultured DRG neurones. The apparent increase in current during 8 min recording from cells treated for 10 min with FCCP was not signigicant. However, this may represent some additional recovery from block with FCCP.

Extracellular application of cyanide (1 μM) by low pressure ejection caused a reduction in the mean I_{max} and mean I_{end} of the Ca^{2+} current (Figure 3). The percentage inhibition of I_{max} and I_{end} was 58% ($P<0.01$) and 27% ($P<0.02$) respectively ($n = 8$). The mean degree of inactivation was 59% for control currents and 28% in the presence of cyanide ions (1 μM) which may reflect less inactivation produced by smaller Ca^{2+} currents or some degree of differential sensitivity of different voltage-activated Ca^{2+} channels with distinct inactivation properties. On removing the pressure ejection pipette no significant recovery in 10 min was seen in any component of the Ca^{2+} current ($n = 8$) (Figure 3).

Intracellular application was achieved by inclusion of sodium cyanide (1 μM) in the patch pipette solution. Calcium currents were activated by 100 ms voltage step commands from a V_h of −90 mV to a V_c of 0 mV immediately on entering the whole cell configuration and at 1 min intervals thereafter for 4 min. Over this time I_{max} was reduced from −1.52 ± 0.17 nA to −1.06 ± 0.20 nA, a mean reduction in maximal Ca^{2+} current amplitude of 30% ($n = 12$, $P<0.02$) and I_{end} was reduced from −0.77 ± 0.12 nA to −0.66 ± 0.16 nA, a 14% reduction in mean I_{end} ($n = 12$). The degree of inactivation of the currents on first entering the whole cell configuration was 49% while that after 4 min recording was 38%, suggesting that the inactivation kinetics were unaffected.

The initial results with both cyanide and FCCP demonstrated that, in cultured DRG neurones, Ca^{2+} homeostatic mechanisms were surprisingly resistant to the effects of metabolic blockade with these agents. However, since ATP was present in the patch solution and because of the rapid onset and achievement of a steady-state Ca^{2+} current inhibition and, in the case of FCCP, rapid but partial recovery was seen, we suggest that both drugs may interact with Ca^{2+} channels or membrane constituents which result in inhibition of the current. Both low and high voltage-activated Ca^{2+} currents were attenuated by FCCP and cyanide. Steady state inactivation of the high voltage-activated Ca^{2+} currents was unaffected. These findings indicate that FCCP and cyanide may interact in a nonselective manner rather than with a distinct type of Ca^{2+} channel. For these reasons we changed the approach to producing metabolic stress by inhibiting glycolysis using 2-deoxyglucose with no ATP or glucose in the recording solutions.

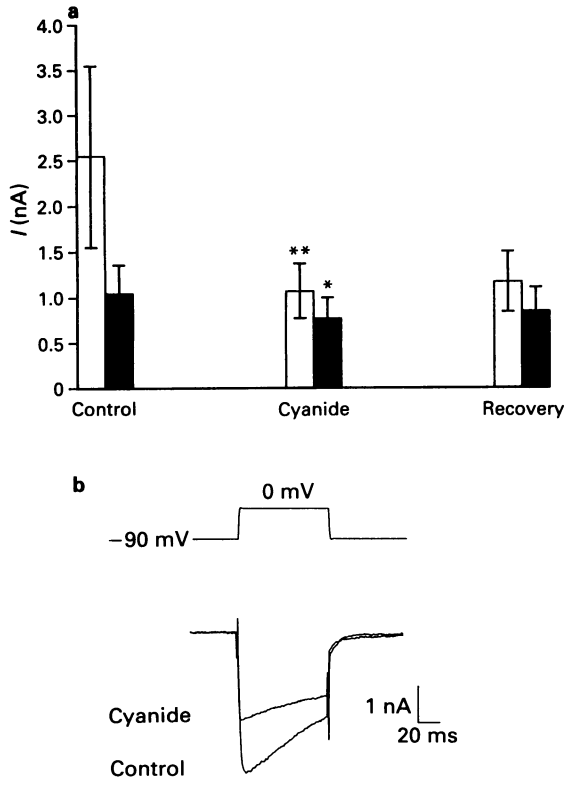

Figure 3 The effect of 1 μM sodium cyanide on high voltage-activated Ca^{2+} currents. (a) Chart showing the inhibitory action of cyanide on high voltage-activated Ca^{2+} currents measured at the peak (I_{max}, open columns) and at the end of the 100 ms voltage step command (I_{end}, solid columns). Data shown are the mean ± s.e.mean from eight experiments. Cyanide (1 μM) was applied extracellularly for 3 to 5 min and no recovery was observed 5 min after removal of the pressure ejection pipette containing cyanide. *$P<0.02$; **$P<0.01$. (b) Traces showing high voltage-activated Ca^{2+} currents recorded under control conditions and following inhibition by cyanide (1 μM). All data was recorded from cells voltage clamped at a holding potential of −90 mV.

Inhibition of glycolysis

No ATP/glucose Experiments were performed on DRG neurones bathed, for between 30 min and 3 h, in recording medium in which no glucose was present, the osmolarity being corrected with mannitol and with patch pipette solution in which ATP was excluded. Again Ca^{2+} currents were activated on entering the whole cell recording configuration and every 3 min thereafter by 100 ms voltage step commands from a V_h of −90 mV to a V_c of 0 mV. No significant change in either I_{max} or I_{end} was seen in the course of 15 min recording (I_{max} −1.79 ± 0.28 nA, I_{end} −1.08 ± 0.26 nA at 3 min; I_{max} −1.82 ± 0.30 nA, I_{end} −0.98 ± 0.42 nA at 15 min, $n = 6$).

2-Deoxy-D-glucose Neuronal glycolysis was also inhibited by the inclusion of 2DG (5 mM) in the glucose-free recording medium. Calcium currents and Ca^{2+}-activated currents were activated by both 100 ms and 500 ms depolarizing voltage step commands again using patch pipette solution without ATP. For each cell, 100 ms depolarizing voltage steps were applied immediately on entering the whole cell recording configuration and at 15 s intervals for 1 min. This was followed after a further minute by 500 ms depolarizing steps at 1 min intervals. Voltage-activated Ca^{2+} currents were studied in addition to Ca^{2+}-activated Cl^- tail currents (Currie & Scott, 1992) measured 20 ms after the end of the depolarizing step command ($I_{Cl(Ca)}$) and by an assessment of the time to 63% decay of this current ($t_{63\%}$) and of the total charge flow during the tail current (Q). Calcium-activated tail currents were seen in approximately 52% (16/31 cells) of cells studied.

In control cells with normal patch and recording media without 2DG, consecutive 100 ms depolarizing voltage step commands every 15 s caused no significant change in the properties of voltage-activated Ca^{2+} currents produced at a V_c of 0 mV ($n = 11$). Likewise with 2DG present, four 100 ms depolarizing voltage steps had no affect on Ca^{2+} currents ($n = 11$). The amplitude of the $I_{Cl(Ca)}$ measured 20 ms after the end of the 100 ms voltage step command was not significantly altered by the activation of consecutive steps. Under control conditions the mean amplitude of $I_{Cl(Ca)}$ changed from −1.09 ± 0.26 nA to −1.18 ± 0.30 nA, and with 2DG present $I_{Cl(Ca)}$ had values of −1.29 ± 0.18 nA and −1.11 ± 0.19 nA at the 1st and 4th steps respectively ($n = 11$). Additionally the times to 63% decay ($t_{63\%}$) of $I_{Cl(Ca)}$ were not significantly changed by 2DG when the current was activated following a 100 ms Ca^{2+} current. Under control conditions $t_{63\%}$ was 680 ± 180 ms and 860 ± 240 ms ($n = 11$) and with 2DG present the mean values were 850 ± 240 ms and 1090 ± 280 ms ($n = 11$) for the 1st and 4th steps respectively. Again the total charge flow (Q) during the full time course of the tail current activated by a 100 ms voltage step command was not significantly altered for both control cells and those treated with 2DG. These data show that 2DG has no significant action on Ca^{2+} currents activated by a 100 ms voltage step command nor on the accompanying Cl^- tail current. As a result, we decided to increase the intracellular

Table 2 Comparison of Ca^{2+} currents and Ca^{2+}-activated Cl^- currents recorded under control conditions and with 2-deoxy-D-glucose (2DG, 5 mM)

Step No.	I_{max} (nA)	I_{end} (nA)	$I_{Cl(Ca)}$ (nA)	$t_{63\%}$ (ms)
Control $n = 7$				
1	-1.10 ± 0.22	-0.49 ± 0.10	-1.66 ± 0.45	2990 ± 660
2	-1.01 ± 0.20	-0.42 ± 0.09	-1.38 ± 0.36	3140 ± 730
3	-1.00 ± 0.20	-0.43 ± 0.09	-1.31 ± 0.34	3040 ± 750
4	-0.96 ± 0.19	-0.41 ± 0.08	-1.29 ± 0.36	2800 ± 690
2-Deoxyglucose (5 mM) $n = 11$				
1	-1.19 ± 0.19	-0.31 ± 0.10	-1.25 ± 0.18	4000 ± 800
2	-1.10 ± 0.20	-0.25 ± 0.09	-1.14 ± 0.18	5580 ± 1100*
3	-0.99 ± 0.18	-0.18 ± 0.10	-1.03 ± 0.17	7610 ± 1790*
4	-0.94 ± 0.17	-0.14 ± 0.10	-0.99 ± 0.18	7350 ± 2080*

I_{max} and I_{end} were the Ca^{2+} current amplitude measured at the peak and end of 500 ms voltage step commands to 0 mV. $I_{Cl(Ca)}$ was the tail current amplitude measured 20 ms after the end of the voltage step command and $t_{63\%}$ was the time for the tail current to deactivate by 63% of $I_{Cl(Ca)}$.
*$P < 0.05$, values compared with step no. 1 with 2DG.

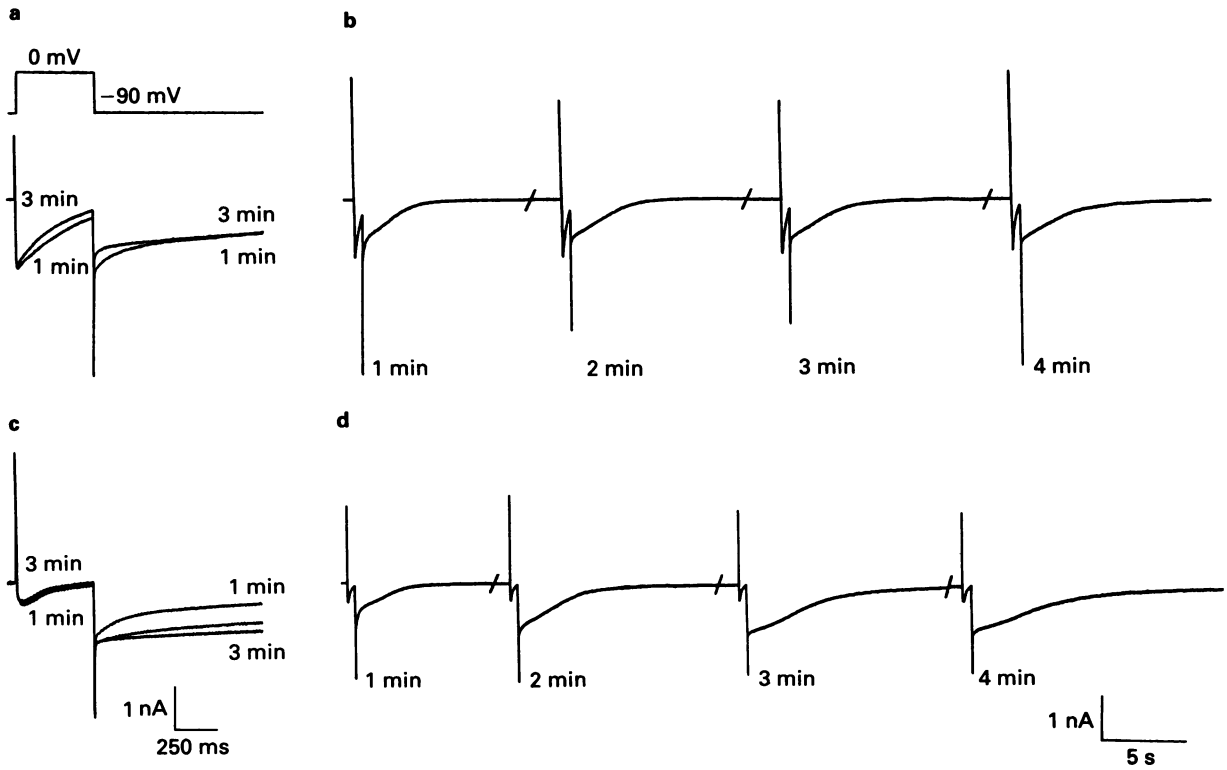

Figure 4 Action of 2-deoxyglucose on voltage-activated Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents. (a) Control currents at 1 and 3 min expanded to show the Ca^{2+} current activated by a 500 ms voltage step command and the initial part of the Cl^- tail current. (b) Series of four currents activated at a frequency of one per min under control conditions. There was no significant change in the Ca^{2+} current and Cl^- tail current. (c) Expanded current records at 1, 2 and 3 min showing no significant change in the Ca^{2+} current but that the tail current deactivation was prolonged by 5 mM 2-deoxyglucose. The tail current at 2 min is the middle record. (d) Series of four currents activated at a frequency of one per min in the presence of 2-deoxyglucose. The Cl^- tail currents were greatly prolonged but the Ca^{2+} currents were not changed significantly. All Ca^{2+} currents were activated from a holding potential of −90 mV by 500 ms voltage step commands to 0 mV. The records presented are not leak subtracted.

Ca^{2+} load by increasing the duration of the voltage-activated Ca^{2+} current to 500 ms.

When 500 ms voltage step commands were applied to DRG neurones every minute, under both control conditions and in the presence of 2DG (5 mM), again there was no significant difference in the values of I_{max} or I_{end} for high voltage-activated Ca^{2+} currents nor for the amplitude of $I_{Cl(Ca)}$ between the first and fourth events (Table 2, Figure 4). However, the $t_{63\%}$ values for $I_{Cl(Ca)}$, in the presence of 2DG (5 mM) became greatly prolonged when compared with controls which remained almost unchanged. The values of the total charge flow showed a significant increase between the first and second steps from 4.4 ± 1.0 nC to 5.5 ± 0.9 nC in the presence of 2DG (5 mM) ($n = 11$, $P < 0.001$) while control values were not significantly affected (Figures 4 and 5).

Finally, in order to investigate whether protracted exposure to 2DG would enhance this prolongation of the decay time to 63% of Ca^{2+}-activated Cl^- tail currents, DRG neurones were incubated in culture for 18 h with 2DG (5 mM). The same recording conditions and voltage command protocols were used as for the previous experiments with 2DG. Only one small amplitude (720 pA) Ca^{2+}-acti-

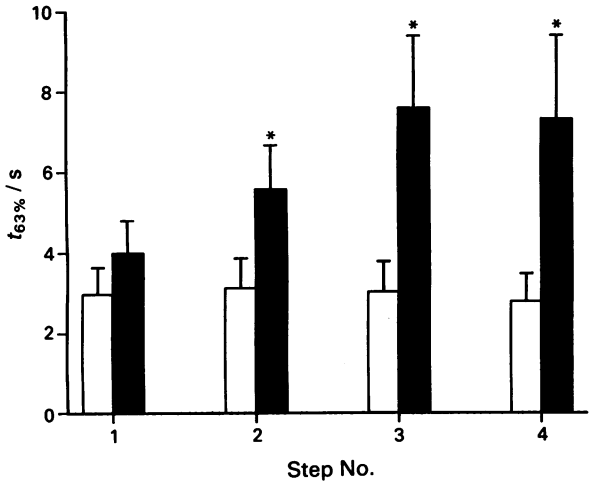

Figure 5 Actions of 2-deoxyglucose on Cl^- tail current decay following activation by a 500 ms high voltage-activated Ca^{2+} current. Chart shows time for Cl^- tail currents to deactivate by 63% ($t_{63\%}$) when activated by four 500 ms Ca^{2+} currents evoked at a rate of one per min. Control data (open columns, $n = 7$) shows no significant change in current decay but in the presence of 5 mM 2-deoxyglucose (solid columns, $n = 11$) Cl^- tail current deactivation is slowed as reflected by the increase in $t_{63\%}$. All data shown are means ± s.e.mean for seven control and 11 2-deoxyglucose experiments. $^*P < 0.05$.

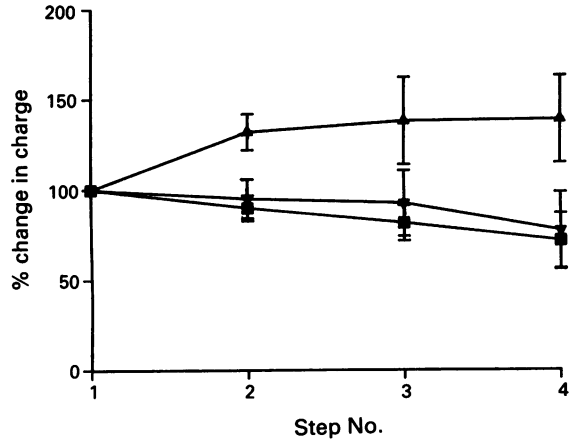

Figure 6 Percentage change in total charge flow during Ca^{2+}-activated Cl^- tail currents following consecutive 500 ms high voltage-activated Ca^{2+} currents. Four 500 ms voltage steps were applied to activate Ca^{2+} currents at a rate of one per min. The step number is plotted against the percentage change in charge relative to the total charge flow during the first Ca^{2+}-activated Cl^- tail current (100%). Under control conditions (■, $n = 7$) and in the presence of fructose 1,6-diphosphate (500 μM) with 2-deoxyglucose (5 mM, $n = 7$) (▼) the mean percentage change in charge declined slightly. In contrast, with 2-deoxyglucose alone (5 mM, $n = 11$, ▲) the percentage change in charge increases relative to both control and fructose 1,6-diphosphate data. Mean percentage change ± s.e.mean are presented.

vated Cl^- tail current was seen in 23 recorded cells. The degree of 'run-down' of I_{max} and I_{end} after consecutive 100 ms voltage step commands was similar to that after brief exposure (12% of I_{max} and 14% of I_{end} after brief exposure, $n = 11$ and 11% of I_{max} and 10% of I_{end} after 18 h exposure, $n = 23$). The degree of inactivation during a 100 ms voltage step command was unaffected by prolonged exposure to 2DG.

Voltage step commands of 500 ms duration were also applied to cells at 1 min intervals after exposure to 2DG (5 mM) for 18 h. Once more, 'run down' in relatively small Ca^{2+} currents was seen with a fall in I_{max} of 37% (from -0.76 ± 0.09 nA to -0.48 ± 0.06 nA, $n = 18$) over the course of 4 min and a reduction of I_{end} by 59% over the same time (-0.22 ± 0.05 nA to -0.09 ± 0.03 nA, $n = 18$). As expected a greater degree of inactivation occurred with the 500 ms depolarizing steps than with 100 ms steps (71% and 81% for the 1st and 4th steps respectively for 500 ms steps compared with 36% and 36% for 100 ms steps).

Fructose 1,6-diphosphate In order to test the ATP dependence of the tail current prolongation and total charge increase seen in the presence of 2DG (5 mM) and absence of glucose or ATP in the patch pipette solution, we performed similar experiments to those described above with the inclusion of fructose, 1,6-diphosphate (500 μM) (F1,6-DP) in the patch pipette solution with 2DG (5 mM) present (again with ATP and glucose excluded). F1,6-DP is a downstream metabolite in the glycolytic pathway, the substrate for aldolase (fructose 1,6-diphosphate: D-glyceraldehyde 3-phosphate lyase) and hence beyond the glycolytic block produced by 2DG. We have used 500 μM F1,6-DP (K_{eq} for this enzyme is approximately 750 μM; Bergmeyer & Bernt, 1974) in order to ensure a suitable supply of substrate and to produce a reasonable diffusion gradient from the patch pipette into the cell. Again 500 ms depolarizing voltage step commands were applied every minute to activate high threshold Ca^{2+} currents and Ca^{2+}-activated Cl^- tail currents. Although some 'run-down' in I_{max} was seen and relatively small total charge flow values for the tail currents were observed (Q at 1 min: 3.9 ± 1.1 nC and 2.1 ± 0.7 nC at 4 min) there was no significant change in I_{end}, $I_{Cl(Ca)}$, $t_{63\%}$ nor in Q ($n = 7$) during the 4 voltage step commands. When the total charge flow was evaluated as a percentage of the initial value for each cell (Q%) the rate of decline in Q% for both controls and F1,6-DP with successive steps was remarkably similar whereas the values for 2DG alone show a substantial increase (Figure 6). These data suggest that in the presence of F1,6-DP the effect of 2DG in prolonging tail current decay time and in increasing total charge flow of the Cl^- tail currents is bypassed, possibly due to the return of the ability to synthesize ATP and hence maintain intracellular Ca^{2+} homeostasis as reflected in the Cl^- tail current parameters.

Discussion

The reduction of an adequate supply of ATP to neurones as occurs clinically in hypoxia, ischaemia or hypoglycaemia may precipitate the loss of intracellular Ca^{2+} homeostasis and the subsequent triggering of a damaging cascade of Ca^{2+}-dependent events leading ultimately to neuronal death (Siesjö, 1981). The two approaches used in this study to induce metabolic stress: inhibition of mitochondrial oxidative phosphorylation and inhibition of glycolysis, demonstrate several important points for the future evaluation of the effects of chemical hypoxia on Ca^{2+} homeostasis. (i) Primary cultures of neonatal rat DRG neurones are remarkably resistant to metabolic stress. (ii) Agents, such as FCCP and cyanide, used to induce chemical hypoxia may themselves interact with Ca^{2+} channels or with intracellular Ca^{2+} homeostatic mechanisms in a reversible manner making them unsuitable agents for the production of chemical hypoxia while studying whole cell Ca^{2+} currents and Ca^{2+}-dependent currents. (iii) Given suitable metabolic stress, such as exposure to 2DG, Ca^{2+} homeostatic mechanisms may be disturbed sufficiently to modulate Ca^{2+}-dependent currents, without directly interacting with either the voltage-activated Ca^{2+} channels or with Ca^{2+}-activated Cl^- channels.

Calcium-activated Cl^- tail currents are observed as slowly decaying inward currents in about 50% of cultured dorsal root ganglion (DRG) neurones loaded with chloride. The duration of the Cl^- tail current is in part determined by the

amount of Ca^{2+} entry through voltage-activated Ca^{2+} channels (Mayer, 1985; Bader *et al.*, 1987; Akasu *et al.*, 1990; Currie & Scott, 1992) but is also modulated by the efficiency with which Ca^{2+} homeostatic mechanisms handle the intracellular Ca^{2+} load (Currie & Scott, 1992; Stapleton *et al.*, 1992). Calcium-activated Cl^- conductances can also be observed following very modest Ca^{2+} entry during a single action potential recorded in the absence of K^+ channel blockers (Mayer, 1985). Furthermore, $I_{Cl(Ca)}$ can be used to identify changes in intracellular Ca^{2+} produced by release of Ca^{2+} from intracellular stores (Currie & Scott, 1992) and to detect photoreleased Ca^{2+} from DM-nitrophen (Currie & Scott, 1993). Recording $I_{Cl(Ca)}$ has a number of benefits including the fact that experiments can be conducted in the presence of K^+ channel blockers so that voltage-activated Ca^{2+} currents can be measured free from contamination by voltage- and Ca^{2+}-activated K^+ conductances which would partially mask the Ca^{2+} currents. Additionally, the reversal potential for $I_{Cl(Ca)}$ is 0 mV under our recording conditions, so high voltage-activated Ca^{2+} currents (activated on stepping to 0 mV) are not contaminated by Cl^- current, which is not observed until repolarization. On repolarization, at the end of the voltage step command, there is a substantial driving force of 90 mV for Cl^- to leave the cell. Therefore Ca^{2+} currents, which reflect the Ca^{2+} load, can be accurately measured under control and experimental conditions and compared with the properties of $I_{Cl(Ca)}$.

Primary cultures of neonatal rat DRG neurones appear to be highly resistant to metabolic stress as reflected by their ability to survive even after long term (up to 18 h) exposure to FCCP and by the apparent lack of disturbance in Ca^{2+} homeostasis as assessed by the degree of inactivation of Ca^{2+} current (Chad & Eckert, 1986). Our data suggest that primary cultures of DRG neurones function on low levels of ATP and can be independent of oxidative phosphorylation. This may not apply to freshly dissociated cells as studied by Duchen and colleagues (Duchen, 1990; Duchen *et al.*, 1990) who found altered intracellular Ca^{2+} homeostasis with FCCP (5 μM) and cyanide (2 mM). It is probable also that central neurones with selective vulnerability to hypoxia such as hippocampal CA1 neurones have a greater sensitivity to such metabolic insults (Simon *et al.*, 1984). Another possibility for the apparent insensitivity of these cultures to chemical hypoxia is that they are neonatal in origin and as such might be expected to withstand more prolonged exposure to hypoxic conditions (Kass & Lipton, 1989; Krnjevic *et al.*, 1989; Friedman & Haddad, 1993). However Kostyuk and colleagues (1993) have recently demonstrated that run-down of Ca^{2+} currents in rat neonatal DRG neurones appears to be more ATP-sensitive than in those seen in adult and aging neurones.

Single electrode voltage clamp studies on hippocampal slice preparations (Krnjevic & Leblond, 1989), dissociated mouse sensory neurones (Duchen, 1990) and in guinea-pig ventricular myocytes (Goldhaber *et al.*, 1991) confirm a reduction in Ca^{2+} current in response to chemical hypoxia and nitrogen perfusion as seen in our experiments. However, care must be taken in the interpretation of changes in Ca^{2+} current due to induced chemical hypoxia since the agents used may themselves interact with Ca^{2+} channels as seems probable with FCCP, since the effects of this substance on Ca^{2+} currents in our system have a rapid onset of action (10–20 s) and are readily reversible even after 18 h exposure. Additionally FCCP rapidly inhibited Ca^{2+} currents even when ATP and HEPES were present in the patch solution, suggesting that the action was not due to reduced intracellular ATP or pH.

A Ca^{2+}-dependent K^+ conductance has been demonstrated during hypoxia in hippocampal slice preparations (Fujiwara *et al.*, 1987; Krnjevic & Leblond, 1989) and in dissociated mouse sensory neurones (Duchen, 1990). Duchen (1990) also described an increased Ca^{2+}-dependent Cl^- conductance in response to metabolic blockade. We have demonstrated a prolongation of a Ca^{2+}-dependent Cl^- current following inhibition of glycolysis with 2DG in rat DRG neurones. This effect suggests that given a suitably severe metabolic insult, Ca^{2+} homeostatic mechanisms begin to fail, possibly due to the lack of ATP. This concept is supported by the finding that in the presence of F1,6-DP, a glycolytic intermediary which bypasses the 2DG-induced block, the tail current changes are no longer seen, suggesting a restoration of ATP synthesis and hence of Ca^{2+} homeostasis. However, tail current amplitudes and decay time (and hence total charge) were highly variable between different cells, possibly reflecting variations in the number of Ca^{2+}-dependent Cl^- channels present, in channel distribution or in their threshold for activation. These channels may also require phosphorylation for proper function by analogy with Ca^{2+} channels themselves (Armstrong & Eckert, 1987) and differences in resting $[Ca^{2+}]_i$ may affect the degree to which this occurs.

Another interesting finding is the virtual absence of tail currents, after prolonged (18 h) exposure to 2DG in culture. This may reflect down-regulation of Ca^{2+}-activated Cl^- channels or a change in their properties resulting in reduced availability, and may be analogous to the dependence of Ca^{2+} channels on phosphorylation for their normal function (Armstrong & Eckert, 1987) but may also be due to an increase in the activity of a Ca^{2+}-dependent phosphatase. Our data suggest that Ca^{2+}-activated Cl^- tail currents are more sensitive to metabolic stress than Ca^{2+} currents themselves.

This study has demonstrated that in primary cultures of neonatal rat DRG neurones, prolongation of the decay time of Ca^{2+}-activated Cl^- tail currents and an increase of the total charge flow occurs in the presence of 2DG after removal of both glucose and ATP from the recording solutions. We believe that the use of $I_{Cl(Ca)}$ and 2DG may prove useful in the study of changes in intracellular Ca^{2+} produced by metabolic stress. Further study will be directed at cells with a greater sensitivity to metabolic stress.

The authors wish to thank the Wellcome Trust for financial support. We also thank Dr A. Hughes for the programme to calculate free Ca^{2+} levels and Mr C. Entwhistle and Mr F. Mitchell for the computer programme used to calculate the total charge flow for tail currents. We thank Ms S. Maddox for help with preparation of the manuscript.

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(Received May 7, 1993
Revised July 29, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 65–72

A study of ATP as a sympathetic cotransmitter in human saphenous vein

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1 Strips of human saphenous veins were superfused with Krebs-Henseleit solution at either 25°C or 37°C. Constrictor responses to electrical stimulation (10 Hz, 40 s) but not to exogenous noradrenaline (0.1, 1 μM) were abolished by guanethidine (10 μM) and tetrodotoxin (1 μM). Hence, responses to electrical stimulation are due to action potential-induced release of sympathetic neurotransmitters.

2 Constrictor responses to electrical stimulation and noradrenaline were reduced by the α_1-adrenoceptor antagonist, prazosin (0.3 μM) as well as by the α_2-adrenoceptor antagonist, rauwolscine (1 μM). The combination of prazosin and rauwolscine abolished constrictor responses to noradrenaline at 25°C and 37°C. However, constrictor responses to electrical stimulation were partly resistant to α-adrenoceptor blockade by prazosin and rauwolscine (at 25°C about 30%). Residual constrictor responses to electrical stimulation were also observed in the presence of the combination of prazosin (3 μM) and rauwolscine (10 μM) as well as in the presence of phenoxybenzamine (10 μM).

3 Veins, incubated with [^{3}H]-noradrenaline, released tritium upon electrical stimulation (10 Hz, 40 s). Moreover, electrical stimulation also induced an overflow of ATP amounting to 4.8 ± 1.5 pmol g^{-1} at 25°C and 2.0 ± 0.5 pmol g^{-1} at 37°C. Both tritium and ATP overflow were abolished by tetrodotoxin (0.5 μM). The combination of prazosin (0.3 μM) and rauwolscine (1 μM) increased tritium overflow at either 25°C or 37°C by about 120%, but reduced ATP overflow by about 70%. Hence, a significant percentage of the electrically evoked ATP overflow seems to be released from non-neuronal cells upon activation of α-adrenoceptors by endogenous noradrenaline. The remaining ATP overflow, which was resistant to α-adrenoceptor blockade, may reflect neuronally released ATP.

4 ATP (300 μM) and α,β-methylene-ATP (1, 10 μM), both induced constrictor responses. The P_2-purinoceptor antagonist, suramin (300 μM) markedly inhibited constrictor responses to ATP and α,β-methylene-ATP, but not those to electrical stimulation and to noradrenaline. Moreover, suramin (300 μM) failed to diminish the α-adrenoceptor blockade-resistant constrictor response to 10 Hz.

5 In conclusion, constrictor responses to sympathetic nerve stimulation in human saphenous veins are mainly but not exclusively mediated by neuronally released noradrenaline. There is a concomitant release of ATP and noradrenaline. P_2-purinoceptors which mediate vasoconstriction are present; however, a role of neuronally released ATP in constrictor responses to electrical stimulation could not be established. Therefore, the nature of the sympathetic transmitter responsible for α-adrenoceptor blockade-resistant constrictor responses remains unknown.

Keywords: α-Adrenoceptors; P_{2X}-purinoceptors; noradrenaline-ATP cotransmission; ATP release; prazosin; rauwolscine; α,β-methylene-ATP; suramin; human saphenous vein

Introduction

Noradrenaline and ATP are cotransmitters in the postganglionic sympathetic nervous system. In numerous tissues of several animal species ATP or a related nucleotide mediates responses to sympathetic nerve stimulation, which are resistant to α-adrenoceptor blockade (see Burnstock, 1986; 1990; von Kügelgen & Starke, 1991a; Hoyle, 1992; Morris & Gibbins, 1992). α-Adrenoceptor blockade-resistant responses have also been observed in man (Taddei *et al.*, 1989; 1990; Kahan *et al.*, 1992). However, it is an open question whether or not ATP is involved in sympathetic neurotransmission in man and, hence, could be responsible for the reported α-adrenoceptor blockade-resistant responses (Pelleg & Burnstock, 1990; Taddei *et al.*, 1990; Stephens *et al.*, 1992).

The saphenous vein is a commonly used preparation to study sympathetic neurotransmission in man (see Docherty, 1987). The smooth muscle cells of these veins possess α_1- and α_2-adrenoceptors both of which mediate vasoconstrictor responses to endogenous and exogenous noradrenaline (Müller-Schweinitzer, 1984; Steen *et al.*, 1984; Docherty & Hyland, 1985a; Docherty, 1987). The same holds true for the saphenous vein of the dog (Sullivan & Drew, 1980; De Mey & Vanhoutte, 1981; Pereira *et al.*, 1991). Vasoconstrictor responses to sympathetic nerve stimulation in the canine saphenous vein are at least partly resistant to blockade by α-adrenoceptor antagonists (Flavahan & Vanhoutte, 1986b). These non-adrenergic constrictor responses were even more prominent at a temperature of 24°C and seem to be mediated by neuronally released ATP (Flavahan & Vanhoutte, 1986b).

In order to study a possible cotransmitter role of ATP in man, constrictor responses to sympathetic nerve stimulation as well as ATP and noradrenaline overflow were measured in human saphenous veins.

Methods

The study was approved by the local ethics committee. Veins were obtained from patients (21 females, 51–76 years of age, mean age 65.1 ± 1.6; 90 males, 42–81 years of age, mean age 62.2 ± 0.9) undergoing open heart surgery for coronary bypass grafting. None of the patients had been treated with drugs known to interact with storage or release mechanism of noradrenaline. Portions of about 3–4 cm of the human saphenous vein were dissected free from surrounding connective tissue and cut into helical strips. The strips were placed

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between two platinum electrodes in a jacketed chamber maintained at either 25°C or 37°C and superfused with Krebs-Henseleit solution at a constant rate of 6 ml min^{-1} (experiments with overflow measurements 1 ml min^{-1}). The strips were connected to a force-displacement transducer (Biegestab K30, Hugo Sachs Electronics, March-Hugstetten, Germany) for measuring isometric tension with a multi-pen recorder (Rikadenki, Freiburg i. Br., Germany). Resting tension was set at 19.6 mN. Vasoconstrictor responses were measured as the maximum increase in tension (mN) above resting tension. The superfusion fluid was gassed continuously with carbogen (95% O_2:5% CO_2) and passed through a heat exchanger before it reached the vessels. A Stimulator II (Hugo Sachs Electronics) operating in the constant current mode was used for electrical field-stimulation.

Protocol A (measurement of postjunctional responses)

After an equilibration period of 90 min vasoconstrictor responses were elicited by two periods of stimulation (60 min apart). In some experiments each stimulation period consisted of three consecutive electrical stimulations at 1, 4 and 10 Hz (40 s duration, 100 mA current strength, 0.5 ms pulse width; applied at intervals of 5 min). In most other experiments each stimulation period consisted of an electrical stimulation (S_1 or S_2, at either 2.5 or 10 Hz, 40 s) followed, after an interval of 5 min, by a stimulation elicited by exogenous noradrenaline (N_1 or N_2, 0.1–1.0 μM). In some further experiments either ATP (300 μM) or the stable ATP analogue, α,β-methylene-ATP (1–10 μM) was used instead of electrical field stimulation to elicit vasoconstrictor responses. Noradrenaline, ATP and α,β-methylene-ATP were added to the superfusion solution until the constrictor response had reached its maximum. The effect of other drugs on the vasoconstrictor responses was tested by adding them to the superfusion solution 50 min before the second stimulation period.

Further experimental details are given in the Results section.

Protocol B (overflow measurement)

The strips were incubated at 37°C for 30 min in 2 ml Krebs-Henseleit solution containing 0.4 μM [^{3}H]-noradrenaline. After a washing period the strips were placed between two parallel platinum electrodes and superfused with Krebs-Henseleit solution. Drugs or solvent were administered by infusion (16 μl min^{-1}) into the superfusion stream. After an equilibration period of 60 min, a priming stimulation (10 Hz for 40 s, 100 mA, 0.3 ms) was given; 58 min after the priming stimulation the superfusate was collected in 1, 5 or 10 min periods. Then there were two electrical stimulations (S_1 and S_2), 60 min apart, at 10 Hz for either 10 or 40 s. The effect of drugs was tested by adding them to the superfusion solution 42 min before S_2. At the end of each experiment, the strips were weighed (104.1 ± 12.7 mg; $n = 29$) and solubilized in 1 ml Soluene-350 (Canberra Packard, Frankfurt a. M., Germany). The ATP content of the superfusate was measured with the luciferase technique (von Kügelgen & Starke, 1991b) using the ATP bioluminescence HS assay kit (Boehringer Mannheim, Mannheim, Germany) and a Biolumat LB 9500 C luminometer (Berthold, Wildbad, Germany). A blank value obtained with fresh solution was subtracted from each experimental value. Calibration curves for ATP were obtained using Krebs-Henseleit solution as solvent. None of the drugs used interfered with the assay. The amount of tritium in the superfusate samples and the solubilized tissues was measured by liquid scintillation counting. The outflow of ATP was expressed in pmol min^{-1} g^{-1} tissue. Outflow of tritium was expressed as a fraction of the amount of tritium present in the tissue at the onset of the respective collection period (fractional rate of outflow; min^{-1}). The electrically evoked overflow of ATP and tritium was calculated as the difference between the total overflow during the 7 min after onset of stimulation, and the estimated basal outflow within these 7 min. The basal outflow of ATP was assumed to be the outflow in the 1 min interval before onset of stimulation, and the electrically evoked overflow of ATP (total minus basal) was expressed as pmol g^{-1} tissue. The basal outflow of tritium was assumed to change linearly from the 1 min interval before to the interval 7–8 min after onset of stimulation, and the electrically evoked overflow of tritium (total minus basal; Bq) was expressed as a percentage of the tritium content (Bq) of the tissue at the start of stimulation.

Materials

The following drugs were used: neuropeptide Y (NPY, human) (Bachem, Heidelberg, Germany), suramin hexasodium salt (Bayer, Wuppertal, Germany), adenosine 5'-triphosphate disodium salt (ATP) (Boehringer Mannheim, Mannheim, Germany), (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 1.62, 1.94 or 2.11 TBq $mmol^{-1}$ (Du Pont, Dreieich, Germany), prazosin HCl (Pfizer, Karlsruhe, Germany), phenoxybenzamine HCl (Röhm Pharma, Weiterstadt, Germany), rauwolscine HCl (Roth, Karlsruhe, Germany), guanethidine sulphate, α,β-methylene-ATP lithium salt, (−)-noradrenaline HCl, tetrodotoxin (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with distilled water (tetrodotoxin: sodium acetate buffer 0.1 M, pH 4.8). The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, glucose 11.1, disodium EDTA 0.07, ascorbic acid 0.07.

Statistics

Means ± s.e.mean are given throughout. Differences between means were tested for statistical significance by Student's t test. $P<0.05$ or lower was taken to be statistically significant.

Results

Human saphenous veins were cut into helical strips and superfused with Krebs-Henseleit solution. Constrictor re-

Table 1 Constrictor responses to electrical field stimulation, exogenous noradrenaline, ATP and α,β-methylene-ATP in human saphenous vein

Stimulus	*Constrictor responses* (mN) *at 25°C*	*Constrictor responses* (mN) *at 37°C*
2.5 Hz	22.2 ± 4.3 (13)	29.3 ± 7.9 (8)
10 Hz	22.4 ± 1.7 (73)	33.7 ± 3.2 (45)
Noradrenaline 0.1 μM	31.2 ± 2.7 (57)	33.6 ± 3.1 (33)
1 μM	45.9 ± 4.7 (29)	56.3 ± 3.7 (49)
ATP 300 μM		8.8 ± 1.6 (16)
α,β-methylene-ATP 1 μM		20.6 ± 3.6 (10)
10 μM		36.3 ± 17.0 (3)

Strips of human saphenous veins were superfused with Krebs-Henseleit solution at 25°C or 37°C. Constrictor responses were elicited by electrical stimulation (2.5 or 10 Hz for 40 s), exogenous noradrenaline, ATP or α,β-methylene-ATP. Means ± s.e.mean of (n) experiments.

sponses to electrical field stimulation, exogenous noradrenaline, ATP and α,β-methylene-ATP in the absence of drugs at 25°C and 37°C are shown in Table 1.

Effect of phenoxybenzamine on constrictor responses to either 1, 4 and 10 Hz or to exogenous noradrenaline

There were two series (60 min apart) of consecutive electrical stimulations at 1, 4 and 10 Hz (each for 40 s) at 37°C. Vasoconstrictor responses in the first series were 1.8 ± 1.1 mN, 5.8 ± 2.1 mN and 12.2 ± 1.6 mN, respectively ($n = 4$). Phenoxybenzamine (10 μM), added to the superfusion solution 50 min before the second series of electrical stimulation at 1, 4 and 10 Hz reduced the respective vasoconstrictor responses to 2.5 ± 0.9, 16.3 ± 1.4 and 21.1 ± 1.9% of control ($n = 4$). The residual responses to electrical stimulation at 4 and 10 Hz in the presence of phenoxybenzamine were almost abolished by further addition of tetrodotoxin (1 μM; $n = 4$; not shown). Constrictor responses to exogenous noradrenaline (1 μM) were abolished by phenyoxybenzamine (10 μM; $n = 5$; not shown).

Effects of guanethidine and tetrodotoxin on constrictor responses to either 10 Hz or exogenous noradrenaline

Guanethidine (10 μM) and tetrodotoxin (1 μM), when added to the superfusion solution 50 min before the second stimulation period, almost abolished the vasoconstrictor responses to electrical stimulation at 25°C and 37°C (Figure 1). Vasoconstrictor responses to exogenous noradrenaline were not significantly altered by either tetrodotoxin or guanethidine (Figure 1).

Effects of prazosin and rauwolscine on constrictor responses to either 10 Hz or exogenous noradrenaline

Prazosin (0.3 μM) as well as rauwolscine (1 μM) reduced constrictor responses to electrical stimulation and to exogenous noradrenaline (0.1, 1 μM) at 25°C and 37°C (Figure 2). The combination of prazosin and rauwolscine abolished constrictor responses to noradrenaline (0.1, 1 μM) at both temperatures. However, constrictor responses to electrical

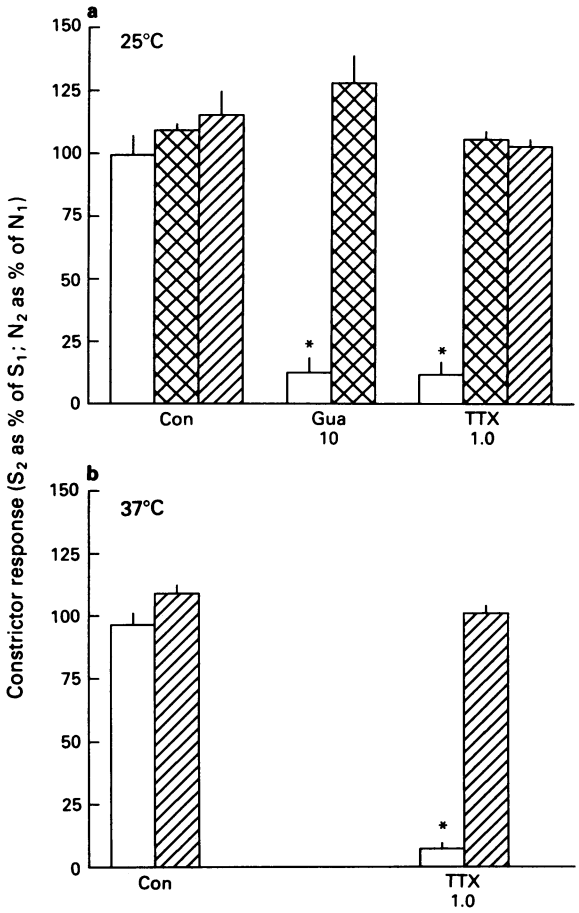

Figure 1 Effects of guanethidine (Gua) and tetrodotoxin (TTX) on constrictor responses to electrical stimulation and exogenous noradrenaline (NA) in human saphenous veins. Veins were superfused with Krebs-Henseleit solution at 25°C (a) or 37°C (b). There were two stimulation periods (60 min apart). Each period consisted of an electrical stimulation (S_1 or S_2; at 10 Hz, 40 s; open columns) followed by a stimulation elicited by noradrenaline (N_1 or N_2; 0.1 μM, cross-hatched columns or 1 μM, hatched columns). Guanethidine (10 μM), tetrodotoxin (1 μM) or their solvents (control, Con) were added to the superfusion solution 50 min before S_2. Constrictor responses in the second stimulation period (S_2 or N_2) are expressed as a percentage of that in the first stimulation period (S_1 or N_1, respectively). Means ± s.e.mean of 3–15 experiments. *Significant differences from corresponding control (solvent; $P < 0.05$).

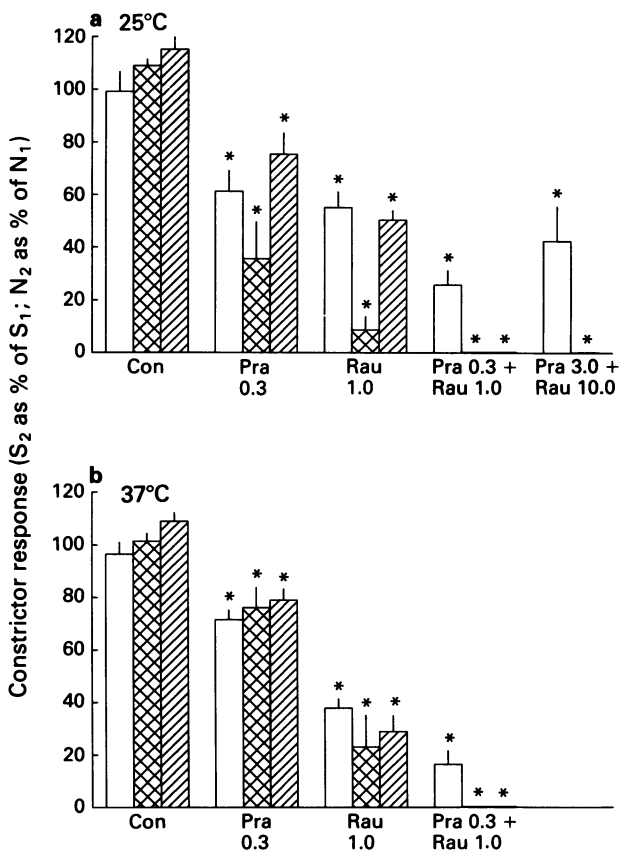

Figure 2 Effects of prazosin (Pra), rauwolscine (Rau) and the combination of prazosin and rauwolscine on constrictor responses to electrical stimulation and exogenous noradrenaline (NA) in human saphenous veins. Veins were superfused with Krebs-Henseleit solution at 25°C (a) or 37°C (b). There were two stimulation periods (60 min apart). Each period consisted of an electrical stimulation (S_1 or S_2; at 10 Hz, 40 s; open columns) followed by a stimulation elicited by noradrenaline (N_1 or N_2; 0.1 μM, cross-hatched columns or 1 μM, hatched columns). Prazosin (0.3 μM or 3 μM), rauwolscine (1 μM or 10 μM) or their solvent (control, Con) were added to the superfusion solution 50 min before S_2. Constrictor responses in the second stimulation period (S_2 or N_2) are expressed as a percentage of that in the first stimulation period (S_1 or N_1, respectively). Means ± s.e.mean of 3–15 experiments. *Significant differences from corresponding control (solvent; $P < 0.05$).

stimulation were further inhibited but not abolished by the combination of prazosin and rauwolscine (Figure 2). Even ten fold higher concentrations of prazosin and rauwolscine (3 μM and 10 μM, respectively) failed to attenuate further the constrictor responses to electrical stimulation (25°C; Figure 2). Tetrodotoxin (1 μM) added in addition to prazosin (3 μM) and rauwolscine (10 μM) markedly inhibited the residual responses to electrical stimulation ($n = 5$; not shown). Residual constrictor responses to electrical stimulation obtained in the presence of prazosin and rauwolscine were greater at 25°C than at 37°C and were about 30% of control at 25°C (Figure 2; see also below Figure 4).

Effects of prazosin and rauwolscine on constrictor responses to either 2.5 Hz or exogenous noradrenaline

Similar results were obtained when the veins were electrically stimulated for 40 s at 2.5 Hz instead of at 10 Hz. The combination of prazosin (0.3 μM) and rauwolscine (1 μM) abolished constrictor responses to exogenous noradrenaline (0.1 μM) at 25°C and 37°C (not shown). Constrictor responses to electrical stimulation were abolished at 37°C, but there were residual constrictor responses (about 22% of control) at 25°C. These residual constrictor responses to electrical stimulation tended to be smaller than those obtained with stimulation at 10 Hz.

Effects of tetrodotoxin, prazosin and rauwolscine on electrically evoked constrictor responses, tritium and ATP overflow

Human saphenous veins were preincubated with [^{3}H]-noradrenaline and then superfused with Kreb-Henseleit solution at 25°C or 37°C. Electrical stimulation elicited constrictor

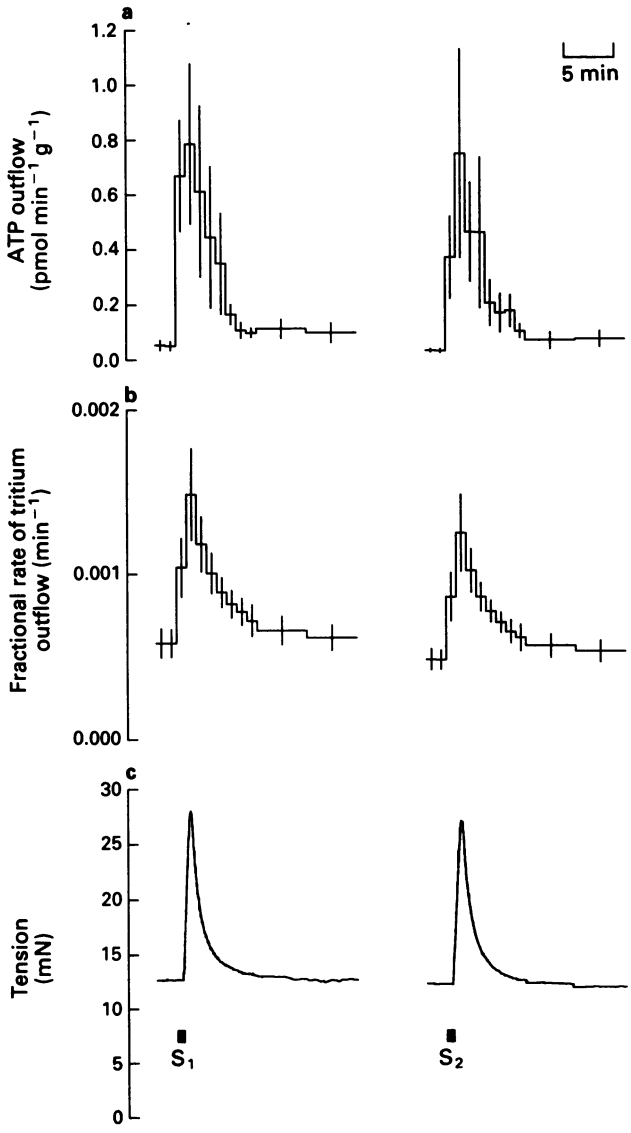

Figure 3 Time course of ATP outflow (a), tritium outflow (b) and smooth muscle tension (c). Human saphenous veins were incubated with [^{3}H]-noradrenaline and then superfused with Krebs-Henseleit solution at 37°C. There were two electrical stimulations (S_1 and S_2, 120 and 180 min after the start of superfusion, each at 10 Hz, 40 s). Solvent was added to the superfusion solution 42 min before S_2. Figure shows ATP and tritium outflow per minute as means ± s.e.mean of 6 experiments and representative smooth muscle tension tracings from one experiment.

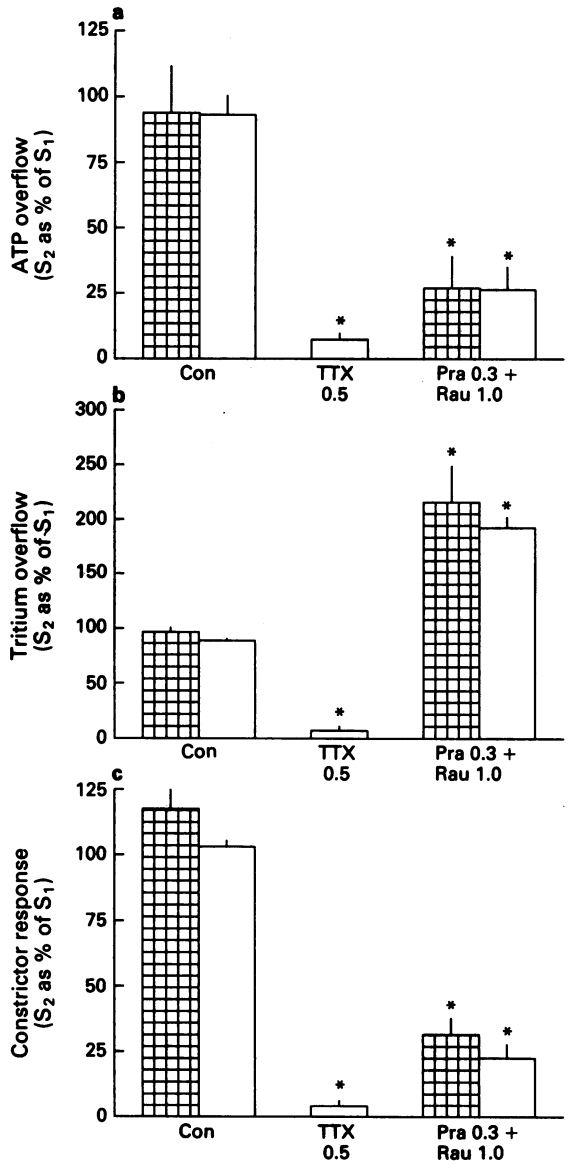

Figure 4 Effects of tetrodotoxin (TTX) and a combination of prazosin and rauwolscine (Pra + Rau) on electrically evoked ATP overflow (a), tritium overflow (b) and constrictor responses (c) in human saphenous veins. Veins were superfused with Krebs-Henseleit solution at 25°C (cross-hatched) or 37°C (open columns). There were two stimulations (S_1 or S_2; 60 min apart, each at 10 Hz, 40 s). Tetrodotoxin (0.5 μM), prazosin (0.3 μM), rauwolscine (1 μM) or their solvents (control, Con) were added to the superfusion solution 42 min before S_2. Responses in the second stimulation period (S_2) were expressed as a percentage of that in the first stimulation period (S_1). Means ± s.e.mean of 4–6 experiments. *Significant differences from corresponding control (solvent; $P < 0.05$).

Table 2 Basal ATP and tritium outflow (b_1) from human saphenous vein, and electrically evoked ATP and tritium overflow and constriction (S_1)

	b_1		S_1			
Stimulus	*ATP outflow* (pmol $min^{-1} g^{-1}$)	*Tritium outflow* (min^{-1})	*ATP overflow* (pmol g^{-1})	*Tritium overflow* (% of tissue tritium)	*Constriction* (mN)	n
			Temperature: 25°C			
10 Hz, 40 s	0.149 ± 0.039	0.00044* ± 0.00005	4.745 ± 1.522	0.319 ± 0.079	20.1 ± 3.6	10
			Temperature: 37°C			
10 Hz, 10 s	0.124 ± 0.023	0.00083* ± 0.00006	0.782 ± 0.291	0.071* ± 0.016	13.1 ± 2.3	5
10 Hz, 40 s	0.094 ± 0.016	0.00064[a] ± 0.00004	1.949 ± 0.496	0.331[b] ± 0.076	20.2 ± 3.5	14

After incubation with [^{3}H]-noradrenaline, human saphenous veins were superfused with Krebs-Henseleit solution at 25°C or 37°C. S_1 was applied after 120 min of superfusion and consisted of either 100 pulses (10 Hz, 10 s) or 400 pulses (10 Hz, 40 s). b_1 refers to the 1 min period immediately before S_1. Means ± s.e.mean of *n* experiments.
[a]Corresponding to 36.8 ± 5.3 Bq min^{-1}; [b]corresponding to 159.0 ± 32.7 Bq.
*Significant differences from experiments with 10 Hz, 40 s at 37°C ($P<0.05$).

responses as well as an overflow of tritium and ATP. The time course is shown for one experimental group in Figure 3 and mean values of the responses elicited by the first stimulation period in the absence of drugs are summarized in Table 2. Electrical stimulation with 400 pulses (10 Hz for 40 s) induced greater constrictor responses, tritium and ATP overflow than stimulation with 100 pulses (10 Hz for 10 s; Table 2; 37°C). Constrictor responses and tritium overflow at either 25°C or 37°C did not differ. The mean value of ATP overflow obtained at 25°C was about twice that obtained at 37°C; however, this difference was not significant ($P = 0.059$). Constrictor responses as well as tritium and ATP overflow to 400 pulses (10 Hz) were well reproducible after an interval of 60 min (Figures 3 and 4). Tetrodotoxin (0.5 μM) added 42 min before the second stimulation period abolished constrictor responses as well as tritium and ATP overflow (Figure 4). The combination of prazosin (0.3 μM) and rauwolscine (1 μM) reduced but did not abolish constrictor responses and ATP overflow at 25°C and 37°C, whereas the combination increased tritium overflow by about 120% at either temperature (Figure 4). In the presence of prazosin and rauwolscine the basal outflow of tritium was increased by about 60% at both temperatures; the basal outflow of ATP remained unchanged (not shown).

Effects of suramin on constrictor responses to 10 Hz, noradrenaline, ATP and α,β-methylene-ATP at 37°C

Exogenous ATP (300 μM) and α,β-methylene-ATP (1–10 μM) elicited fast constrictor responses (mean values of the maximum increase in tension are shown in Table 1). The responses to ATP were transient; the smooth muscle tended to return to baseline within 5 min after addition of ATP. The responses to α,β-methylene-ATP, in contrast, were more sustained (most prominently at 25°C, not shown). Suramin (300 μM), when added to the superfusion solution 50 min before the second stimulation period, almost abolished the constrictor responses to ATP (300 μM) and α,β-methylene-ATP (1 μM) (Figure 5). Suramin did not significantly reduce constrictor responses to either 10 Hz or noradrenaline (0.1 μM) (Figure 5).

Effects of suramin on α-adrenoceptor blockade-resistant constrictor responses to 10 Hz at 25°C

The combination of prazosin (0.3 μM) and rauwolscine (1 μM) abolished the constrictor responses to noradrenaline, but only partly reduced the constrictor responses to electrical stimulation at 10 Hz (Figure 2). Suramin (300 μM), given in addition to prazosin and rauwolscine, did not further diminish the α-adrenoceptor blockade-resistant constrictor responses to 10 Hz (Figure 6).

Constrictor responses to exogenous neuropeptide Y at 37°C

Neuropeptide Y (0.01, 0.1 and 1 μM) induced concentration-dependent constrictor responses of 2.5 ± 1.0 mN ($n = 13$), 3.3 ± 1.1 mN ($n = 14$) and 8.0 ± 3.6 mN ($n = 11$), respectively.

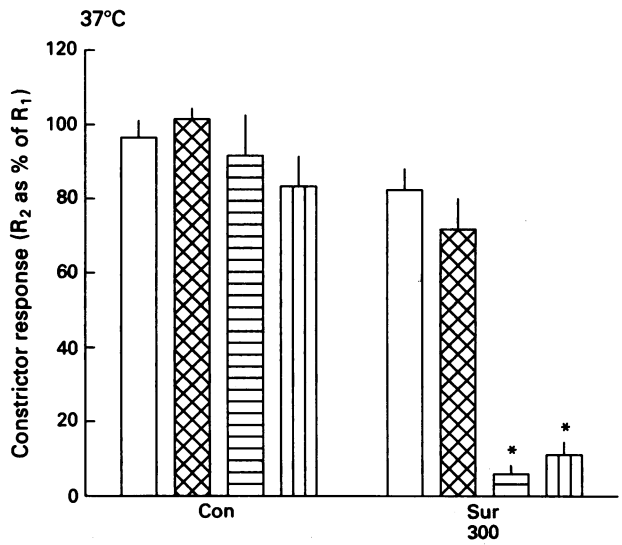

Figure 5 Effects of suramin (Sur) on constrictor responses (R) to electrical stimulation, exogenous noradrenaline (NA), ATP and α,β-methylene-ATP (mATP) in human saphenous veins. Veins were superfused with Krebs-Henseleit solution at 37°C. There were two stimulation periods (60 min apart). Each period consisted of a stimulation elicited by electrical pulses (10 Hz, 40 s; open columns), ATP (300 μM; horizontally striped columns) or mATP (1 μM; vertically striped columns) followed by a stimulation elicited by noradrenaline (0.1 μM, cross-hatched columns). Suramin (300 μM) or its solvent (control, Con) was added to the superfusion solution 50 min before S_2. Constrictor responses in the second stimulation period (R_2) are expressed as a percentage of that in the first stimulation period (R_1). Means ± s.e.mean of 4–12 experiments. *Significant differences from corresponding control (solvent; $P<0.05$).

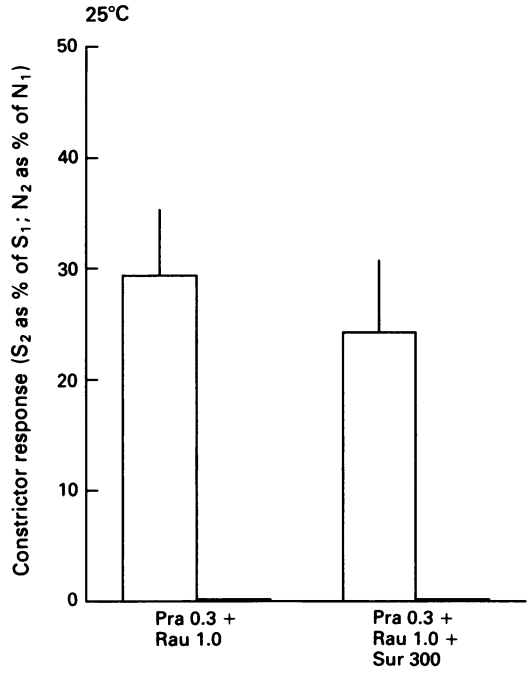

Figure 6 Effects of suramin (Sur) on constrictor responses to electrical stimulation and exogenous noradrenaline (NA) in the presence of prazosin (Pra) and rauwolscine (Rau). Veins were superfused with Krebs-Henseleit solution at 25°C. There were two stimulation periods (60 min apart). Each period consisted of an electrical stimulation (S_1 or S_2; at 10 Hz, 40 s; open columns) followed by a stimulation elicited by noradrenaline (N_1 or N_2; 0.1 μM, hatched columns). Suramin (300 μM) was added to the superfusion solution together with prazosin (0.3 μM) and rauwolscine (1 μM) 50 min before S_2. Constrictor responses in the second stimulation period (S_2 or N_2) are expressed as a percentage of that in the first stimulation period (S_1 or N_1, respectively). Means ± s.e.mean of 6–11 experiments.

Discussion

ATP plays an important role as a cotransmitter of noradrenaline in sympathetically innervated tissues of various animals species (Burnstock, 1990; von Kügelgen & Starke, 1991a; Hoyle, 1992). The aim of the present study was to investigate whether this may also be the case in human saphenous veins. Electrical stimulation elicited constrictor responses of human saphenous veins; these responses were sensitive to tetrodotoxin and guanethidine and thus mediated by transmitter substances released from sympathetic nerve varicosities (see Burnstock, 1990; von Kügelgen & Starke, 1991a).

α-Adrenoceptor-mediated constrictor responses

In human saphenous veins constrictor responses to exogenous noradrenaline were inhibited by both the preferential α_1-adrenoceptor antagonist, prazosin and the preferential α_2-adrenoceptor antagonist, rauwolscine but only a combination of both drugs abolished these responses. This is in accord with previous findings that smooth muscle cells of the human saphenous vein possess both α_1- and α_2-adrenoceptors (Müller-Schweinitzer, 1984; Steen *et al.*, 1984; Docherty & Hyland, 1985a; Docherty, 1987). When the human saphenous veins were stimulated with a frequency of 10 Hz, the constrictor responses to electrical stimulation were also diminished by prazosin as well as by rauwolscine. Moreover, the combination of prazosin and rauwolscine caused a greater reduction of constrictor responses to electrical stimulation at 10 Hz than each of the antagonists alone. This confirms previous findings that in the human saphenous vein both smooth muscle α_1-adrenoceptors and smooth muscle α_2-adrenoceptors are activated by endogenous noradrenaline and mediate a portion of the effector responses to sympathetic nerve stimulation (Docherty & Hyland, 1985a).

α-Adrenoceptor blockade resistant constrictor responses

The combination of prazosin and rauwolscine as well as phenoxybenzamine abolished constrictor responses to exogenous noradrenline (0.1 μM and 1 μM). Even though the constrictor responses to exogenous noradrenaline were higher than those to electrical stimulation (Table 1), the α-adrenoceptor antagonists failed to cause complete blockade of the responses to electrical stimulation. A ten fold increase in the concentrations of prazosin and rauwolscine did not further attenuate the constrictor responses to electrical stimulation. This, in addition, excludes the possibility that the α-adrenoceptor antagonists failed to block completetly the responses to electrical stimulation due to the use of too low concentrations. Hence, these findings indicate that in the human saphenous vein some components of neurogenic constriction are resistant to blockade of α-adrenoceptors.

Responses to sympathetic nerve stimulation which are resistant to α-adrenoceptor blockade are well known in sympathetically innervated smooth muscle tissues of animals including the canine saphenous vein and have also been observed in human tissues (see Introduction). These α-adrenoceptor blockade-resistant constrictor responses vary with frequency of stimulation (Burnstock, 1990; von Kügelgen & Starke, 1991a). Therefore, the frequency-dependence was tested in the present study. When all α-adrenoceptors were blocked by phenoxybenzamine, constrictor responses to lower stimulation frequencies were abolished (1 Hz) or markedly inhibited (4 Hz). In contrast, at higher stimulation frequency (10 Hz) there were significant residual constrictor responses. A similar pattern of α-adrenoceptor blockade-resistant constrictor responses to stimulation at high but not at low frequencies has been observed in the canine saphenous vein (Flavahan & Vanhoutte, 1986b). The more pronounced α-adrenoceptor blockade resistant constrictor responses at 10 Hz as compared to 1 and 4 Hz may be due to a greater 'per pulse' release of sympathetic cotransmitters at higher frequencies. An increase in the release of the sympathetic cotransmitters ATP and neuropeptide Y with increasing frequency has been demonstrated in guinea-pig vas deferens (Kirkpatrick & Burnstock, 1987; von Kügelgen & Starke, 1991b), canine gracilis muscle (Pernow, 1988), pig nasal mucosa (Lacroix *et al.*, 1989) and pig spleen (Lundberg *et al.*, 1989). Since the present study in human saphenous veins is focussed on possible non-adrenergic mechanisms of sympathetic neurotransmission, most of the experiments discussed were performed at a stimulation frequency of 10 Hz.

Cooling from 37°C to 25°C augmented α-adrenoceptor blockade-resistant constrictor responses to electrical stimulation in human saphenous veins. A temperature lower than 37°C may be a more physiological temperature for superficial veins such as the vena saphena. Thus, the importance of non-adrenergic components observed at 37°C in these tissues is likely to be underestimated compared with the *in vivo* situation. An augmented residual constrictor response at 24°C as compared to 37°C has also been observed in the canine saphenous vein (Flavahan & Vanhoutte, 1986b) and, in addition, not only in venous preparations but also in the rat isolated mesenteric vasculature (Yamamoto *et al.*, 1992). It is not known why cooling enhances α-adrenoceptor blockade-resistant constrictor responses. α-Adrenoceptor responsiveness may be reduced at lower temperatures (Flavahan & Vanhoutte, 1986a). Another likely explanation is that sympathetic cotransmitters are inactivated more slowly at lower temperatures as has been shown for ATP in the guinea-pig vas deferens (Cunnane & Manchanda, 1988).

ATP-overflow and P_2-purinoceptors

If ATP is the transmitter responsible for the observed α-adrenoceptor blockade-resistant constrictor responses, then ATP should be released upon electrical stimulation and should be a vasoconstrictor agent in the human saphenous veins. This was investigated further. Electrical stimulation of veins preincubated with [^{3}H]-noradrenaline induced an overflow of tritium. Tetrodotoxin abolished this tritium overflow. This in agreement with previous findings indicates that the tritium overflow represents neuronal release of [^{3}H]-noradrenaline in the human saphenous vein (Janssens & Verhaeghe, 1983; Göthert *et al.*, 1984; Docherty & Hyland, 1985b; see Starke, 1977). Electrical stimulation caused not only an overflow of tritium but also an overflow of ATP. A concomitant release of noradrenaline and ATP upon electrical stimulation has previously been demonstrated mainly in vasa deferentia: guinea-pig (Kirkpatric & Burnstock, 1987; Lew & White, 1987; Kasakov *et al.*, 1988; von Kügelgen & Starke, 1991b; Vizi *et al.*, 1992), rat (Vizi & Burnstock, 1988) and mouse (Drake & Petersen, 1992) but also in arterial blood vessels: rat caudal artery (Westfall *et al.*, 1987) and rabbit aorta (Sedaa *et al.*, 1990).

Tetrodotoxin abolished both ATP and tritium overflow which seems to support the idea that ATP released from human saphenous veins is neuronal in origin. However, the sources of ATP released upon stimulation of nerve fibres are not exclusively neuronal. The combination of prazosin and rauwolscine markedly reduced the overflow of ATP. This indicates that a major part of the electrically evoked ATP overflow derives from non-neuronal cells upon activation of postjunctional α-adrenoceptors by endogenous noradrenaline. Accordingly, ATP release from smooth muscle or endothelium cells induced by endogenous noradrenaline contributes to the total electrically evoked ATP overflow in the rat caudal artery (Westfall *et al.*, 1987), the rabbit aorta (Sedaa *et al.*, 1990) and the guinea-pig (von Kügelgen & Starke, 1991b; Vizi *et al.*, 1992) and rat vas deferens (Vizi & Burnstock, 1988). The physiological consequences of this non-neuronal ATP release are not known but it has been proposed that ATP released from non-neuronal sources may contribute to the constrictor responses (Vizi *et al.*, 1992). The remaining overflow of ATP in the presence of α-adrenoceptor blockade by prazosin and rauwolscine may reflect neuronally released ATP (see Sedaa *et al.*, 1990).

Are there vasoconstriction-mediating P_2-purinoceptors which could be activated by released ATP? Exogenous ATP and the metabolically stable and P_{2X}-purinoceptor selective analogue α,β-methylene-ATP caused constrictor responses of the human saphenous vein. The P_2-purinoceptor antagonist, suramin (Dunn & Blakeley, 1988; Schlicker *et al.*, 1989; von Kügelgen *et al.*, 1989) markedly attenuated the responses to ATP and α,β-methylene-ATP. These findings indicate that the human saphenous vein smooth muscle possesses P_{2X}-purinoceptors as has been shown for many blood vessels of animal species (see Kennedy, 1990; Hoyle, 1992) as well as for human pial (Hardebo *et al.*, 1987), pulmonary (Liu *et al.*, 1989), subcutaneous and omental arteries (Martin *et al.*, 1991).

If neuronally released ATP plays a role in neurogenic constriction of the human saphenous vein, blockade of P_{2X}-purinoceptors by suramin should attenuate the effector responses to electrical stimulation as has been shown, for example, in mouse vas deferens (von Kügelgen *et al.*, 1989), guinea-pig submucosal arterioles (Evans & Surprenant, 1992) and the pithed rat (Schlicker *et al.*, 1989). However, this was not the case. Suramin did not change the constrictor responses to electrical stimulation in the absence of other drugs and in the presence of α-adrenoceptor blockade by prazosin and rauwolscine. Desensitization of P_{2X}-purinoceptors by prolonged addition of α,β-methylene-ATP has also been used to identify P_{2X}-purinoceptor-mediated components of neurogenic vasoconstriction in isolated blood vessels (von Kügelgen & Starke, 1985; Flavahan & Vanhoutte, 1986b) and perfused organs such as the rat isolated kidney (Schwartz & Malik, 1989; Rump *et al.*, 1990; 1992). In human saphenous veins, α,β-methylene-ATP caused long-lasting increases in resting tone. At 25°C the smooth muscle tone did not return to baseline within 60 min after addition of α,β-methylene-ATP. Therefore, in our study this approach could not be used.

In conclusion, constrictor responses to sympathetic nerve stimulation in human saphenous veins are mainly but not exclusively mediated by neuronally released noradrenaline. Sympathetic nerve stimulation induces a concomitant release of ATP and noradrenaline. There are vasoconstriction-mediating P_2-purinoceptors in the human saphenous vein, but they seem not to be activated by endogenous ATP. Hence, our study failed to identify ATP as the responsible transmitter for α-adrenoceptor blockade-resistant constrictor responses. The nature of this sympathetic transmitter remains unknown. Neuropeptide Y is a vasoconstrictor agent in the human saphenous vein and therefore a likely candidate.

We thank Miss U. Schaible for her skilful and excellent assistance. Samples of saphenous veins were kindly provided by Drs R. Fasol, G. Fraedrich, R. Seitelberger and G. Spillner of the Abteilung für Herzchirurgie, Universitätsklinik Freiburg i. Br. (Dir. Prof. Dr V. Schlosser). Drugs were donated by Bayer (Wuppertal, Germany), Pfizer (Karlsruhe, Germany) and Röhm Pharma (Weiterstadt, Germany). The study was supported by the Deutsche Forschungsgemeinschaft (Ru 401/1-3; SFB 325).

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(Received August 25, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 73–76

The dose-related hyper-and-hypokalaemic effects of salbutamol and its arrhythmogenic potential

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1 The hypokalaemic effect of salbutamol after more than 30 min of administration has been well described. A hyper-and-hypokalaemic effect for adrenaline has been reported, but no such hyperkalaemic effect for salbutamol.

2 The possible hyper-and-hypokalaemic effects of salbutamol with the concomitant potential for pro-arrhythmia were assessed in the baboon (*Papio ursinus*).

3 Male and femal baboons were anaesthetized with ketamine (15 mg kg^{-1}) and maintained with 6% pentobarbitone as spontaneously breathing animals. Six baboons in each group received either 10, 100 or 500 $\mu g\ kg^{-1}$ salbutamol i.v. Lead II of the ECG and femoral i.a. blood pressure were recorded continuously for 10 min. Arterial blood samples were collected at 0 min and then after 3 and 10 min of salbutamol administration.

4 All the animals developed sinus tachycardia (above 200 beats min^{-1}) within 30 s of each dose of salbutamol administration and the high heart rate persisted throughout the experiment. All the animals were hyperkalaemic after 3 min and hypokalaemic after 10 min for each dose of salbutamol. Left ventricular conduction defects were seen in 3 animals during the hyperkalaemic phase. No arrhythmia was seen during the hypokalaemic phase.

5 Salbutamol has a transient hyperkalaemic and a more prolonged hypokalaemic effect in the baboon. The hypokalaemia could not be associated with arrhythmia although conduction defects were associated with the hyperkalaemia.

6 Since salbutamol is used as a bronchodilator in asthmatic patients and to treat acute hyperkalaemia, it is suggested that caution should be exercised when using salbutamol in high doses to treat acute asthma especially during the first few minutes of administration. The finding of hyperkalaemia with salbutamol questions its use in the treatment of hyperkalaemia.

Keywords: Salbutamol; hyperkalaemia; hypokalaemia; arrhythmia; asthma

Introduction

In 1971 the biphasic response of plasma potassium after stimulation with adrenaline was reported. The initial potassium increase occurred within 1 min of a continuous adrenaline infusion and kept on increasing for 6 min followed by a decrease to below control values within 14 min (Todd & Vick, 1971). The first response was shown to be a combined α-adrenoceptor and β-adrenoceptor effect and the second response a β-adrenoceptor effect. The hypokalaemic effect of the β_2-adrenoceptor agonist, salbutamol, was reported in normal volunteers within 30, 60 and 120 min of administration respectively (Smith *et al.*, 1984; Whyte *et al.*, 1987; 1988), within 15 min in cats (Clapham & Hamilton, 1992), and within 15 min in asthmatics (Bremner *et al.*, 1992). However, no hypokalaemia was reported in asthmatics on long-term treatment (12 months) (Hock *et al.*, 1992). The incidence of hypokalaemia occurred irrespective of intravenous infusion, aerosol or nebulized delivery.

From the literature it was suggested that hypokalaemia might be a predisposing factor for tachyarrhythmia (Levy & Wiseman, 1991) but especially in asthmatics if they use β_2-agonists (Leitch *et al.*, 1976). β-Adrenoceptor stimulant drugs are essential for the management of asthma. Yet, if used injudiciously it has been suggested they might be cardiotoxic and lead to death (Morild & Giertzen, 1989; Chazan *et al.*, 1993). This is true for both β_2-selective and non-selective agents (Strunk *et al.*, 1985). However, it is still controversial whether arrhythmias are due to the reported hypokalaemia. In some studies where hypokalaemia was reported no arrhythmias were seen (Smith *et al.*, 1984; Whyte *et al.*, 1987; Bremner *et al.*, 1992; Clapham & Hamilton, 1992). In another study hypokalaemia was reported after 2 h and only one subject had premature supraventricular beats (Whyte *et al.*, 1988). In contrast ventricular ectopic beats were reported in asthmatics who were on long-term (12 months) salbutamol therapy but no hypokalaemia was seen (Hoyer *et al.*, 1992). In none of the above mentioned reports was reference made to a possible dose-dependency of the effects of salbutamol although this has been described by others (Smith *et al.*, 1984; Crane *et al.*, 1989). No hyperkalaemic effects were reported.

It was therefore decided to investigate possible dose-related hyper-and-hypokalaemic effects of salbutamol and its arrhythmogenic potential in the baboon (*Papio ursinus*) as a primate model. The doses were chosen to correspond to that of a therapeutic bronchodilator dose (Hochhaus & Möllmann, 1992) and to a cardiotoxic dose (Chazan *et al.*, 1993).

Method

Male and female baboons with a body weight of between 10 and 15 kg were anaesthetized with ketamine (15 mg kg^{-1}, i.m.) and maintained with 6% pentobarbitone as spontaneously breathing animals. Lead II of the ECG and femoral intra-arterial blood pressure were recorded continuously for 10 min after an i.v. bolus injection of salbutamol. The recordings were made with a Hewlett Packard (HP) 7758 recording system and stored on magnetic tape (HP 3968 instrumentation recorder) for later analyses.

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Blood pressure was measured with an HP Quarts pressure transducer. Data were subsequently analysed with an HP computer. Three groups of baboons were used, each group consisting of six animals. The baboons in each group received either 10, 100 or 500 $\mu g\ kg^{-1}$ salbutamol (SA Druggists) respectively. Baseline values of the parameters measured before each dose served as control values. A placebo group of six baboons each received 2 ml saline which contained 100 μl 70% ethanol. The latter was also used as a solvent for salbutamol. Arterial serum electrolytes and blood gases were determined before drug administration, and 3 and 10 min thereafter. A CX 5 Analyzer (Beckman) and an ABL Blood Gas Analyzer (Radiometer) were used respectively for analyses.

The data were analysed and tested for significance by use of an ANOVA and performing Duncan's test. A P value of less than 0.05 was accepted as significant.

The protocol was approved by the Research and Animal Ethics Committee of the Medical University of Southern Africa. An independent veterinarian monitored the proceedings on random occasions.

Table 1 The dose-response of intravenously administered salbutamol on arterial serum potassium and arterial Po_2 in the baboon

	K^+ (mmol l^{-1})	Po_2 (kPa)
Placebo	3.35 ± 0.45	9.26 ± 0.41
Salbutamol 10 $\mu g\ kg^{-1}$		
Control	3.58 ± 0.33	10.38 ± 1.01
3 min	4.23 ± 0.57*	11.93 ± 1.37*
10 min	3.42 ± 0.21†	11.17 ± 0.99*
Salbutamol 100 $\mu g\ kg^{-1}$		
Control	3.65 ± 0.90	9.85 ± 1.23
3 min	4.13 ± 0.38*	11.63 ± 1.64*
10 min	3.25 ± 0.31†	11.71 ± 1.71*
Salbutamol 500 $\mu g\ kg^{-1}$		
Control	3.17 ± 0.41	9.74 ± 1.58
3 min	3.75 ± 0.62*	12.59 ± 1.78*
10 min	2.02 ± 0.29†	11.28 ± 1.30*

Values are given as means ± s.d. for $n = 6$.
*Significantly different from control values: $P < 0.05$.
†Significantly different from the 3 min interval: $P < 0.05$ (ANOVA, Duncan's test).

Results

Arterial serum potassium and electrolytes

The arterial serum potassium levels increased significantly ($P < 0.05$) at the 3 min interval for each of the salbutamol doses and returned to below base-line values at the 10 min interval. The largest increase was 0.65 mmol l^{-1} (after administration of 10 $\mu g\ kg^{-1}$ salbutamol) and the largest decrease was 1.15 mmol l^{-1} (after administration of 500 $\mu g\ kg^{-1}$ salbutamol). The potassium increase was not dose-related while the decrease was dose-related (Table 1). Serum Ca^{2+}, Mg^{2+} and Na^+ were unchanged.

T wave amplitude

The T wave amplitude started to increase within 10 s of salbutamol administration and reached a maximum after 30 s for each dose. The duration of the effect was dose-related. The magnitude of the effect after 500 $\mu g\ kg^{-1}$ salbutamol was almost the same at 30 s and 10 min, while the magnitude of the effect after 10 $\mu g\ kg^{-1}$ salbutamol was back to base-line values after 10 min (Table 2).

Heart rate and arrhythmia

The initial sinus tachycardia after each dose of salbutamol remained relatively high throughout the experiment (10 min). A maximum of 202 ± 7, 206 ± 10, and 221 ± 15 beats min^{-1} was reached after 10, 100 and 500 $\mu g\ kg^{-1}$ salbutamol respectively. Except for the 10 $\mu g\ kg^{-1}$ dose for which the maximum effect was reached after 30 s, the maxima for the 100 and 500 μg doses were reached after 3 min (Table 2). Despite the tachycardia, only two baboons developed isolated abberrant ventricular conduction defects after 100 $\mu g\ kg^{-1}$ salbutamol at the 1 min (3 complexes) and 2 min (1 complex) intervals respectively and one baboon developed 46 successive such complexes after 500 $\mu g\ kg^{-1}$ salbutamol at the 3 min interval. All the animals were hyperkalaemic at that stage. However, for each dose of salbutamol, all the animals were hypokalaemic at the 10 min interval but no arrhythmia was seen.

Table 2 The dose-response of intravenously administered salbutamol on heart rate, blood pressure and T wave amplitude in the baboon

	Heart rate (beats min^{-1})	*Systolic pressure* (mmHg)	*Diastolic pressure* (mmHg)	*T wave* (mV)
Placebo	141 ± 6	175 ± 12	114 ± 7	0.077 ± 0.043
Salbutamol 10 $\mu g\ kg^{-1}$				
Control	129 ± 8	172 ± 14	102 ± 11	0.076 ± 0.052
30 s	202 ± 7*	164 ± 13*	79 ± 18*	0.196 ± 0.089*
3 min	183 ± 12*	184 ± 9	107 ± 8	0.093 ± 0.074*
10 min	160 ± 17*	189 ± 11	107 ± 8	0.057 ± 0.083
Salbutamol 100 $\mu g\ kg^{-1}$				
Control	128 ± 13	158 ± 15	91 ± 16	0.050 ± 0.095
30 s	203 ± 13*	119 ± 26*	46 ± 14*	0.257 ± 0.127
3 min	206 ± 10*	158 ± 32	77 ± 24	0.250 ± 0.121*
10 min	192 ± 11*	172 ± 24	92 ± 13	0.156 ± 0.131
Salbutamol 500 $\mu g\ kg^{-1}$				
Control	146 ± 4	179 ± 14	107 ± 13	0.090 ± 0.023
30 s	218 ± 13*	114 ± 30	46 ± 17*	0.245 ± 0.082
3 min	221 ± 15*	149 ± 30	74 ± 25*	0.253 ± 0.039*
10 min	211 ± 13*	177 ± 10	87 ± 14*	0.240 ± 0.132

Values are given as means ± s.d. for $n = 6$.
*Significantly different from control values: $P < 0.05$ (ANOVA, Duncan's test).

Blood pressure

In all cases blood pressure decreased rapidly and dose-dependently within the first 10 s and reached a maximum after 30 s after which it returned to base-line values. The difference between 100 and 500 $\mu g\,kg^{-1}$ salbutamol on the maximum decrease in diastolic pressure was not significant, indicating that a maximal decrease was reached after 100 $\mu g\,kg^{-1}$ salbutamol (Table 2). The diastolic pressure changed more in magnitude than the systolic pressure and was dose-related (Table 2).

Arterial blood gases

The Po_2 increased significantly for each of the doses and was only slightly lower at the 10 min interval compared to the 3 min interval. No other blood gas parameters showed any change (Table 1).

The data were compared to control values. The placebo had no effect.

Discussion

Arterial serum potassium and arrhythmia

It was clearly shown that an i.v. bolus injection of salbutamol had a biphasic effect on arterial serum potassium: first a transient hyperkalaemic effect followed by a more prolonged hypokalaemic effect. The hyperkalaemic effect was not dose-dependent since no significant differences could be found between the different doses. However, the hypokalaemic effect appeared to be dose-dependent. Although no difference could be found in effect between 10 and 100 $\mu g\,kg^{-1}$ salbutamol, both differed significantly from the 500 $\mu g\,kg^{-1}$ dose. A similar biphasic response has been reported for adrenaline (Todd & Vick, 1971; Vick *et al.*, 1972) but only a hypokalaemic effect has been reported for salbutamol (Smith *et al.*, 1984; Whyte *et al.*, 1987; 1988) perhaps because the blood samples were taken after the occurrence of the earlier hyperkalaemia. The present results show that the hyperkalaemic effect occurs within 3 min after salbutamol administration. It occurred within 1 min after adrenaline as reported by others (Todd & Vick, 1971; Vick *et al.*, 1972).

Although we did not measure potassium levels within 1 min the possibility exists that the hyperkalaemic effect of salbutamol might also occur within a minute as reported for adrenaline. To support this possibility the results show that the T wave amplitude began to increase within 30 s after salbutamol administration (Table 2) (Mudge & Weiner, 1990).

Our study shows that salbutamol causes hypokalaemia after 10 min in the baboon, while other studies showed the hypokalaemic effect only after 15 min (Bremner *et al.*, 1992; Clapham & Hamilton, 1992) and longer (Whyte *et al.*, 1987; 1988). The hypokalaemic effect could conceivably be mediated by the stimulation of β-adrenoceptors in the liver and skeletal muscle through the activation of Na^+-K^+-ATPase, which increased the uptake of potassium (Vick *et al.*, 1972; Clapham & Hamilton, 1992) and more specifically by β_2-receptors (Brown *et al.*, 1983).

Although it has been shown that sympathomimetic hyperkalaemia is mediated through α-adrenoceptors (Williams *et al.*, 1984), a hyperkalaemic effect after salbutamol administration was shown to be dependent on β_2-adrenoceptor stimulation, but no reference was made to the origin of the potassium (Reverte *et al.*, 1993). This dual mechanism remains unexplained. In addition it is speculated that the increase in potassium might be the result of potassium release from skeletal muscle after β_2-adrenoceptor stimulation similar to that seen during exercise (Hutchinson *et al.*, 1992).

The suggested use of salbutamol to treat hyperkalaemia during dialysis (Leanza *et al.*, 1992) or renal failure (Mocan *et al.*, 1993) could therefore be extremely dangerous if induction of hyperkalaemia occurs in man also, since this study suggests that salbutamol, although only transiently, causes hyperkalaemia.

Furthermore it was suggested that hypokalaemia might be a predisposing factor for tachyarrhythmia (Steinfath *et al.*, 1992) but our study showed only an abberrant ventricular conduction defect during the hyperkalaemic phase and tachycardia during all the phases. The abberrant ventricular conduction could be secondary to the hyperkalaemia but the possibility that the precipitous decrease in afterload could lead to myocardial ischaemia, and in turn to arrhythmia cannot be excluded. The tachycardia is probably a combined result of reflex-mediation due to the drop in blood pressure and a direct β_2-adrenoceptor stimulation (Steinfath *et al.*, 1992).

T wave amplitude

Since the T wave amplitude followed more or less the same pattern as the changes in the potassium concentrations, with an initial increase followed by a decrease in amplitude, it is suggested that the T wave changes reflect the changes in the serum potassium concentration.

Blood pressure and blood gases

The fall in diastolic and systolic pressure recovered within 2 min due in part to a reflex-mediated tachycardia. However, after 10 min the blood pressure was back to base-line values while the tachycardia persisted. This latter response could be a direct β_2-mediated chronotropy (Steinfath *et al.*, 1992) together with a sustained reflex response.

In conclusion, salbutamol has a transient hyperkalaemic effect as well as a more prolonged hypokalaemic effect, both of which could be responsible for cardiotoxic effects. Conduction defects might occur during the transient hyperkalaemia. It would therefore be extremely important to monitor an asthmatic patient especially during the first few minutes of emergency treatment with β_2-agonists. Furthermore, caution should be taken when treating acute hyperkalaemia with β_2-agonists.

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(Received June 15, 1993
Revised August 31, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 77–82

Mechanism underlying substance P-induced relaxation in dog isolated superficial temporal arteries

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1 In helical strips of dog superficial temporal arteries with intact endothelium, substance P elicited a concentration-related relaxation with an EC_{50} of 2.8 (2.4–3.2) $\times 10^{-10}$ M.

2 The relaxant response to the peptide in low concentrations ($1-4 \times 10^{-10}$ M) sufficient to produce approximately half maximal relaxation was not inhibited by indomethacin, but was markedly suppressed by N^G-nitro-L-arginine (L-NOARG), a nitric oxide (NO) synthase inhibitor, and by endothelium denudation.

3 High concentration (10^{-7} M) of substance P produced marked relaxations in endothelium-intact strips. Removal of the endothelium attenuated the relaxation, and indomethacin or tranylcypromine suppressed the endothelium-independent relaxation. In indomethacin-treated strips with intact endothelium, L-NOARG attenuated but did not abolish the relaxation. The residual, L-NOARG-resistant relaxation was not significantly inhibited by ouabain, glibenclamide or tetraethylammonium.

4 Substance P (10^{-7} M) increased the levels of cyclic GMP and cyclic AMP. The increase in cyclic GMP was abolished by endothelium denudation and treatment with L-NOARG, whereas the cyclic AMP increment was abolished by indomethacin.

5 Three different mechanisms may be involved in the substance P-induced relaxation: (1) an endothelium-dependent relaxation mediated by the release of NO from the endothelium, resulting in an increase of cyclic GMP (low and high concentrations of the peptide); (2) an endothelium-independent relaxation in association with cyclic AMP increment caused by prostaglandin I_2 released from sub-endothelial tissues (high concentration), and (3) another endothelium-dependent relaxation possibly mediated by unidentified mediator(s) released from the endothelium (high concentration).

Keywords: Superficial temporal artery; endothelium; nitric oxide; substance P; prostaglandin I_2; EDRF

Introduction

Peptides, amines and other endogenous vasoactive substances are implicated in the pathophysiology of various forms of headache, local pain reactions and vasospasms following subarachnoid haemorrhage. It is postulated that one of the main causes of migraine headache is dilatation of extracranial arteries, such as superficial temporal arteries (Drummond & Lance, 1983; Olesen, 1991). Histochemical studies on extracranial arteries have demonstrated that there are abundant nerve fibres containing vasoactive peptides, such as substance P, calcitonin gene-related peptide, and vasoactive intestinal polypeptide (Uddman *et al.*, 1986; 1989; Edvinsson *et al.*, 1988; Uddman & Edvinsson, 1989), mediating vasodilatation of intra- and extra-cranial arteries.

Substance P dilates blood vessels mainly via a release of relaxing substances from the endothelium (Furchgott, 1983; Toda *et al.*, 1991). Goadsby *et al.* (1988) have reported a marked elevation of plasma level of substance P in external jugular venous blood during stimulation of the trigeminal ganglion, which contributes to the innervation of superficial temporal arteries and is involved in the modulation of cranial pain (Moskowitz, 1984). The peptide release may produce vasodilatation in extracranial arteries, including the superficial temporal artery, and lead to the provocation of vascular headache. Nevertheless, effects of substance P in superficial temporal arteries have not been clarified.

The present study was undertaken to analyse the mechanism of action of substance P in the dog superficial temporal arteries, with special reference to the endothelium, nitric oxide (NO) and prostaglandin I_2 (PGI_2). We found that the mechanism of action differ with low and high concentrations of the peptide, and endothelium-derived relaxing factor (EDRF), as distinct from NO, is partially involved in the substance P-induced relaxation.

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Methods

Preparation

Mongrel dogs of either sex, weighing 7–15 kg, were anaesthetized with i.v. injections of sodium pentobarbitone (30 mg kg^{-1}) and killed by bleeding from the carotid arteries. Superficial temporal arteries were rapidly isolated. The arteries (0.6–1.5 mm o.d.) were cut helically into strips, approx. 15 mm long. The strips were fixed vertically between hooks in a muscle bath of 20 ml capacity containing the modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Koden Kogyo, Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing the maximal contraction. Constituents of the solution were as follows (mM): NaCl 120, KCl 5.4, $NaHCO_3$ 25.0, $CaCl_2$ 2.2, $MgCl_2$ 1.0 and dextrose 5.6. The pH of the solution was 7.35–7.41. Before the start of experiments, the arterial strips were allowed to equilibrate for 60–90 min in the control medium, during which time the solution was replaced every 10–15 min.

Tension recording

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. Contractions induced by 30 mM K^+ were obtained first and then the strips were washed three times with fresh media. Relaxant responses to the agents were obtained in the strips partially contracted with $PGF_{2\alpha}$, the contraction being in a range between 20 and 35% of the contractions induced by 30 mM K^+. At the end of each series of experiment, 10^{-4} M papaverine was added to attain the maximal relaxation. Since tachyphylaxis to subtance P rapidly developed, the peptide (10^{-7} M) was applied

repeatedly until the response was stabilized. Then, substance P in a single concentration (10^{-10}–10^{-7} M) was applied to obtain the concentration-response relationship. In some experiments, substance P, sodium nitroprusside and NO in single concentrations were applied successively. The relaxant response to the drug was expressed as the relative value to that induced by papaverine. After the responses to the agents were stabilized, the effects of blocking agents were examined by treatment of strips for 20 min or longer. In other experiments, one to three pairs of strips with and without the endothelium were obtained from each dog, and paired comparisons were made in the responses of endothelium-intact and -denuded strips. The endothelium was removed by gently rubbing the intimal surface with a cotton ball. The endothelium denudation was confirmed by the abolition or marked suppression of the relaxation produced by 10^{-6} M acetylcholine, and also by silver staining procedure (Abrol *et al.*, 1984).

Measurement of cyclic AMP and cylic GMP

Contents of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in dog superficial temporal artery strips with and without the endothelium were measured. After 20 min of equilibration in the control bathing medium, and that containing indomethacin or N^G-nitro-L-arginine (L-NOARG), the strips were exposed to 10^{-7} M substance P, then plunged into liquid nitrogen. The tissues were homogenized in 1.5 ml of 6% trichloroacetic acid at 0°C with a glass homogenizer. After centrifugation at 800 *g* for 10 min, an ether extraction procedure was carried out on the supernatant. An aliquot of the extract was then used for determination of cyclic AMP and cylic GMP by the use of commercial radioimmunoassay kit (Yamasa Co., Chiba, Japan).

Statistics and drugs used

The results shown in the text and figures are expressed as mean values ± s.e.mean. EC_{50} is expressed as geometric means with 95% confidence limits. Statistical analyses were made with Student's unpaired *t* test and Tukey's method after one-way analysis of variance. Drugs used were substance P, N^G-nitro-L-arginine (L-NOARG), N^G-nitro-D-arginine (D-NOARG) (Peptide Institute Inc., Minoh, Japan), L- and D-Arginine (L- and D-Arg), tetraethylammonium bromide, tranylcypromine hydrochloride (Nacalai Tesque, Kyoto, Japan), indomethacin, glibenclamide (Sigma, St. Louis, MO., U.S.A.), sodium nitroprusside, ouabain octahydrate (Merck, Darmstadt, Germany), beraprost sodium (Toray-Kaken Pharmaceutical Co., Tokyo), prostaglandin $F_{2\alpha}$ (Ono Co., Osaka, Japan), papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka), and acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo). Responses to nitric oxide (NO) were obtained by adding the $NaNO_2$ solution adjusted to pH 2 (Furchgott, 1988).

Results

Mechanical responses to substance P

The addition of substance P (10^{-10}–10^{-7} M) produced a concentration-related relaxation in dog superficial temporal artery strips partially contracted with $PGF_{2\alpha}$; the EC_{50} was 2.8 $(2.4-3.2) \times 10^{-10}$ M ($n = 6$) (Figure 1, left panel). Removal of the endothelium markedly inhibited the relaxant response to the peptide in concentrations from 1 to 4×10^{-10} M, and moderately attenuated the response to the higher concentrations. The EC_{50} of the substance P-induced relaxations in endothelium-denuded strips was 1.3 $(1.0-1.7) \times 10^{-9}$ M (n = 6). Substance P in low concentrations produced only endothelium-dependent relaxation, whereas the peptide in concentrations higher than 4×10^{-10} M produced both endothelium-dependent and -independent relaxations. Modifications by indomethacin (10^{-6} M) and L-NOARG (3×10^{-4} M) of the concentration-related relaxant responses to substance P in the endothelium-intact strips are shown in right panel of Figure 1. In endothelium-intact artery strips, indomethacin did not influence the relaxant responses, but additional treatment with 3×10^{-4} M L-NOARG markedly suppressed the relaxant responses to the peptide in low concentrations, and moderately attenuated the response to the higher concentrations. Indomethacin (10^{-6} M) or L-NOARG (3×10^{-4} M) slightly increased the basal tone and augmented the contractile response to $PGF_{2\alpha}$. However, the precontraction levels were adjusted to a magnitude similar to that in control media by decreasing the amount of $PGF_{2\alpha}$ applied. In the remainder of this paper, mechanisms of the substance P-induced relaxation were analysed separately by using low ($1-4 \times 10^{-10}$ M, producing approximately half maximal relaxation) and high (10^{-7} M) concentrations.

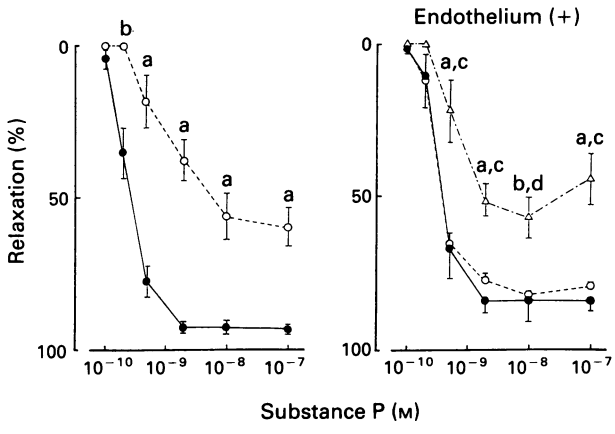

Figure 1 Modification by endothelium denudation (left panel), indomethacin (10^{-6} M) and indomethacin plus N^G-nitro-L-arginine (L-NOARG, 3×10^{-4} M) (right panel) of relaxant responses to substance P in dog superficial temporal artery strips partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $3-10 \times 10^{-7}$ M). Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values at 10^{-10} M substance P in the strips with (●) and without the endothelium (○) were 240 ± 11 and 196 ± 21 mg ($n = 6$), respectively (left panel) and those in control arterial strips (●), treated with indomethacin (○) and indomethacin plus L-NOARG (△) were 230 ± 26, 200 ± 19 and 248 ± 38 mg ($n = 5$), respectively (right panel). There were no significant differences in the size of the relaxant response to 10^{-4} M papaverine in each concentration of substance P (10^{-10}, 2×10^{-10}, 5×10^{-10}, 2×10^{-9}, 10^{-8} and 10^{-7} M). Left panel; significantly different from the values in endothelium-intact arteries. $^aP<0.01$; $^bP<0.05$. Right panel; significantly different from control, $^aP<0.01$; $^bP<0.05$. Significantly different from the value treated with indomethacin, $^cP<0.01$; $^dP<0.05$ (Tukey's method). Values are means ± s.e.mean.

In endothelium-intact artery strips partially contracted with $PGF_{2\alpha}$, substance P ($1-4 \times 10^{-10}$ M) and sodium nitroprusside (SNP, 3×10^{-8} M) produced moderate relaxations (Figure 2). Indomethacin (10^{-6} M) did not alter the relaxation, but additional treatment with L-NOARG (10^{-4} M) abolished the peptide-induced relaxation, whereas the response to SNP was not inhibited. L-NOARG inhibited the peptide-induced relaxation in a concentration-dependent manner; mean values of the relaxation relative to that caused by 10^{-4} M papaverine in control media and those containing 10^{-6} M indomethacin and indomethacin plus 10^{-5} or 10^{-4} M L-NOARG were 47.3 ± 2.6% ($n = 17$), 44.9 ± 2.5% ($n = 23$), 10.4 ± 1.9% ($P<0.01$ vs. control and indomethacin, $n = 18$) and 1.8 ± 0.9% ($P<0.01$ vs. control, indomethacin and indomethacin plus 10^{-5} M L-NOARG, $n = 9$), respectively.

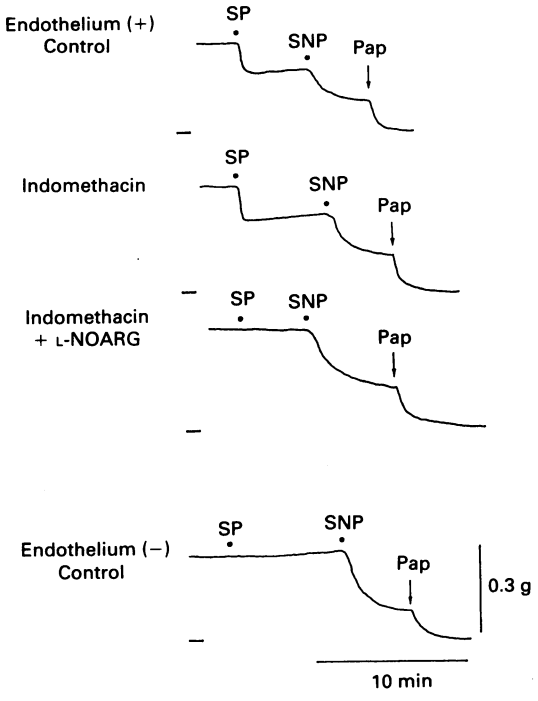

Figure 2 Typical recordings of the response to substance P (SP, 2×10^{-10} M) and sodium nitroprusside (SNP, 3×10^{-8} M) of dog superficial temporal artery strips with and without the endothelium obtained from the same dog. Responses of the endothelium-intact strip were obtained before (control) and after treatment with 10^{-6} M indomethacin or indomethacin plus 10^{-4} M N^G-nitro-L-arginine (L-NOARG). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $2-5 \times 10^{-7}$ M): the level before the addition of $PGF_{2\alpha}$ is shown as a horizontal line just left of each tracing. Pap represents 10^{-4} M papaverine that produced maximal relaxation.

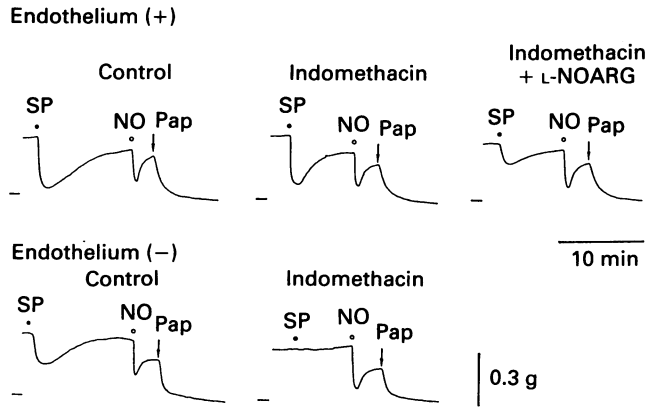

Figure 3 Typical tracings of the response to substance P (SP, 10^{-7} M) and nitric oxide (NO, 10^{-6} M) of dog superficial temporal artery strips with and without the endothelium. Responses of the endothelium-intact strips were obtained before (control) and after treatment with 10^{-6} M indomethacin or indomethacin plus 10^{-4} M N^G-nitro-L-arginine (L-NOARG) (upper tracing). Responses of the endothelium-denuded strips were obtained before (control) and after treatment with indomethacin (lower tracing). The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $2-5 \times 10^{-7}$ M): the level before the addition of $PGF_{2\alpha}$ is shown as a horizontal line just to the left of each tracing. Pap represents 10^{-4} M papaverine that produced maximal relaxation.

D-NOARG was without effect; mean values of the relaxation in control media and those containing 10^{-5} and 10^{-4} M D-NOARG were 44.5 ± 5.2%, 40.3 ± 6.6%, and 37.3 ± 8.3% ($n = 6$), respectively. The inhibitory effect of 10^{-5} M L-NOARG was reversed by the addition of 3×10^{-3} M L-arginine (L-Arg) but not by 3×10^{-3} M D-Arg; mean values of the relaxation in control media and those containing 10^{-5} M L-NOARG, 10^{-5} M L-NOARG plus 3×10^{-3} M D-Arg and 10^{-5} M L-NOARG plus 3×10^{-3} M L-Arg were 42.3 ± 4.4%, 4.2 ± 1.4% ($P<0.01$ vs. control), 0.8 ± 0.4% ($P<0.01$ vs. control) and 29.5 ± 4.5% ($P<0.05$ vs. control, $P<0.01$ vs. 10^{-5} M L-NOARG) ($n = 4$), respectively. Removal of the endothelium abolished the relaxation caused by substance P in low concentrations (Figure 2, lower); mean values of the relaxation in endothelium-intact and -denuded strips were 45.1 ± 3.8% and 1.9 ± 1.4% ($P<0.01$, $n = 9$), respectively.

The addition of substance P (10^{-7} M) produced marked relaxations in the endothelium-intact artery strips. Typical responses to substance P and NO are illustrated in the upper tracing of Figure 3. Treatment with indomethacin (10^{-6} M) did not affect the magnitude of the relaxation, but accelerated the recovery. Additional treatment with 10^{-4} M L-NOARG significantly suppressed but did not abolish the relaxation. Relaxations caused by exogenous NO were not influenced by indomethacin and L-NOARG. Increase in the concentration of L-NOARG to 3×10^{-4} M did not produce the additional inhibition; mean values of the relaxation in control media and those containing 10^{-6} M indomethacin and indomethacin plus 10^{-5} M, 10^{-4} M or 3×10^{-4} M L-NOARG were 75.3 ± 2.0% ($n = 29$), 74.9 ± 2.6% ($n = 31$), 44.1 ± 7.4% ($P<0.01$ vs. control and indomethacin, $n = 15$), 43.6 ± 4.3% ($P<0.01$ vs. control and indomethacin, $n = 31$) and 40.7 ± 4.2% ($P<0.01$ vs. control and indomethacin, $n = 23$), respectively. The inhibitory effect of 10^{-5} M L-NOARG was reversed by the addition of 3×10^{-3} M L-Arg but not by D-Arg ($n = 3$, data not shown). The residual relaxation under combined treatment with indomethacin and 3×10^{-4} M L-NOARG was not significantly influenced by 10^{-6} M ouabain (from 47.2 ± 5.4% to 43.3 ± 5.9%, $n = 8$), 10^{-6} M glibenclamide (from 55.0 ± 8.7% to 42.7 ± 11.5%, $n = 5$), 10^{-3} M (from 39.8 ± 9.5% to 49.0 ± 7.4%, $n = 5$) or 10^{-2} M tetraethylammonium (from 40.1 ± 4.8% to 29.5 ± 6.2%, $n = 8$). Although, ouabain and tetraethylammonium *per se* evoked a contraction and augmented the response to $PGF_{2\alpha}$, the precontraction level was matched to that in the absence of the inhibitors. On the other hand, glibenclamide had no effect on the basal tone nor the contractile response to $PGF_{2\alpha}$. In the endothelium-denuded artery strips, substance P produced moderate relaxation, which was significantly less than the relaxation in endothelium-intact strips (Figure 1, left). The relaxation in the strips without endothelium was abolished by treatment with 10^{-6} M indomethacin (Figure 3, lower) and suppressed by 3×10^{-4} M tranylcypromine; mean values of the relaxation in control media and those containing 10^{-6} M indomethacin and 3×10^{-4} M tranylcypromine were 48.7 ± 2.4% ($n = 28$), 3.9 ± 0.7% ($P<0.01$ vs. control, $n = 24$) and 24.4 ± 6.2% ($P<0.01$ vs. control, $n = 12$), respectively. Tranylcypromine in the same concentration attenuated the relaxant response to beraprost, a stable analogue of PGI_2 (Toda, 1988), in concentrations of $1-3 \times 10^{-8}$ M, sufficient to produce similar magnitude of relaxation to those elicited by substance P; however, the inhibition (30.9 ± 10.2%, $n = 12$) was significantly less than that induced by substance P (53.8 ± 8.6% inhibition, $n = 12$; $P<0.05$).

Cyclic AMP and cyclic GMP levels

The addition of 10^{-7} M substance P significantly increased the content of cyclic AMP and cyclic GMP in the endothelium-intact artery strips. The peak effect on cyclic GMP was attained 1 min later (94.3 ± 28.1% increase, $n = 6$; $P<0.01$) and the plateau of cyclic AMP increment was obtained from 30 s to 2 min (80.0 ± 22.6%, $n = 5$, $P<0.01$).

Therefore, contents of cyclic AMP and cyclic GMP were measured 1 min after the peptide application in the same samples from different individuals.

In endothelium-intact and denuded artery strips, basal levels of cyclic AMP averaged 166.6 ± 26.3 ($n = 7$) and 112.5 ± 20.5 fmol mg^{-1} frozen tissue ($n = 5$), respectively. Substance P (10^{-7} M) significantly increased the cyclic AMP levels in both strips (Figure 4). The stimulating effect was not influenced by treatment with 5×10^{-6} M L-NOARG, but was abolished by 10^{-6} M indomethacin. Indomethacin alone decreased the basal level of cyclic AMP in endothelium-intact artery strips (11.4 ± 3.8% decrease $n = 7$; $P < 0.05$).

The level of cyclic GMP in endothelium-intact artery strips (255.8 ± 43.8 fmol mg^{-1} frozen tissue, $n = 7$) was increased by 149.7 ± 14.6% following treatment with 10^{-7} M substance P. L-NOARG (3×10^{-4} M) significantly decreased the basal level of cyclic GMP (89.2 ± 2.5% decrease, $n = 7$; $P < 0.01$) and abolished the stimulating effect of substance P on the cyclic GMP level. Removal of the endothelium decreased the cyclic GMP level to 24.2 ± 9.9 fmol mg^{-1} frozen tissue (90.5 ± 2.7% decrease, $n = 4$; $P < 0.01$) and abolished the effect of substance P (Figure 5).

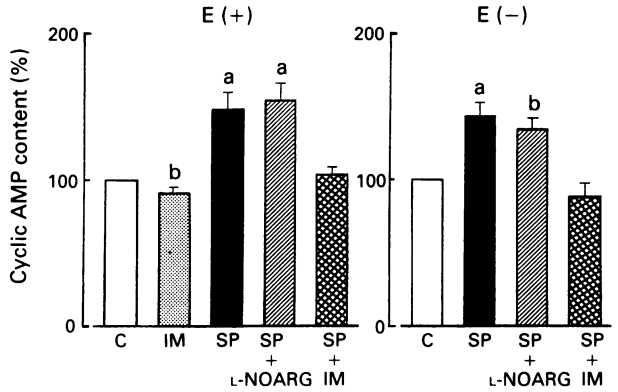

Figure 4 Effect of substance P (SP, 10^{-7} M) in the absence and presence of N^G-nitro-L-arginine (L-NOARG, 5×10^{-6} M) or indomethacin (IM, 10^{-6} M) on the cyclic AMP formation in dog superficial temporal artery strips with (left panel) and without the endothelium (E) (right panel). Basal levels (C) of the nucleotide in endothelium-intact and -denuded strip were 166.6 ± 26.3 ($n = 7$) and 112.5 ± 20.5 ($n = 5$) fmol mg^{-1} frozen tissue, respectively. Significantly different from control, $^aP < 0.01$; $^bP < 0.05$ (Tukey's method). Columns show mean values with s.e.mean.

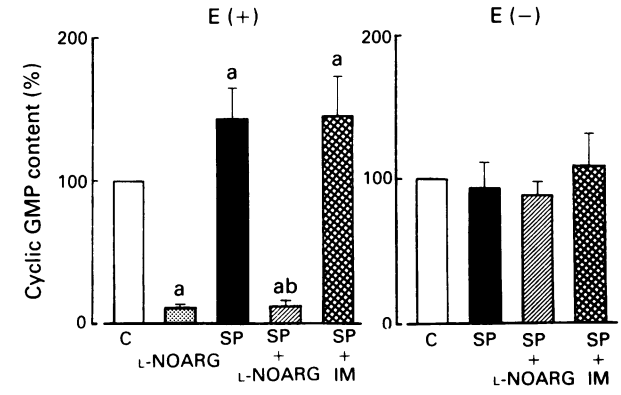

Figure 5 Effect of substance P (SP, 10^{-7} M) in the absence and presence of N^G-nitro-L-arginine (L-NOARG, 3×10^{-4} M) on the cyclic GMP formation in dog superficial temporal artery strips with the endothelium (E) (left panel); and effect of the peptide in the absence and presence of L-NOARG (5×10^{-6} M) or indomethacin (IM, 10^{-6} M) on the cyclic GMP formation in the strips without the endothelium (right panel). Basal levels (C) of the nucleotide in the endothelium-intact and -denuded strips were 255.8 ± 43.8 ($n = 7$) and 24.2 ± 9.9 ($n = 4$) fmol mg^{-1} frozen tissue, respectively. Significantly different from control, $^aP < 0.01$; and from the value treated with substance P, $^bP < 0.01$ (Tukey's method). Columns show mean values with s.e.mean.

Discussion

The present study revealed that low concentrations of substance P elicited only an endothelium-dependent relaxation in dog isolated superficial temporal arteries, which was not influenced by treatment with indomethacin but was abolished by L-NOARG, an NO synthase inhibitor (Moore *et al.*, 1990; Toda *et al.*, 1990). These findings suggest that the peptide releases NO from the endothelium that acts on smooth muscle to increase the production of cyclic GMP, responsible for a muscle relaxation. Similar results were also obtained in monkey temporal arteries (Toda *et al.*, 1991), dog cerebral arteries (Onoue *et al.*, 1988; Toda & Okamura, 1991) and various arteries from different species (Zawadzki *et al.*, 1981; Furchgott, 1983).

High concentrations of substance P produced endothelium-dependent and -independent relaxations. The addition of 10^{-7} M substance P elicited a marked relaxation in endothelium-intact artery strips. Indomethacin did not influence the relaxation, and additional treatment with L-NOARG suppressed the relaxation. In endothelium-denuded artery strips, substance P (10^{-7} M) produced a moderate relaxation, which was abolished by treatment with indomethacin or markedly suppressed by tranylcypromine, a PGI_2 synthase inhibitor (Gryglewski *et al.*, 1970). Substance P increased the cyclic AMP level in endothelium-intact and denuded strips, the increase being abolished by indomethacin. Therefore, endothelium-independent relaxations observed in the arteries stimulated by the peptide may be produced by vasodilator prostaglandins, possibly PGI_2, that are released from subendothelial tissues and are responsible for cyclic AMP formation.

On the other hand, endothelium-dependent relaxation caused by a high concentration of substance P in endothelium-intact strips treated with indomethacin was partially inhibited but not abolished by L-NOARG even when the arteries were treated with high concentrations (up to 3×10^{-4} M). However, the stimulating effect of the peptide on the production of cyclic GMP was completely abolished by 3×10^{-4} M L-NOARG. Therefore, the relaxation appears to be associated partly with endothelium-derived NO, as observed in the arteries stimulated by low concentrations of substance P, and also with a mechanism other than that via NO and cyclic GMP. Similar responses, resistant to NO synthase inhibitors, have been reported in porcine coronary (Richard *et al.*, 1990; Cowan & Cohen, 1991; Matsumoto *et al.*, 1993), human gastroepiploic (O'Neil *et al.*, 1991) and coronary arteries (Chester *et al.*, 1990) and canine renal (Pawloski & Chapnick, 1991) and femoral veins (Miller, 1991) in response to substance P, bradykinin, leukotriene D_4, acetylcholine, ADP or thrombin. In the present study, the peptide-induced relaxation resistant to L-NOARG, was not influenced by ouabain, glibenclamide and tetraethylammonium, suggesting that the electrogenic sodium pump, ATP-activated K^+ channel and voltage-dependent K^+ channel are not involved. The inability of substance P to liberate endothelium-derived hyperpolarizing factors from guinea-pig small mesenteric arteries has been demonstrated by Bolton & Clapp (1986). On the other hand, Vanhoutte and coworkers have suggested the involvement of sodium pump activation and K^+ channel opening, because relaxations of canine coronary arteries induced by arachidonic acid and acetylcholine are susceptible to ouabain and glibenclamide (Rubanyi & Vanhoutte, 1985; Feletou & Vanhoutte, 1988; Hoeffner *et al.*, 1989).

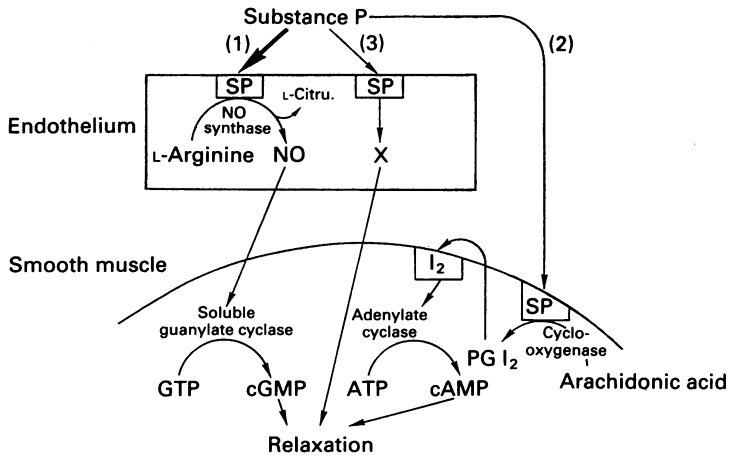

Figure 6 Schematic presentation of possible mechanisms of action of substance P in dog superficial temporal artery strips. SP, substance P; NO, nitric oxide; PGI_2, prostaglandin I_2; X, unknown mediator(s); Squares in the endothelium and smooth muscle represent receptors.

Figure 6 is a hypothetical diagram on the mechanism of action of substance P in dog superficial temporal arteries. The peptide acts (1) on receptors in the endothelium to promote the synthesis and release of NO, which activates soluble guanylate cyclase in smooth muscle, resulting in the increase of cyclic GMP responsible for an endothelium-dependent relaxation; (2) on smooth muscle to synthesize and release PGI_2, which generates cyclic AMP in smooth muscle and produces an endothelium-independent relaxation; and (3) on the endothelium to produce another endothelium-dependent relaxation by releasing relaxing factor(s) distinct from NO ('X' in Figure 6), which does not appear to be mediated by cyclic nucleotides nor associated with sodium pump activation or K^+ channel opening. Substance P is one of the important vasodilator substances which are thought to be involved in the modulation of cranial vessel tone.

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(Received June 8, 1993
Revised September 1, 1993
Accepted September 7, 1993)

Br. J. Pharmacol. (1994), **111**, 83–88

Nitric oxide pathway-mediated relaxant effect of bradykinin in the guinea-pig isolated trachea

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1 The effects of two nitric oxide (NO) biosynthesis-inhibitors N^G-nitro-L-arginine (L-NOARG) and N^G-monomethyl-L-arginine (L-NMMA) on the relaxation induced by bradykinin (BK, 100 nM), isoprenaline (Iso, 1 μM) and sodium nitroprusside (SNP, 1 μM) were investigated in epithelium-intact strips of guinea-pig isolated trachea.

2 Relaxations induced by BK (100 nM) in guinea-pig tracheal strips under spontaneous tone were inhibited in a concentration-related manner by L-NOARG and L-NMMA (1 to 100 μM), with IC_{50}s (and 95% confidence limits) of 9.1 (6.9– 11.6) μM and 7.0 (4.2– 12.3) μM, respectively. However, at the maximal concentration (100 μM) used, neither of these drugs inhibited completely BK-induced relaxation (maximal inhibition of 74 ± 7 and 67 ± 7%, respectively). On the other hand, D-NMMA, the D-enantiomer of L-NMMA, up to 100 μM failed to inhibit BK-induced relaxation. The relaxation induced by Iso (1 μM) and SNP (1 μM) were not affected by either L-NOARG or L-NMMA (30 μM).

3 The inhibition of BK-induced relaxation caused by L-NOARG and L-NMMA was partially reversed by addition of excess of L-arginine but not D-arginine (1 mM).

4 Like L-NOARG and L-NMMA, methylene blue (10 μM), an agent that inhibits the activation of soluble guanylate cyclase by NO, also significantly inhibited BK-induced relaxation, leaving responses to Iso unaffected.

5 Indomethacin (0.3 nM to 10 nM), a cyclo-oxygenase inhibitor, concentration-dependently inhibited BK-mediated relaxation, with an IC_{50} of 2.6 (1.7– 3.8) nM, without affecting Iso and SNP-mediated relaxant responses.

6 A combination of a very low concentration of indomethacin (1 nM) and either L-NOARG or L-NMMA (100 μM) changed the response of tracheal preparations to BK (100 nM) from a relaxation to a sustained contraction.

7 These findings indicate that BK-induced relaxation in guinea-pig trachea is mediated jointly by the release of NO or a NO-related substance and a prostanoid, probably prostaglandin E_2.

Keywords: Guinea-pig trachea; epithelium; bradykinin; nitric oxide; nitric oxide inhibitors; indomethacin

Introduction

It is now widely established that the vasoactive peptide bradykinin (BK) and related peptides produce potent endothelium-dependent vasodilatation by releasing endothelium-derived relaxant factor, now identified as nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). The endothelium-dependent vasorelaxant response to BK is mediated through either B_1 or B_2 receptor subtypes (Sung *et al.*, 1988; Wiemer & Wirth, 1992) and in some vascular beds, BK can release oxygen-derived free radicals as a result of the stimulation of phospholipase A_2 which in turn determines the release of prostanoids (Kontos *et al.*, 1984).

There are now several pieces of evidence suggesting the involvement of BK in physiological and pathological control of bronchoalveolar function (for review see: Barnes *et al.*, 1988; Farmer, 1991; Hall, 1992; Barnes, 1992). Thus, BK may cause contraction or relaxation of the guinea-pig trachea, an action that is mediated, at least in part, by generation of an eicosanoid-derived product of arachidonic acid metabolism released by the epithelium (Bramley *et al.*, 1990; Farmer *et al.*, 1991). It has recently been demonstrated that NO is present in many tissues (for review see Moncada *et al.*, 1991) and that the relaxant responses to BK in several tissues, such as pig coronary artery (Cowan & Cohen, 1991; Vials & Burnstock, 1992), rat kidney (Fulton *et al.*, 1992) and guinea-pig isolated heart (Kelm & Schrader, 1988) are mediated by release of NO or a NO-related substance. In addition, evidence now suggests that NO acts as the non-adrenergic and non-cholinergic inhibitory transmitter in a number of tissues, including trachea (Li & Rand, 1991; Belvisi *et al.*, 1991; Kannan & Johnson, 1992; Fischer *et al.*, 1993).

The present study was therefore, designed to investigate whether or not the L-arginine-NO pathway is involved in BK-mediated relaxation in the guinea-pig isolated trachea by examining the effect of two selective NO synthase inhibitors, N^G-nitro-L-arginine (L-NOARG) and N^G-monomethyl-L-arginine (L-NMMA), the D-enantiomer of L-NMMA, D-NMMA and methylene blue, an agent that inhibits the activation of guanylate cyclase by NO. In addition, we have also assessed the effect of indomethacin either alone, or in combination with L-NOARG and L-NMMA, on the epithelium-dependent relaxation caused by BK.

Methods

Tissue preparation

Guinea-pigs of either sex (200–350 g) were killed by a blow on the head and were exsanguinated from carotid arteries. The trachea was rapidly removed and carefully dissected from adhering fat and connective tissues. Usually four transverse rings (3–4 mm wide) were obtained from each animal. The rings were opened and strips of about 8–10 mm in length with intact epithelium were suspended in individual 5 ml jacketed organ baths containing Krebs-Henseleit solution maintained at 37°C, pH 7.2, and gassed with 95% of O_2

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and 5% CO_2 with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_2$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.1 and glucose 11. Preparations were allowed to equilibrate for at least 120 min before drug additions, under a resting tension of 1 g, during which the bath solution was renewed each 15 min. Isometric tension changes were recorded by means of F-60 force transducer (Narco Biosystem). The integrity of the epithelium was assessed by the ability of BK (100 nM) to induce relaxation. Tissues were considered to contain a viable epithelium when BK caused a relaxation of over 80% (approximately 300–400 mg) in preparations under spontaneous tonus.

Experimental procedures

Following the equilibration period and under spontaneous tonus, non-cumulative complete concentration-response curves were obtained by the addition of increasing single concentrations of BK (0.3 to 1000 nM), for 5 to 10 min at 20 min intervals between concentrations. To test the effect of inhibition of NO synthesis, single relaxation responses to BK (100 nM) were obtained in the absence or in the presence of L-NOARG and L-NMMA (1–100 μM), incubated with the preparations 20 min beforehand. All BK experiments were carried out in the presence of captopril (3 μM) to avoid degradation of BK by the action of kininase II. In separate sets of experiments, we investigated the effect of D-NMMA (100 μM) and methylene blue (10 μM), an agent which inhibits the activation of guanylate cyclase by NO, incubated with the preparations 20 min beforehand. In other experiments, the effect of NO inhibitors on BK-induced relaxation was analysed in the presence of excess of either L-arginine or D-arginine (1 mM) or in presence of the D-enantiomer D-NMMA (100 μM), incubated simultaneously in the bath for 20 min. Isoprenaline (Iso) and the NO-generating vasodilator drug, sodium nitroprusside (SNP) were used as reference relaxant drugs to evaluate the non-specific effects of NO synthesis inhibitors.

To assess further the relative contribution of cyclo-oxygenase products of arachidonic acid on BK-induced relaxation in guinea-pig trachea, after obtaining stable relaxant responses to BK (100 nM), preparations were incubated for 30 min with indomethacin (0.3–10 nM) alone or in combination with either L-NOARG or L-NMMA and new relaxant responses to BK (100 nM) were obtained in its presence. To correct the decrease of the spontaneous tonus of the preparations in the presence of indomethacin, resting tone was adjusted by addition of low concentrations of the stable thromboxane A_2/prostaglandin H_2-mimetic analogue U 46619 (0.1 to 10 nM). The relaxant response to BK and other agonists were expressed in mg of tension.

The IC_{50} values, i.e., the concentration of inhibitors producing 50% inhibition of spontaneous tone relative to control values, were determined for individual experiments from complete concentration-response curves using the least-squares method. Control experiments (preparation taken from the same animal) were carried out in parallel in the presence of phosphate-buffered solution (PBS, concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer 10 mM).

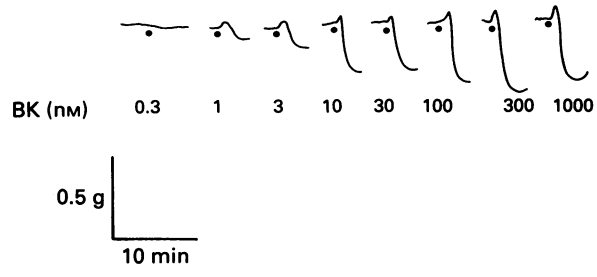

Figure 1 Representative isometric tension recording showing the relaxant effect of bradykinin (BK) in epithelium-intact strips of guinea-pig isolated trachea under spontaneous tone. Similar results were obtained in seven additional experiments.

Statistical analysis

Data are presented as mean ± s.e.mean, except the IC_{50}s that are given as geometric mean accompanied by their respective 95% confidence limits. Statistical analysis was performed either by means of unpaired Student's t test or by analysis of variance followed by Dunnett's multicomparison test when appropriate. $P<0.05$ or less was considered as indicative of significance.

Drugs

Drugs used were: bradykinin, indomethacin, L-isoprenaline bitartrate, sodium nitroprusside HCl, methylene blue HCl, L-NOARG, L-NMMA, D-NMMA acetate salt, L-arginine, D-arginine (Sigma Chemical, St Louis, U.S.A.). U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$, UpJohn, Kalamazoo, MI, U.S.A.). Most drugs (bradykinin, isoprenaline, sodium nitroprusside, L-NMMA, U 46619) were stored (1 to 100 mM) at −20°C. All other drugs were prepared daily just before the experiments. Bradykinin, L-NOARG, L-NMMA, D-NMMA, methylene blue and U46619 were dissolved in phosphate-buffered saline. Indomethacin was dissolved in absolute ethanol, and isoprenaline was dissolved in HCl 0.01 N and diluted in 0.9% of NaCl containing

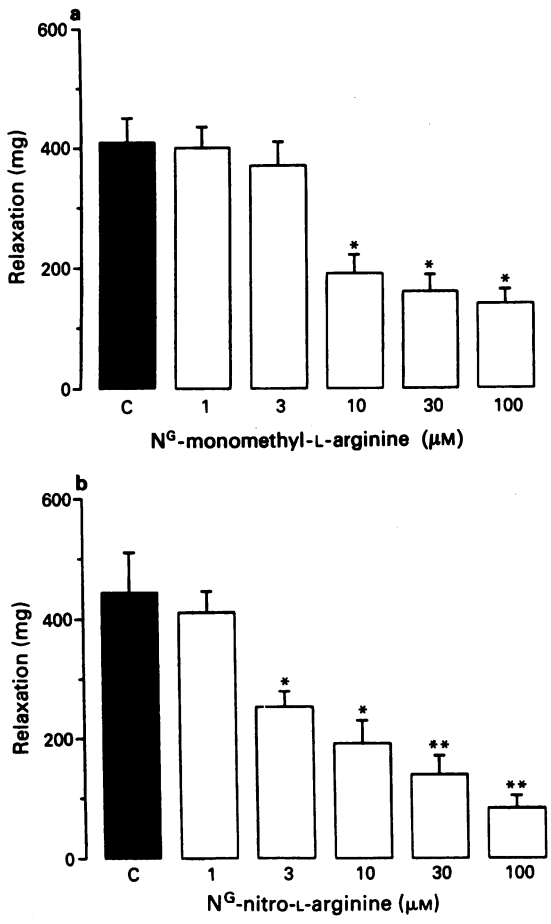

Figure 2 Effects of N^G-monomethyl-L-arginine (a) and N^G-nitro-L-arginine (b) on bradykinin-induced relaxation of the guinea-pig isolated tracheal strips with intact epithelium. Each column represents the mean of 8 experiments together with the s.e.means. *$P<0.05$; **$P<0.01$ versus control responses (C).

ascorbic acid at 50 $\mu g\ ml^{-1}$. All other drugs were dissolved in PBS just before use at the desired concentrations. The final concentration of ethanol did not exceed 0.02%.

Results

Concentration-response curve for bradykinin

Addition of a single BK concentration (0.3–1000 nM) to the bath caused a transient contraction followed by concentration-dependent relaxations of slow onset with an IC_{50} (95% confidence limits) of 21.1 (6.4–69.6) nM and maximal relaxation of 380 ± 49 mg of tension (Figure 1). The relaxation induced by BK was reproducible with no evidence of tachyphylaxis.

Effect of L-NOARG, L-NMMA and methylene blue on the relaxation caused by bradykinin and other agonists

Pretreatment with L-NOARG or L-NMMA (1 to 100 μM) did not promote significant changes in the spontaneous tonus (data not shown), but caused a concentration-dependent inhibition of BK (100 nM)-induced relaxations (Figure 2a and b). The IC_{50} (and 95% confidence limits) were: 9.1 (6.9–11.6) μM and 7.0 (4.0–12.1) μM for L-NOARG and L-NMMA, respectively. L-NOARG and L-NMMA at 100 μM inhibited BK-induced relaxation by 74 ± 7 and 67 ± 7%, respectively. However, D-NMMA had no effect on BK-induced relaxation (Figure 3). Figure 3 also shows that addition of excess of L-arginine but not D-arginine (1 mM) partially reversed the inhibition of BK (100 nM)-induced relaxation by L-NOARG and L-NMMA. L- and D-arginine did not significantly affect the tone of the tissues (data not shown). However, both L-NOARG (30 μM) and L-NMMA (30 μM), at a concentration at which they significantly inhibited BK (100 nM)-induced relaxation, failed to affect the relaxation caused by either Iso (1 μM) or SNP (1 μM) (Figure 4).

Preincubation of the preparations with methylene blue (10 μM, a soluble guanylate cyclase inhibitor) increased slightly the tone of the preparations (3 ± 1.5 mg), and like L-NOARG and L-NMMA also significantly inhibited BK-induced relaxations (Figure 5), leaving the relaxation responses induced by Iso (1 μM) unaffected.

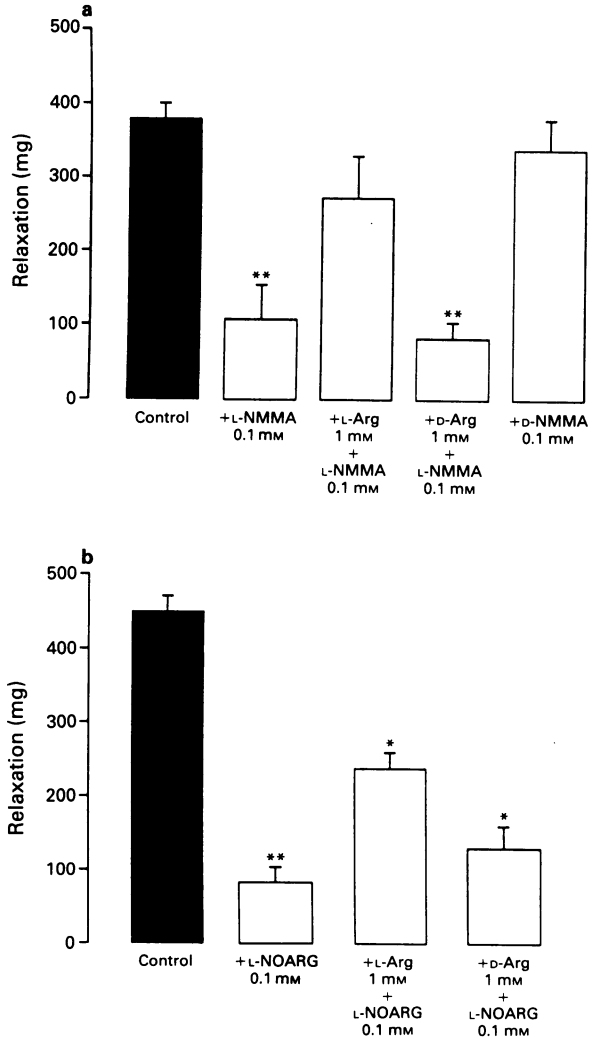

Figure 3 Effects of N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (a) and N^G-nitro-L-arginine (L-NOARG) (b) on bradykinin-induced relaxation in guinea-pig isolated tracheal strips with intact epithelium in absence or in presence of L-arginine (L-Arg) and D-arginine (D-Arg). Each column represents the mean of 6–8 experiments together with s.e.means. $*P<0.05$; $**P<0.01$ versus control group.

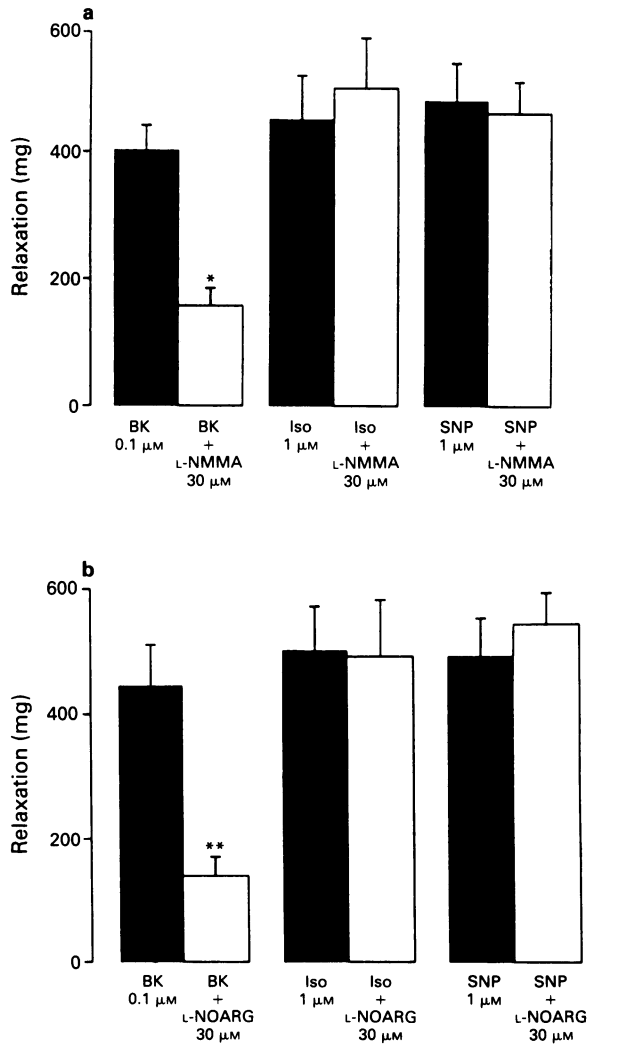

Figure 4 Effect of N^G-monomethyl-L-arginine (L-NMMA) (a) and N^G-nitro-L-arginine (L-NOARG) (b) on bradykinin (BK), isoprenaline (Iso) and sodium nitroprusside (SNP)-induced relaxation of guinea-pig isolated tracheal strips with intact epithelium. Each column represents the mean of 6–8 experiments with s.e.means. $*P<0.05$; $**P<0.01$ versus control group.

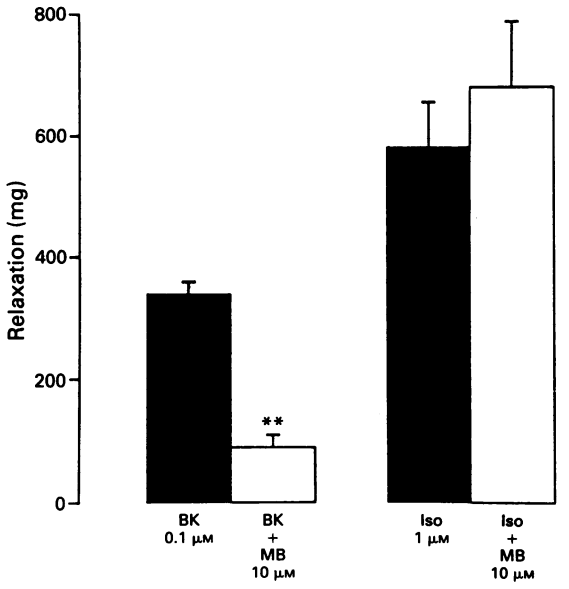

Figure 5 Effect of methylene blue (MB) on bradykinin (BK) and isoprenaline (Iso)-induced relaxation in epithelium intact strips of guinea-pig isolated trachea. Each column represents the mean of 6–8 experiments with s.e.means. $^{**}P<0.01$ versus control group.

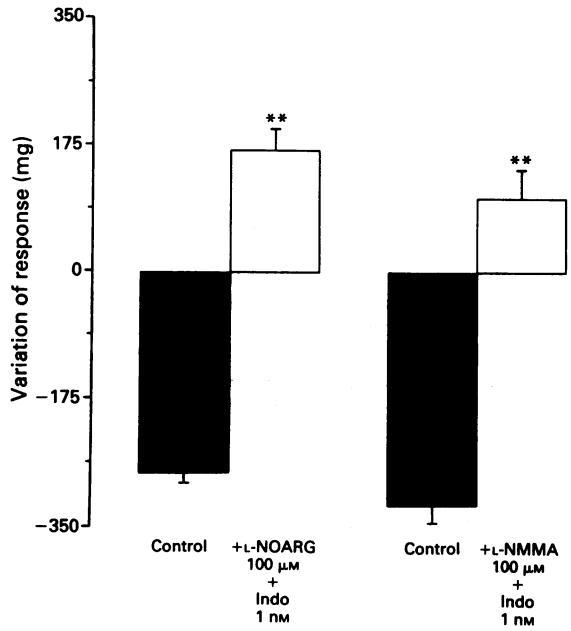

Figure 7 Mean inhibitory effect of combination of indomethacin with N^G-nitro-L-arginine (L-NOARG) or with N^G-monomethyl-L-arginine (L-NMMA) on bradykinin-induced relaxation of guinea-pig isolated tracheal strips with intact epithelium. Each column represents the mean of 5 experiments with s.e.means. $^{**}P<0.01$ versus control group.

Effect of indomethacin on bradykinin and other agonist-induced relaxation

Preincubation of tracheal strips with indomethacin (1, 3 and 10 nM) 20 min beforehand caused a progressive decrease of the tones of the preparations (-64 ± 15; -144 ± 37 and -371 ± 43 mg) respectively, and caused a graded inhibition of BK (100 nM)-induced relaxation (Figure 6). In the presence of 10 nM indomethacin, BK (100 nM) caused a marked contraction (291 ± 43 mg) instead of relaxation. In contrast, indomethacin up to 3 μM did not significantly affect Iso (1 μM) or SNP (1 μM)-mediated relaxations (data not shown). The results illustrated in figure 7 show that the combination of a very low concentration of indomethacin (1 nM) with either L-NOARG or L-NMMA (100 μM) changed the response of tracheal preparations to BK (100 nM) from a relaxation to a sustained contraction.

Discussion

The results of the present study have demonstrated for the first time that besides releasing cyclo-oxygenase products

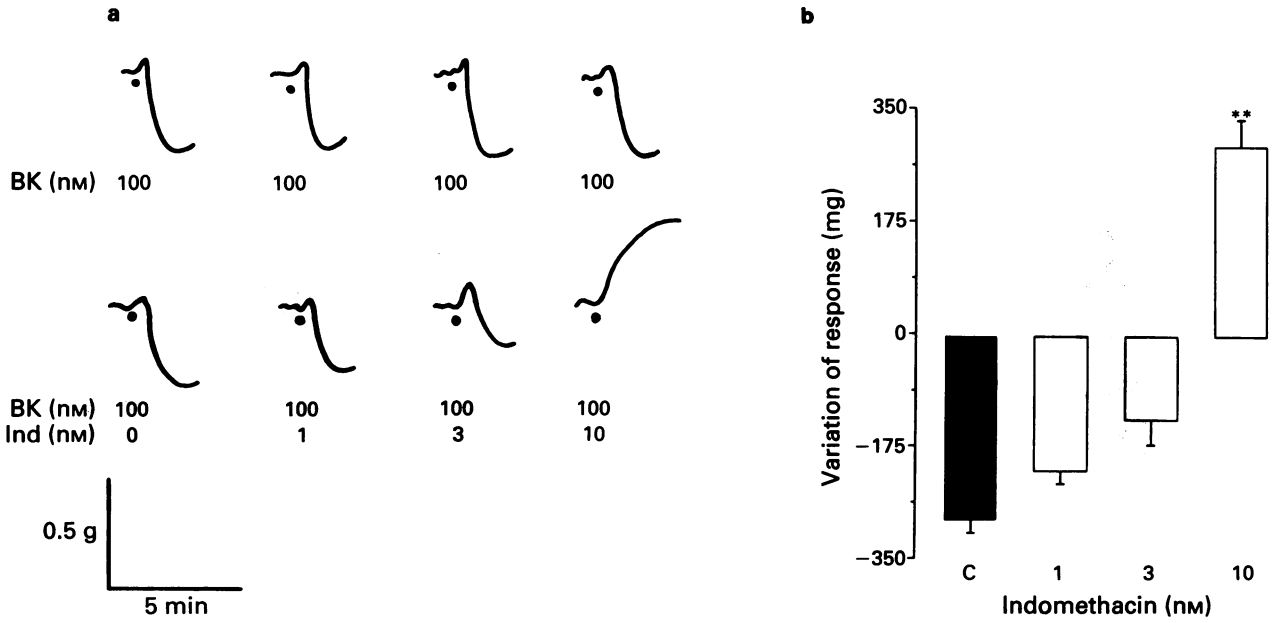

Figure 6 (a) Typical isometric tracing showing the relaxant effect of bradykinin (100 nM) in epithelium-intact strips of guinea-pig trachea in absence or in presence of indomethacin. (b) Mean inhibitory effect of indomethacin on bradykinin (100 nM)-induced relaxations in epithelium intact strips on guinea-pig trachea. Each column represents the mean of 6 experiments with s.e.means. $^{**}P<0.01$ versus control group (C).

from arachidonic acid metabolism, BK relaxes epithelium-intact strips of guinea-pig isolated trachea largely by the release of NO or a NO-related substance. This view is substantiated by several observations. First, the incubation of guinea-pig trachea with L-NOARG and L-NMMA, two inhibitors of NO synthesis, (Palmer *et al.*, 1988; Moore *et al.*, 1990; Ischii *et al.*, 1990), but not with the D-enantiomer of L-NMMA, D-NMMA, was able to inhibit in a concentration-dependent manner BK-induced relaxations in guinea-pig trachea without interfering with relaxations induced by Iso or SNP. Secondly, the inhibitory effect of both NO biosynthesis inhibitors, L-NOARG and L-NMMA, was reversed in a stereospecific manner by the addition of excess L-arginine but not D-arginine, in conditions where L-arginine failed to affect the relaxation induced by Iso or SNP. Thirdly, methylene blue, which inhibits the soluble guanylate cyclase induced by NO (Gruetter *et al.*, 1981), like L-NOARG and L-NMMA, produced a qualitatively similar inhibition of the relaxant response of the guinea-pig trachea to BK, leaving relaxation responses to Iso unaffected. Taken together, these findings are consistent with the view that the L-arginine-NO pathway or NO-releasing substances are constitutively present in this tissue and seem to be crucial in mediating epithelium-dependent BK relaxation. Recently reported data indicate that NO-dependent mechanisms mediate BK responses in several preparations, including pig coronary artery (Cowan & Cohen, 1991; Vials & Burnstock, 1992), rat kidney (Fulton *et al.*, 1992), pig endothelial cells (Boulanger *et al.*, 1990), rat skin microvasculature (Khalil & Helme, 1992), rabbit coronary bed (Lamontague *et al.*, 1992) and rat mesenteric bed (Berguer *et al.*, 1993). Interestingly, higher concentrations of L-arginine only partially reversed the inhibitions caused by both L-NOARG and L-NMMA, suggesting that BK may induce relaxation by another NO-independent mechanism, probably by releasing prostanoids which, together with NO, completely relax the guinea-pig isolated trachea. However, we cannot fully discard the possibility that the amount of L-arginine available for NO synthesis in guinea-pig trachea during BK-mediated relaxation is rate-limited.

Another important finding of the present study was the fact that very low concentrations of indomethacin concentration-dependently inhibited both the tonus of the preparations and BK-induced relaxations in guinea-pig trachea. At 10 nM, indomethacin was able to change the BK-induced relaxation into a contraction. Furthermore, the combination of a very low concentration of indomethacin (1 nM), which alone caused only a small inhibition of BK-mediated relaxation, with either L-NOARG or L-NMMA changed the BK-induced response of the preparations from relaxation to sustained contraction. These results further extend previous evidence from the literature, indicating that the release, by the epithelium, of cyclo-oxygenase products derived from arachidonic acid pathway in response to BK, possibly prostaglandin E_2 (PGE_2) (Mizrahi *et al.*, 1982; Bramley *et al.*, 1990; Calixto *et al.*, 1992), together with NO are largely responsible for the relaxation response caused by this peptide. However, to date, our results cannot answer the question as to whether or not NO and PGE_2 are released simultaneously by BK, or whether their actions involve different mechanisms. Recent reports have demonstrated that NO or NO-related compound is an inhibitory neurotransmitter in several tissues of the gastrointestinal, pulmonary and urinary tracts and in blood vessels (see for recent review, Rand, 1992). Immunohistochemical studies have shown the presence of NO synthase in mammalian myenteric neurones. Recent evidence also suggests the presence of NO in smooth muscle cells. Thus, a great part of the relaxant response caused by vasoactive intestinal polypeptide (VIP) in the gut is mediated by release of NO from smooth muscle cells (Grider *et al.*, 1992). Several lines of evidence suggest that NO may play a role as an inhibitory neurotransmitter in the trachea (Li & Rand, 1990; Tucker *et al.*, 1990; Belvisi *et al.*, 1991). Recently Fischer *et al.* (1993) demonstrated the presence of NO synthase in guinea-pig lower airway innervation. However, histochemical studies are lacking to assess whether NADP-diaphorase-containing nerves are present in the vicinity of epithelial and/or smooth cells of guinea-pig trachea. Thus, the origin of the NO released by BK remains to be clearly established.

Relaxation induced by BK in guinea-pig trachea is mediated by activation of B_2 receptors (Bramley *et al.*, 1989). Furthermore, it has been shown (Bramley *et al.*, 1990) that low concentrations of BK, cause graded relaxations of epithelium-intact preparations of guinea-pig trachea associated with marked increase of bronchodilator PGE_2 levels. The same authors also demonstrated that BK-mediated relaxation and PGE_2 synthesis in guinea-pig trachea were attenuated either by removal of the epithelium or by exposure of the preparations to indomethacin, suggesting that the epithelial-dependent cyclo-oxygenase products derived from arachidonic acid are important in controlling BK-mediated responses in this preparation. Recent radioligand binding studies, carried out on guinea-pig tracheal epithelial cells, demonstrated two types of B_2 kinin receptors, a high affinity site with a K_d of 0.44 nM and a low affinity site with K_d of 10 nM (Proud *et al.*, 1993). Hoe 140, a potent selective B_2 receptor antagonist, displaced labelled BK from both sites and inhibited BK-induced PGE_2 production.

In summary, the current results provide consistent evidence supporting the view that L-arginine-derived NO or a NO-related pathway modulates the epithelium-dependent relaxation response to BK in guinea-pig trachea. In addition, we have also demonstrated that a cyclo-oxygenase-derived product from arachidonic acid pathway, possibly PGE_2, also largely contributes to the relaxant response to BK in guinea-pig trachea. The physiopathological relevance of these findings is unclear at the moment. However, it is possible that the lack of epithelium, and consequently the absence of a relaxant response to BK, by the release of NO and/or prostanoids in inflamed airway tissues, may account for increase airway responsiveness in conditions such as asthma.

The authors are grateful to Elizabet Ramos Ganzer and Josane Andrada for secretarial help in preparing this manuscript. This work was supported by grants from CNPq and FINEP (Brazil). V.S. is an M.Sc. student receiving a grant from CAPES (Brazil).

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(Received June 4, 1993
Revised September 2, 1993
Accepted September 7, 1993)

Br. J. Pharmacol. (1994), **111**, 89–96

Possible involvement of NMDA receptor-mediated transmission in barbiturate physical dependence

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1 The competitive antagonists at the N-methyl-D-aspartate (NMDA) receptor, CGP39551 and CGP37849, protected against the barbiturate withdrawal syndrome in mice, as measured by ratings of convulsive behaviour on handling.

2 The effective doses of these compounds were lower than those required to prevent seizures due to NMDA in naive animals; these were in turn lower than those needed to prevent the convulsive effects of the α-aminobutyric acid (GABA) antagonist, bicuculline.

3 The NMDA-receptor antagonists did not alter the increase in the incidence of convulsions due to the $GABA_A$ antagonist, bicuculline, that is seen during barbiturate withdrawal, although the latencies to these convulsions during barbital withdrawal were significantly increased after CGP39551.

4 Barbiturate withdrawal did not affect the convulsive actions of NMDA, whether measured by the incidence of convulsions or by intravenous infusion.

5 The B_{max} for [^{3}H]-dizocilpine ([^{3}H]-MK801) binding was significantly increased by chronic barbital treatment in cerebrocortical but not in hippocampal tissues, while the K_d remained unaltered in either case.

6 At 1 h and 24 h after administration of a single dose of barbitone, the B_{max} for [^{3}H]-dizocilpine binding was unaltered in cerebrocortical tissue. Acute addition of barbitone *in vitro* did not alter [^{3}H]-dizocilpine binding or the displacement of binding of thienylcyclohexylpyridine.

Keywords: NMDA; barbiturate; withdrawal; binding; dizocilpine; anticonvulsant

Introduction

The barbiturates produce tolerance and a physical dependence that resembles that which develops to ethanol but the physiological changes that are responsible for these effects are not fully understood. Acutely, the barbiturates have actions at several receptor sites or complexes. They are well known to potentiate the effects of γ-aminobutyric acid (GABA), acting at their own receptor sites on the GABA receptor/ionophore complex (Nicoll *et al.*, 1975; Ticku & Olsen, 1978; Macdonald & Barker, 1979). They also block the L-subtype of neuronal calcium channels, and increase inactivation of the N-subtype (Gross & Macdonald, 1988).

Fewer studies have been made on the interactions of barbiturates with excitatory amino acids. The central receptors for these compounds have been divided into subtypes, named after the selective agonists, N-methyl-D-aspartate (NMDA), AMPA (this subtype was previously known as the quisqualate subtype) and kainate. Until recently, antagonists for NMDA receptors were not systemically active, but more lipid soluble compounds are now available, such as CGP39551 or CGP37849 (Fagg *et al.*, 1990; Schmutz *et al.*, 1990). The NMDA receptor complex also bears other receptor sites that interact allosterically, including one that binds glycine, which was found to be an essential co-agonist in the action of NMDA (Johnson & Ascher, 1987).

Early reports described antagonism of responses to glutamate by barbiturates (Richards & Smaje, 1976; Barker & Ransom, 1978; Macdonald & Barker, 1979), and recent papers have examined this effect with respect to the subtypes of excitatory amino acid receptors. The blocking action appears to be selective for the AMPA and kainate receptor subtypes (Teichberg *et al.*, 1984; Sawada & Yamamoto, 1985), although some antagonist actions against aspartate have been reported (Miljkovic & Macdonald, 1986). A recent study (Zeman & Lodge, 1992), in which the effects of low concentrations of kainate were investigated, suggested that pentobarbitone had a selective blocking action on kainate receptors; higher concentrations of kainate may act at the AMPA receptor. The involvement of excitatory amino acid transmission in barbiturate dependence was suggested by McCaslin & Morgan (1988) who demonstrated that certain antagonists, given intracerebroventricularly, decreased the incidence of abstinence convulsions in rats. The compounds used were 2-amino-7-phosphonoheptanoic acid (APH), that is selective for NMDA sites, glutamyldiethyl ester (GDEE), said to have some selectivity for quisqualate receptors, and *cis*-2,3-piperidine dicarboxylic acid (PDA), a nonselective blocker. The increase in cerebellar guanosine 3′:5′-cyclic monophosphate (cyclic GMP) seen during barbiturate withdrawal was decreased by APH, but not by the other compounds. However, it was not clear from this study whether the effect of APH represented a selective action on the withdrawal syndrome, that might suggest an involvement of NMDA in the origin of the seizure state, or a general anticonvulsant action, that might be exerted against convulsions due to any cause.

In the present study we have investigated the effects of the systemically active NMDA antagonists, CGP39551 and CGP37849 on the barbiturate withdrawal syndrome. Because the effects of anticonvulsant agents on withdrawal syndromes are not very informative unless their effects are directly compared with those against other convulsive syndromes (Little, 1991), the actions of these compounds against seizures due to the selective agonist, NMDA, and the GABA antagonist, bicuculline were measured. Their effects on spontaneous locomotor activity were also investigated, in order to see whether or not any effects were due to sedative properties. In order to see whether or not changes occurred at the NMDA receptor after chronic barbiturate treatment, binding of the noncompetitive antagonist, [^{3}H]-dizocilpine was measured.

Preliminary accounts of this work have been presented to the British Pharmacological Society (Rabbani & Little, 1991; Wright *et al.*, 1991).

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Methods

Chronic drug treatment

Barbitone was used for all the chronic barbiturate treatment, because this compound is little metabolized and so does not induce microsomal enzymes. Male mice, TO strain were used, between 20–35 g, with not more than 5 g range in any one experiment and they were housed 10 mice per cage. They were given barbitone in powdered food for 7 days: 3 mg barbitone per g food for 2 days, 4 mg g^{-1} food for 2 days and 5 mg g^{-1} food for 3 days. Controls received a matched amount of powdered food only. All mice were weighed regularly during the treatments and no significant differences in weights were found. In all studies the amount of food, and hence barbitone, taken in by the mice was measured daily. The barbitone intake rose during the treatment from 400 mg $kg^{-1}24\ h^{-1}$ on the first day to 700 mg $kg^{-1}24\ h^{-1}$ on the last day of treatment. For the withdrawal measurements, the mice were placed in clean cages at the end of the 7 day treatment, and given powdered food without barbitone for at least 19 h before testing.

Measurement of the barbiturate withdrawal syndrome

The barbiturate withdrawal syndrome was measured by ratings of convulsive behaviour on handling. This method was first established by Goldstein & Pal (1971) for ethanol withdrawal. Our method was similar to this, with slight modifications (Green *et al.*, 1990). Mice were gently picked up by the tail and turned, first in one direction then the other. Rating numbers were allocated as follows: 0, no signs of tremor or hyperexcitability; 1, occasional signs of tremor; 2, continuous tremor; 3, intermittent clonic convulsions, consisting of repeated contractions of the limbs, particularly the hind legs; 4, continuous clonic convulsions.

The ratings of convulsive behaviour on handling were carried out once an hour, over periods of time after the removal of the barbitone. All the mice were coded so that the observer was unaware of the prior drug treatment. After removal of barbitone from the food, the mice were put into clean cages, to avoid intake of any food in the sawdust. A minimum of 10 animals were used in each treatment group.

The severity of the barbiturate withdrawal syndrome was measured from 20 h to 28 h after barbitone withdrawal. Our previous studies have shown that this period covers the time of maximum changes in behaviour following cessation of the barbitone treatment. Single i.p. injections of CGP39551 and CGP37849 were made at 19 h into the withdrawal period.

Single dose of barbitone

For comparison in the binding studies with the effects of chronic barbitone treatment, the effects of a single intraperitoneal dose of barbitone, or saline, were measured on [^{3}H]-dizocilpine binding. The dose of barbitone used was 250 mg kg^{-1}, which produced moderate ataxia, and the cerebrocortical tissues were prepared at 1 h and at 24 h after the injections.

The effects of bicuculline during barbitone withdrawal

We have previously shown that the convulsive response to bicuculline is increased during barbiturate withdrawal (Rabbani & Little, 1990). The effects of CGP39551 on the actions of bicuculline during barbitone withdrawal were examined at pretreatment times of 1 h and 5 h for CGP39551. The bicuculline was injected i.p., 24 h after barbitone withdrawal and the incidence of seizures noted for the next 45 min. The dose of bicuculline chosen for the barbitone withdrawn mice was 3.5 mg kg^{-1}, which we had previously found to cause clonic convulsions in approximately 90% of mice in these circumstances. For comparison, the effects of the CGP39551 on bicuculline seizures were examined in control mice. Doses of 5 mg kg^{-1} bicuculline were used in these animals, that gave a response in about 90% of the mice. The effects of CGP37849 against bicuculline convulsions were tested when this compound was given 1 h before the bicuculline administration, with bicuculline given 24 h into withdrawal, and 9 h before bicuculline, when this convulsant was given 28 h into withdrawal (after the handling tests). The mice were killed humanely as soon as the first signs of a convulsion were evident. In all cases the observer was blind to the prior drug-treatment.

Convulsive effects of NMDA

The convulsive effects of NMDA were studied during barbiturate withdrawal by administration of either 250 or 325 mg kg^{-1} NMDA i.p. 24 h after the withdrawal of the barbitone. The incidence of full seizures, defined as above, and their latencies, were noted for 45 min, by an observer who was not aware of which drug-treatment the animals had received.

The actions of NMDA were also measured by determination of the seizure thresholds by intravenous infusion (Singh *et al.*, 1991). NMDA, dissolved in saline at 50 mg ml^{-1}, was infused into the tail vein at 1.6 ml min^{-1}. The response times for the first muscle twitch, and for the first signs of a full convulsion (which usually took the form of a tonic seizure) were measured by an observer who did not know the prior drug-treatment. Animals were killed humanely as soon as the second endpoint was reached. The seizure thresholds were then calculated in mg kg^{-1} from the response times and the mouse weights.

Anticonvulsant actions against bicuculline or NMDA in naive mice

The selectivity of the protective effects of the compounds on the barbiturate withdrawal syndrome were examined by their effects on seizures produced by bicuculline, or by NMDA, in naive animals. The doses used of these convulsants, bicuculline 5 mg kg^{-1} and NMDA 325 mg kg^{-1}, were chosen on the basis of earlier studies, to produce convulsions on 80–90% of a group of mice. The anticonvulsant compounds were given, i.p. 1 h or 5 h before administration of either convulsant, to provide comparison with the withdrawal experiments. The seizure incidence was noted for 40 min following the convulsant injections. A full seizure was recorded when clonic movements of the limbs were observed, accompanied by loss of posture. The mice were killed humanely as soon as the first signs of a convulsion were evident. Statistical comparisons were made only between results from animals tested concurrently. In all cases the observer was blind to the prior drug treatment.

Locomotor activity

The actions of the compounds on spontaneous locomotor activity were measured automatically by breaking of infra-red beams. Pairs of mice, receiving the same drug treatment, were placed in novel cages, and the locomotor activity measured at 5 min intervals for the next 30 min. Six pairs of mice were used for each treatment group. The treatments were randomized throughout the day, between 09 h 00 min and 17 h 00 min, to control for diurnal variations in activity.

[^{3}H]-dizocilpine binding

Following the chronic treatment, the animals were killed by cervical dislocation and the cerebrocortical and hippocampal tissues rapidly dissected out and frozen at −25°C, for a maximum of four weeks. For the studies on cortical tissues, the cortices from two mice were pooled for each experimental

determination, and in the case of hippocampal tissue, the hippocampi from 6 to 8 mice were pooled.

On the day of binding measurement, the frozen tissues were thawed, weighed and suspended in assay buffer (5 mM Tris HCl, pH 7.4 at 25°C) at 55 ml per g of wet tissue, at 0–4°C. They were then hand homogenized, and the homogenate centrifuged at 1,000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 20,000 *g* for 20 min. The supernatant was discarded and the pellet was resuspended in the original volume. The suspension was finally centrifuged at 20,000 *g* for 20 min. The resultant pellet was resuspended in 5 mM Tris HCl to given 0.5 mg ml^{-1} tissue and kept on ice. The radioligand [^{3}H]-dizocilpine (1.0 mCi ml^{-1}) ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cycloheptane-5,10-imine maleate), was diluted in glass containers with 5 mM Tris HCl, to 1.0 to 15.0 nM. Triplicate determinations were made for each concentration. The non-specific binding was assessed by use of 10 μM cold thienylcyclohexylpiperidine (TCP). Total binding was measured in 1 ml total volume of samples containing: (1) 50 μl assay buffer, 5 mM Tris HCl; (2) 100 μl assay buffer, 5 mM Tris HCl; (3) 100 μl above concentrations of [^{3}H]-dizocilpine; (4) 750 μl tissue aliquot.

Non-specific binding was measured using the following: (1) 50 μl assay buffer, 5 mM Tris HCl; (2) 100 μl TCP at final concentration of 10 μM; (3) 100 μl above concentrations of [^{3}H]-dizocilpine; (4) 750 μl tissue aliquot.

The tubes were gently mixed and placed in a water bath at 25°C for 45 min. Following the incubation of the samples, the tubes were rapidly filtered using 30-place Brandel cell harvester and Whatman GF/B filters. A total of two washes were applied during each filtration, using ice-cold 5 mM Tris HCl. Individual filter papers were removed and placed in vials, then 4 ml of scintillation fluid was added to each vial and counted on the scintillation counter, for 5 min each. In order to measure the amount of radioactivity put into each test tube, 50 μl of the stock and diluted hot [^{3}H]-dizocilpine was added into a scintillation vial and counted as above.

Action of barbitone in vitro *on [^{3}H]-dizocilpine binding*

In order to check whether or not the barbitone could have had a direct effect on [^{3}H]-dizocilpine binding during the chronic treatment, its actions were tested *in vitro*. The effect on total binding was measured when barbitone was added to the incubation medium at a range of concentrations between 1 nM and 1 mM (at ten fold intervals of concentration). The effect of barbitone on the displacement of [^{3}H]-dizocilpine by TCP were also studied, by addition of barbitone with each concentration of TCP to barbitone concentrations of 1 and 100 nM, 10 μM and 1 mM. For the saturation experiments, the prepared tissue was incubated as above for 45 min at 25°C, in the presence of either TCP 10 μM or TCP plus barbitone at 1 nM, 1 μM or 100 μM.

Drugs used

CGP39551 (DL-(E)-2-amino-4-methyl-5-phosphonopentanoate carboxy-ethylester, Ciba-Geigy), and CGP37849 (DL-(E)-2-amino-4-methyl-5-phosphonopentanoic acid, Ciba-Geigy) were dissolved in distilled water. Fresh solutions of CGP39551 and CGP37849 were made daily. NMDA was dissolved in saline. Bicuculline was dissolved in glacial acetic acid, brought to pH 3 with NaOH and diluted with saline brought to pH 3 with HCl.

Statistical analysis

The ratings of convulsive behaviour during withdrawal were compared by two-way non-parametric analysis of variance, designed for repeat measurements on the same animals and multiple comparisons with controls (Meddis, 1984). Seizure incidences were compared by Fisher's Exact probability test; comparisons were made only between concurrently tested groups. Comparisons in the measurements of seizure thresholds, latencies and locomotor activity were made by Student's unpaired *t* test. The binding results were analysed using Scatchard analysis, and statistical comparisons made with Student's unpaired *t* test. Either five or six tissue samples, prepared from different animals, with tissues pooled as described above, were used for each set of binding measurements.

Results

Effects of CGP39551 and CGP37849 on the barbiturate withdrawal syndrome

The first experiment showed that CGP39551, at 20 mg kg^{-1} completely prevented the barbitone withdrawal syndrome

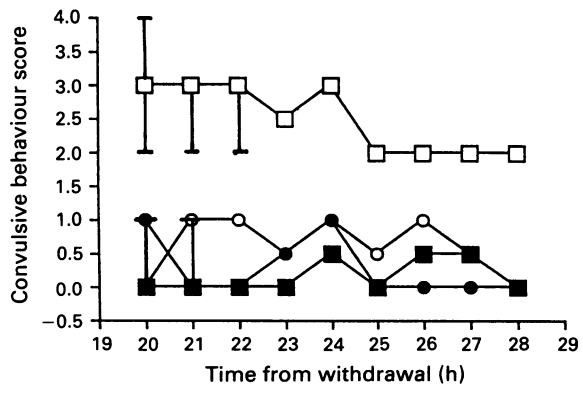

Figure 1 The effects of CGP39551 in decreasing the ratings of convulsive behaviour on handling. Values are medians with representative interquartile ranges. The measurements were made from 19 h to 28 h from cessation of chronic barbitone treatment. (○) Control diet + acute saline; (●) control diet + acute CGP39551, 20 mg kg^{-1}; (□) barbitone diet + acute saline; (■) barbitone diet + acute CGP39551, 20 mg kg^{-1}. The effect of 20 mg kg^{-1} CGP39551 on the withdrawal hyperexcitability was significant, $P<0.001$ for comparison between saline and CGP39551 after barbitone treatment, over the whole test period.

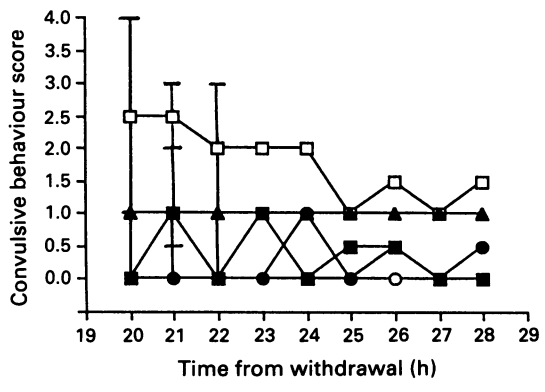

Figure 2 The effectiveness of a range of low doses of CGP39551, 1, 2.5 and 5 mg kg^{-1} against the withdrawal hyperexcitability; the actions of all doses were significant ($P<0.01$ in each case), when compared with the effects of saline administration, over the whole testing period. (○) Control diet + acute saline; (□) barbitone diet + acute saline; (▲) barbitone diet + acute CGP39551, 2.5 mg kg^{-1}; (■) barbitone diet + acute CGP39551, 5 mg kg^{-1}; (●) barbitone diet + acute CGP39551, 10 mg kg^{-1}. Values are medians with representative interquartile ranges. In all cases the ratings after barbitone withdrawal, with acute saline administration, were significantly higher than in controls that did not receive barbitone ($P<0.001$).

($P<0.001$ for comparison between results from barbitone withdrawn animals given saline or CGP39551), over the whole of the testing period (Figure 1). A further study, carried out with lower doses of CGP39551, showed that this compound significantly decreased the withdrawal ratings, at doses of 2.5, 5 or 10 mg kg^{-1} ($P<0.01$). These results are illustrated in Figure 2.

CGP37849 also had a protective action on the barbitone withdrawal syndrome, but this was of shorter duration than that of CGP39551 (Figure 3). The effect of the 10 mg kg^{-1} dose was significant when compared over the first 5 h of testing ($P<0.05$).

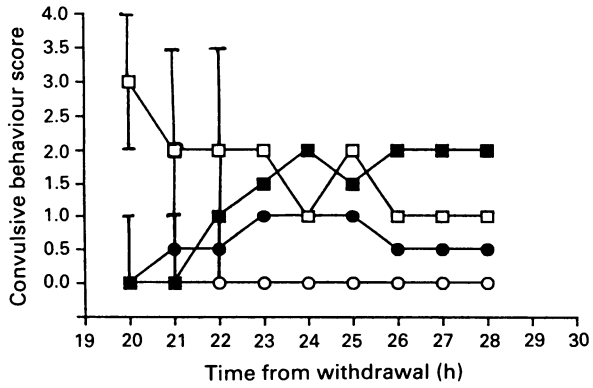

Figure 3 The effects of CGP37849,10 mg kg^{-1}, on the ratings of convulsive behaviour (± interquartile range) during barbitone withdrawal. Values are medians with representative interquartile ranges. (○) Control diet + acute saline; (●) control diet + acute CGP37849, 10 mg kg^{-1}; (□) barbitone diet + acute saline; (■) barbitone diet + acute CGP37849, 10 mg kg^{-1}. The ratings of behaviour after barbitone withdrawal and saline administration were significantly greater than after the control diet and saline administration, as expected. When comparison was made over the first 5 h of testing, this dose of CGP37849 significantly reduced the ratings ($P<0.005$).

Neither CGP39551 or CGP37849 had any effect on the behavioural ratings in control animals.

Effects of bicuculline during barbitone withdrawal

The responses to bicuculline during barbitone withdrawal are given in Table 1. The administration of 2.5, 5, 10 or 20 mg kg^{-1} CGP39551, 1 h or 5 h before bicuculline administration, did not alter the incidence of convulsions in response to bicuculline, in either barbitone withdrawn or control mice (Table 1A). The latencies to the convulsions were significantly increased after CGP39551, during barbitone withdrawal only. When the effects of CGP37849 were tested, given 1 h before the administration of bicuculline, there were no significant differences in the responses to bicuculline, either in control animals or those withdrawn from barbitone treatment (Table 1B).

Convulsive effects of NMDA during barbitone withdrawal

The seizure incidence in response to NMDA, shown in Table 2, was unaffected by barbiturate withdrawal. The latencies of the seizures after the 325 mg kg^{-1} dose were also unaltered. There was a small, but just significant ($P = 0.05$), increase in the latencies after the 250 mg kg^{-1} dose.

The convulsion thresholds to NMDA measured by the infusion method, are illustrated in Figure 4. There was no significant difference between the thresholds in animals given chronic barbitone treatment and those of controls ($P>0.1$).

The anticonvulsant actions of CGP39551 and CGP37849 in naive mice

The effects of CGP39551 and CGP37849 against convulsions due to NMDA or to bicuculline were studied in naive mice, for comparison with the effects on the barbiturate withdrawal syndrome. The results are given in Table 3. CGP39551 provided significant protection against NMDA seizures when

Table 1 The actions of CGP39551 and CGP37849 against bicuculline in control and barbitone-withdrawn mice

A CGP39551 was given 1 h or 5 h prior to the bicuculline. In each case the convulsant was given 24 h after the cessation of chronic barbitone treatment

Chronic treatment	*Bicuculline dose* (mg kg^{-1})	*Pretreatment time*	*CGP39551 dose* (mg kg^{-1})	*Seizure incidence*	*Seizure latencies*
Controls	5	1 h	Vehicle	6/8	119 ± 19
Controls	5	1 h	10	7/8	201 ± 67
Barbitone	3.5	1 h	Vehicle	7/8	85 ± 17
Barbitone	3.5	1 h	10	8/8	81 ± 9
Controls	5	5 h	Vehicle	6/10	168 ± 46
Controls	5	5 h	20	9/10	178 ± 42
Barbitone	3.5	5 h	Vehicle	9/9	67 ± 7
Barbitone	3.5	5 h	5	7/10	110* ± 27
Barbitone	3.5	5 h	10	8/10	172* ± 57
Barbitone	3.5	5 h	20	7/9	112* ± 24

Latencies are mean ± s.e.mean (s) for the number of mice that convulsed in each group.
*$P<0.05$ compared with results after vehicle treatment.

B CGP37849 was given 1 h prior to the bicuculline; the convulsant was given 24 h after the cessation of chronic barbitone treatment

Chronic treatment	*Bicuculline dose* (mg kg^{-1})	*Pretreatment time*	*CGP37849 dose* (mg kg^{-1})	*Seizure incidence*	*Seizure latencies*
Controls	5	1 h	Vehicle	6/8	119 ± 19
Controls	5	1 h	10	8/8	81 ± 9
Barbitone	3.5	1 h	Vehicle	7/8	85 ± 17
Barbitone	3.5	1 h	10	6/8	185 ± 39

Latencies are mean ± s.e.mean (s) for the number of mice that convulsed in each group.

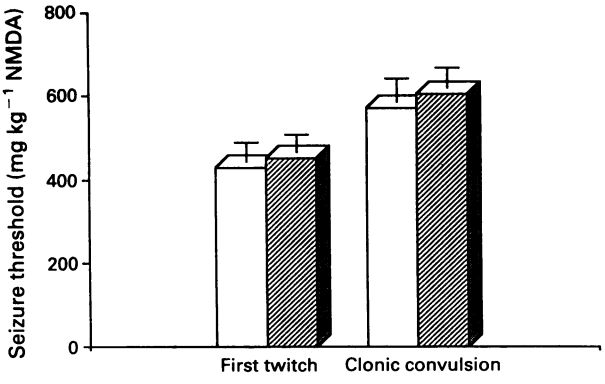

Figure 4 Convulsion thresholds to N-methyl-D-aspartate (NMDA) during barbitone withdrawal, measured by intravenous infusion of NMDA. Results are expressed as mg kg^{-1} NMDA (mean ± s.e.mean), the open columns indicate the values for control animals, the hatched columns those obtained during barbiturate withdrawal. The barbiturate withdrawal did not significantly affect either the threshold for the first twitch or the threshold for a full convulsion ($P > 0.1$).

Table 2 Responses to NMDA during barbiturate withdrawal

Chronic treatment	*NMDA dose* (mg kg^{-1})	*Seizure incidence*	*Seizure latencies (s)*
Control	250	5/10	688 ± 150
Barbitone	250	5/10	1152 ± 127*
Control	325	6/10	393 ± 28
Barbitone	325	7/10	513 ± 99

Latencies are mean ± s.e.mean (s) for the number of mice that convulsed in each group. Measurements were made 24 h after withdrawal from chronic barbitone treatment.
*$P = 0.05$ compared with results after vehicle treatment.

Table 3 Anticonvulsant actions of CGP39551 in naive mice

Convulsant	*CGP39551 dose* (mg kg^{-1})	*Pretreatment time* (h)	*Seizure incidence*	*Latencies*
NMDA	Saline	1	5/8	754 ± 122
NMDA	10	1	0/8*	–
NMDA	20	1	0/8*	–
NMDA	Saline	5	7/8	511 ± 31
NMDA	10	5	5/8	728 ± 106*
NMDA	Saline	5	8/8	542 ± 82
NMDA	20	5	2/8*	865 ± 385
NMDA	Vehicle	1	6/8	886 ± 170
NMDA	1	1	6/8	839 ± 110
NMDA	2.5	1	6/8	800 ± 165
NMDA	5	1	6/8	1009 ± 196
Bicuculline	Saline	1	4/8	179 ± 54
Bicuculline	10	1	4/8	118 ± 17
Bicuculline	20	1	4/8	127 ± 39
Bicuculline	Saline	5	7/8	121 ± 19
Bicuculline	10	5	3/8	298 ± 153
Bicuculline	Saline	5	5/8	171 ± 79
Bicuculline	20	5	5/8	257 ± 61

Latencies are mean ± s.e.mean for the number of mice that convulsed. N-methyl-D-aspartate (NMDA) was given at 325 mg kg^{-1} and bicuculline at 5 mg kg^{-1}.
*$P < 0.05$ compared with results after vehicle treatment.

Table 4 Anticonvulsant actions of CGP37849 in naive mice

Convulsant	*CGP dose* (mg kg^{-1})	*Pretreatment time* (h)	*Seizure incidence*	*Latencies*
NMDA	Saline	1	6/8	675 ± 122
NMDA	10	1	0/8**	–
NMDA	Saline	5	6/8	572 ± 158
NMDA	10	5	5/8	1541 ± 662
NMDA	Saline	5	7/8	511 ± 31
NMDA	20	5	3/8	939 ± 54 **
Bicuculline	Saline	1	4/8	195 ± 34
Bicuculline	10	1	5/8	233 ± 98
Bicuculline	Saline	1	5/8	171 ± 79
Bicuculline	20	1	2/8	190 ± 3
Bicuculline	Saline	5	7/8	121 ± 19
Bicuculline	10	5	4/8	259 ± 124
Bicuculline	Saline	5	5/8	171 ± 79
Bicuculline	20	5	2/8	190 ± 31

Latencies are mean ± s.e.mean for the number of mice that convulsed in each group. N-methyl-D-aspartate (NMDA) was given at 325 mg kg^{-1} and bicuculline at 5 mg kg^{-1}.
*$P < 0.05$ compared with results after vehicle treatment.

Table 5 The effects of CGP39551 and CGP37849 on spontaneous locomotor activity in naive mice

Drug	*Dose* (mg kg^{-1})	*Pretreatment time* (h)	*Cumulative activity counts*
Saline	–	1	5626 ± 538
CGP39551	10	1	3837 ± 809*
CGP37849	10	1	1864 ± 740**
Saline	–	1	6325 ± 624
CGP39551	20	1	2466 ± 550**
CGP37849	20	1	1442 ± 424**
Saline	–	1	6183 ± 314
CGP39551	2.5	1	4695 ± 739
Saline	–	5	6297 ± 476
CGP39551	10	5	3916 ± 448**

Cumulative activity was measured for 30 min, starting at the pretreatment times given below. Results are expressed as mean ± s.e.mean.
*$P < 0.05$, **$P < 0.01$, compared with concurrently tested controls.

given at 10 mg kg^{-1}, if a 1 h pretreatment time was used but not if given 5 h prior to the convulsant (Table 3). At 20 mg kg^{-1}, this compound decreased the effects of NMDA at both 1 h and 5 h pretreatment times. Neither of these doses of CGP39551, at either pretreatment time, significantly affected the responses to bicuculline. At lower doses, 1, 2.5 or 5 mg kg^{-1}, CGP39551 did not alter the responses to bicuculline (Table 3).

A similar pattern was seen when CGP37849 was used, except that the effects of this compound were of shorter duration, as was found in the withdrawal studies (Table 4). Significant protection against NMDA seizures, but not those due to bicuculline, was seen 1 h after CGP37849. The decrease in seizure incidence, 5 h after the 20 mg kg^{-1} dose of CGP37849, was not significant, although the latencies of the NMDA seizures were significantly increased.

Effects of CGP39551 and CGP37849 on spontaneous locomotor activity

Both CGP39551 and CGP37849, at 10 and 20 mg kg^{-1}, significantly decreased spontaneous locomotor activity in

naive mice (Table 5); CGP37849 appeared to have a greater effect than CGP39551 at each dose, although the differences were not significant ($P > 0.1$). The 2.5 mg kg^{-1} dose of CGP39551, that gave significant protection against the barbiturate withdrawal syndrome, did not affect the spontaneous locomotor activity.

Decreases in spontaneous locomotor activity do not always indicate sedation, but after administration of the above doses of these drugs the animals appeared limp and sleepy and showed reduced responses to external stimuli, such as loud noises.

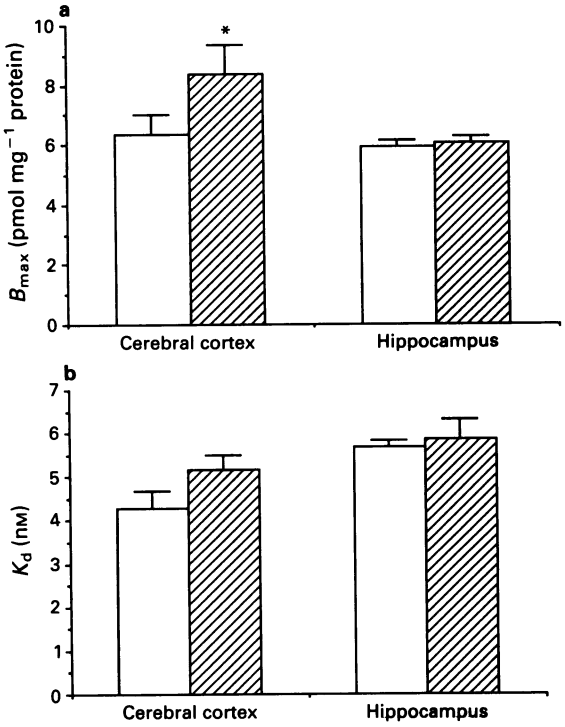

Figure 5 (a) B_{max} values measured in tissues prepared 24 h after withdrawal from chronic barbitone treatment. Open columns indicate control values; hatched columns values obtained after chronic barbitone treatment. Values are mean ± s.e.mean. *$P < 0.05$ for comparison between control and barbitone treatment. (b) K_d values in tissues prepared 24 h after withdrawal from chronic barbitone treatment. Values are mean ± s.e.mean. Open columns indicate control values; hatched columns values obtained after chronic barbitone treatment. No significant differences were seen.

[^{3}H]-dizocilpine binding after chronic barbiturate treatment

The chronic barbiturate administration significantly ($P < 0.05$) increased the B_{max} values for [^{3}H]-dizocilpine binding in cerebrocortical tissue, by 32% (Figure 5a), when the tissues were prepared 24 h after withdrawal of the animals

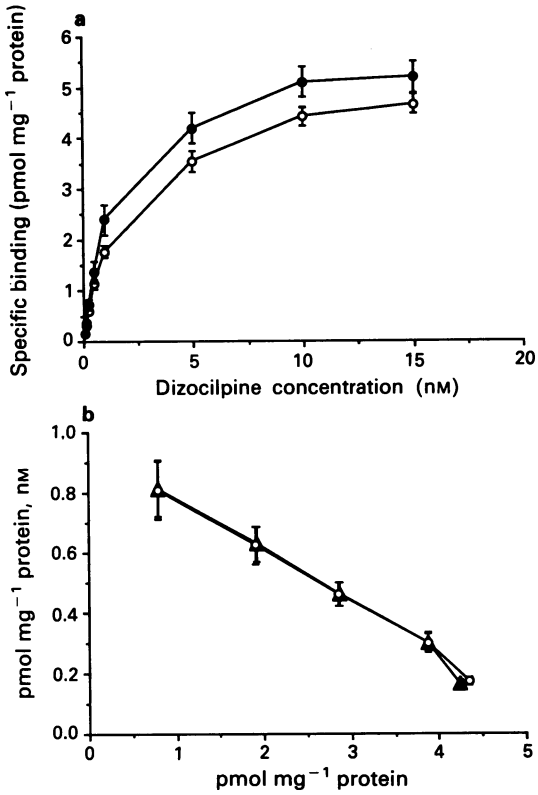

Figure 6 (a) Specific binding at a range of concentrations of [^{3}H]-dizocilpine, measured in cerebrocortical tissue from controls and from mice withdrawn for 24 h from the chronic barbitone treatment: (○) controls; (●) chronic barbitone. Values are mean ± s.e.mean, from six determinations, using tissues from six animals, in each case. (b) Scatchard plots obtained in the presence and absence of barbitone (100 μM) added to the incubation medium *in vitro*: (○) controls; (▲) barbitone. Values are mean ± s.e.mean, from six determinations in each case. There were no significant differences between the results obtained in the presence and absence of barbitone.

Table 6 Lack of effect of a single dose of barbitone (250 mg kg^{-1}) on K_d and B_{max} values for [^{3}H]-dizocilpine binding in cerebrocortical tissue

Pretreatment time (h)	K_d (nM) *Control*	*Barbitone*	B_{max} (pmol mg^{-1} protein) *Control*	*Barbitone*
1	6.98 ± 1.46	6.68 ± 0.84	6.00 ± 0.68	5.67 ± 0.43
24	4.55 ± 0.33	4.39 ± 0.32	7.31 ± 1.48	6.04 ± 0.85

The effects of a single i.p. dose of barbitone, 250 mg kg^{-1}, on K_d and B_{max} values for [^{3}H]-dizocilpine binding, compared with administration of saline. Results are expressed as mean ± s.e.mean. There were no significant differences between the values at either 1 h or 24 h after the injections ($n = 6$ per group).

Table 7 Lack of effect of barbitone added *in vitro* on K_d and B_{max} values for [^{3}H]-dizocilpine binding

Barbitone (μM)	K_d (nM) *Control*	*Barbitone*	B_{max} (pmol mg^{-1} protein) *Control*	*Barbitone*
1	6.84 ± 0.90	6.60 ± 0.50	5.73 ± 0.81	5.62 ± 0.69
100	5.91 ± 0.91	5.69 ± 0.79	5.51 ± 0.72	5.40 ± 0.77

The effects of barbitone, 1 and 100 μM, added *in vitro* on K_d and B_{max} values for [^{3}H]-dizocilpine binding, expressed as mean ± s.e.mean. There were no significant differences between the groups.

from the treatment. The K_d was not significantly altered (Figure 5b). The mean values calculated for the specific binding in these studies are illustrated in Figure 6a.

In contrast to the results obtained in the cortex, the chronic barbitone treatment left unaltered [^{3}H]-dizocilpine binding in hippocampal tissues (Figures 5a and b). There were no significant differences in either the B_{max} or the K_d values measured in tissues from controls and barbitone-treated animals ($P<0.05$).

Administration of a single dose of barbitone

At 1 h and 24 h after administration of 250 mg kg^{-1} barbitone, the B_{max} and K_d values for binding remained unaltered, compared with those after a vehicle injection. The results are given in Table 6.

Addition of barbitone in vitro

Addition of barbitone to the incubation medium did not change the total binding of [^{3}H]-dizocilpine, at any of the barbitone concentrations used (results not shown). The displacement of [^{3}H]-dizocilpine by TCP was not significantly altered by the addition of barbitone, although the mean IC_{50} values for TCP were lower in each experiment when measured in the presence of barbitone. The K_d and B_{max} values measured in the presence and absence of 1 and 100 μM barbitone are given in Table 7, and the Scatchard plots obtained during the study of the effects of barbitone, 100 μM, are illustrated in Figure 6b.

Discussion

The competitive NMDA antagonists, CGP39551 and CGP-37849, prevented all the signs of the barbiturate withdrawal syndrome measured by ratings of convulsive behaviour. This method measures both tremor and convulsions and is the standard method used for estimating withdrawal severity for sedative/hypnotic drugs such as barbiturates and alcohol (Goldstein & Pal, 1971; Green *et al.*, 1990). The method measures the reactions of the animals to a gentle disturbance and the response is characteristic of withdrawal from this type of drug. The effects of the drugs were seen at low doses, down to 2.5 mg kg^{-1} CGP39551. The effective doses of CGP39551 were lower than those needed to prevent seizures due to NMDA in naive mice. These in turn were lower than the doses of this compound required to prevent seizures caused by the GABA antagonist, bicuculline, in naive mice. A similar pattern was seen in the effects of CGP37849. Decreases in locomotor activity were seen in naive mice after doses of 10 and 20 mg kg^{-1} of either CGP39551 or CGP37849, but at 2.5 mg kg^{-1} CGP39551 did not significantly affect locomotor activity. We have previously demonstrated that similarly low doses of CGP39551 and CGP37849 protect against the ethanol withdrawal syndrome (Ripley *et al.*, 1991).

The method of oral administration of barbitone used in this study provides a model of the human oral intake of this type of drug. The intake of barbitone was monitored throughout the chronic treatment and did not differ between cages. Although the exact doses received by individual mice were not known precisely, the animals were randomly allocated to acute treatment groups at the beginning of the withdrawal measurements and it was clear from the results that all the mice received sufficient drug for a sufficient length of time to produce a withdrawal syndrome.

The effectiveness of the NMDA antagonists in preventing the barbiturate withdrawal signs raised the possibility that changes at the NMDA receptor complex may be involved in the genesis of the withdrawal hyperexcitability. Evidence that neurochemical changes do occur at this receptor complex was provided by the demonstration that the density of NMDA receptors, measured by binding to the noncompetitive antagonist dizocilpine, was increased after the chronic barbitone treatment.

The selectivity of the action of the NMDA antagonists during barbiturate withdrawal, and the low doses required to produce the protective effect, strongly suggested that the underlying causes of the withdrawal hyperexcitability involve increased activity at NMDA receptors. However, the convulsive effects of NMDA were unaltered during barbiturate withdrawal, despite the increase in binding site number.

In the present study, the distinction between the doses of CGP39551 and CGP37849 that were effective against barbiturate withdrawal hyperexcitability and those with anticonvulsant action in other models was clear, but protection against other types of seizure has been reported at the former doses. Antagonism of NMDA receptor activity is well-established to have anticonvulsant actions in many models, although much of the work so far has been on the non-competitive antagonist, dizocilpine. Schmutz *et al.* (1990) demonstrated protective actions of CGP39551 and CGP-37849 against maximal electroshock seizures in rats, with ED_{50} values of 2.8 mg kg^{-1} and 2.0 mg kg^{-1}, respectively, after intraperitoneal administration. The times at which the effects were maximal were found to be 4 h for CGP39551 and 2 h for CGP37849. In our animals, the protective effects of CGP39551 against NMDA seizures in naive mice were clearly greater at 1 h than at 5 h after treatment. Fagg *et al.* (1990) showed the competitive antagonists used in the present study to be effective against electroshock seizures, with oral ED_{50} values of 4 mg kg^{-1} for CGP39551 and 21 mg kg^{-1} for CGP37849. Comparison between these values and those used in the present study is difficult because of the different route of administration, and the fact that differences between the two compounds in bioavailability have been suggested (Loscher & Honack, 1991). The latter authors studied the effects of CGP39551 and CGP37849 on kindled seizures in rats, when these compounds were given by the intraperitoneal route at 1, 2.5 and 5 mg kg^{-1}. At these doses, both compounds were ineffective against kindled seizures in this model. In the actions of the competitive NMDA antagonists, therefore, the barbiturate withdrawal syndrome and that of ethanol, resemble maximal electroshock seizures, rather than kindled seizures. Kindled seizures are regarded as a model for complex partial epilepsy, while the effects of drugs against maximal electroshock resemble those against generalized tonic-clonic seizure states in humans. The underlying causes of maximal electroshock seizures are unclear, but are likely to include increased NMDA receptor activity.

Loscher & Honack (1991) described excitatory actions of CGP39551 and CGP37849 in the kindled rats, including increased locomotor activity, head-weaving and circling. These were attributed to an increased function of NMDA receptors reported to occur after kindling (Mody & Heinemann, 1987), as they are not seen in nonkindled animals. None of these behaviours was seen in mice during barbiturate withdrawal in the present study, even though upregulation of NMDA receptor sites occurred after the barbiturate chronic treatment. The evidence for increased NDMA receptor activity during the ethanol withdrawal syndrome is greater than for barbiturate withdrawal, as many more studies have been carried out (see Grant *et al.*, 1990; Whittington *et al.*, 1992; and the review, Little, 1991), but no such abnormal behaviours were seen with CGP39551 or CGP37849 during our studies on this syndrome.

The chronic barbitone treatment caused an increase in the density of binding to [^{3}H]-dizocilpine in cerebrocortical tissue but not in the hippocampus. This ligand acts at the non-competitive site on the NMDA complex and was used as a marker for NMDA receptor. As described in the introduction, the acute action of barbiturates is to block responses at the AMPA and kainate subtypes of receptor, with less effect at NMDA receptors. This differential effect has been described in both hippocampal (Sawada & Yamamoto, 1985;

Miljkovic & Macdonald, 1986) and cortical tissue (Harrison & Simmonds, 1985), as well as in striatal preparations (Teichberg *et al.*, 1984). Barbitone added *in vitro*, over a wide range of concentrations, did not alter [^{3}H]-dizocilpine binding. The upregulation of the number of NMDA receptors in the cerebral cortex after chronic treatment may therefore represent the involvement of these receptors in neuronal plasticity, rather than a direct adaptive response to initial blockade. Upregulation of [^{3}H]-dizocilpine binding has also been seen after chronic phenobarbitone administration (Short & Tabakoff, 1993) and after chronic ethanol treatment (Grant *et al.*, 1990).

No upregulation of [^{3}H]-dizocilpine binding was seen in hippocampal tissue after chronic barbitone treatment. It has not been established whether the hyperexcitability seen during barbiturate withdrawal originates in one area of the CNS or if many areas become hyperexcitable simultaneously. Hinman & Okamoto (1984) recorded EEG patterns *in vivo* during 'moderate intensity' barbiturate withdrawal. Rhythmical burst of activity were seen in the hippocampus, amygdala and visual cortex and spike and wave activity in the hippocampus, accompanied by burst activity in the motor cortex when spontaneous generalized seizures occurred. These data suggest that both the hippocampus and cortical areas are involved in the withdrawal syndrome, but the patterns of adaptation may vary in different brain areas.

In conclusion, the evidence from the behavioural studies, demonstrating a potent protective effect of NMDA-receptor antagonists against withdrawal signs, and the receptor binding studies, showing upregulation of dizocilpine binding sites, suggests that increased activity at the NMDA receptors may contribute to the physical signs of withdrawal from chronic barbitone treatment. However, the lack of effect of the NMDA antagonists on the increased sensitivity to bicuculline-induced convulsions during the withdrawal period, and the lack of increased sensitivity to NMDA seen at this time show that other factors may be involved.

We thank the Mental Health Foundation and the Wellcome Trust for financial assistance. We are very grateful to Ciba-Geigy for gifts of CGP39551 and CGP37849. H.J.L. is a Wellcome Trust Senior Lecturer. X.Z. was the holder of a British Council Fellowship during this work.

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(Received January 11, 1993
Revised August 24, 1993
Accepted September 7, 1993)

Br. J. Pharmacol. (1994), **111**, 97–102

The effects of BTS 54 505, a metabolite of sibutramine, on monoamine and excitatory amino acid-evoked responses in the rat dorsolateral geniculate nucleus *in vivo*

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1 The effects of BTS 54 505, the primary amine metabolite of the *non-tricyclic* putative antidepressant sibutramine, on the responses evoked by visual stimulation and ionophoretic application of noradrenaline (NA), 5-hydroxytryptamine (5-HT) and excitatory amino acids (EAAs) in the rat dorsolateral geniculate nucleus (dLGN) have been investigated.

2 Ionophoretic application of 5-HT to dLGN neurones attenuated visually-evoked ($n = 46$), NMDA-evoked ($n = 21$) and AMPA-evoked responses ($n = 21$), while ionophoretic application of NA potentiated visually-evoked activity in these cells ($n = 27$).

3 Simultaneous application of BTS 54 505 with 5-HT (over 120 s) resulted in a prolongation of the recovery time (i.e. the period required by a neurone to recover by 50%, RT_{50}) from the 5-HT-mediated suppression of discharge activity ($\approx$275% increase in RT_{50}). BTS 54 505 also prolonged the recovery time from a NA-mediated potentiation of firing ($\approx$450% increase in RT_{50}). These effects on recovery time are attributed to the inhibition of uptake of both 5-HT and NA by BTS 54 505. The *amplitude* of the response to 5-HT or NA was unaffected by co-ejection of BTS 54 505.

4 Ionophoretic application of N-methyl-D-aspartate (NMDA) produced a current-dependent increase in neuronal firing, as did application of the non-NMDA receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). A simultaneous 120 s application of BTS 54 505 inhibited the NMDA response in all cells studied (mean $ED_{50} = 16 \pm 5$ nA) but had no effect on AMPA-evoked activity in the majority of the same cells ($n = 15/21$).

5 Short 10 s applications of BTS 54 505, at ejecting currents ($\geqslant 30$ nA) that attenuated NMDA-evoked activity in all cells studied, had no effect on either response amplitude or recovery time from ionophoretic application of 5-HT, suggesting that inhibition of NMDA-evoked activity by BTS 54 505 was not mediated by 5-HT uptake blockade.

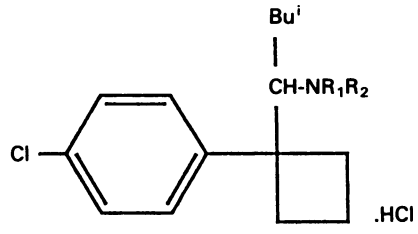
6 These results suggest that BTS 54 505 inhibits NMDA-evoked activity, and the observation that this effect is unlikely to be due to raised levels of endogenous 5-HT following monoamine uptake blockade indicate that BTS 54 505 may interact directly with the NMDA receptor ionophore complex.

Keywords: Dorsolateral geniculate nucleus; excitatory amino acids; antidepressants; sibutramine HCl; BTS 54 505

Introduction

Sibutramine HCl (BTS 54 524; N-1-(1-(4-chlorophenyl)cyclobutyl)-3-methylbutyl-N,N-dimethylamine hydrochloride monohydrate) (Figure 1) is a non-tricyclic putative antidepressant. In models indicative of potential antidepressant activity, sibutramine potently counteracted the behavioural effects of reserpine and downregulated cortical β-adrenoceptors after only three days treatment (Buckett *et al.*, 1988a). This profile is characteristic of monoamine reuptake inhibitors, but sibutramine is a relatively weak inhibitor of noradrenaline (NA), 5-hydroxytryptamine (5-HT) and dopamine uptake *in vitro* (Cheetham *et al.*, 1990). The primary and secondary amine metabolites (BTS 54 505 and BTS 54 354 respectively; Figure 1) of sibutramine have a similar pharmacological profile to the parent compound *in vivo*, but are up to 100 fold more potent than sibutramine as monoamine uptake inhibitors *in vitro* (Luscombe *et al.*, 1989; Cheetham *et al.*, 1990). This indicates that the pharmacological effects of sibutramine *in vivo* are mainly due to the activity of its primary and secondary amine metabolites, so the present studies have utilised the primary amine metabolite, BTS 54 505.

It has recently been reported that tricyclic antidepressants have antagonistic behaviour at N-methyl-D-aspartate (NMDA) receptors. Reynolds & Miller (1988) demonstrated that imipramine and desmethylimipramine (DMI) inhibited [^{3}H]-MK-801 binding in rat brain membranes and prevented the NMDA-induced Ca^{2+} influx into cultured rat cortical neurones. It has also been demonstrated, in whole-cell and single-channel recording techniques, that DMI attenuates NMDA receptor channel activity in a dose- and voltage-dependent manner, suggesting that DMI inhibits NMDA receptors by open channel block in a manner similar to MK-801 (Sernagor *et al.*, 1989; White *et al.*, 1990). Tricyclic



BTS	NR_1R_2
54 524	NMe_2
54 354	NHMe
54 505	NH_2

Figure 1 Chemical structure of sibutramine (BTS 54 524) and its active amine metabolites.

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antidepressants have also been shown to block NMDA-induced lethality in mice (Leander, 1989) and the NMDA receptor antagonists MK-801 and 2-amino-7-phosphonoheptanoic acid (AP7) have been shown to exhibit antidepressant-like actions in an inescapable stress paradigm (Trullas & Skolnick, 1990). Since these observations suggest that tricyclic antidepressants may act as NMDA receptor channel blockers, we have used electrophysiological techniques to elucidate whether BTS 54 505, an active metabolite of the non-tricyclic putative antidepressant sibutramine (Luscombe *et al.*, 1989), has any effect on NMDA receptor activity.

In this study we examined the effects of BTS 54 505 on NMDA-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, 5-HT- and NA-evoked activity in the dorsolateral geniculate nucleus (dLGN) of the rat thalamus. It has been shown that direct application of NA to geniculate visual relay cells in the rat by microiontophoresis produces a facilitation of firing (Rogawski & Aghajanian, 1980), whereas in contrast to NA, ionophoretic application of 5-HT depresses the spontaneous and evoked activity of dLGN neurones in the rat (Rogawski & Aghajanian, 1980). These contrasting responses to NA and 5-HT in this nucleus therefore allow the effects of BTS 54 505 on both 5-HT and NA uptake to be readily investigated. It has been demonstrated that retinogeniculate neurotransmission is mediated by excitatory amino acid receptors in the cat (Sillito *et al.*, 1990a,b) and in the rat (Scott & Mason, 1992b,c). Recording from the dLGN thus affords the opportunity to investigate the effects of BTS 54 505 both on monoamine and on NMDA- and AMPA-evoked responses in one site. Preliminary reports of these studies have appeared elsewhere in abstract form (Scott *et al.*, 1991; 1992).

Methods

Male Lister hooded rat (200–250 g) were housed in groups of 2–5 with a 12 h light:12 h dark lighting cycle (lights on at 07h 00 min GMT). The animals were anaesthetized with urethane (1.3–1.5 $g.kg^{-1}$, i.p.; Sigma) and mounted in a David Kopf stereotaxic frame. Following subcutaneous injection under the scalp of local anaesthetic (2% w/v xylocaine; Astra), a mid-line scalp incision was made and the scalp muscle retracted. A craniotomy of 4–5 mm diameter was then performed with a hand-held troephine, 3 mm posterior to Bregma and 3.6 mm lateral to the midline suture in accordance with the stereotaxic atlas of Pellegrino *et al.* (1979). Silicone-coated seven-barrelled microelectrodes were used to record extracellular neuronal discharge activity and to eject drugs by the process of ionophoresis (Scott & Mason, 1992a). The silicone-coated seven-barrelled microelectrodes were made from borosilicate glass tubing with an inner filament ('Kwik-fil', 1.5 mm o.d. × 0.86 mm i.d.; Clark Electromedical Instruments, UK) and the tips broken back to ≈4–6 μm. The centre barrel and one outer barrel were filled with pontamine sky blue (5% w/v in 500 mM NaCl) for extracellular recording and automatic current balancing respectively. The remaining barrels were each filled with one of the following: N-methyl-D-aspartate (NMDA: 10 mM: Sigma), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA: 1 mM: Tocris Neuramin), L-glutamate (10 mM: Sigma), 5-hydroxytryptamine (5-HT: 10 mM: Sigma), noradrenaline (NA: 10 mM: Sigma) or N-1-(1-[4-chlorophenyl]-cyclobutyl)-3-methylbutylamine hydrochloride (BTS 54 505: 10 mM: Boots Pharmaceuticals Research Department); all drugs were dissolved in 165 mM NaCl.

After surgical preparation of the animals for recording, the multibarrelled electrode was stereotaxically positioned and driven under remote hydraulic control to the dLGN. Hippocampal neurones were encountered 2–4 mm below the cortical surface and physiologically identified from their large spike amplitude (≥ 800 μV) and regular spontaneous firing rate (Kandel & Spencer, 1961). The dLGN neurones were recorded below the level of the hippocampus (between 4.5–6 mm below the cortical surface), and were physiologically identified by their response to visual stimulation. This comprised a diffuse whole-field flash of 1 s duration followed by an off period of 1 s presented to the contralateral eye. Cells were classified according to their response as either light-activated (ON-cells), light suppressed (OFF-cells) or ON-OFF responsive cells. The recording sites were marked by ejection of pontamine sky blue (5% w/v in 500 mM NaCl; 10 μA over 20 min) from the recording electrode and subsequent histological preparation of 50 μm thick coronal brain sections to enable identification of the recording sites.

Timing of ionophoretic ejection of drugs was controlled by an electronic clock and retaining currents of ± 10 nA were used between ejections with continuous automatic current balancing. The effect of ionophoresed compounds on EAA-evoked responses in the dLGN were evaluated from integrated firing rate histograms. Spike waveform was monitored to ensure drugs did not exert anaesthetic effects. For each cell recorded a 'dose' (ejection current)-response profile was plotted for the various agonists used (e.g. Figure 2a for 5-HT; Figure 2b for NA). Subsequent modulatory effects of BTS 54 505 on agonist-evoked responses were then routinely assessed using agonist ejection currents that elicited 50–80% maximal response. Electrophysiological recording, display and processing of discriminated signals was conventional (Mason, 1986). Discriminated neuronal activity was plotted as integrated firing rate histograms over successive 5 s epochs onto a chart recorder. The chart records were then digitised with the aid of a digitising tablet (Summasketch Professional) using Sigmascan (Jandel, U.S.A.) software and imported into Sigmaplot (Jandel, U.S.A.) for data analysis.

Results

Effects of BTS 54 505 on 5-HT- and NA-evoked responses in dLGN

Ionophoretic application of 5-HT and NA onto dLGN neurones revealed opposite effects on visually-evoked firing, similar to their reported actions on electrical stimulation of the optic nerve in rat dLGN (Rogawski & Aghajanian, 1980). Ejection of 5-HT (−4 to 50 nA; 10–120 s duration) potently inhibited visually-evoked firing in a current-dependent, reversible manner with a rapid onset (1–10 s) in all cells studied ($n = 46$) (Figure 2a). In contrast to 5-HT, ionophoretic application of NA (0 to 32 nA; 10–30 s duration), produced a current-dependent, reversible and rapid (1–10 s onset) potentiation of visually-evoked firing in all cells studied ($n = 27$) (Figure 2b). Both 5-HT- and NA-evoked responses were evident on the same dLGN cells.

As BTS 54 505, the primary amine metabolite of the monoamine uptake inhibitor sibutramine, is a more potent inhibitor of NA uptake than of 5-HT uptake (Cheetham *et al.*, 1990), ionophoretic application of BTS 54 505 might be predicted to have a more profound effect on exogenously applied NA than on exogenously applied 5-HT. This effect on monoamine uptake processes may be reflected in changes in response amplitude and/or recovery from agonist-evoked responses. The recovery time (RT), i.e. the period required by the neurone to recover by 50% (RT_{50}) from termination of the ionophoretic ejection, was used as an index of the efficacy of the transmitter uptake process (Wang *et al.*, 1979). In order to dissociate clearly the effects of BTS 54 505 on 5-HT receptor from NMDA receptor (see later results) responses, we examined the effects of BTS 54 505 on 5-HT-evoked responses in OFF cells, since visually-evoked responses in light-suppressed (OFF) cells in the rat *in vivo* appear to be mediated selectively by non-NMDA receptors (Scott & Mason, 1992b,c). BTS 54 505 (0 to 30 nA; 120 s duration) was ejected continuously at a current that had no effect on the basal visually-evoked firing rate (Figures 3a,b) while

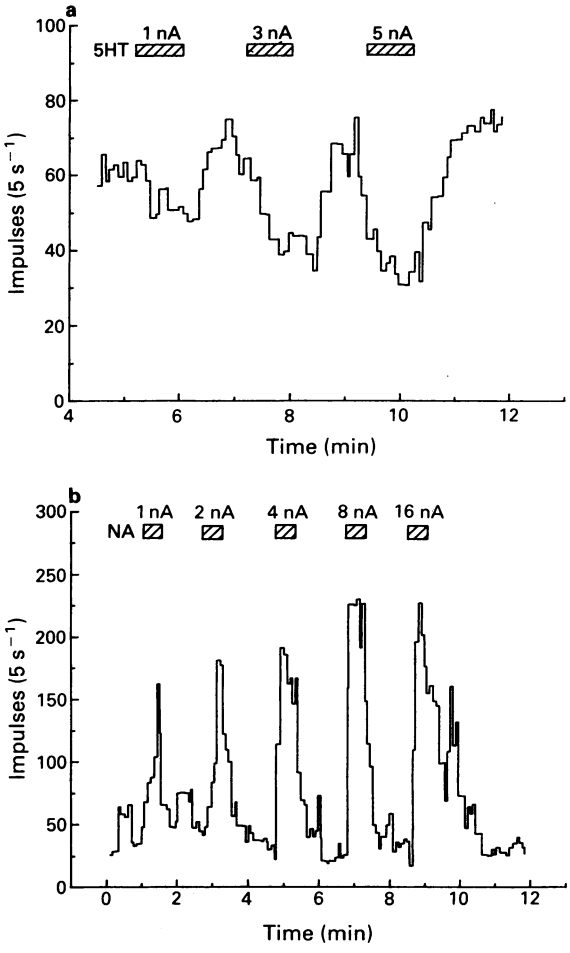

Figure 2 (a) Integrated firing rate histogram accumulated over successive 5 s epochs for a visually-driven dLGN cell. Ionophoretic application of 5-hydroxytryptamine (5-HT, 1 to 5 nA; 60 s duration) resulted in a current-dependent and reversible inhibition of the visually-evoked response. In this and subsequent figures the duration of drug ejection is indicated by the bars above the histogram record; the numbers above the bars indicate the ionophoretic current used. (b) Integrated firing rate histogram accumulated over successive 5 s epochs for a visually-driven dLGN cell. Ionophoretic application of noradrenaline (NA, 1 to 16 nA; 30 s duration) resulted in a current-dependent and reversible potentiation of the visually-evoked response.

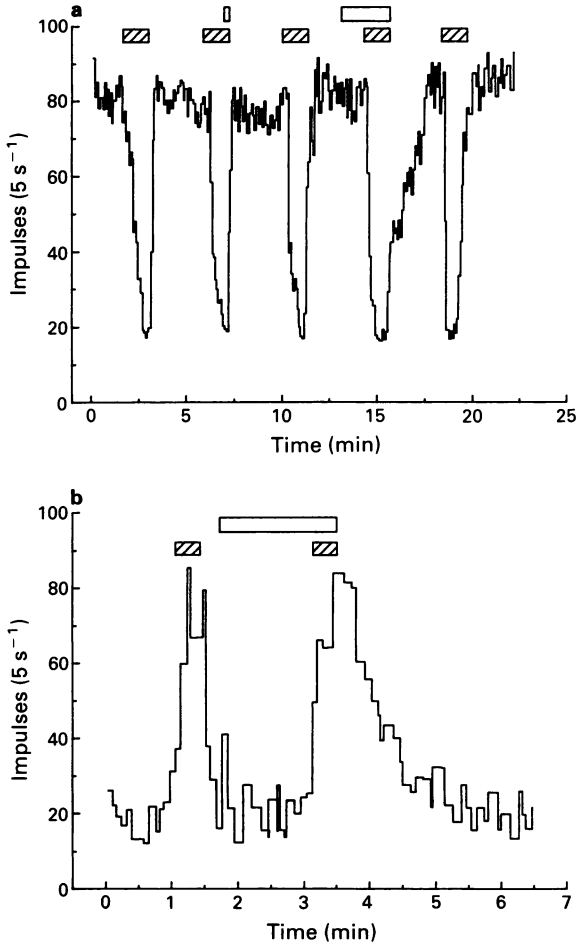

Figure 3 (a) Integrated firing rate histogram accumulated over successive 5 s epochs for a visually-driven dLGN cell. Ionophoretic application of 5-hydroxytryptamine (5-HT, 20 nA; 30 s duration) (hatched bars) resulted in a reversible inhibition of the visually-evoked response. Application of BTS 54 505 (open bar) (30 nA for 10 s) had no affect on either response amplitude or recovery time of the 5-HT-evoked response, whereas application of BTS 54 505 (30 nA) for 120 s prolonged the total recovery time from the 5-HT-evoked inhibition, but had no effect on response amplitude. (b) Integrated firing rate histogram accumulated over successive 5 s epochs for a visually-driven dLGN cell. Ionophoretic application of noradrenaline (NA, 16 nA; 30 s duration) (hatched bars) resulted in a reversible potentiation of the visually-evoked response. Application of BTS 54 505 (20 nA; 120 s) (open bar) prolonged the total recovery time from NA-evoked excitation, but had no effect on response amplitude.

regular ejections of either 5-HT or NA were repeated at 2 or 4 min intervals. BTS 54 505 prolonged the suppression of firing induced by ionophoretically applied 5-HT [mean $RT_{50} \pm$ s.e.mean: (control) 14 ± 4 s; (BTS 54 505) 35 ± 4 s; $n = 13$] (Figure 3a). Similarly, BTS 54 505 prolonged the potentiation of firing by ionophoretically applied NA [mean $RT_{50} \pm$ s.e.mean: (control) 29 ± 2 s; (BTS 54 505) 131 ± 7 s; $n = 6$] (Figure 3b). In all of these cells, it is notable that *only* the recovery time of the response to both 5-HT and NA was affected by application of BTS 54 505; the amplitude of the 5-HT- and NA-evoked responses remained unaltered (Figures 3a, b). The observation that the response amplitudes were unaffected is unlikely to be due to saturation of agonist receptors since the effects of BTS 54 505 were assessed on 5-HT and NA responses which were between 50–80% of maximum (see Methods). In the same cells when 120 s applications of BTS 54 505 prolonged the recovery time following cessation of 5-HT ejection, short 10 s application of BTS 54 505, had no effect on the recovery time (0–30 nA; $n = 6$ cells) (Figure 3a).

Effect of BTS 54 505 on excitatory amino acid-evoked responses in the dLGN

The effects of BTS 54 505 on NMDA- and AMPA-evoked responses were compared in order to evaluate the specificity of BTS 54 505 on NMDA and non-NMDA receptors. Ionophoretic application of NMDA (0 to 40 nA; 10 s duration; $n = 21$), AMPA (0 to 40 nA; 10 s duration; $n = 21$) or glutamate (20 to 100 nA; 10 s duration; $n = 9$) (data not shown) produced a current-dependent potentiation of activity in all cells studied.

BTS 54 505 (0 to 40 nA; 120 s duration) produced a current-dependent and reversible inhibition of the NMDA-evoked (2 to 40 nA; 10 s duration) response in all cells studied (mean $ED_{50} \pm$ s.e.mean: 16 ± 5 nA; $n = 21$) (Figure 4a). Ionophoretically applied 5-HT (5 to 20 nA; 30 s duration) also resulted in a current-dependent attenuation of the

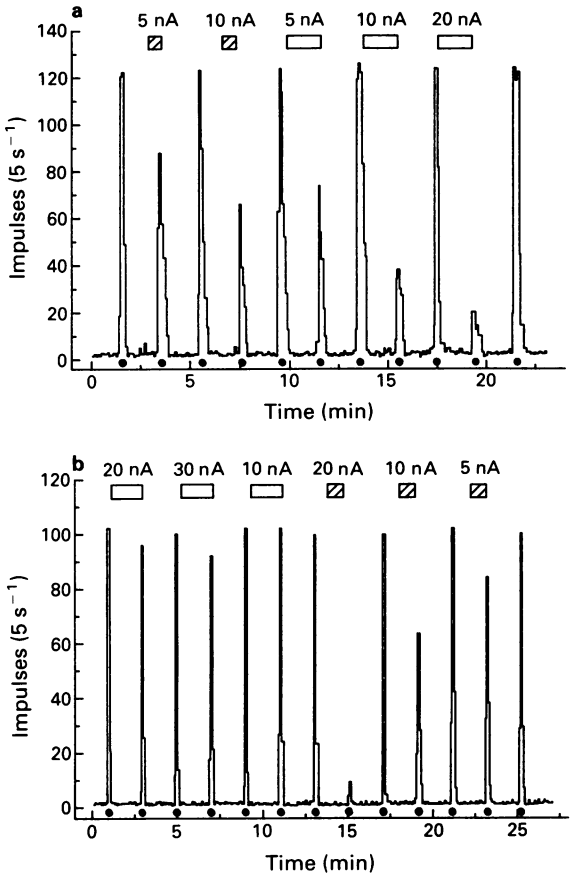

Figure 4 (a) Integrated firing rate histogram accumulated over successive 5 s epochs for a dLGN cell. Ionophoretic application of N-methyl-D-aspartate (NMDA, 20 nA; 10 s duration) (●) at regular 120 s intervals resulted in a consistent potentiation of baseline activity. Co-application of 5-HT (5 to 10 nA; 30 s duration) (hatched bars) resulted in a current-dependent and reversible inhibition of the NMDA-evoked response. Co-application of BTS 54 505 (BTS; 5 to 20 nA; 120 s duration) (open bars) also resulted in a current-dependent and reversible inhibition of the NMDA-evoked response. (b) Integrated firing rate histogram accumulated over successive 5 s epochs for the same dLGN cell as in (a). Ionophoretic application of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 20 nA; 10 s duration) at regular 120 s intervals resulted in a consistent potentiation of baseline activity. AMPA applications are denoted by the filled circles below the histogram. Co-application of 5-HT (5 to 20 nA; 30 s duration) (hatched bars) resulted in a current-dependent and reversible inhibition of the NMDA-evoked response. Co-application of BTS 54 505 (BTS; 10 to 30 nA; 120 s duration) (open bars) had no effect on AMPA-evoked activity.

NMDA-evoked response in the same cells (mean $ED_{50} \pm$ s.e.mean: 9 ± 3 nA; $n = 21$) (Figure 4a).

The process mediating the block of NMDA-evoked activity by BTS 54 505 was addressed by applying BTS 54 505 and NMDA *simultaneously* for the 10 s period of NMDA ejection only. It would be unlikely that a 10 s application of BTS 54 505 would raise synaptic levels of endogenous 5-HT to a level sufficient to inhibit the simultaneous NMDA-evoked response, since a 10 s application of BTS 54 505 had no effect on the response amplitude or recovery time to ionophoretically applied 5-HT (Figure 3a). As illustrated in Figure 5, co-ejection of NMDA and BTS 54 505 (12–25 nA; 10 s duration) produced a current-dependent and reversible inhibition of the NMDA-evoked potentiation of firing in all cells studied (mean $ED_{50} \pm$ s.e.mean: 18 ± 4 nA; $n = 11$). Therefore, our observations that BTS 54 505 produced a current-dependent inhibition of the NMDA-evoked response following simultaneous application, strongly suggest that

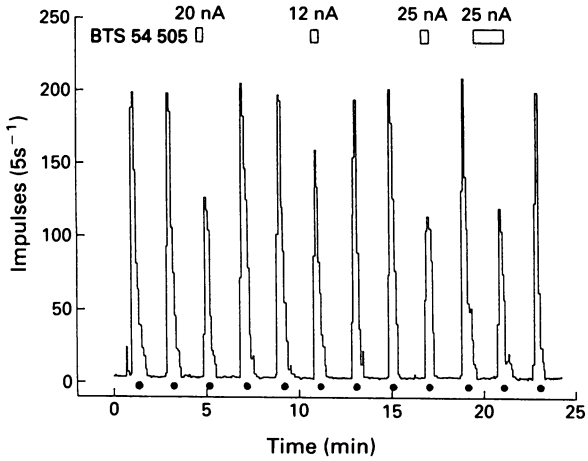

Figure 5 Integrated firing rate histogram accumulated over successive 5 s epochs for a dLGN cell. Ionophoretic application of N-methyl-D-aspartate (NMDA, 40 nA; 10 s duration) at regular 120 s intervals (●) resulted in a consistent potentiation of baseline activity. Co-application of BTS 54 505 (25 nA; 120 s duration) (open bars) resulted in a reversible inhibition of the NMDA-evoked response. Shorter applications of BTS 54 505 (12 to 25 nA; 10 s duration) had the same effect as the longer application.

BTS 54 505 blockade of 5-HT uptake alone is unlikely to be responsible for this effect on NMDA-evoked responses.

If BTS 54 505 acts specifically at the NMDA receptor, then one prediction is that the responses evoked by the non-NMDA receptor agonist, AMPA, should remain unaffected by simultaneous application of BTS 54 505. In the same cells ($n = 21$) that 5-HT and BTS 54 505 attenuated NMDA-evoked activity (Figure 4a) ionophoresed 5-HT (5–20 nA; 30 s duration) produced a current-dependent and reversible inhibition of the AMPA-evoked response (mean $ED_{50} \pm$ s.e.mean: 11 ± 4 nA) (Figure 4b). Ejection of BTS 54 505 (10–30 nA; 120 s duration), however, had no effect on the AMPA-evoked response in ≈75% of the same 21 cells. BTS 54 505 did show a weak (<15%) current-dependent and reversible inhibition of the AMPA-evoked response in the remaining cells ($n = 6$) (Figure 4b).

Discussion

The present studies have shown that ionophoretic application of 5-HT attenuates both EAA- and visually-evoked firing in the rat dLGN, whereas NA potentiates EAA- and visually-evoked firing in these cells. Such activity has been reported previously (Rogawski & Aghajanian, 1980). BTS 54 505 prolonged the recovery time of dLGN cells from responses elicited by application of 5-HT and NA, but did not alter the amplitude of the responses to 5-HT and NA. Ionophoretic application of NMDA, AMPA or glutamate produced a current-dependent and reversible potentiation of firing in all cells studied. BTS 54 505 inhibited NMDA-evoked activity in a current-dependent manner, but had no effect on AMPA-evoked activity in ≈75% of cells studied.

It may be considered that monoamine uptake inhibition by BTS 54 505 raises synaptic levels of 5-HT sufficiently to inhibit the NMDA-evoked or AMPA-evoked responses. However, BTS 54 505 is more potent at inhibiting NA uptake than 5-HT uptake (Cheetham *et al.*, 1990), yet at no time was there a potentiation of visually-evoked or NMDA-evoked firing by BTS 54 505, which would be mediated by blockade of uptake of endogenously released NA from the locus coeruleus noradrenergic innervation of the dLGN. It is possible that a dominance in 5-hydroxytryptaminergic over noradrenergic neurotransmission to the dLGN may explain

the absence of a potentiation of firing by BTS 54 505, but it is known that under anaesthesia, locus coeruleus neurones fire spontaneously at 0.5–5 Hz (Marwaha & Aghajanian, 1982) while dorsal raphé neurones fire spontaneously at 0.5–2.5 Hz (Marwaha & Aghajanian, 1982). The similarity between the firing rates, and hence the presumed release of neurotransmitter by the two monoaminergic projections, alone cannot be used as an index of the efficacy of synaptic activation in the dLGN. Nerve impulse traffic in the locus coeruleus or dorsal raphé pathways afferent to the dLGN does not necessarily imply release of neurotransmitter in the dLGN, since there may be physiological or modulatory synaptic influences that may prevent the ultimate release of transmitter at the terminal site. Even if there were release of transmitter in the dLGN, a similar frequency of host cell activation cannot imply a similar quantitative measure of neurotransmitter release. However, the observation that a 10 s application of BTS 54 505, at an ejecting current (30 nA) that was sufficient to inhibit NMDA-evoked activity in the cells studied (Figure 4a), had no effect on the response amplitude or recovery time from 5-HT application (Figure 3a), suggests that BTS 54 505 does not exert this attenuation of NMDA-evoked activity via 5-HT uptake blockade and that this rapid effect is probably mediated via NMDA receptor blockade.

In electrophysiological studies in the rat dLGN it has been observed that ionophoretic application of the tricyclic antidepressant desipramine also attenuated NMDA-evoked responses (Scott & Mason, unpublished observations), and that these inhibitory effects were equivalent to those observed using BTS 54 505. DMI is well established as a NMDA receptor antagonist; recent studies using [^{3}H]-MK-801 binding (Reynolds & Miller, 1988; Scott *et al.*, unpublished observations), whole-cell and single-channel recording techniques (Sernagor *et al.*, 1989; White *et al.*, 1990) suggest that DMI blocks the open channel of the NMDA receptor ionophore. In addition, substitution of a single amino acid in the NMDA receptor channel by site-directed mutagenesis markedly alters the sensitivity to MK-801, Mg^{2+} and DMI (Sakurada *et al.*, 1993), providing further evidence that DMI acts in the NMDA receptor channel. It has also been demonstrated that DMI and imipramine prevent the Ca^{2+} influx produced by NMDA in rat cultured cortical neurones (Reynolds & Miller, 1988), that imipramine, amitriptyline and nortriptyline protect against NMDA-induced toxicity in rat cultured cerebellar granule cells (McCaslin *et al.*, 1992) and that TCA's block the induction of long-term potentiation in the rat hippocampus by inhibiting activity at NMDA receptors (Watanabe *et al.*, 1993).

The inhibition of EAA-evoked responses by BTS 54 505 may suggest that BTS 54 505 is a non-specific EAA antagonist, blocking both NMDA and AMPA receptor channels. However, the present studies have demonstrated that BTS 54 505 and 5-HT attenuate NMDA-evoked activity in all cells studied (Figure 4a). In contrast, 5-HT attenuated AMPA-evoked activity in the same cells (Figure 4b), whereas BTS 54 505 was ineffective at inhibiting the AMPA-evoked response in the majority of these cells (Figure 4b). In addition, the NMDA receptor antagonist, APV, was observed to attenuate the AMPA-evoked response in a number of dLGN cells ($n = 4/9$; data not shown). Therefore a more plausible explanation may be that part of the AMPA-evoked response is in fact due to the recruitment of NMDA receptors following the removal of the voltage-dependent block by physiological levels of Mg^{2+} after AMPA-mediated depolarization, thereby allowing the activation of the NMDA receptors by endogenous EAAs. This postulated NMDA receptor-mediated component of the AMPA-evoked response would then be open to blockade by NMDA antagonists. While both NMDA- and AMPA-evoked responses were similarly affected by 5-HT, the EAA-evoked responses displayed differential sensitivity to application of BTS 54 505 in the same cells. This observation further supports the notion that attenuation of the NMDA-evoked activity is not due to a blockade of 5-HT uptake but is mediated by a direct action at the NMDA receptor. Indeed, this view is supported by receptor binding studies that show a displacement of [^{3}H]-MK-801 binding to rat hippocampal membranes by BTS 54 505 with a K_i value equivalent to those of the known NMDA receptor channel blockers DMI and ketamine (Scott *et al.*, unpublished observations).

Open channel blockers and compounds such as DMI may be useful in protecting against NMDA-induced toxicity and in the treatment of neurodegenerative diseases induced by NMDA receptor hyperactivity (Lodge, 1988). These compounds, due to their use-dependent activity (Kemp *et al.*, 1987; Sernagor *et al.*, 1989), would have little or no effect on NMDA receptor mediated neurotransmission in situations where the NMDA receptor would be inactive due to the voltage-dependent block by Mg^{2+} (Mayer *et al.*, 1984; Nowak *et al.*, 1984). Alleviation of the voltage-dependent Mg^{2+} block of NMDA receptor activity by membrane depolarization would then allow channel blockers to exert their antagonistic properties. However, it is known that the channel blockers have psychotomimetic side-effects (Wheal & Thomson, 1991; Oye *et al.*, 1992). Tricyclic antidepressants also have a number of side effects which arise from the affinity of these drugs for muscarinic cholinoceptors (Synder & Yamamura, 1977; Nomura *et al.*, 1987) and histamine receptors (Alvares *et al.*, 1988). These side-effects may therefore limit their therapeutic use in the treatment and/or prevention of NMDA-induced toxicity and neurodegeneration. Sibutramine and its active metabolite BTS 54 505, however, have no significant affinity for muscarinic receptors, α_1, α_2 and β adrenoceptors, dopamine D_1 and D_2 receptors, and 5-HT_1 and 5-HT_2 receptors (Buckett *et al.*, 1988b; Luscombe, unpublished observations), suggesting that sibutramine and BTS 54 505 may result in fewer and less pronounced side-effects than the tricyclic antidepressants. Further investigations, including [^{3}H]-MK-801 receptor binding and single-channel studies, are presently in progress to elucidate the precise mechanism of action of BTS 54 505 on NMDA receptor activity.

G.S. is a MRC collaborative student with Boots Pharmaceuticals.

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(Received June 8, 1993
Revised September 1, 1993
Accepted September 9, 1993)

Br. J. Pharmacol. (1994), 111, 103-110

Facilitatory effects of selective agonists for tachykinin receptors on cholinergic neurotransmission: evidence for species differences

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1 Exogenous tachykinins modulate cholinergic neurotransmission in rabbit and guinea-pig airways. We have investigated the effect of selective tachykinin receptor agonists and antagonists on cholinergic neurotransmission evoked by electrical field stimulation (EFS) of bronchial rings in rabbit, guinea-pig and human airways *in vitro* to assess which type of tachykinin receptor is mediating this facilitatory effect.

2 Bronchial rings were set up for isometric tension recording. Contractile responses to EFS (60 V, 0.4 ms, 2 Hz for 10 s every min) and exogenous acetylcholine (ACh) were obtained and the effects of selective tachykinin agonists and antagonists were investigated.

3 In rabbit bronchi the endogenous tachykinins, substance P (SP) and neurokinin A (NKA) (10 nM) potentiated cholinergic responses to EFS (by 287.6 ± 121%, $P<0.01$ and 181.4 ± 56.5%, $P<0.001$ respectively).

4 The NK_1 receptor selective agonist, [Sar^9]SP sulphone (10 nM) evoked a maximal facilitatory action on cholinergic responses of 334.9 ± 63% ($P<0.01$) ($pD_2 = 8.5 \pm 0.06$) an effect which was blocked by the selective NK_1-receptor antagonist, CP 96,345 (100 nM) ($P<0.05$) but not by the NK_2 receptor antagonist, MEN 10,376 (100 nM). The NK_2 receptor selective agonist, [βAla^8]NKA(4-10) (10 nM), produced a maximum enhancement of 278 ± 83.5% ($P<0.01$) ($pD_2 = 8.7 \pm 0.1$) an effect which was blocked by MEN 10,376 (100 nM) ($P<0.05$) and not by CP 96,345. [MePhe^7]NKB, an NK_3 receptor selective agonist was without effect.

5 The rank order of potency of NK_2 receptor antagonists against enhancement of cholinergic responses by [βAla^8]NKA(4-10) was MEN 10,376 > L 659,877 > R 396. This pattern together with the observation of the full agonist activity of MDL 28,564 indicates that the NK_2 receptors in the rabbit bronchus are similar to those which are present in the rabbit pulmonary artery.

6 Neither [Sar^9]SP sulphone (5 nM) nor [βAla^8]NKA(4-10) (1 nM) had any effect on contractile responses to ACh (10 μM) suggesting a pre-junctional mechanism of action.

7 By contrast, in guinea-pig bronchi only the NK_1-receptor agonist [Sar^9]SP sulphone (3 nM) was effective in enhancing cholinergic neurotransmission but the effect was relatively small (maximal enhancement 25.7 ± 5.5%, $P<0.01$). In human bronchial rings all the selective neurokinin agonists were without effect on cholinergic neurotransmission.

8 These results suggest that tachykinins may play an important role in modulating cholinergic neurotransmission in rabbit (via NK_1 and NK_2 receptors) and guinea-pig airways (via NK_1 receptor) but have no demonstrable effect on human airways

Keywords: Tachykinins; cholinergic neurotransmission; bronchial smooth muscle; contraction

Introduction

The tachykinins, substance P (SP) and neurokinin A (NKA) are localized to sensory nerves in the airways of several species, including man (Lundberg *et al.*, 1984; Martling *et al.*, 1987; Uddman *et al.*, 1987; Takeda *et al.*, 1990). Tachykinins have been shown to increase mucus secretion (Rogers *et al.*, 1989), vascular permeability (Rogers *et al.*, 1988), increase airway blood flow (Salonen *et al.*, 1988) and produce bronchoconstriction (Advenier *et al.*, 1987).

A neuromodulatory role has also been proposed for neuropeptides in animal airways. In rabbit trachea, SP-induced bronchoconstriction is significantly reduced by atropine suggesting that SP releases acetylcholine (ACh) from cholinergic nerve terminals (Tanaka *et al.*, 1986). In addition, SP potentiates cholinergic nerve-induced contractions in rabbit trachea *in vitro* via a postganglionic, prejunctional mechanism (Armour *et al.*, 1991). Exogenous tachykinins also potentiate cholinergic neurotransmission in guinea-pig trachea (Hall *et al.*, 1989; Watson *et al.*, 1993).

Endogenous tachykinins may also facilitate cholinergic neurotransmission because capsaicin pretreatment, which depletes sensory nerves of tachykinins, results in a significant reduction in cholinergic responses both *in vitro* and *in vivo* in guinea-pig airways (Stretton *et al.*, 1992). Capsaicin, at a sub-threshold concentration, acutely releases tachykinins which enhance cholinergic neurotransmission in guinea-pig trachea *in vitro* (Aizawa *et al.*, 1990). In addition, the inhibitor of neutral endopeptidase (NEP), phosphoramidon, increased the amplitude of contractions produced by preganglionic vagal stimulation in guinea-pig trachea and this effect is blocked by capsaicin pretreatment (Watson *et al.*, 1993). However, Aizawa *et al.* (1990) reported that phosphoramidon also enhanced contractions to electrical field stimulation (EFS) in guinea-pig trachea without changing responses to exogenous ACh. In addition, Sekizawa and colleagues have demonstrated that NEP inhibitors increase

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the vagally-mediated contractions in ferret trachea (Sekizawa *et al.*, 1987). These results suggest that endogenous tachykinins released from afferent sensory nerves may normally facilitate cholinergic neurotransmission pre- and post-ganglionically.

Finally, in human bronchus, NKA potentiates cholinergic neural responses but only in the presence of K^+-channel blockade and this modulation occurs prejunctionally (Black *et al.*, 1990).

The aim of this study was to investigate the effect of receptor-selective tachykinin agonists and antagonists for NK_1 or NK_2 receptors on cholinergic neurotransmission evoked by electrical field stimulation of bronchial rings in rabbit, guinea-pig and human airway smooth muscle in order to define more clearly the nature of the prejunctional tachykinin receptor on airway cholinergic nerves.

Methods

Animal studies

Male albino rabbits (3.0–3.5 kg) and male albino guinea-pigs (240–280 g) were stunned and bled. The lungs, trachea and main bronchi were removed and placed in standard Krebs solution containing indomethacin (5 μM) of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11; it was gassed continuously with a mixture of 95% O_2/5% CO_2 to give pH 7.4.

Indomethacin was present throughout to prevent fading of neural responses due to endogenous prostaglandin production. Under these experimental conditions reproducible contractile responses to electrical field stimualtion could be achieved for several hours. The main bronchi were isolated and gently rubbed several times on their internal surface by means of a cotton-tipped applicator in order to remove the epithelium, as described previously (Maggi *et al.*, 1990b). The epithelium was removed as it is known that in animal and human airways removal of this layer increases the *in vitro* responsiveness of smooth muscle stimulation by numerous bronchoconstrictor agents including tachykinins (Devillier *et al.*, 1988; Naline *et al.*, 1989). Several theories have been postulated to explain this inhibitory effect exerted by the epithelium on smooth muscle contraction, such as the release of an epithelial derived ralaxant factor (Barnes *et al.*, 1985). In addition, the epithelial inhibitory effect can be, at least in part, due to the degradation of peptides by enkephalinase (endopeptidase 24.11) (Devillier *et al.*, 1988). Therefore, in these experiments we chose to remove the epithelial layer and to conduct the experiments in the presence of peptidase inhibitors (as described below). Each bronchial ring was suspended in a 5 ml organ bath containing Krebs solution and maintained at 37°C. The tissues were allowed to equilibrate for 1 h with frequent washing, under a resting tension of 0.5 g, which was found to be optimal for measuring changes in tension. Contractile responses were measured by means of an isometric transducer connected to a Basile 7050 pen recorder.

Human studies

We studied airways from 6 patients (between 20–47 years, 5 male) obtained from heart or single lung donor patients with brain death. There was no evidence of heart or lung disease in these donors.

Lung tissue was immediately placed in oxygenated Krebs solution and cooled to 4°C and transported to the laboratory. Bronchi (from the level of distal lobar to segmental with internal diameters of 7–9 mm and 3–5 mm respectively) from normal donors were dissected free from adjacent tissue. The bronchi were macroscopically removed and bronchial preparations were then suspended in 10 ml organ baths containing Krebs solution at 37°C with 95% O_2/5% CO_2 (pH 7.4). Indomethacin (5 μM) was present throughout. Bronchial rings were connected via silk threads to Grass FT.03 force-displacement transducers and recorded on a polygraph for the measurement of isometric changes in tension. The tissues were allowed to equilibrate for 1 h with frequent washing under a resting tension of 2 g which was found to be optimal for measuring changes in tension.

In additional experiments, strips of smooth muscle were taken from the major bronchus of three non-smoking donor patients (between 25–42 years, one male) for heart-lung, heart or single lung transplantation. The epithelium was removed by careful dissection, minimizing damage to the smooth muscle; this was confirmed later by macroscopic histology. These experiments were performed so that the studies in human tissue could be compared with those in animal tissue in terms of the airway level studied and the epithelial removal.

Electrical field stimulation

Electrical field stimulation was performed by means of two wire platinum electrodes placed at the top and the bottom of the organ bath for animal tissue and parallel to each other for human tissue, connected to a Grass S88 stimulator (Grass Instruments, Quincy, MA, U.S.A.). Trains of stimuli (2 Hz; 60 V; 0.4 ms pulse width for 10 s) were delivered at 1 min intervals until reproducible responses were obtained. Control experiments showed that there was no significant fading of the response to field stimulation during the experimental period. Contractile responses to field stimulation were inhibited by both tetrodotoxin (0.3 μM) and atropine (1 μM) in all species suggesting that the contractile response was due to stimulation of cholinergic nerves. Experiments were carried out in the presence of peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each, to inhibit aminopeptidase, angiotensin converting enzyme and endopeptidase 24.11, respectively). Peptidase inhibitors were added 15 min prior to the start of the following experiments. In each experiment, the response to KCl (80 mM added to the bath) was used as an internal standard.

Capsaicin treatment

In some experiments, to avoid any modulation of cholinergic responses to EFS by endogenous tachykinins, guinea-pig and rabbit bronchi were pretreated with capsaicin to deplete neuropeptides. *In vitro* capsaicin desensitization was achieved by prolonged (30 min) exposure of bronchial rings to 10 μM capsaicin, followed by washing and re-equilibration (Geppetti *et al.*, 1990).

Effects of agonists

In these experiments concentrations of SP, NKA, [Sar^9]SP sulphone, [βAla^8]NKA(4–10), [$MePhe^7$]NKB (each between 0.1 nM–30 nM) and MDL 28,564 (0.1 nM–1 μM) were added in a randomized fashion and the effect on responses to subsequent electrical field stimulation investigated. Tissues were washed between concentrations. If the agonist produced a contractile response which resulted in a rise in baseline, EFS was continued and the measurement taken when the contractile response had subsided. Only one agonist was tested per tissue. [Sar^9]SP sulphone is a potent and selective NK_1 receptor agonist (Drapeau *et al.*, 1987) while [βAla^8]NKA(4–10) is a selective NK_2 receptor agonist (Patacchini *et al.*, 1989). MDL 26,564 is a highly selective NK_2 receptor agonist which behaves as a full agonist at one NK_2 receptor subtype while being a competitive antagonist at the other (Buck *et al.*, 1990). [$MePhe^7$]NKB is a selective NK_3 receptor agonist (Drapeau *et al.*, 1987).

In a separate series of experiments the effects of the selective agonists, which were effective at enhancing contractile

responses to EFS, were investigated on responses to the postjunctional effects of acetylcholine (ACh). The effects of the selective neurokinin agonists were studied on the contractile response evoked by a concentration of ACh (10 μM) that produced a response similar in magnitude as that evoked by EFS at the above parameters.

Effect of antagonists

In the next series of experiments the effects of tachykinin antagonists ((±)-CP-96,345 for NK_1 receptors, MEN 10,376 for NK_2 receptors) against the enhancement of cholinergic contractile responses produced by a NK_1-selective ([Sar^9]SP sulphone) (5 nM) or a NK_2-selective ([βAla$_8$]NKA(4–10) (1 nM) agonist was investigated at concentrations that approximated the EC_{50} values for the agonists. The contact time for the antagonists was 15 min. Two reproducible responses to the agonist were obtained prior to administration of the antagonist.

In another series of experiments the effects of other competitive antagonists (0.3 μM–1 μM) for NK_2 receptors were investigated on the enhancement of cholinergic responses produced by the NK_2 agonist, [βAla^8]NKA(4–10) (1 nM). L659,877 is a cyclic peptide introduced by Williams *et al.* (1988) which is selective for NK_2 receptors. MEN 10,376 and R396 are two linear peptides endowed with marked selectivity for NK_2 over NK_1 or NK_3 tachykinin receptors (Maggi *et al.*, 1990a; 1991a; Dion *et al.*, 1990) which also discriminate between different NK_2 receptor subtypes (Maggi *et al.*, 1990a; Van Giesbergen *et al.*, 1991).

Drugs

Drugs used were: acetylcholine chloride, capsaicin, indomethacin, bestatin, thiorphan, (Sigma, St Louis, MO, U.S.A.), captopril (Squibb), tetrodotoxin (Sankyo, Tokyo, Japan), atropine (Serva, Heidelberg, Germany). All drugs were dissolved in distilled water and stored in aliquots at 20°C. Capsaicin was dissolved in absolute ethanol and diluted in distilled water.

Peptides

Subtance P (SP), neurokinin A (NKA), [βAla^8]NKA(4–10), [Tyr^5, D-Trp^{6,8,9}, Lys^{11}]-NKA(4–10) (MEN 10,376) and [MePhe^7]neurokinin B were synthesized at Menarini Laboratories (Florence, Italy) by conventional solid-phase methods. L659,877 was obtained from Cambridge R.B. (Cambridge, U.K.). MDL 28,564 and R 396 were kind gifts from Dr S.H. Buck, Marion Merrell Dow Research Institute, and Prof. D. Regoli, Department of Physiology and Pharmacology, University of Sherbrooke, Canada, respectively. The selective NK_1 receptor agonist, [Sar^9]SP sulphone, was purchased from Peninsula (San Carlos, U.S.A.). The selective NK_1 receptor antagonist ([(2S, 3S)-*cis*-2-(diphenylmethyl)-N-[methoxyphenyl)-methyl]-1-azabicyclo(2,2,2]octan3-amine]) ((±)-CP-96,345) was synthesized as previously described (Lecci *et al.*, 1991). The amino acid sequence of the peptide agonist and antagonists used in this study is shown in Table 1.

Aliquots of SP, NKA, [MePhe^7]NKB, [Sar^9]SP sulphone were dissolved in distilled water and stored at −20°C. Stock solutions (1–10 mM) of all the other peptides were made in dimethyl sulphoxide (DMSO) then diluted in water.

Analysis of results

Contractile responses were expressed as absolute changes in tension, and then transformed to a mean response for 3 control stimulations obtained to EFS in each tissue. The effect of the tachykinin agonists and antagonists on the mean responses was then expressed as a percentage increase. A mean value for 2 successive contractile responses to an ACh bolus was calculated in mg tension and the effect of tachykinins on the mean response analysed. The effects of exogenous drug additions on contractile responses evoked by EFS and acetylcholine in each tissue were assessed by use of the Student's *t* test (one-tailed) for paired data. Probability values of <0.05 were considered significant. EC_{50} values were calculated by using a non-linear iterative curve fitting programme, Inplot (Graphpad Inc. CA, U.S.A.).

Results

Effect of the endogenous tachykinins SP and NKA on cholinergic neurotransmission in rabbit bronchi

SP (0.1–30 nM) significantly potentiated cholinergic contractile responses evoked by EFS in a concentration-dependent manner (maximum enhancement evoked by SP (10 nM) 287 ± 121%, $P<0.01$, $n=6$) (Figure 1). This amounted to an increase from 204 ± 67.2 mg to 445 ± 47.6 mg of evoked contraction in absolute values. At this concentration, SP

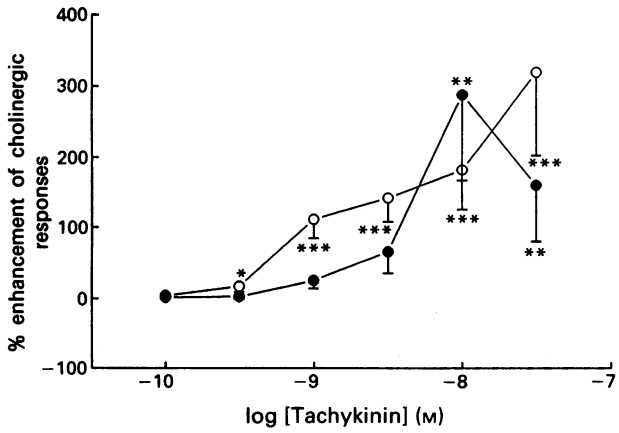


Figure 1 Concentration-dependent facilitation of cholinergic responses to electrical field stimulation (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every min) in rabbit bronchi by neurokinin A (0.1–30 nM, ○) and substance P (0.1–30 nM, ●). Values are mean ($n=4$–9 observations) ± s.e.mean; significance of enhancement: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

Table 1 Amino acid sequences of peptides used in this study

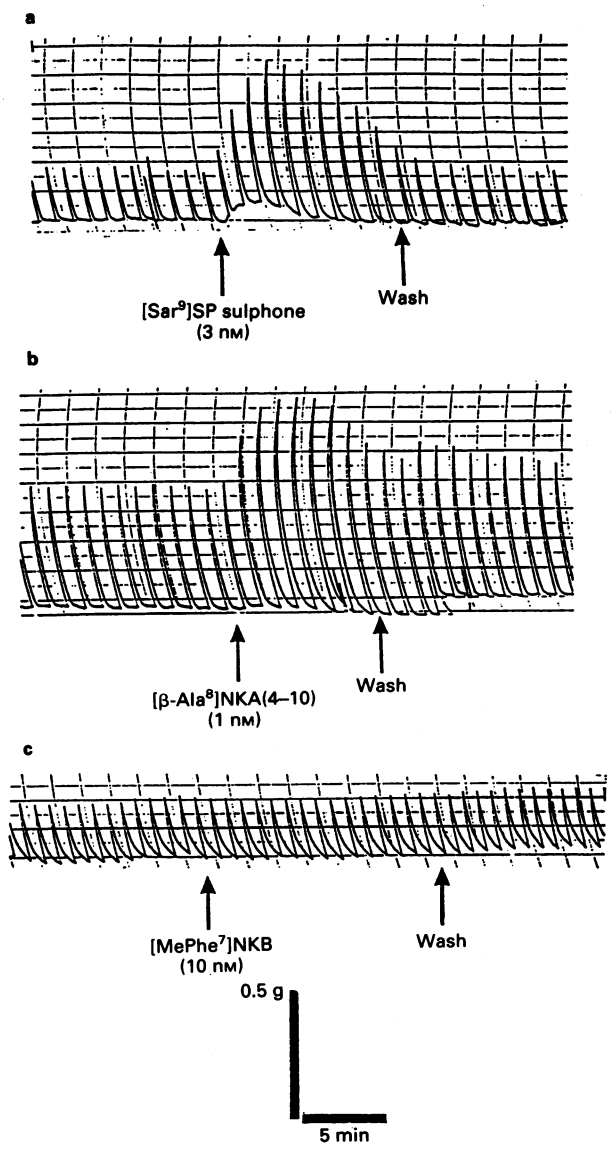
Agonists	
[Sar^9,Met(O$_2$)^{11}]SP	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Sar-Leu-Met(O$_2$)-NH$_2$
[βAla^8]NKA(4–10)	H-Asp-Ser-Phe-Val-βAla-Leu-Met-NH$_2$
[MePhe^7]NKB	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH$_2$
MDL 28,564	H-Asp-Ser-Phe-Val-Gly-Leu (CH$_2$NH)Leu-NH$_2$
Antagonists	
MEN10,376	H-Asp-Tyr-D-Trp-val-D-Trp-D-Trp-Lys-NH$_2$
L659,877	cyclo(Leu-Met-Gln-Trp-Phe-Gly)
R 396	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH$_2$

produced contraction of rabbit bronchi (222.5 ± 50.4 mg, $n = 5$). However, this increase in tension cannot account for the enhancement in the cholinergic contractile response as this was measured when the initial contractile response had returned to baseline values. In fact, other workers have previously shown that the rise in baseline alone cannot account for the potentiation of EFS-induced contraction in rabbit airway smooth muscle (Armour *et al.*, 1988).

NKA (0.1–30 nM) also significantly enhanced cholinergic neurotransmission in a concentration-dependent manner (maximum enhancement evoked by NKA (30 nM) 319.2 ± 117%, $P < 0.001$, $n = 9$) (Figure 1b). This corresponds to an increase from 156 ± 52.7 mg to 406 ± 75.3 mg of evoked contraction in absolute values. At this concentration NKA evoked a contractile response in rabbit bronchi (942.4 ± 127.5 mg). However, NKA (0.3 nM) enhanced contractile responses to EFS by 16.9 ± 7.8% ($P < 0.05$, $n = 9$) and at this concentration produced no increase in baseline tone.

Effect of selective agonists for tachykinin receptors on cholinergic responses to EFS in rabbit bronchi

The NK_1 receptor-selective agonist, [Sar^9]SP sulphone (0.1–30 nM) evoked a concentration-dependent increase in cholinergic responses to EFS (Figure 2a and Figure 3). [Sar^9]SP sulphone (10 nM) produced a maximal increase in cholinergic responses of 334.9 ± 63% ($P < 0.01$, $n = 8$) with a pD_2 value of 8.5 ± 0.06. In further experiments involving the use of selective tachykinin receptor antagonists a concentration of [Sar^9]SP sulphone was chosen (5 nM) which approximated the pD_2 value. A concentration of 10 nM [Sar^9]SP sulphone produced contraction of rabbit bronchi (378.6 ± 90.8 mg, $n = 7$). However, [Sar^9]SP sulphone (3 nM) still produced considerable enhancement of cholinergic responses to EFS (186.2 ± 62.9% enhancement, $P < 0.05$, $n = 7$) at concentrations that produced negligible bronchoconstriction (27.1 ± 14.6 mg, $n = 6$).



The NK_2 receptor-selective agonist, [βAla^8]NKA(4–10) (0.1–30 nM), also evoked a concentration-dependent increase in cholinergic responses to EFS (Figure 2b and Figure 3). [βAla^8]NKA(4–10) (10 nM) produced a maximum enhancement of 278 ± 83.5% ($P < 0.01$, $n = 4$) with a pD_2 value of 8.7 ± 0.1. In the next set of experiments involving the use of selective tachykinin antagonists, a concentration of [βAla^8]NKA(4–10) was chosen (1 nM) which approximated the pD_2 value. In addition to producing a maximum enhancement of the cholinergic response, [βAla^8]NKA(4–10) (10 nM) also produced contraction of rabbit bronchi (340 ± 41.7 mg, $n = 5$). However, [βAla^8]NKA(4–10) (1 nM) evoked no change in the baseline tone (6.25 ± 6.25 mg, $n = 6$) while the enhancement of cholinergic contractile responses evoked by EFS was considerable (125.6 ± 39.6%, $P < 0.05$, $n = 7$).

[MePhe^7]NKB (0.1–30 nM), an NK_3 receptor-selective agonist, produced no direct contractile response and no enhancement of cholinergic responses to EFS in rabbit bronchi (Figure 2c and Figure 3).

Finally, in the rabbit, MDL 28,564 (10 nM), a highly selective NK_2 receptor ligand, enhanced cholinergic responses by 12.5 ± 4.7% ($P < 0.05$) ($n = 5$) ($pD_2 = 6.7 \pm 0.22$, maximum enhancement 441.3 ± 159% at 1 μM, $P < 0.05$, $n = 6$) (Figure 3). In further experiments involving the use of selective tachykinin receptor antagonists, a concentration of MDL 28,564 was chosen (0.1 μM) which approximated the pD_2 value.

All cholinergic contractile responses evoked to EFS were abolished by atropine (1 μM) or tetrodotoxin (0.3 μM).

Effect of NK_1 and NK_2 receptor agonists on responses to ACh in rabbit bronchi

Both the NK_1 receptor-selective agonist, [Sar^9]SP sulphone (5 nM) and the NK_2 receptor-selective agonist [βAla^8]NKA (4–10) (1 nM), had no effect on contractile responses to ACh (10 μM) in rabbit bronchi ($n = 6$). This suggests that the augmentation of neurally-mediated responses to EFS by agonists selective for the NK_1 and NK_2 receptor involves a prejunctional mechanism.

Effect of NK_1 and NK_2 receptor antagonists on rabbit bronchi

[Sar^9]SP sulphone (5 nM), the NK_1-selective agonist, produced 111.3 ± 37.5% ($n = 7$) enhancement of the cholinergic response, an effect which was not affected by the NK_2-selective antagonist, MEN 10,376 (0.1 μM) (114.9 ± 44.7% enhancement, $n = 7$, NS) and completely blocked by the selective NK_1 receptor antagonist, CP 96,345 (0.1 μM) (4.61 ± 3.2% enhancement, $n = 6$, $P < 0.05$) (Figure 4a).

Figure 2 Trace illustrating the effect of selective tachykinin receptor agonists on cholinergic responses to EFS (60 V, 0.4 ms, 2 Hz for 10 s every 1 min) in rabbit bronchi. (a) Effect of NK_1-receptor agonist, [Sar^9]SP sulphone (3 nM) at a concentration that approximated the EC_{50} value for enhancement of responses. (b) Effect of NK_2-receptor agonist, [βAla^8]NKA(4–10) (1 nM) at a concentration that approximated the EC_{50} value for enhancement of responses. (c) Effect of a maximal concentration of the NK_3-receptor agonist, [MePhe^7]NKB (10 nM).

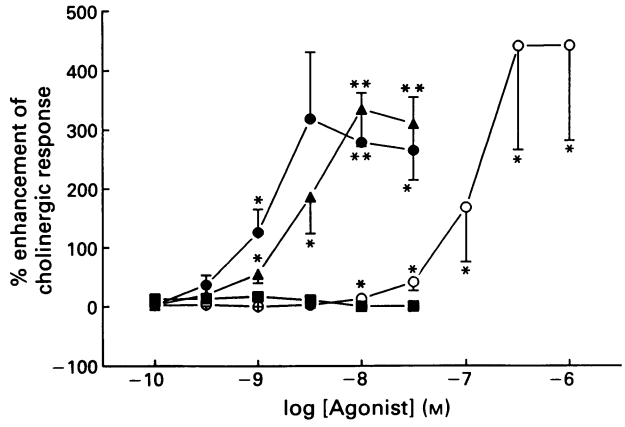


Figure 3 Concentration-dependent facilitation of cholinergic responses to electrical field stimulation (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every min) in rabbit bronchi by [βAla^8]NKA(4–10) (0.1–30 nM) (●), [Sar^9]SP sulphone (0.1–30 nM) (▲), [MePhe^7]NKB (0.1–30 nM) (■) and MDL 28,564 (0.1 nM–1 μM) (○). Values are mean (n = 3–7 observations) ± s.e.mean; significance of enhancement: $^{**}P<0.01$, $^{*}P<0.05$.

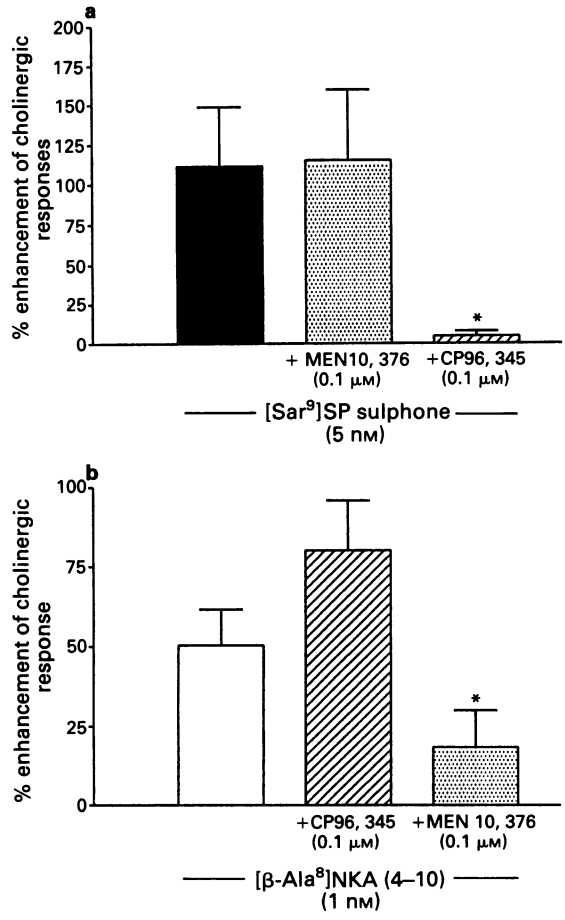


Figure 4 Histogram demonstrating the inhibitory effects of tachykinin antagonists on facilitation of cholinergic responses evoked by EFS produced by selective NK_1 and NK_2 agonists. (a) Enhancement of cholinergic response produced by [Sar^9]SP sulphone (5 nM) (solid column) and the effect of the NK_2 antagonist, MEN 10,376 (0.1 μM) (stippled column) and the NK_1 antagonist CP 96,345 (0.1 μM) (hatched column). (b) Enhancement of cholinergic response produced by [βAla^8]NKA(4–10) (1 nM) (open column) and the effect of the NK_1 antagonist, CP 96,345 (0.1 μM) (hatched column) and the NK_2 antagonist, MEN 10,376 (0.1 μM) (stippled column). Values are mean (n = 6 or 7), with s.e. of mean; significance of inhibition: $^{*}P<0.05$.

[βAla^8]NKA(4–10) (1 nM), the NK_2 receptor-selective agonist, produced 50.4 ± 11.3% (n = 7) enhancement of cholinergic responses evoked by EFS, an effect which was not affected by the NK_1-selective antagonist, CP 96,345 (0.1 μM) (80.2 ± 15.8% enhancement, n = 7, NS) and significantly inhibited by the NK_2 receptor antagonist, MEN 10,376 (0.1 μM) (18.4 ± 11.6% enhancement, n = 7, $P<0.05$) (Figure 4b) and completely inhibited by MEN 10,376 (0.3 μM) (0% enhancement, n = 4, $P<0.05$) (Figure 6a).

MDL 28,564 (0.1 μM), a selective NK_2 receptor agonist, produced 193.2 ± 108% (n = 5) enhancement of cholinergic responses evoked by EFS an effect which was not blocked by the NK_1 receptor-selective antagonist, CP 96,345 (0.1 μM) (256.9 ± 183.2% enhancement, n = 5, NS). However, the NK_2 antagonist, MEN 10,376 (0.1 μM) inhibited this response (83.8 ± 50% enhancement, n = 5, NS) and MEN 10, 376 (0.3 μM) completely abolished this enhancement (0% enhancement, n = 4, $P<0.05$) (Figure 5).

[βAla^8]NKA(4–10) (1 nM) evoked a 264.9 ± 133.2% enhancement of cholinergic responses to EFS, an effect which was completely antagonized by, MEN 10,376 (0.3 μM) (0% enhancement, n = 4, $P<0.05$) (Figure 6a). However, L659,877 (0.3 μM) was without effect on enhancement of responses produced by the same concentration of [βAla^8]NKA(4–10) (1 nM) (98.7 ± 16.2% enhancement in the absence and 100.1 ± 16.3% enhancement in the presence of L659,877, n = 5, NS) (Figure 6a). Finally, R396 (0.3 μM) (another NK_2 antagonist) was also without effect (81.1 ± 22.1% enhancement by [βAla^8]NKA(4–10) in the absence and 148.7 ± 66.6% enhancement in the presence of R 396, n = 5, NS) (Figure 6a). The response to [βAla^8]NKA(4–10) (1 nM) was variable (the range of potentiation evoked being between 81 to 264%). However, the variation was probably due to the fact that in the first group (the data with MEN 10,376) the maximum potentiation of the cholinergic responses evoked was 550% and the minimum 21.4% leading to a large standard error in this group. However, as each tissue was used as its own control this does not influence the results obtained with the antagonists as the inhibition achieved was not dependent upon the magnitude of the initial facilitation of the cholinergic contractile response.

L659,877 (1 μM) antagonized the enhancement of the response to [βAla^8]NKA(4–10) (1 nM) (147.5 ± 68.6% enhancement in the absence and 8.4 ± 5.0 in the presence of L659, 877, n = 4, $P<0.05$) (Figure 6b). R 396 was inactive at concentrations up to 1 μM; [βAla^8]NKA(4–10) (1 nM) evoked a 69.2 ± 23% increase in cholinergic responses to EFS in the absence and 87.1 ± 41.6% increase in the presence of R 396 (1 μM) (Figure 6b).

The antagonists used in this study, had no effect on cholinergic responses to EFS at the concentrations stated.

Effect of selective agonists on neurally-evoked, atropine-sensitive contractions in guinea-pig bronchi

In guinea-pig bronchi, there is a prominent excitatory non-adrenergic, non-cholinergic contraction which is due to the release of tachykinins (NKA and SP) (Maggi *et al.*, 1991a,b). Tissues were pretreated with capsaicin (10 μM) to deplete tachykinins so that any results obtained were not affected by the release of endogenous tachykinins. In guinea-pig bronchi. only the NK_1 receptor agonist [Sar^9]SP sulphone (3 nM) was effective at enhancing cholinergic neurotransmission but the effect was relatively small (maximal enhancement 27.5 ± 5.5%, n = 4, $P<0.01$) (Figure 7).

Effect of selective agonists on neurally-evoked, atropine-sensitive contractions of human airways

In human bronchial rings all the selective tachykinin receptor agonists (0.1–30 nM) ([Sar^9]SP sulphone, [βAla^8]NKA(4–10), [MePhe^7]NKB) were without effect on cholinergic neural re-

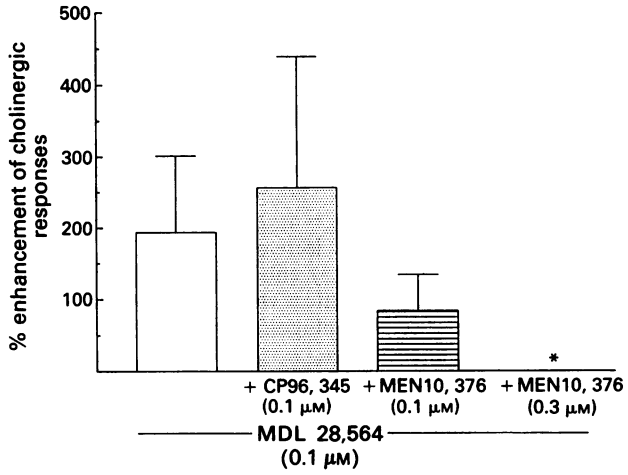


Figure 5 Histogram demonstrating the facilitatory effect of the selective NK_2 agonist MDL 28,564 (0.1 μM) (open column) on cholinergic responses in rabbit bronchi in the presence of the selective NK_1 antagonist, CP 96,345 (0.1 μM) (stippled column), and the NK_2 antagonist, MEN 10,376 (0.1 μM) (horizontal lined column) and (0.3 μM) (complete inhibition). Values are mean ($n = 5$ or 4), with s.e.mean; significance of inhibition: $*P < 0.05$.

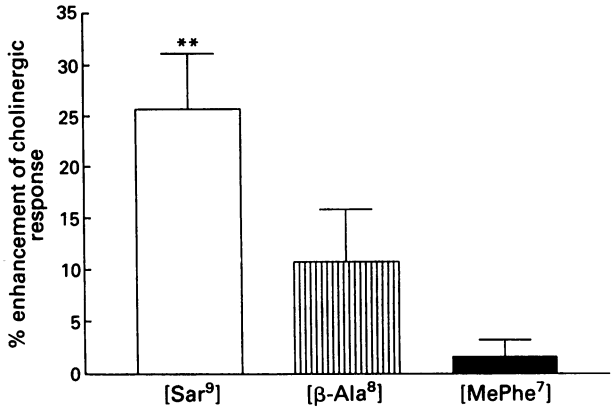


Figure 7 Histogram illustrating the effects of selective tachykinin agonists (each at 3 nM) for the NK_1 receptor ([Sar^9]SP sulphone, open column), the NK_2 receptor ([βAla^8]NKA(4–10, vertical lined column) and the NK_3 receptor ([MePhe^7]NKB, solid column) on cholinergic responses to EFS (EFS: 60 V, 0.4 ms, 2 Hz for 10 s every 10 min) in guinea-pig bronchi. Values are mean ($n = 4$ or 5 observations) with s.e.mean; significance of inhibition: $*P < 0.01$.

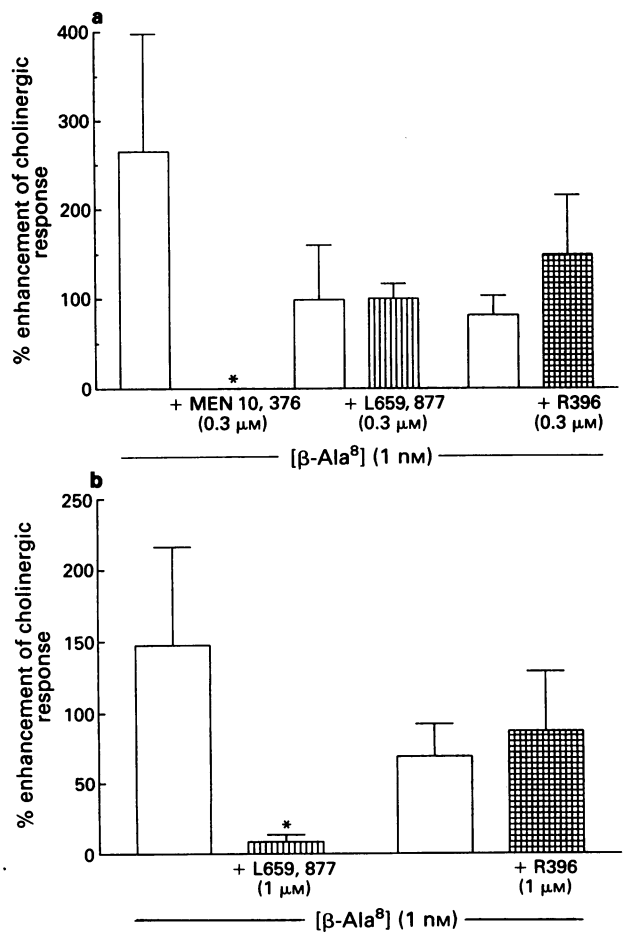


Figure 6 Histogram demonstrating the facilitatory effects of the NK_2 agonist [βAla^8]NKA(4–10) (1 nM) (open column) on cholinergic responses in rabbit bronchi in the presence of (a) the NK_2 antagonist L 659,877 (0.3 μM) (vertical lined column) and another NK_2 antagonist R 396 (0.3 μM) (checked column). (b) L 659,877 (1 μM) (vertical lined column) and R 396 (1 μM) (checked column). Values are mean ($n = 4$ or 5 observations), with s.e.mean; significance of inhibition: $*P < 0.05$.

sponses at any of the concentrations studied ($n = 6$ patients).

In strips of human major bronchus with the epithelium removed all the above selective tachykinin receptor agonists, at the same concentrations as were used above, were still ineffective on cholinergic neural contractile responses ($n = 3$ patients).

Discussion

The first report that suggested that tachykinins may have a neuromodulatory role in the peripheral nervous system was in the guinea-pig myenteric plexus where SP was found to evoke the release of ACh in a concentration-dependent manner (Yau & Youther, 1982). More recently, it has been suggested that tachykinins may play an important role in modulating cholinergic neurotransmission in airway smooth muscle on the basis of immunohistochemical (Dey *et al.*, 1991) and functional studies.

Exogenous tachykinins have been previously shown to facilitate cholinergic neurotransmission in airway smooth muscle. In rabbit isolated trachea, SP potentiated, in a concentration-dependent manner, contractile responses evoked by cholinergic nerve stimulation via a postganglionic, prejunctional mechanism (Tanaka *et al.*, 1986; Armour *et al.*, 1991). However, in rabbit trachea the tachykinin receptors mediating this effect were not investigated. Exogenous tachykinins also potentiate cholinergic neurotransmission at pre- and postganglionic nerve terminals in guinea-pig trachea (Hall *et al.*, 1989; Watson *et al.*, 1993). The tachykinin receptor mediating these effects appeared to be of the NK_1 receptor subtype (Watson *et al.*, 1993). However, in addition, NKA, which preferentially stimulates NK_2 receptors facilitated contractions evoked by pre- and postganglionic nerve stimulation (Hall *et al.*, 1989; Watson *et al.*, 1993). Therefore, these data do not exclude the involvement of NK_2 receptors. However, since a range of selective agonists and antagonists for NK_2 receptors were not investigated, the receptor classification in these two studies was not conclusive. In human bronchus NKA in the presence of K^+-channel blockade potentiates cholinergic neural responses and this modulation occurs prejunctionally (Black *et al.*, 1990).

The present results demonstrate that the tachykinins NKA and SP can produce concentration-dependent enhancement of contractile responses evoked by EFS of rabbit trachea *in*

vitro, confirming previous data on SP-evoked facilitatory effects (Tanaka *et al.*, 1986; Armour *et al.*, 1991). Further experiments were performed to assess the effects of receptor-selective neurokinin agonists and antagonists on cholinergic neurotransmission to investigate which tachykinin receptors were involved. The selective NK_1 tachykinin receptor agonist, [Sar^9]SP sulphone, was very effective at enhancing responses to EFS, an effect which was blocked by the selective NK_1 receptor antagonist CP 96,345 but not by the NK_2 receptor antagonist, MEN 10,376 suggesting that NK_1 receptor activation may be important in this response. However, the NK_2 receptor-selective agonist [βAla^8]NKA(4–10), also enhanced cholinergic responses to EFS in rabbit bronchi and this effect was blocked by MEN 10,376 but not by CP 96,345 suggesting involvement of NK_2 receptors. NK_3 receptor activation does not seem to be involved in the enhancement of cholinergic neurotransmission by tachykinins as the selective NK_3-receptor agonist, [MePhe^7] NKB, was without effect. Augmented contractile responses evoked by EFS in the presence of the tachykinins e.g. SP and NKA or the selective tachykinin receptor agonists. e.g. [Sar^9]SP sulphone or [βAla^8]NKA(4–10), were completely inhibited by tetrodotoxin and atropine indicating that the augmented airway contractile response was neural in origin and cholinergic in nature. In addition, the potentiating effects of tachykinins (SP and NKA) or the selective agonists for NK_1 and NK_2 receptors were observed even at very low concentrations where there was very little or no change in the contractile state of the tissue.

Neither [Sar^9]SP sulphone nor [βAla^8]NKA(4–10) had any effect on contractile responses to exogenous ACh. This is in agreement with an earlier study in which contractile responses to methacholine in rabbit trachea were unaltered by SP (Tanaka *et al.*, 1986). This implies that the potentiation of contractile responses to EFS by tachykinins is not related to a postjunctional change in airway smooth muscle function, such as changes in the rate of ACh degradation, enhanced muscarinic receptor binding, potentiation of intrinsic contractile processes. Therefore, these results indicate that the enhancement of the cholinergic response produced by tachykinins is likely to be due to an increased prejunctional release of ACh.

The results obtained in the rabbit bronchi suggest the presence of both NK_1 and NK_2 receptors on cholinergic nerves in rabbit bronchi. The full agonist activity of MDL 28,564 and the rank order of potency of the NK_2 receptor antagonists, MEN 10,376, L 659,877 and R 396, indicates that the NK_2 receptors mediating facilitation of ACh release in rabbit bronchi belong to the same subtype that mediates contraction of the endothelium-deprived rabbit pulmonary artery (termed NK_{2A}) (Maggi *et al.*, 1990a).

In guinea-pig bronchi only the NK_1 receptor agonist, [Sar^9]SP sulphone, was effective in enhancing cholinergic neurotransmission but the effect was relatively small. These data are in agreement with Watson *et al.* (1993) who demonstrated that the NK_1 agonist, GR73632, facilitated preganglionic and postganglionic contractile responses to electrical stimulation in guinea-pig trachea. However, the ineffectiveness of the selective NK_2 agonist, [βAla^8]NKA(4–10), seems to suggest that NK_2 receptors are not involved in the facilitation of cholinergic constrictor responses in the guinea-pig bronchi in contrast to the suggestions made in previous reports (Hall *et al.*, 1989; Watson *et al.*, 1993).

In human bronchial rings, none of the selective tachykinin agonists had any effect on cholinergic neurotransmission. In fact, it has been shown previously that NKA produces potentiation of the response to EFS in human bronchi, but only in the presence of K^+ channel blockade. This points to a neuromodulatory role for NKA in human airways, only in situations where the K^+ channel activity is decreased (Black *et al.*, 1990).

Facilitatory effects of tachykinins on cholinergic neurotransmission may have physiological relevance as there has been some suggestion that endogenous tachykinins facilitate cholinergic contractile responses in airway smooth muscle. The metallopeptidase neutral endopeptidase 24.11 is a major enzyme involved in the breakdown of tachykinins (Erdos & Skidgel, 1989). Inhibition of this enzyme by phosphoramidon (an inhibitor of neutral endopeptidase) would be expected to augment the actions of endogenously released tachykinins. In guinea-pig trachea phosphoramidon facilitates contractile responses evoked by preganglionic vagal nerve stimualtion but not transmural stimulation in a concentration-dependent manner and this effect is blocked by capsaicin pretreatment (Watson *et al.*, 1993). These results indicate that there is release of endogenous tachykinins during pre- but not postganglionic nerve stimulation in guinea-pig trachea suggsting that there are facilitatory tachykinin receptors (probably of the NK_1 receptor subtype) at the level of the parasympathetic ganglia (Watson *et al.*, 1993). However, Aizawa *et al.* (1990) have reported that phosphoramidon also enhances contractions to EFS in guinea-pig trachea without changing responses to exogenous ACh. In addition, Sekizawa *et al.* (1987) have demonstrated that NEP inhibitors increase the contractions of the ferret trachea induced by EFS. Some of these discrepancies may be due to species differences. Capsaicin pretreatment, which depletes sensory nerves of tachykinins, results in a significant reduction in cholinergic responses both *in vivo* and *in vitro* in guinea-pig airways (Stretton *et al.*, 1992) suggesting a role for endogenous tachykinins in the facilitation of cholinergic neurotransmission. In addition, capsaicin, at a sub-threshold concentration, acutely releases tachykinins which enhance cholinergic responses in guinea-pig trachea *in vitro* again indicative of endogenous tachykinin-induced modulation of cholinergic responses (Aizawa *et al.*, 1990). In this study endogenous tachykinins failed to facilitate cholinergic responses to field stimulation in rabbit bronchi as there was no effect of the selective tachykinin antagonists in the absence of exogenous agonist. These data are in agreement with Watson *et al.* (1993) who demonstrated that endogenous tachykinins facilitate cholinergic nerve-induced contractions at the level of the parasympathetic ganglia in guinea-pig airways and, in addition, that facilitatory tachykinin receptors on postganglionic nerve terminals can only be demonstrated by exogenous agonists. Other workers (mentioned above) have demonstrated an effect of endogenous tachykinins on facilitation of cholinergic contractile responses but they have demonstrated these effects with tools such as phosphoramidon and capsaicin which may have actions other than those on endogenous tachykinins.

These results suggest that tachykinins may play an important role in modulating cholinergic neurotransmission in rabbit (via NK_1 and NK_2 receptors) and guinea-pig (via NK_1 receptors) airways with no demonstrable effects on human airways. However, this does not rule out a role for endogenous tachykinins in the modulation of cholinergic neurotransmission in human airways. Another consideration is that while this may not be important under normal conditions, the system may be active in disease. For example, if K^+ channels were impaired in disease then modulatory effects of tachykinins on cholinergic neurotransmission may become evident.

The authors thank A. Menarini Pharmaceuticals for funding this study. Many thanks to Dr P. Santicioli, Dr S. Giuliani and Dr A. Lecci for all their help and advice during the time spent working at A. Menarini Pharmaceuticals. We also acknowledge Fisons plc and The Wellcome Trust for supporting this study.

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(Received March 3, 1993
Revised August 24, 1993
Accepted September 14, 1993)

Br. J. Pharmacol. (1994), **111**, 111–116

Effect of nitrendipine on autoregulation of perfusion in the cortex and papilla of kidneys from Wistar and stroke prone spontaneously hypertensive rats

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1 This investigation examined the autoregulatory efficiency of different vascular regions of the normotensive and stroke prone-spontaneously hypertensive rat (SP-SHR) kidney and determined how these myogenic responses were dependent upon extracellular calcium. In acute studies, renal autoregulatory blood perfusion curves for cortex and papilla were generated, autoregulatory indices (AI's) calculated as a ratio of the perfusion change divided by the ratio of the pressure difference where zero represents perfect and 1 equates to no autoregulation. The influence of a calcium channel antagonist on this AI was measured at both cortex and papilla.

2 Rats were anaesthetized with sodium pentobarbitone, the kidney exposed and cortical and papillary perfusion measured by Laser-Doppler flowmetry. Groups of rats either received no drug or nitrendipine at either 0.125 or 0.25 $\mu g\,kg^{-1}\,min^{-1}$.

3 In the Wistar normotensive rats there was efficient autoregulation in the cortex (AI = 0.21 ± 0.17), from 127 to 90 mmHg, but not in the papilla (AI = 0.89 ± 0.08), while below 90 mmHg perfusion in both regions decreased with renal perfusion pressure. Nitrendipine attenuated cortical autoregulation at the higher pressure range (AI = 0.62 ± 0.13 and 0.92 ± 0.10 at the low and high dose, respectively) while having no effect on the papillary pressure perfusion pattern.

4 In the SP-SHR, reduction in renal perfusion pressure, from 150 to 100 mmHg, gave a cortical AI of 0.49 ± 0.10, indicating impaired autoregulation, whereas the papilla demonstrated little myogenic response. Over the high pressure range in the presence of both doses of nitrendipine there was neither cortical (AI of 0.75 ± 0.11 and 0.94 ± 0.12, respectively) nor papillary autoregulation.

5 Autoregulation in the renal cortex but not papilla of the young Wistar rats is well developed. The myogenic responses are attenuated by the calcium channel antagonists suggesting that they are dependent upon the availability of extracellular calcium. Cortical autoregulation in the SP-SHR is deficient compared to the normotensive rats and is further impaired by the calcium channel antagonists.

Keywords: Renal autoregulation; laser-Doppler flowmetry; calcium channel antagonists; kidney haemodynamic function; genetic hypertension

Introduction

The dihydropyridine calcium channel blocking drugs are able to prevent the inward movement of calcium into vascular smooth muscle cells via ligand and/or voltage gated L-type channels and as a consequence they cause vasodilatation in many vascular beds (Godfraind *et al.*, 1986). At the level of the kidney, Loutzenhiser & Epstein (1985) in an early review reported that although the calcium channel antagonists had variable effects on renal blood flow, either no change or an increase, there was a general concensus that glomerular filtration rate was raised. Importantly, there have been a number of reports, including those from this laboratory (Johns, 1985; Johns & Manitius, 1987) showing that administration of mildly depressor doses of calcium channel antagonists, causes a natriuresis and diuresis. The mechanisms underlying this action of the calcium channel blockers is not apparent although it is clear that the renal sympathetic nerves have no role to play (Herod & Johns, 1985). Two further possibilities exist, firstly that the compounds have a direct tubular action and secondly, that the excretory response is an indirect consequence of changes in intra-renal haemodynamics (Granger, 1992).

The evidence for a tubular site of action of the dihydropyridine calcium channel antagonists remains controversial. Haberle *et al.* (1987), using micropuncture techniques in the rat, suggested a proximal site of action, whereas DiBona (1990) utilising a similar approach, provided data to support a distal tubular site of action. Our own whole kidney studies using the lithium clearance technique (Johns, 1988) failed to support a proximal tubular action, but did suggest a site further down the nephron, either at the loop of Henle or beyond. However, in all these studies the possibility existed that changes in intrarenal haemodynamics could indirectly alter fluid transport.

There are a number of intriguing reports which seem to suggest that the calcium channel antagonists may have a differential action on the afferent and efferent resistance arterioles of the kidney. Fleming *et al.* (1987) using the hydronephrotic kidney, were able to show that calcium channel blockade had a vasodilator action at the afferent but not efferent arteriole. Furthermore, Carmines & Navar (1989), using the blood-perfused kidney and measuring the diameter of afferent and efferent arterioles, found the former dilated far more following administration of calcium channel antagonists than the latter and that the afferent arteriolar constrictor responses to angiotensin II were also blunted. These actions of the calcium channel antagonists described *in vitro* help explain the observations that at a whole animal level the autoregulatory ability of the kidney was reduced (Navar *et al.*, 1986; Ogawa, 1990).

The aim of the present study was to explore the effects of the calcium channel antagonists on renal autoregulation and whether there was a differential action on the cortex and papillary regions and to compare what occurs in an experimental model of genetic hypertension. This was done by measuring cortical blood flow and papillary blood flow

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separately by laser-Doppler flowmetry, and determining how autoregulation was affected by the calcium channel antagonist, nitrendipine, when given at two dose levels. Comparative studies were undertaken in the stroke prone spontaneously hypertensive rat to determine firstly, whether autoregulation occurred in this hypertensive situation and secondly, to discover whether the calcium channel antagonists had any action on intrarenal haemodynamics.

Methods

Experimental procedure

On the day of the experiment, rats were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and cannulae were placed in the left femoral vein and the left jugular vein for infusion of normal saline, drugs, supplementary doses of anaesthetic and transfusion of blood. Cannulae were inserted in the right carotid artery and the left femoral artery for measurements of arterial pressure above and below the left renal artery (Statham P23ID linked to a Grass model 7 polygraph). The left kidney was exposed via an abdominal incision and ligatures were placed loosely around the aorta above and below the left renal artery and attached to a screw device such that the aorta could be constricted, thereby either increasing or reducing pressure at the kidney; further ligatures were loosely placed around mesenteric and coeliac arteries. The kidney was then cleared of peri-renal fat, its artery, vein and ureter cleared and it was placed in a plastic holder to expose its dorsal surface. The ureter was sectioned and dissected towards the renal hilus to expose the papilla which protrudes into the papilla at this age.

Cortical and papillary perfusion measurements

Laser-Doppler perfusion was measured with a Perimed PF3 flowmeter and a PF303 probe of 1 mm diameter as previously described (Huang *et al.*, 1992). The laser-Doppler flowmeter was calibrated so that one perfusion unit (PU) was equivalent to 10 mV and this was recorded on the Grass polygraph where the output was damped to give an average level. Perfusion measured in this way represents the product of the velocity of moving blood cells and the concentration of moving blood cells (CMBC) in the volume of tissue under the probe. The probe was closely applied to the tissue (approximately 1 mm) and four measurements of 1–2 min duration were made at different sites over the cortex and the average values calculated while one measurement was taken over the papilla. At the end of the experiment a ligature was tied around the renal artery and vein and measurements were again taken to obtain background readings at each site of cortex and papilla. These background values were subtracted from all measurements taken.

Renal perfusion pressure (RPP) was first increased to a maximal level, approximately 25 mmHg, in two ways, firstly by occluding the mesenteric and coeliac arteries, secondly by intravenous transfusion of 1–2 ml of blood collected previously from donor rats. Cortical and papillary perfusion was measured at the highest renal perfusion pressure (RPP) and once completed, RPP was lowered in steps of 10 mmHg until 50 mmHg was reached. At each step in pressure, 5–10 min was allowed for a stable level to be achieved and cortical and papillary perfusions were again measured. An autoregulation index (AI) was calculated for the cortex and papilla by use of the following formula:

$$AI = [(Perfusion1 - Perfusion2)/Perfusion1]/[(RPP1 - RPP2)/RPP1]$$

where Perfusion1 and RPP1 represent the initial perfusion and pressure values and perfusion2 and RPP2 represent the respective values achieved after the 10 mmHg step reduction in pressure. Average values were then calculated over the high and low pressure ranges studied. This ratio was devised by Semple & DeWardener (1959) and an index of 0 indicates perfect autoregulation of perfusion whereas an index of 1 indicates no autoregulation and that the system has a fixed resistance.

Hormone infusion

Modification of both systemic and renal perfusion pressures could lead to large changes in the endogenous secretion of vasoactive hormones which themselves could influence basal resistance of blood vessels within the kidney. Therefore the approach of Roman & Cowley (1985) was adopted in which various hormones were infused exogenously to achieve high but stable levels. Thus, the following hormones were given as a cocktail: aldosterone (66 ng kg^{-1} min^{-1}), hydrocortisone (60 μg kg^{-1} min^{-1}), vasopressin (0.17 ng kg^{-1} min^{-1}), noradrenaline (333 ng kg^{-1} min^{-1}). The hormone cocktail was dissolved in a saline (150 mM NaCl) and infused at 33 μl min^{-1} 100 g^{-1} body weight.

Drugs

Nitrendipine was dissolved at 1 mg ml^{-1} in a 969:60:100 polyethylene-glycol-400:glycerol:water mixture and aliquots were stored deep frozen for no longer than one week. When nitrendipine was infused, all syringes, containers and cannulae were covered with aluminium foil to prevent breakdown of the nitrendipine due to exposure to light.

Experimental protocol

An intravenous infusion of normal saline was started at a rate of 33 μl min^{-1} 100 g^{-1} body weight immediately after the venous cannula had been inserted. The first measurements of cortical and papillary perfusions were taken 1 h after exposure of the papilla which were taken to give baseline values. The hormone cocktail was then infused and continued throughout the experiment. Once a new stable level of blood pressure had been achieved (approximately 30 min) further perfusion measurements were taken to give the starting levels for the experiment.

Six groups of rats were studied: Group I ($n = 9$) were Wistar rats, 129 ± 5 g, in which saline was infused throughout and acted as a control group. Once the highest blood pressure was achieved, perfusion measurements and pressure reductions were undertaken as described above. Group II ($n = 9$) were Wistar rats, 127 ± 3 g, which were infused with nitrendipine, 0.125 μg kg^{-1} min^{-1} i.v. for 30 min before the perfusion measurements at various renal perfusion pressures were undertaken. Group III ($n = 6$) were Wistar rats, 137 ± 2 g, and this group was identical to group II except that nitrendipine was given at 0.25 μg kg^{-1} min^{-1}. Groups IV ($n = 9$), V ($n = 6$) and VI ($n = 6$) were composed of SP-SHR rats, 133 ± 3 g, which were given either vehicle (group IV), or 0.125 μg kg^{-1} min^{-1} nitrendipine (group V) or 0.25 μg kg^{-1} min^{-1} nitrendipine (group VI).

Statistics

Renal perfusion pressure, cortical and papillary perfusions were recorded at each pressure level and absolute changes calculated. The absolute changes in each of the variables induced by nitrendipine was calculated and examined by a Student's paired t test. Autoregulatory curves were drawn up using a 'set-point' which was closest to the initial baseline pressure for each of the group of rats (90 and 110 mmHg for the Wistar and SP-SHR, respectively) and percentage deviations from this level were calculated and presented in the tables and figures. Autoregulatory indices (AI) were calculated over defined ranges and differences from 1 (no autoregulation) were examined and comparisons between groups were undertaken by use of a one way ANOVA followed by a

post-hoc Bonferroni-Dunn test. Analysis was undertaken with SuperANOVA software and significant differences were assumed when P was less than 0.05.

Results

The baseline values of body weight, age, renal perfusion pressure, cortical and papillary perfusions for the groups I to VI are presented in Table 1. There were no significant differences in the weight or age ranges, blood pressure, cortical or papillary perfusion between groups I, II or III of Wistar rats. In group I rats, initial blood pressure was 92 ± 4 mmHg (Table 1) and the highest pressure achieved following hormone infusion, occlusion of the mesenteric and coeliac arteries and transfusion of blood, was approximately 127 mmHg. As renal perfusion pressure was gradually reduced in group I animals to 90 mmHg, there was only a small fall in cortical perfusion and the AI (Table 2), of 0.20 ± 0.17, which was significantly different from 1 ($P < 0.05$), indicated that there was effective autoregulation whereas papillary perfusion decreased steadily over this range with an AI of 1.11 ± 0.24 (Table 2). Further reduction of perfusion pressure, from 90 to 50 mmHg, was associated with AI's (Table 2) not different from 1 which demonstrated that neither cortex nor papilla were capable of autoregulating over this pressure range. The full pressure/perfusion curves for the group I animals are shown in Figure 1.

The group II rats (Table 1) had baseline variables comparable to group I and responded similarly to the pressure elevations and although the administration of nitrendipine at $0.125\ \mu g\ kg^{-1}\ min^{-1}$ had no effect on either cortical or papillary perfusions, blood pressure fell by 7 ± 3 mmHg ($P < 0.05$). Following elevation of blood pressure to its maximal level, decreasing renal perfusion pressure in the range 120 to 90 mmHg was associated with gradual falls in cortical and papillary perfusions and calculation of the AI's showed them not to be significantly different from 1 (Table 2) indicating no autoregulation, and over the pressure range 90 to 50 mmHg similar autoregulatory indices were observed (Table 2). The AI of the cortex in the group II rats over the higher pressure range was significantly ($P < 0.01$) larger than in the group I rats (0.62 ± 0.13 versus 0.20 ± 0.17). The full autoregulatory curves are shown in Figure 1 which more clearly illustrates the relationship between pressure and perfusion.

Administration of the higher dose of nitrendipine, $0.25\ \mu g\ kg^{-1}\ min^{-1}$ into group III animals reduced blood pressure by 9 ± 4 mmHg ($P < 0.05$) but had no effect on basal levels of either cortical or papillary perfusions. Over the pressure range 120 to 90 mmHg and 90 to 50 mmHg perfusion in both regions fell proportionately with pressure and neither cortex nor papilla gave AI's indicative of autoregulation (Table 2) which were values significantly ($P < 0.01$ and 0.05, respectively) different from those observed in the animals not receiving nitrendipine (group I). The full autoregulatory curves for group III animals are given in Figure 1.

The baseline levels of various variables of the SP-SHR group IV to VI are given in Table 1 and the animals were matched in weight, but not age, with the normotensive rats. These groups had baseline blood pressures approximately 10 mmHg higher than the small Wistar rats and the highest pressure achieved at the start the autoregulatory curves was 150 mmHg. Over the pressure range 150 to 100 mmHg the saline control (group IV) exhibited a gradual fall in cortical perfusion and gave an AI, of 0.49 ± 0.10 (Table 2) which was significantly ($P < 0.01$) less than 1, but it was significantly ($P < 0.01$) larger than 0, which was taken to reflect an impaired autoregulation. Under these conditions papillary perfusion fell directly as pressure was reduced and there was no indication of autoregulation (Table 2). The full autoregulatory curves for both regions are shown in Figure 2.

The group V animals were infused with the low dose of nitrendipine which had no effect on either blood pressure, cortical or papillary perfusions. Reducing renal perfusion pressure in the range 150 to 100 mmHg and then 100 to 50 mmHg caused progressive reductions in both cortical and papillary perfusions and resulted in AI's (Table 2) which were

Table 1 Basal variables of group I to VI

Group	n	*Body wt* (g)	*Age* (wk)	*Base line* *RPP* (mmHg)	*Base line* *Cortical perfusion* (PU)	*Base line* *Papillary perfusion* (PU)
Group I	9	129 ± 5	5.8 ± 0.1	92 ± 4	146 ± 8	213 ± 17
Group II	9	127 ± 3	5.5 ± 0.2	93 ± 2	135 ± 7	191 ± 14
Group III	6	137 ± 2	4.7 ± 0.2	97 ± 2	153 ± 8	238 ± 16
Group IV	9	132 ± 2	8.3 ± 0.1	106 ± 3	189 ± 12	227 ± 13
Group V	6	133 ± 2	8.0 ± 0.1	104 ± 5	185 ± 5	213 ± 12
Group VI	6	133 ± 3	8.8 ± 0.1	108 ± 2	142 ± 7	196 ± 16

n: Number of rats tested. Base line: data obtained before infusion of hormone cocktail. RPP: renal perfusion pressure, reported as a mean value. PU: perfusion unit. Groups I, II and III are Wistar rats infused with vehicle, 0.125 and $0.25\ \mu g\ kg^{-1}\ min^{-1}$ nitrendipine, respectively while groups IV, V and VI are SP-SHR infused with vehicle, 0.125 and $0.25\ \mu g\ kg^{-1}\ min^{-1}$ nitrendipine, respectively.

Table 2 Autoregulatory indices of groups I to VI

Group	*RPP range (mmHg)* *Range 1*	*Range 2*	*Cortical AI* *In range 1*	*In range 2*	*Papillary AI* *In range 1*	*In range 2*
Group I	127–90	90–50	0.20 ± 0.17†	1.11 ± 0.24	0.89 ± 0.08	1.05 ± 0.09
Group II	130–90	90–50	0.62 ± 0.13	1.23 ± 0.11	0.89 ± 0.10	1.16 ± 0.57
Group III	125–90	90–50	0.92 ± 0.10**	0.99 ± 0.21	1.18 ± 0.06*	1.39 ± 0.10*
Group IV	150–100	100–50	0.49 ± 0.10‡	1.02 ± 0.03	0.74 ± 0.13	0.75 ± 0.07
Group V	163–100	100–50	0.75 ± 0.11	1.34 ± 0.11*	0.71 ± 0.09	1.12 ± 0.13*
Group VI	150–100	100–50	0.94 ± 0.12*	1.33 ± 0.12*	0.99 ± 0.15	1.14 ± 0.07*

RPP: Renal perfusion pressure, reported as a mean value. AI: autoregulatory index. †Significantly lower than 1; *Significantly different from control. † and * $P < 0.05$, ‡ and ** $P < 0.01$. Groups I, II and III are Wistar rats infused with vehicle, 0.125 and $0.25\ \mu g\ kg^{-1}\ min^{-1}$ nitrendipine, respectively while groups IV, V and VI are SP-SHR infused with vehicle, 0.125 and $0.25\ \mu g\ kg^{-1}\ min^{-1}$ nitrendipine, respectively.

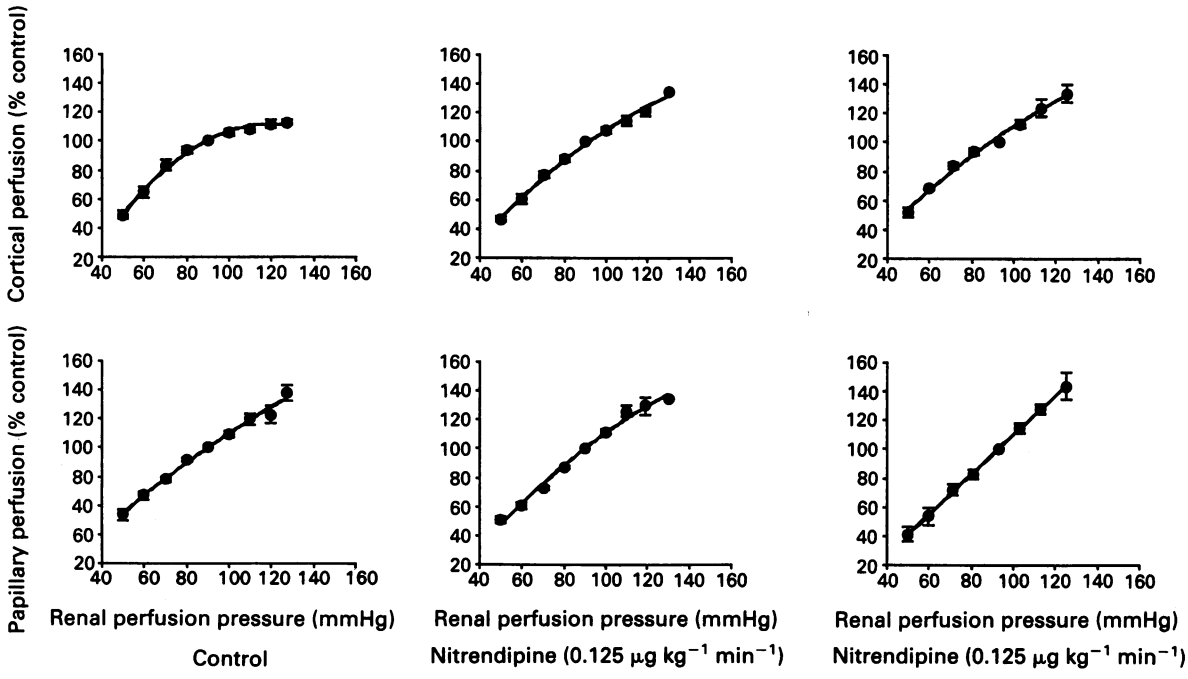

Figure 1 This shows the percentage change in cortical and papillary perfusions from baseline pressure (90 mmHg) at each pressure level achieved in Wistar rats without drug (control) or receiving nitrendipine at 0.125 $\mu g\ kg^{-1}\ min^{-1}$ or 0.25 $\mu g\ kg^{-1}\ min^{-1}$. Each value is displayed as a mean ± s.e.mean.

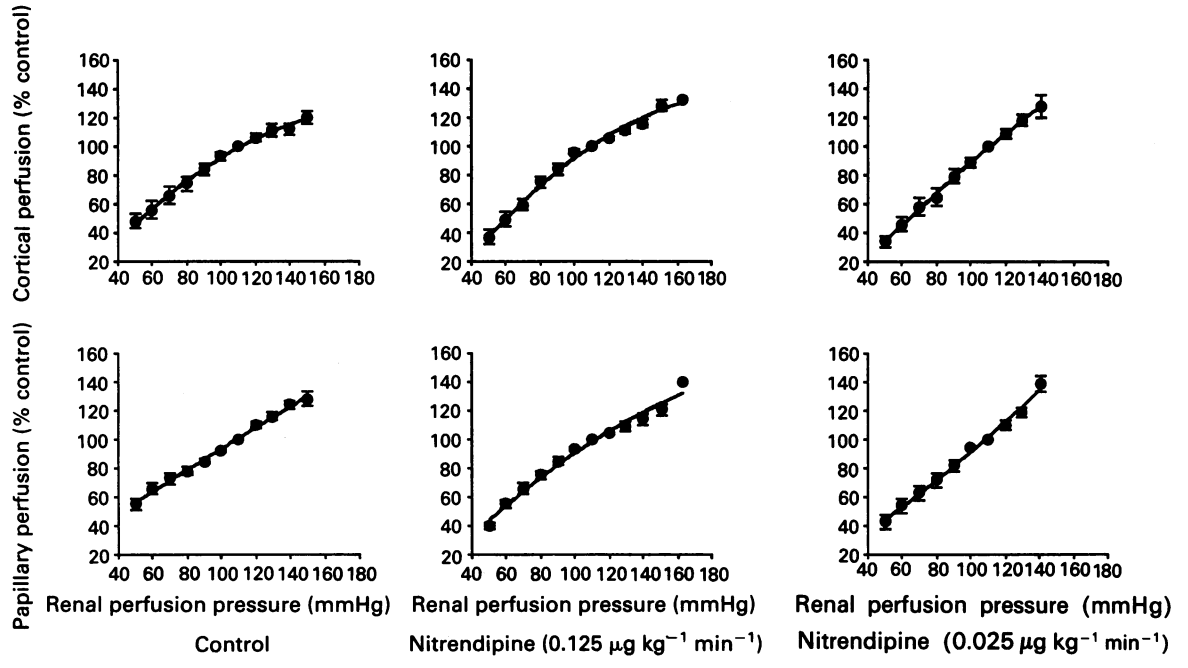

Figure 2 This shows the percentage change in cortical and papillary perfusions from baseline (110 mmHg) at each pressure level achieved in SP-SHR without drug (control) or receiving nitrendipine at 0.125 $\mu g\ kg^{-1}\ min^{-1}$ or 0.25 $\mu g\ kg^{-1}\ min^{-1}$. Each value is displayed as a mean ± s.e.mean.

not significantly different from 1. These observations indicated that autoregulation did not occur in either region over either pressure range in the presence of the low dose nitrendipine and the full autoregulatory curves for group V are given in Figure 2. The infusion of the high dose of nitrendipine (group VI) caused a significant reduction in blood pressure (9 ± 2 mmHg; $P<0.01$) which was associated with a significant ($P<0.05$) increase in cortical (33 ± 9 PU) but not papillary perfusion. In the group VI rats, reduction in renal perfusion pressure, over the ranges 150 to 100 mmHg, and then 100 to 50 mmHg caused steady falls in both cortical and papillary perfusion which resulted in AI's (Table 2) consistent with no autoregulation at any pressure level and the full autoregulatory curves are shown in Figure 2.

Discussion

This study was designed to investigate the influence of calcium channel antagonists on the ability of the kidney to

autoregulate blood flow through the different vascular beds of the kidney, the cortex and papilla, in normotensive rats and a genetic rat model of hypertension. Only a limited number of techniques exist which allow dynamic changes in local blood flow to be measured but one method offering a qualitative assessment is laser-Doppler flowmetry (Shepherd & Oberg, 1990) and its use in the kidney has been validated by Roman and co-workers (Smits *et al.*, 1986; Roman & Smits, 1986). This methodology does not measure blood flow per unit time, but rather records perfusion, given as the product of red cell velocity times the concentration of moving red cells in the volume of measurement. It can thus be used to examine the physiological control of perfusion within different regions of the kidney, primarily the cortex and papilla (Huang *et al.*, 1991a,b).

A second difficulty was generating a model in which blood pressure was elevated to a sufficient degree that the kidney could be exposed to a range of pressure in order that autoregulation could be tested adequately. The approach adopted was based on that of Roman & Cowley (1985) in which replacement hormones and catecholamines were given, which resulted in 4 to 5 times normal endogenous levels, and the mesenteric and coeliac arteries were tied off to increase peripheral resistance. In spite of these manoeuvres, often it was still not possible to achieve a sufficiently high blood pressure and thus the alternative strategy of transfusing 1 to 2 ml of blood from donor rats was developed. This was particularly important as nitrendipine was being used at doses which caused quite marked falls in blood pressure and it was therefore imperative that once this had occurred, pressure was still sufficiently high for an autoregulatory curve to be generated. It is important to acknowledge that the data were obtained under conditions in which vasoactive compounds were given and vascular tone was elevated, as shown by the increase in blood pressure. The possibility exists that if the studies were undertaken in the absence of this hormone cocktail infusion, different results might have been obtained. However, the overall influence of the sustaining hormone infusion was likely to be minor as the increase in blood pressure was small.

The studies with both the normotensive rats showed that cortical perfusion was reasonably constant down to approximately 100 mmHg, and calculation of the autoregulatory index, as developed by Semple and DeWardener (1959), showed that good autoregulation was taking place over this pressure range. It was apparent that below 100 mmHg, cortical perfusion began to fall more rapidly with pressure, and indeed no autoregulation occurred from about 90 to 50 mmHg. These results, therefore, suggest that cortical autoregulation was lost between approximately 85 to 95 mmHg. These observations are comparable to those reported by Roman & Kaldunski (1988) for slightly older rats (7–9 weeks versus 5 weeks of the present study) using laser-Doppler flowmetry, although the animals appeared to autoregulate to a slightly lower pressure (80 versus 90 mmHg in the present study). The pressure at which cortical autoregulation failed was somewhat lower than the values for the limit of autoregulation of total renal blood flow in the anaesthetized rat (electromagnetic flowmetry) measured by Arendshorst (1979), but was somewhat higher than the values for normotensive rats reported by Iversen *et al.* (1986). It is reassuring that changes in cortical perfusion should parallel those occurring in total renal blood flow as some 90% of this blood flows through the cortex with only a minor proportion flowing through the deeper region of the kidney (Jamison & Kriz, 1982). A further observation was that under the experimental conditions of the present study, papillary perfusion did not exhibit autoregulation over any pressure range. There is conflicting evidence in the literature regarding autoregulation in the papilla and whereas a report by Cohen *et al.* (1983) using the dual slit videomicroscopic method to measure red cell velocity showed autoregulation to exist, Roman & Kaldunski (1988) utilising the laser-Doppler technique suggested that papillary autoregulation only occurred under hydropenic conditions. Because the present experimental approach used a relatively high rate of saline infusion combined with blood transfusion, it was very likely that the animals were not hydropenic and therefore not in a state in which papillary autoregulation might be expected to occur.

Administration of nitrendipine into the Wistar rats reduced blood pressure at both dose levels, indicating that the compound was having an action at vascular smooth muscle, although it had no measurable effect on either cortical or papillary perfusion. There is no easy end-point for determining the degree of action of these drugs but the hypotensive action is one indicator. Indeed, in previous studies using nipendipine (Johns, 1985) and amlodipine (Johns, 1988) mildly vasodepressor doses of the dihydropyridine calcium channel antagonists had marked renal effects in terms of increasing sodium and water excretion. Furthermore, larger doses of the nitrendipine would have caused such marked falls in blood pressure that it would not be possible to undertake the studies using a wide range of renal perfusion pressure. It was striking that in both groups of Wistar rats given nitrendipine, cortical autoregulation could not be measured over the pressure range 120 to 90 mmHg. Interestingly, in a comparable study by Fenoy and colleagues, nisoldipine was found not to change the relationship between cortical perfusion pressure and renal perfusion pressure and their results therefore do not support the present observations. However, the findings presented herein do support and extend observations in the anaesthetized dog in which both verapamil (Navar *et al.*, 1986) and nicardipine (Ogawa, 1990) attenuated autoregulation of total renal blood flow. This attenuation of autoregulation would suggest that the primary resistance vessels of the kidney, the afferent arterioles, were no longer able to exert their appopriate myogenic response to reductions in perfusion pressure. Such an action is compatible with the reports in the hydronephrotic kidney (Fleming *et al.*, 1987) and in the *in vitro* blood perfused kidney (Carmines & Navar, 1986) showing that calcium channel blockers had a preferential vasodilator action at the afferent arterioles.

Baseline levels of blood pressure recorded in the SP-SHR were some 10 mmHg higher than in the Wistar controls while there were no meaningful differences in the baseline values of either cortical or papillary perfusions between the nomotensive and hypertensive group of rats. Following the hormone replacement infusion, artery occlusion and blood transfusion, the highest pressures achieved at the start of the experiment in the SP-SHR were somewhat higher than in the Wistar, 150 versus 127 mmHg, while there were only minor changes in cortical and papillary perfusion. Under these conditions, it was interesting that at any particular perfusion pressure papillary, but not cortical, perfusion was some 15 to 20% lower in the SP-SHR. This feature was also remarked upon by Roman & Kaldunski (1988) in the SHR, of which the SP-SHR is a sub-strain; they concluded that the papillary resistance in the SHR was elevated compared with the normotensive rats. However, an alternative possibility is that the papillary vasculature of the hypertensive rats was more sensitive to the experimental manoeuvres to increase perfusion pressure rather than having an inherently elevated resistance. The data generated in the SP-SHR indicated that autoregulation within the cortex was impaired compared to the normotensive animals but was not totally absent. This could possibly suggest that in the growing phase there is some deficiency in the myogenic properties of the vasculature of the SP-SHR. This observation in 5 week old SP-SHR is similar to that of Roman & Kaldunski (1988) who found that in young SHR (3 to 5 weeks) cortical autoregulation was not effective although at 6–9 weeks there appeared to be a degree of autoregulation that was comparable to the present studies in the SP-SHR. Indeed, there is controversy in the literature concerning the autoregulatory powers of the SHR, of which the SP-SHR is a related substrain. Thus, whereas Adrendshorst (1979) claimed good autoregulation of total

renal blood flow in the SHR, DiBona & Rios (1988) reported a marked deficiency in the SHR even though both groups used similar experimental approaches and age of rat.

Administration of nitrendipine at the high but not low doses caused small falls in blood pressure and increase in cortical perfusion in the SP-SHR (except for group V rats). Further, in the presence of both low and high doses of nitrendipine no autoregulatory response could be demonstrated within the cortex. These studies clearly showed that the cortical vasculature of the SP-SHR, was sensitive to the calcium channel blocker in a way very comparable to that of the cortex of the Wistar rats which contrast with the observations in the SHR given nisoldipine (Fenoy *et al.*, 1992). It was also worthy of note that in neither group of SP-SHR was there evidence of papillary autoregulation either before or following nitrendipine and as indicated above, the volume expansion necessitated by the experimental procedure could have suppressed the autoregulatory response of the papilla. Indeed, in both Wistar and SP-SHR no differences in renal perfusion pressure and papillary perfusion could be detected either in the absence or presence of nitrendipine which contrasts with the observations of Fenoy *et al.* (1992) who demonstrated that the SHR papillary perfusion became identical to that observed for the WKY controls.

In summary, this investigation set out to examine how nitrendipine, a dihydropyridine calcium channel antagonist, affected the ability of two vascular beds in the kidney, the cortex and papilla, to maintain perfusion over a normal pressure range, i.e. to autoregulate. The studies showed that in normotensive rats there was good autoregulation in the cortex over normal physiological ranges but the SP-SHR appeared to have a deficient autoregulatory response in the cortex. By contrast, there was little evidence of papillary autoregulation over any range in either Wistar or SP-SHR but this probably was a consequence of the experimental approach. In both normotensive and SP-SHR the calcium channel antagonist markedly attenuated the ability of the cortex to autoregulate. The underlying mechanisms which caused this effect are not apparent at the present time but deserve to be investigated. Nevertheless, the attenuation of the normal physiological myogenic responses of the cortical vasculature by the calcium channel antagonists could have a major impact on the renal handling of sodium and water and these compounds have been shown to have a natriuretic and diuretic action.

It is a pleasure to acknowledge a grant from Bayer, A.G., Wuppertal, Germany which made this work possible.

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(Received May 10, 1993
Revised August 29, 1993
Accepted September 16, 1993)

Br. J. Pharmacol. (1994), **111**, 117–122

Role of adhesion glycoproteins CD18 and intercellular adhesion molecule-1 in complement-mediated reactions of rabbit skin

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1 The role of the adhesion glycoproteins CD18 and intercellular adhesion molecule-1 (ICAM-1) in inflammatory responses produced during a reversed passive Arthus (RPA) reaction and induced by zymosan and zymosan-activated plasma (ZAP) were studied in rabbit skin.

2 Oedema formation and haemorrhage were quantified by measuring accumulation of ^{125}I-albumin and ^{111}In-labelled red blood cells (^{111}In-RBC) respectively.

3 Monoclonal antibody (mAb) R15.7 (anti-CD18), administered intravenously, abolished accumulation of ^{125}I-albumin and ^{111}In-RBC in dermal RPA reactions and in response to locally injected zymosan and ZAP.

4 When administered intravenously, the mAb RR1/1 (anti-ICAM-1) suppressed ^{125}I-albumin and ^{111}In-RBC accumulation in dermal RPA reactions and at sites treated with zymosan and ZAP.

5 Oedema formation in response to platelet-activating factor (PAF) and bradykinin (BK) either in the presence or absence of prostaglandin E_2 (PGE_2) were not affected by mAb R15.7 or by mAb RR1/1.1.1.

6 We conclude that oedema formation and haemorrhage associated with RPA reactions and in responses to zymosan and ZAP are completely CD18-dependent, and are mediated, at least in part, via ICAM-1. Responses to the neutrophil-independent oedema forming mediators, PAF and BK are not dependent upon CD18 or ICAM-1.

Keywords: Arthus reaction; complement; oedema formation; haemorrhage; adhesion; CD18; intercellular adhesion molecule-1 (ICAM-1)

Introduction

Neutrophil-dependent inflammatory responses rely upon the selective adherence of neutrophils to vascular endothelium, followed by migration to and activation at the inflammatory locus. Neutrophil adherence is mediated by the interaction of adhesion molecules on the vascular endothelium and circulating leukocytes. CD18 is the common subunit of the β_2 integrin family of adhesion molecules. This group of adhesion molecules include CD11a/CD18 lymphocyte function associated antigen-1 (LFA-1) which is expressed constitutively on the neutrophil surface, and CD11b/CD18 (Mac-1), which is present at low levels constitutively and rapidly induced on stimulated neutrophils (Jutila *et al.*, 1989; Smith *et al.*, 1989; Springer, 1990). Intercellular adhesion molecule-1 (ICAM-1), a ligand for CD11a/CD18 and CD11b/CD18, is an inducible adhesion molecule, expressed on a variety of cells, including endothelial cells, at sites of inflammation (Rothlein *et al.*, 1986; Springer, 1990). A circulating form of ICAM-1 has also been identified in human serum (Rothlein *et al.*, 1991).

The adhesion molecules described above have been well characterized *in vitro* (Springer, 1990). Studies have shown that antibodies raised against these molecules can be effective blockers of neutrophil adhesion *in vitro* (Sanchez-Madrid *et al.*, 1983; Rothlein *et al.*, 1986), and of inflammation *in vivo* (Arfors *et al.*, 1987; Rampart & Williams, 1988; Barton *et al.*, 1989; Argenbright *et al.*, 1991). Recently it has been shown that the Shwartzman reaction, a phenomenon that models disseminated intravascular coagulation, and non-immune complex mediated vasculitis, is both CD18 and ICAM-1 dependent (Argenbright & Barton, 1991).

The Arthus reaction, as described at the turn of the century (Arthus, 1903), is an acute inflammatory and haemorrhagic reaction that develops in the skin of a sensitized rabbit following i.d. injection of antigen. It is more convenient to produce a reversed passive Arthus (RPA) reaction, giving antibody i.d., and antigen i.v. The RPA reaction is relevant clinically, modelling immune complex mediated microvascular injury, and type III hypersensitivity. It is also useful experimentally as a reliable and reproducible means of producing complement-mediated inflammation, primarily by the classical pathway of complement activation. Depletion of neutrophils with nitrogen mustard or anti-neutrophil antiserum has been shown to inhibit the Arthus reaction (Stetson & Good, 1951; Humphrey, 1955), thus demonstrating neutrophil-dependence. Zymosan particles given i.d. also induce inflammatory responses in rabbit skin. Zymosan activates complement, typically by the alternative pathway, without a requirement for prior sensitization or i.v. antigen challenge. RPA reactions and responses to preformed immune complexes or zymosan can be distinguished pharmacologically (Rossi *et al.*, 1992; Hellewell & Williams, 1986), which may reflect differences in the location of neutrophil contact with the particulate stimulus in the tissue.

The aim of this study was to investigate the involvement of β_2 integrins and intercellular adhesion molecule-1 (ICAM-1), in the RPA, and other, complement-mediated, inflammatory reactions using monoclonal antibodies (mAbs) R15.7 and RR1/1, that have previously been shown to block selectively rabbit leucocyte CD18 (Rothlein *et al.*, 1986) and rabbit endothelial cell ICAM-1 respectively, (Argenbright *et al.*, 1991; Horgan *et al.*, 1991). We monitored accumulation of ^{125}I-labelled albumin and ^{111}In-labelled red blood cells (RBC), as measures of oedema formation and haemorrhage respectively, in RPA reactions and responses to zymosan and zymosan-activated plasma (ZAP), a source of preformed $C5a_{desArg}$. For comparison, the effects of mAbs R15.7 and RR1/1 on responses to platelet-activating factor (PAF) and

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bradykinin (BK), alone or in the presence of the vasodilator prostaglandin in E_2 (PGE_2), were also studied.

Methods

Animals

NZW rabbits (2.5–3 kg) were purchased from Froxfield Farm, Hampshire.

Monoclonal antibodies

The mouse mAb R15.7 (IgG_1) was raised against canine CD18 adhesion glycoprotein and the mouse mAb RR1/1 (IgG_1) was raised against human ICAM-1. Their specificity and binding properties were tested as described previously (Argenbright & Barton, 1992; Rothlein *et al.*, 1986). Purified mouse IgG was used as the control. Purified antibodies were dissolved in sterile saline and injected i.v. at 2 mg kg^{-1}. This dose has been previously shown to be effective for both R15.7 and RR1/1 (Welbourn *et al.*, 1990; Van Osselaer *et al.*, 1991; Hill *et al.*, 1993).

Generation of antiserum for the RPA reaction

Arthus antiserum, anti-bovine-γ-globulin (BGG), was raised in rabbits as previously described (Hellewell & Williams, 1986). Briefly, subcutaneous injections (4 × 0.25 ml) of BGG (2 mg ml^{-1} in saline) emulsified in an equal volume of Freund's complete adjuvant were administered. This was followed 14 and 28 days later by booster subcutaneous injections (4 × 0.25 ml) of the same concentration of BGG in an equal volume of Freund's incomplete adjuvant. Blood was collected by carotid artery cannulation at day 38. Serum from six rabbits was pooled and stored in aliquots at −20°C. Heat-inactivated normal serum was used as a control.

Preparation of zymosan-activated plasma (ZAP)

ZAP, a source of preformed $C5a_{desArg}$, was prepared by incubating heparinized (10 u ml^{-1}) rabbit plasma with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min; 2500 *g*) and ZAP stored in 1 ml aliquots at −20°C. The $C5a_{desArg}$ content of ZAP was approximately 5×10^{-7} M as determined by radioimmunoassay (Collins *et al.*, 1991).

Preparation of ^{111}In-labelled RBC

Rabbit blood collected by carotid artery cannulation into tri-sodium citrate (0.38%, w/v) was centrifuged at 250 *g* for 20 min; 1 ml of red cell pellet was added to 10–15 μl (~100–400 μCi) of $^{111}InCl_3$ chelated with 2-mercaptopyridine-*N*-oxide (40 μg in 0.1 ml) in PBS (50 mM; pH 7.4) for 15 min at room temperature. Following incubation, the ^{111}In-RBC were washed three times, resuspended in 5 ml of Tyrode solution and centrifuged at 250 *g* for 10 min. The ^{111}In-RBC were finally resuspended to a final volume of 9 ml in Tyrode solution. Each of 3 rabbits received approximately 10^9 ^{111}In-labelled RBC.

Preparation of agents for injection

All agents were diluted to the stated concentration in 0.9% sterile, pyrogen-free saline, except PAF, which was prepared in 0.9% sterile, pyrogen-free saline containing 0.1% BSA.

Measurement of RPA reactions and responses to zymosan, ZAP, BK and PAF in rabbit skin

Rabbits were anaesthetized with i.v. sodium pentobarbitone and their dorsal skin shaved and marked out with 6 replicates of 12 treatment sites according to a balanced site plan. ^{111}In-RBC and ^{125}I-albumin (5 μCi kg^{-1}) mixed with Evans blue dye as a visual marker were injected i.v., followed 5 min later by either mAb R15.7, mAb RR1/1, or non-reactive mouse IgG (all at 2 mg kg^{-1}). After a further 15 min, Arthus antiserum (anti-BGG, 25, 50 and 100%), zymosan (30, 100 and 300 μg per site), ZAP (100%), PAF (10^{-9} mol per site) ± PGE_2 (3×10^{-10} mol per site) and BK (10^{-10} mol per site) ± PGE_2 (3×10^{-10} mol per site) were injected i.d. Arthus (BGG) antigen, was injected i.v. 5 min after the i.d. injections. Blood was collected by cardiac puncture 4 h after i.v. BGG, and animals were killed by anaesthetic overdose. The dorsal skin was removed and treatment sites excised with a 17 mm diameter punch. Skin sites were counted, with blood and plasma samples, in a gamma counter with automatic spill-over and cross talk correction (Packard Cobra, Meridian, Ct, U.S.A.). Albumin accumulation was expressed as equivalent μl plasma per skin site by dividing skin sample ^{125}I-counts by ^{125}I-counts in 1 μl of plasma. RBC accumulation was expressed as equivalent μl of blood per skin site by dividing skin sample ^{111}In-counts by the ^{111}In-counts in 1 μl of blood. Blood samples were also used to determine the level of free ^{111}In, which was below 4% in all experiments.

Materials

Freund's complete and incomplete adjuvants, BSA (low endotoxin and fatty acid-free), bovine gamma globulin (BGG), BK, zymosan and PGE_2 were purchased from Sigma Chemical Co., Poole, Dorset. ^{125}I-human serum albumin (20 mg ml^{-1} albumin in sterile isotonic saline, 50 μCi ml^{-1}) and $^{111}InCl_3$ (2 mCi in 0.2 ml sterile pyrogen free 0.04 M HCl) were from Amersham International, Amersham, Buckinghamshire. Sagatal (sodium pentobarbitone, 60 mg ml^{-1}) was from May and Baker, Dagenham, Essex. Evans Blue Dye was from BDH, Poole, Dorset. Viaflex (sterile, pyrogen-free isotonic saline solution) was from Baxter Healthcare Ltd., Thetford, Norfolk. PAF was from Bachem, Saffron, Walden, Essex. Mouse IgG was from Rockland, PO Box 316, Gilbertsville, PA 19525, U.S.A.

Statistical analysis

All data are presented as mean ± s.e.mean of the indicated number of experiments. Data were analyzed by 1 or 2 way analysis of variance, and significant differences ($*P<0.05$, $**P<0.01$) between means were determined by the Neuman Keuls procedure.

Results

Effect of mAb R15.7 and mAb RR1/1 on oedema formation and ^{111}In-RBC accumulation in the RPA reaction

The inflammatory response associated with the RPA reaction in animals treated with i.v. control Ab (mouse; whole IgG) measured after 4 h is shown in Figure 1. Administration (i.d.) of increasing doses of Arthus antiserum (25, 50 and 100%) followed by i.v. injection of antigen elicited a dose-dependent accumulation of ^{125}I-albumin (Figure 1a; open squares) and ^{111}In-RBC (Figure 1b; open squares). In order to determine whether the adhesion glycoproteins CD18 and ICAM-1 are involved in this complement-mediated vasculitis we pretreated (2 mg kg^{-1}, i.v.) rabbits with mAb R15.7 or mAb RR1/1, respectively, and measured the inflammatory response. Accumulation of both ^{125}I-albumin (Figure 1a; filled squares) and ^{111}In-RBC (Figure 1b; filled squares) was attenuated by mAb R15.7. In contrast to the results obtained with mAb R15.7, RR1/1 produced only partial inhibition.

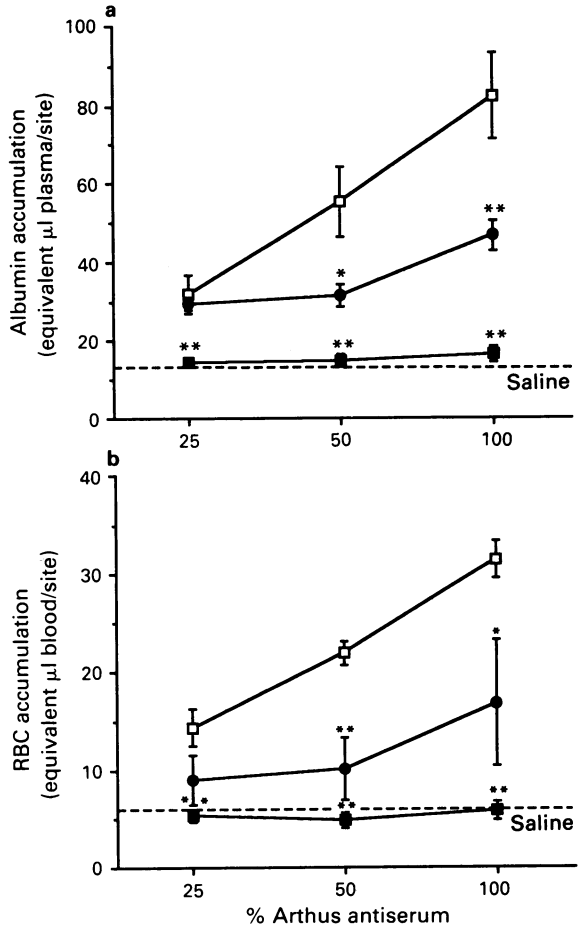

Figure 1 Effect of monoclonal antibodies (mAbs) R15.7 and RR1/1 on ^{125}I-albumin (a) and ^{111}In-RBC accumulation (b) in the reversed passive Arthus (RPA) reaction in rabbit skin. Rabbits received i.v. injections of either non-reactive mouse IgG (□), mAb R15.7 (■), or mAb RR1/1 (●), all at 2 mg kg^{-1}, 15 min later, different titres (25, 50 and 100%) of Arthus antiserum were injected i.d., followed by bovine-γ-globulin (5 mg kg^{-1}) i.v. Animals were killed after 4 h and oedema formation and haemorrhage quantified as detailed in the methods section. Data are presented as mean values ± s.e.mean of responses obtained in $n = 5$ animals. Responses to normal rabbit serum were not significantly different from responses to saline. Significant difference from the appropriate control is indicated by: $^*P<0.05$ or $^{**}P<0.01$.

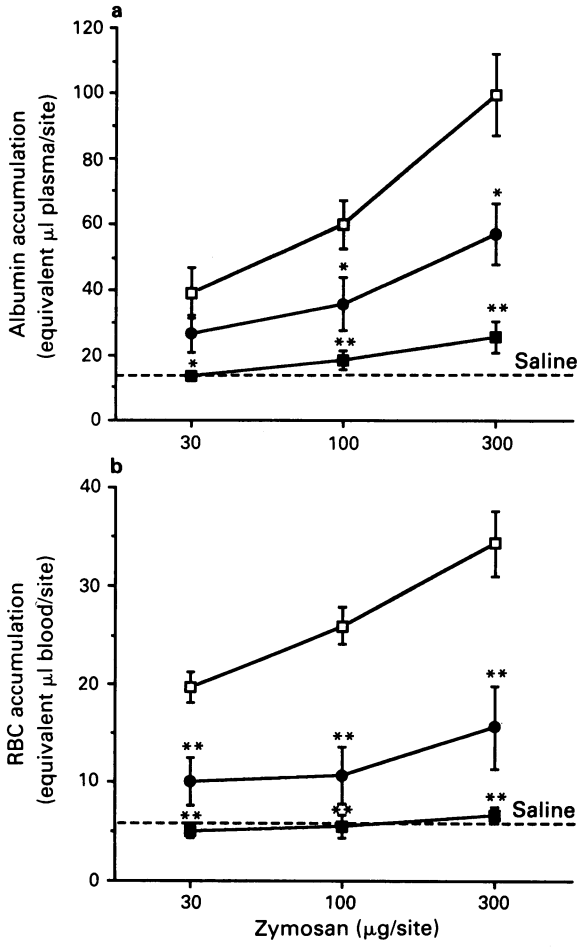

Figure 2 Effect of monoclonal antibodies (mAbs) R15.7 and RR1/1 on ^{125}I-albumin (a) and ^{111}In-RBC accumulation (b) in response to zymosan in rabbit skin. Rabbits received i.v. injections of either non-reactive mouse IgG (□), mAb R15.7 (■), or mAb RR1/1 (●), all at 2 mg kg^{-1}. Increasing doses (30, 100 and 300 μg/site) of zymosan were injected i.d. 15 min later. Animals were killed after 4 h and oedema formation and haemorrhage quantified as detailed in the methods section. Data are presented as mean values ± s.e.mean of responses obtained in $n = 5$ animals. The dotted line represents saline-injected sites. Significant difference from the appropriate control is indicated by: $^*P<0.05$ or $^{**}P<0.01$.

Effect of mAb R15.7 and mAb RR1/1 on oedema formation and ^{111}In-RBC accumulation induced by zymosan particles

Having shown that responses induced by immune complex formation which primarily trigger the classical pathway of complement activation (the RPA reaction) are inhibited to varying degrees by the above mAbs we next examined their effect on responses induced by zymosan particles which typically activate complement by the alternative pathway. Zymosan particles (30,100 and 300 μg per site) caused dose-dependent responses measured 4 h after i.d. injections (Figure 2, open squares). Intravenous mAb R15.7 (2 mg kg^{-1}) inhibited ^{125}I-albumin and ^{111}In-RBC accumulation induced by all doses of zymosan (Figure 2; filled squares) by >90%. Treatment with mAb RR1/1 (2 mg kg^{-1}) significantly reduced, but did not abolish, ^{111}In-RBC accumulation to all doses of zymosan (Figure 2b; filled circles). Accumulation of ^{125}I-albumin in response to 100 and 300 μg per site zymosan was also significantly inhibited by RR1/1 (Figure 2a; filled circles).

Effect of mAb R15.7 and RR1/1 on oedema formation and RBC accumulation in responses to ZAP and other mediators

ZAP, a source of preformed $C5a_{desArg}$, caused marked accumulation of both ^{125}I-albumin (Figure 3a) and ^{111}In-RBC (Figure 3b). These responses were abolished by mAb R15.7. ^{111}In-RBC accumulation in response to ZAP was partially inhibited by RR1/1 (Figure 3b). The neutrophil-independent oedema forming mediators PAF and BK induced accumulation of ^{125}I-albumin, which was potentiated in the presence of PGE_2. These responses were unaffected by mAb R15.7 and RR1/1. Little or no accumulation of ^{125}I-albumin or ^{111}In-RBC, above that caused by saline, was caused by PGE_2 alone.

Discussion

When locally injected antibodies come into contact with systemically administered antigen, as in RPA reactions of rabbit skin, the immune complexes formed cause activation of complement. This sets in motion the chain of events that

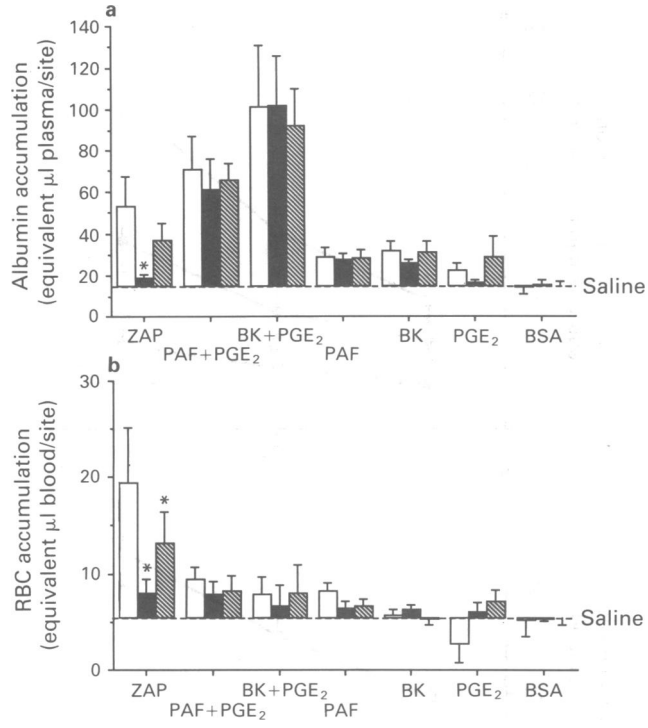

Figure 3 Effect of mAbs R15.7 and RR1/1 on ^{125}I-albumin (a) and ^{111}In-RBC accumulation (b) in responses to zymosan activated plasma (ZAP), platelet activating factor (PAF) and bradykinin (BK) in rabbit skin. Rabbits received i.v. injections of either non-reactive mouse IgG (open columns), mAb R15.7 (solid columns), or mAb RR1/1 (hatched columns), all at 2 mg kg^{-1}; 15 min later ZAP (100%), PAF (10^{-9} mol/site) ± PGE_2 (3×10^{-10} mol/site) and BK (10^{-10} mol/site) ± PGE_2 (3×10^{-10} mol/site) were injected i.d. Animals were killed after 4 h and oedema formation and haemorrhage quantified as detailed in the methods section. Data are presented as mean ± s.e.mean of responses obtained in $n = 4-6$ animals. Responses to PGE_2 (3×10^{-10} mol/site) and BSA were not significantly different from responses to saline. Significant difference from the appropriate control is indicated by: $^*P < 0.05$.

lead to the well described pathology that includes accumulation and activation of inflammatory cells, leakage of plasma proteins, haemorrhage, platelet deposition and in severe cases necrosis (Cochrane & Janoff, 1974; Williams *et al.*, 1986; Pons *et al.*, 1993). Extensive study has revealed much about the processes that underlie the Arthus reaction. The reaction is, as mentioned above, dependent on complement, and can be inhibited by decomplementation with cobra venom factor, anti-complement antibodies or by soluble complement receptors (Stetson & Good, 1951; Humphrey, 1955; Ward & Cochrane, 1965; Cochrane *et al.*, 1970; Cochrane & Janoff, 1974; Rossi *et al.*, 1992). We have previously demonstrated a role for platelet-activating factor (Hellewell & Williams, 1986; Rossi *et al.*, 1992), and for vasodilator prostaglandins (Williams *et al.*, 1986) in the plasma extravasation associated with the RPA reaction. Neutrophil dependence has been demonstrated by the fact that RPA reactions are suppressed in neutrophil-depleted animals (Stetson & Good, 1951; Humphrey, 1955).

The CD18/β_2 integrin family of adhesion molecules has been extensively studied both *in vitro* (Springer, 1990) and *in vivo* (Rampart & Williams, 1988; Nourshargh *et al.*, 1989; Mulligan *et al.*, 1992; 1993a,b; Argenbright & Barton, 1992; Williams & Hellewell, 1992). Two members of the β_2 integrin family, CD11a/CD18 (LFA-1) and C11b/CD18 (Mac-1) are widely held to be important in neutrophil adhesion at and migration to inflammatory sites. Increased adhesion via LFA-1, which is expressed constitutively on resting leukocytes can be seen with no measurable increase in LFA-1 molecule numbers. This is mediated via a change in the avidity of the molecule for its ligand, ICAM-1, a rapid process that occurs within minutes (Springer, 1990). Mac-1 expression, is rapidly increased upon exposure to a variety of inflammatory stimuli (Jutila *et al.*, 1989), a response thought to be important in neutrophil aggregation (Vedder & Harlan, 1988), rather than in adherence to endothelium which is mediated by a conformational change like that of LFA-1. Pretreatment, *in vitro*, of radiolabelled neutrophils with anti-CD18 mAB 60.3 has been shown to inhibit their accumulation in skin, in response to various inflammatory stimuli including; RPA reactions, zymosan, and ZAP (Nourshargh *et al.*, 1989).

In this paper we have demonstrated that oedema formation and ^{111}In-RBC accumulation produced in the RPA reaction, and by zymosan and ZAP in rabbit skin is abolished by systemic treatment with the anti-CD18 mAb R15.7. We have found that mAb R15.7 did not reduce circulating leukocyte numbers. At 0.5, 2 and 4 h in a typical experiment, circulating leukocyte numbers were 3.0, 4.8 and 3.2×10^6 cells per ml blood, comparing with 2.9, 3.7 and 2.0×10^6 cells ml^{-1} blood in a parallel control. This is in agreement with the findings of Lindbom *et al.* (1990). This indicates that oedema formation and haemorrhage produced in the RPA reaction and in response to zymosan and ZAP are totally dependent upon neutrophil accumulation and/or activation via a pathway that involves β_2 integrins. Treatment with anti-CD18 mAbs partially inhibits immune complex-mediated injury of rat lungs (Mulligan *et al.*, 1992). This inhibition is incomplete, as compared to the total block seen in our rabbit skin experiments, a fact which may be attributed to recruitment of neutrophils in lung by CD18-independent adhesion pathways. It has been demonstrated previously that the cytokines interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) are both involved in immune complex mediated injury of rat lung (Mulligan & Ward, 1992), and that IL-1, but not TNFα, is involved in RPA reactions of rat (Mulligan & Ward, 1992) and rabbit (Issekutz *et al.*, 1992) skin. These cytokines, via direct effects on leukocytes, induction of adhesion pathways, and other actions may permit the cell accumulation and activation responsible for the pathology of complement-mediated lung injury, even in the presence of CD18 blockade.

ICAM-1 is a widely distributed adhesion molecule, existing on a variety of cell types including fibroblasts and endothelial cells (Rothlein *et al.*, 1986), which is important as a receptor for LFA-1 and Mac-1. Expression of ICAM-1 can be upregulated by cytokines including IL-1 and TNF-α (Rothlein *et al.*, 1986). Antibodies to ICAM-1 have been shown to inhibit leukocyte adhesion to and migration through endothelial monolayers *in vitro* (Smith *et al.*, 1988; 1989) and inflammation *in vivo* (Barton *et al.*, 1989; Wegner *et al.*, 1989; Argenbright & Barton, 1991; 1992) We have demonstrated that anti-ICAM-1 mAb RR1/1 inhibits oedema formation and haemorrhage caused by the RPA reaction and zymosan, and the haemorrhage caused by ZAP. In a typical experiment, at 0.5, 2 and 4 h after RR1/1 treatment, circulating leukocyte numbers were 2.7, 2.5 and 2.9×10^6 cells ml^{-1} blood respectively, comparing with values in a parallel control of 2.7, 2.2 and 2.0×10^6 cells ml^{-1} blood. Thus, it is unlikely that any inhibitory effect of mAb RR1/1 is due to depletion of circulating leucocyte numbers. The inhibition of the above responses seen with mAb RR1/1 was significant, but was not total when compared to the block seen with mAb R15.7. The fact that this inhibition is not complete may be due to a number of factors including limited antibody potency, or involvement of other ligands such as components of the complement cascade (Springer, 1990), or other ICAMs (Vazeux *et al.*, 1992; Fawcett *et al.*, 1992). A recent work (Noonan *et al.*, 1993), using another anti-ICAM-1 antibody, R6.5, has shown a non significant, 32% reduction of haemorrhage in the Arthus reaction, which is compared with a significant 83% reduction of haemorrhage in the Shwartz-

man reaction, suggesting greater relative importance of ICAM-1 in the Shwartzman than in the Arthus reaction. A partial effect of anti-ICAM-1 treatment in complement and immune complex-mediated rat lung injury has recently been demonstrated (Mulligan *et al.*, 1993a,b). Another endothelial cell adhesion molecule, ELAM-1, has been shown to play a role in immune complex-mediated reactions in both the lungs and skin of rats (Mulligan *et al.*, 1991).

In addition to our studies with complement-mediated reactions, we have shown that plasma protein leakage in response to PAF and BK both in the presence and absence of PGE_2 are unaffected by anti-CD18 or anti-ICAM-1 treatment. This finding is in accordance with the classification of these mediators as neutrophil independent (Wedmore & Williams, 1981a,b). In conclusion, we find that different complement-mediated reactions of rabbit skin developing over 4 h are totally CD18-dependent, and are also, at least partly ICAM-1-dependent.

We thank the National Asthma Campaign and the Wellcome Trust for financial support. K.E.N. is a Ono Pharmaceuticals UK scholar.

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(Received June 7, 1993
Revised September 6, 1993
Accepted September 17, 1993)

Br. J. Pharmacol. (1994), **111**, 123–130

Pharmacological analysis of neutrophil chemotactic factor production by leucocytes and roles of PAF in allergic inflammation in rats

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1 The role of platelet activating factor (PAF) in neutrophil infiltration in air pouch type allergic inflammation in rats was investigated.

2 Neutrophil infiltration into the pouch fluid 8 h after injection of the antigen (azobenzenearsonate-conjugated acetyl bovine serum albumin) solution into the air pouch of immunized rats was inhibited dose-dependently by treatment with PAF antagonists (CV-3988, L-652,731 and Y-24,180) in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid.

3 Four hours after injection of the antigen solution into the air pouch of immunized and non-immunized rats, there was no significant difference between the two groups in the number of total leucocytes, neutrophils, mononuclear cells and eosinophils in the pouch fluid. However, when the infiltrated leucocytes were incubated in medium, chemotactic factor production by leucocytes from immunized rats was greater than that from non-immunized rats.

4 When leucocytes from non-immunized rats were preincubated for various periods in the medium containing 10 or 50 nM of PAF, washed, and further incubated in the medium containing no PAF, chemotactic factor production was not stimulated.

5 The increase in the chemotactic activity in the conditioned medium was not suppressed by the 5-lipoxygenase inhibitor, AA861. In addition, the chemotactic activity in the conditioned medium was not inhibited by the PAF antagonists.

6 Incubation of the infiltrated leucocytes in the medium containing the protein synthesis inhibitor, cycloheximide, inhibited chemotactic factor production in a concentration-dependent manner in parallel with the decrease in uptake of [^{3}H]-leucine into the acid-insoluble fraction of leucocytes.

7 Separation of the chemotactic activity in the conditioned medium by isoelectric focusing revealed that the leucocyte infiltrated into the pouch fluid produce two kinds of factors, viz. leucocyte-derived neutrophil chemotactic factor-1 (LDNCF-1) and LDNCF-2 of which pI values are 4–5 and above 8, respectively.

8 The results indicate that PAF has no significant role in leucocyte activation to produce chemotactic factors, and that neutrophil chemotactic factors produced by infiltrated leucocytes are not PAF or leukotriene B_4 but are produced through a protein synthesis mechanism.

9 The mechanism of action of PAF antagonists on neutrophil infiltration into the inflammatory locus is discussed.

Keywords: Allergic inflammation; leucocytes; neutrophils; chemotactic factor; platelet activating factor; leukotriene B_4; CV-3988; L-652,731; Y-24,180; AA861

Introduction

Using an air pouch type allergic inflammation model in rats, we have demonstrated (Watanabe *et al.*, 1990) that neutrophil infiltration into the allergic inflammatory site is inhibited in a dose-dependent fashion by treatment with receptor antagonists of platelet activating factor (PAF). Inhibition of leucocyte infiltration by PAF antagonists is also reported in interleukin-1-induced inflammation in rabbit eyes (Rubin & Rosenbaum, 1988). Eosinophil infiltration into bronchial walls after the administration of antigen to passively sensitized guinea-pigs (Lellouch-Tubiana *et al.*, 1987) is also inhibited by treatment with PAF antagonists (Lellouch-Tubiana *et al.*, 1988), as is an antigen-induced bronchial hyper-reactivity model in guinea-pigs (Coyle *et al.*, 1988). These observations suggest that PAF has a significant role in leucocyte infiltration into the inflammatory site. During a series of experiments to clarify the role of PAF in allergic inflammation, we found that treatment with PAF antagonists suppressed neutrophil chemotactic activity in the pouch fluid (the present paper) in parallel with the decrease in neutrophil infiltration into the pouch fluid (Watanabe *et al.*, 1990). This finding encouraged us to extend our research to obtain further insight into the role of PAF in allergic inflammation. The present investigation was designed to determine whether infiltrated leucocytes produce neutrophil chemotactic factors, to determine whether PAF that might be produced in the inflammatory site stimulates neutrophil chemotactic factor production by infiltrated leucocytes, and to characterize pharmacologically the chemotactic factors produced by infiltrated leucocytes. The mechanism of action of PAF antagonists on neutrophil infiltration into the inflammatory locus is also discussed.

Methods

Induction of allergic inflammation

The immunization and induction of allergic inflammation of the air pouch type in rats were carried out as described

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previously (Tsurufuji *et al.*, 1982) with a slight modification. Male Sprague-Dawley rats, specific pathogen-free weighing 140–150 g (Charles River Japan Inc., Kanagawa, Japan) were used. An antigen, azobenzenearsonate-conjugated acetyl bovine serum albumin (ABA-AcBSA) was prepared according to the procedure described by Tabachnick & Sobotka (1962). The rats were immunized by i.d. injection of the antigen and Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Nine days after immunization, 8 ml of air was injected s.c. on the dorsum to form an air pouch in the shape of an ellipsoid. Twenty-four hours after the injection of air, 2 mg of the antigen dissolved in 4 ml of a sterilized solution of 0.8% (w/v) sodium carboxymethylcellulose (CMC-Na, Cellogen F3H; Daiichi Kogyo Seiyaku, Niigata, Japan) in 0.9% NaCl solution was injected into the air pouch to provoke allergic inflammation. A group of rats that were injected i.d. with Freund's complete adjuvant emulsion containing no antigen was challenged in the same way and served as the 'non-immunized rats'. The CMC-Na solution was supplemented with penicillin G potassium and dihydrostreptomycin sulphate (Meiji Seika Co., Tokyo, Japan) each at 0.1 mg ml^{-1} concentration.

In vivo *treatment with PAF antagonists*

Four hours after the antigen challenge, 0.5 ml of saline solution containing various doses of PAF antagonists were injected into the pouches. Rats were then killed 8 h after the antigen challenge by cutting the carotid artery under diethyl ether anaesthesia, and total pouch fluid was collected and measured. A portion of the pouch fluid was used for counting the number of infiltrated leucocytes. The rest of the pouch fluid was diluted four fold with RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.25% (w/v) bovine serum albumin (BSA, essentially fatty acid-free, Sigma Chemical Co., St. Louis, MO, U.S.A.) and centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction was collected and finally diluted six fold with RPMI 1640 medium containing 0.25% (w/v) BSA, and used for measurement of chemotactic activity for neutrophils. The precipitate was suspended in a small volume of the medium and smeared onto a slide. Differential cell counts were performed microscopically after May-Giemsa staining.

Leucocyte collection from the pouch fluid

Four hours after the antigen solution was injected into the air pouch, rats were killed by cutting the carotid artery under diethyl ether anaesthesia and the entire pouch fluid was collected and measured. The pouch fluid was diluted four fold with RPMI 1640 medium containing 0.25% (w/v) BSA and centrifuged at 1,500 *g* and 4°C for 5 min. The precipitate was washed three times with the medium and finally suspended in RPMI 1640 medium containing 0.25% (w/v) BSA at 1×10^7 cells ml^{-1}. A portion of the cell suspension was smeared onto a slide and a differential cell count performed as described above.

Leucocyte culture

Four millilitres of the cell suspension (4×10^7 cells) was incubated at 37°C for various times in the presence or absence of drugs. After appropriate times of incubation, the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction was obtained, finally diluted eight fold with RPMI 1640 medium containing 0.25% (w/v) BSA, and used for the assay of chemotactic activity for neutrophils.

Measurement of chemotactic activity for neutrophils

Chemotactic activity of the diluted pouch fluid or conditioned medium was assayed in a modified Boyden chamber (Watanabe *et al.*, 1985). The upper and lower chambers of multiwell-type Boyden chambers were separated by a polycarbonate filter with pores 2 μm in diameter (Nucleopore Corp., Pleasanton, CA, U.S.A.). Rat peritoneal neutrophils were harvested 12–15 h after injection of 20 ml of Ca^{2+}-free Krebs-Ringer solution containing 1% (w/v) casein (casein from milk, vitamin-free, Wako Pure Chemical Ind., Osaka, Japan), which had been sterilized by autoclaving at 120°C for 15 min. The peritoneal neutrophils were washed twice and suspended in RPMI 1640 medium containing 0.25% (w/v) BSA at 1×10^7 cells ml^{-1}; 300 μl of the cell suspension was applied to the upper chamber. In the lower chamber, 400 μl of the diluted pouch fluid or conditioned medium was added and incubated at 37°C for 80 min in a humidified atmosphere containing 5% CO_2. After incubation, the total number of neutrophils that migrated into the lower chamber was counted with a Coulter counter (Coulter Electronics Ltd., Luton, Beds.). The migration rate was calculated as follows: migration rate (%) = (number of neutrophils in the lower chamber/number of neutrophils applied in the upper chamber) × 100. As a positive control, 400 μl of RPMI 1640 medium containing 0.25% (w/v) BSA and 10 nM PAF (a mixture of C_{16} and C_{18} forms, Avanti Polar Lipid Inc., Birmingham, AL, U.S.A.) was added in the lower chamber, and the migration rate was measured in each experiment. The migration index for each sample was obtained from the following equation and used as an index of chemotactic activity: Migration index (%) = (migration rate for each sample/migration rate for 10 nM PAF) × 100.

Measurement of protein synthesis

Protein synthesis was determined by measuring the incorporation of L-[4,5-^{3}H(N)]-leucine (55.9 Ci $mmol^{-1}$, Du Pont/New England Nuclear, Boston, MA, U.S.A.) into an acid-insoluble fraction (Ohuchi *et al.*, 1981). Four millilitres of leucocyte suspension (1×10^7 cells ml^{-1}) in RPMI 1640 medium containing 0.25% (w/v) BSA, 5 μCi of [^{3}H]-leucine and various concentrations of cycloheximide (Wako Pure Chemical Ind.) was incubated at 37°C for 4 h, and the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction was obtained, diluted eight fold with the medium and used for measurement of chemotactic activity for neutrophils. The precipitate was suspended in 1 M perchloric acid (PCA) solution and centrifuged at 2,500 *g* and 4°C for 15 min; the precipitate was washed three times with 1 M PCA solution. Then the precipitate was dissolved in 0.1 M NaOH solution and transferred into scintillation vials for quantification of radioactivity.

Drug treatment

For *in vivo* treatment, PAF antagonists CV-3988 ((**RS**)-2-methoxy-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazolio) ethyl phosphate) (Terashita *et al.*, 1983), L-652,731 (*trans*-2,5-bis-(3,4,5-trimethoxyphenyl)tetrahydrofuran) (Hwang *et al.*, 1985) and Y-24,180 (4-(2-chlorophenyl)-2-[2-(4-isobutyl-phenyl)ethyl]-6,9-dimethyl-6H-thieno[3,2-f] [1,2,4]triazolo[4,3-a] [1,4]diazepine) (Terasawa *et al.*, 1990) were dissolved in ethanol and diluted with saline solution (final 5% (v/v) ethanol); 0.5 ml of the solution were injected into the pouch. For *in vitro* experiments, the 5-lipoxygenase inhibitor AA861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) (Yoshimoto *et al.*, 1982), PAF antagonists CV-3988, L-652,731 and Y-24,180, and the protein synthesis inhibitor cycloheximide were dissolved in ethanol and an aliquot of the ethanol solution was added into RPMI 1640 medium containing 0.25% (w/v) BSA. The final ethanol concentration in the medium was adjusted to 0.1% (v/v).

Measurement of leukotriene B_4 in the conditioned medium

Leukotriene B_4 levels in the conditioned medium were radioimmunoassayed with a leukotriene B_4 ^{3}H-RIA kit (Du Pont/New England Nuclear). Three millilitres of the conditioned medium to which was added 1×10^{-3} μCi of [^{3}H]-leukotriene B_4 (210 Ci mmol^{-1}, Du Pont, New England Nuclear) were applied to an octadecylsilyl silica cartridge (SEP-PAK C_{18}, Waters Associates, Inc., Milford, MA, U.S.A.). The cartridge was then washed with 10 ml of H_2O, and lipophilic components were eluted with 10 ml of methanol. After evaporation of methanol under reduced pressure, the residue was dissolved in 100 μl of the assay buffer, and an aliquot was used for radioimmunoassay. The rest of the solution was used for the correction of recoveries during the extraction procedure by measuring radioactivity of [^{3}H]-leukotriene B_4.

Separation of chemotactic activity for neutrophils in the conditioned medium by isoelectric focusing

Leucocytes in the pouch fluid from eight immunized rats were collected and combined 4 h after the antigen challenge, and incubated for 4 h at 37°C in RPMI 1640 medium containing 0.25% (w/v) BSA at 1×10^7 cells ml^{-1} concentration. After incubation, the cell suspension was centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant fraction (49 ml) was dialyzed against 10 mM NaCl and mixed with 1 ml of Bio-Lyte ampholytes (pH range 3–10, Bio-Rad Lab., Richmond, CA, U.S.A.). The mixture was loaded into the focusing chamber of the Rotofor cell (Bio-Rad Lab.). Isoelectric focusing was carried out at 12 W constant power for 3 h at 4°C. Twenty fractions were harvested and their pH values measured. Each fraction (2.5 ml) was dialyzed against 1 M NaCl to remove ampholytes, and further dialyzed against phosphate-buffered saline (pH 7.4) at 4°C. Chemotactic activity in each fraction was then determined after six fold dilution with RPMI 1640 medium containing 0.25% (w/v) BSA.

Table 1 Effects of PAF antagonists on the number of infiltrated neutrophils and neutrophil chemotactic activity in the pouch fluid at 8 h

Treatment		*No. of neutrophils* ($\times 10^{-8}$ cells)	*Migration index* (%)
Non-immunized		1.35 ± 0.11**	25.6 ± 2.2**
Immunized		2.91 ± 0.15	76.2 ± 2.5
CV-3988	30 μg	2.21 ± 0.12*	58.9 ± 1.9**
	100 μg	2.05 ± 0.11**	53.1 ± 2.1**
	300 μg	1.72 ± 0.09**	48.2 ± 1.8**
L-652,731	3 μg	2.41 ± 0.08*	62.5 ± 3.1**
	10 μg	2.15 ± 0.15**	55.1 ± 2.1**
	30 μg	1.81 ± 0.12**	46.4 ± 1.9**
Y-24,180	0.03 μg	2.35 ± 0.07*	60.1 ± 2.2**
	0.1 μg	1.90 ± 0.11**	53.2 ± 1.9**
	0.3 μg	1.70 ± 0.13**	45.2 ± 1.7**

Chemotactic activity for neutrophils in the pouch fluid was measured after six fold dilution. Values are the means ± s.e.mean from 5–6 rats. Statistical significance: *$P<0.01$, **$P<0.001$ vs. immunized control.

Statistical analysis

Results were analysed for statistical significance by Dunnett's test for multiple comparison or Student's *t* test for unpaired observations.

Results

Effects of PAF antagonists on neutrophil infiltration and neutrophil chemotactic activity in the pouch fluid at 8 h

When PAF antagonists were injected into the pouch of immunized rats 4 h after injection of the antigen solution, neutrophil infiltration into the pouch fluid at 8 h was suppressed in a dose-dependent manner (Table 1). In parallel with the decrease in neutrophil infiltration, neutrophil chemotactic activity in the pouch fluid was decreased (Table 1). Gradient analysis for neutrophil migration with the diluted pouch fluid demonstrated that movement of neutrophils was not chemokinetic but chemotactic, since migrations in a positive gradient were more effective compared with those in a negative gradient (data not shown). Among the three PAF antagonists, Y-24,180 was the most potent. Because the number of infiltrated mononuclear cells and eosinophils was much smaller than that of neutrophils, the effects of PAF antagonists on the infiltration of these cells were inconclusive (data not shown). When the PAF antagonists were injected into the pouch at the time of antigen challenge, neutrophil infiltration into the pouch fluid at 4 h was not suppressed at all either in immunized or non-immunized rats (number of neutrophils [$\times 10^{-8}$ cells per pouch]: 1.43 ± 0.06 for control, 1.38 ± 0.05 for CV-3988 (100 μg)-treated group, 1.46 ± 0.06 for L-652,731 (10 μg)-treated group, and 1.35 ± 0.06 for Y-24,180 (0.1 μg)-treated group, means ± s.e.mean from 5–7 rats).

Total number of leucocytes in pouch fluid at 4 h

There was no significant difference between immunized and non-immunized rats in total number of leucocytes, neutrophils, mononuclear cells and eosinophils in the pouch fluid collected 4 h after injection of the antigen solution (Table 2). Pouch fluid volume in the immunized rats was significantly higher than that in the non-immunized rats (Table 2).

Neutrophil chemotactic factor production by infiltrated leucocytes

When leucocytes (4×10^7 cells) in the pouch fluid collected 4 h after injection of the antigen solution into the air pouch of immunized and non-immunized rats were incubated at 37°C in 4 ml of RPMI 1640 medium containing 0.25% (w/v) BSA, chemotactic activity for neutrophils in the conditioned medium was increased in a time-dependent manner in both groups (Figure 1). Chemotactic factor production by leucocytes from immunized rats was significantly higher than that from non-immunized rats. The difference between the two groups was apparent even at 0.5 h incubation (Figure 1). Gradient analysis of neutrophil migration with the diluted conditioned medium demonstrated that movement was not chemokinetic but chemotactic, since migrations in a positive gradient were more effective than those in a negative gradient (data not shown).

Table 2 Total number of leucocytes in the pouch fluid collected 4 h after injection of the antigen solution

	Pouch fluid (ml)	*Total number of cells* ($\times 10^{-8}$ cells per pouch) *Total leucocytes*	*Neutrophils*	*Mononuclear cells*	*Eosinophils*
Immunized	3.25 ± 0.14	1.57 ± 0.11	1.32 ± 0.04	0.17 ± 0.02	0.07 ± 0.01
Non-immunized	2.54 ± 0.22*	1.49 ± 0.22	1.28 ± 0.05	0.18 ± 0.01	0.07 ± 0.01

Values are the means ± s.e.mean from 6 rats. Statistical significance: *$P<0.05$ vs. immunized group.

Effects of PAF on chemotactic factor production

To clarify whether leucocytes in the pouch fluid of non-immunized rats are stimulated to produce neutrophil chemotactic factors by PAF treatment, the following experiments were performed. Leucocytes (4×10^7 cells) in the pouch fluid collected 4 h after injection of the antigen solution into the air pouch of non-immunized rats were preincubated at 37°C for 5 to 30 min in 4 ml of the medium containing various concentrations of PAF, washed three times with the medium containing no PAF and further incubated for 4 h in 4 ml of RPMI 1640 medium containing 0.25% (w/v) BSA; chemotactic activity for neutrophils in the conditioned medium was then measured. As shown in Table 3, chemotactic factor production by leucocytes from non-immunized rats was not stimulated by pretreatment with PAF at concentrations of 10 or 50 nM. Failure of stimulation of chemotactic factor production by PAF also was observed in leucocytes from immunized rats (Table 3). In another experiment, leucocytes from non-immunized rats were incubated in the medium containing 10 or 50 nM PAF for 30 min, 1, 2 and 4 h, then neutrophil chemotactic activity in the conditioned medium was measured. In this case, chemotactic activity was measured after addition of the PAF antagonist, L-652,731 to the conditioned medium at a concentration of 20 μM, at which concentration, PAF (50 nM)-induced neutrophil chemotaxis was almost completely suppressed. The results also indicated that PAF treatment did not induce chemotactic factor production (data not shown).

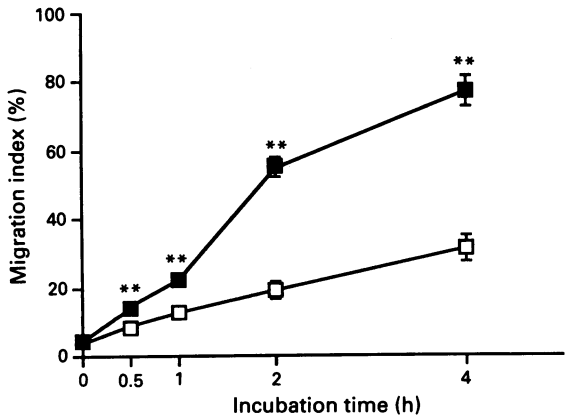

Figure 1 Neutrophil chemotactic factor production by leucocytes in the pouch fluid. Leucocytes were collected from 28 immunized rats (■) and 28 non-immunized rats (□) 4 h after injection of the antigen solution. Leucocytes (4×10^7 cells) from each rat were incubated at 37°C for indicated periods in 4 ml of the medium. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means ± s.e.mean from 5 to 6 rats. Statistical significance: $^{**}P < 0.001$ vs. corresponding non-immunized rats.

Table 3 Effects of PAF on chemotactic factor production by leucocytes in the pouch fluid of non-immunized rats and immunized rats

Leucocytes	*Pretreatment with PAF*	*Migration index (%)*
Non-immunized	None	28.1 ± 2.1
	10 nM, 5 min	29.3 ± 3.1
	10 min	28.8 ± 2.5
	30 min	27.9 ± 3.5
	50 nM, 5 min	30.1 ± 2.2
	10 min	28.9 ± 3.5
	30 min	27.5 ± 2.4
Immunized	None	76.3 ± 4.3*
	10 nM, 5 min	74.9 ± 3.5*
	10 min	75.5 ± 3.9*
	30 min	71.6 ± 4.3*
	50 nM, 5 min	73.2 ± 3.6*
	10 min	77.5 ± 4.2*
	30 min	76.9 ± 5.0*

Leucocytes (4×10^7 cells) from 7 non-immunized rats or 7 immunized rats were preincubated at 37°C for indicated period in 4 ml of the medium containing 10 or 50 nM PAF. Cells were then washed and incubated for 4 h in the medium containing no PAF. Chemotactic activity for neutrophils in eight fold diluted conditioned medium was assayed. Values are the means ± s.e.mean from 4 samples. Statistical significance: $^{*}P < 0.001$ vs. non-immunized control.

Effects of AA861 on chemotactic factor production

Neutrophil chemotactic factor production by leucocytes from immunized rats was not inhibited when various amounts of AA861, a 5-lipoxygenase inhibitor, were added to the incubation medium (Figure 2). Measurement of leukotriene B_4 levels in the conditioned medium by radioimmunoassay revealed that AA861 at these concentrations suppressed leukotriene B_4 production almost completely (leukotriene B_4 levels: 14.2 ± 0.5 pg ml^{-1} for control, compared with less than the detectable amount for AA861-treated groups, mean ± s.e.mean from five samples). Similarly, AA861 failed to suppress chemotactic factor production by leucocytes from non-immunized rats (migration index: 37.5 ± 2.2. for control, 38.9 ± 1.9 for AA861 30.6 μM, mean ± s.e.mean from six samples). AA861 by itself at concentrations up to 30.6 μM showed no effect on neutrophil chemotaxis (data not shown).

Effects of PAF antagonists on neutrophil chemotactic activity in the conditioned medium

If PAF is produced by leucocytes and released into the conditioned medium, the newly synthesized PAF might account for the increased chemotactic activity in the conditioned medium. However, when various amounts of PAF antagonists were added to the diluted conditioned medium of leucocytes from immunized rats and chemotactic activity for neutrophils measured, no significant inhibition was observed at concentrations sufficient to inhibit PAF (10 nM)-induced neutrophil chemotaxis (Figure 3). Among the three PAF antagonists, Y-24,180 was the most potent in inhibiting PAF (10 nM)-induced neutrophil chemotaxis (Figure 3). The addi-

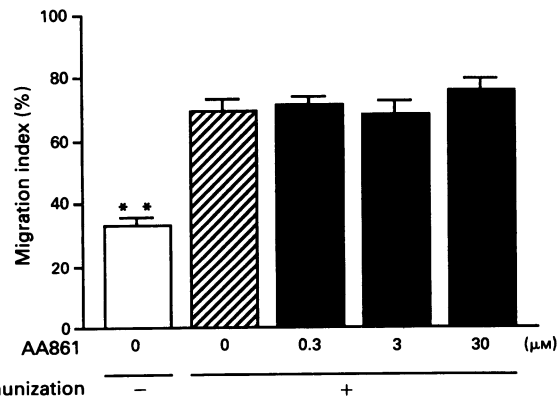

Figure 2 Effects of AA861 on chemotactic factor production by leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (+) and 4 non-immunized rats (−), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing the indicated concentrations of AA861. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means from 4 to 5 samples with s.e.mean. Statistical significance: $^{**}P < 0.001$ vs. immunized control.

tion of PAF antagonists to the conditioned medium of leucocytes from non-immunized rats also failed to inhibit neutrophil chemotaxis (% of control: Y-24,180 at 10^{-8} M; 98.9 ± 1.1, L-652,731 at 10^{-5} M; 99.2 ± 2.2, CV-3988 at 10^{-4} M; 101.2 ± 2.5, mean ± s.e.mean from 5–6 samples).

Chemotactic activity in the lipid fraction of the conditioned medium

Four hours after incubation of the leucocytes from immunized and non-immunized rats, total lipids were extracted from the conditioned medium and reconstituted in the medium. Chemotactic activity of the lipid fraction in the conditioned medium of leucocytes from immunized and non-immunized rats was much lower than that of the original conditioned medium (Figure 4). These results also indicate that participation of lipophilic chemoattractants such as PAF and leukotriene B_4 in the chemotactic activity of the conditioned medium is very low.

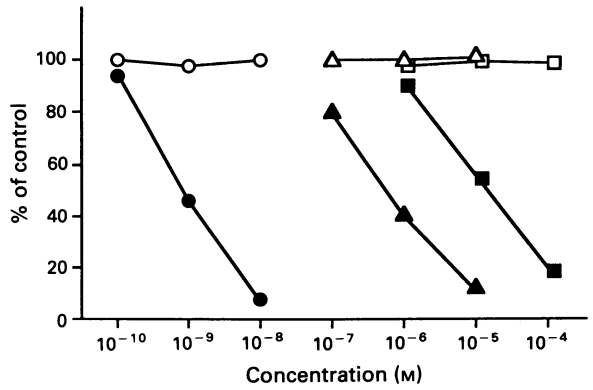

Figure 3 Effects of PAF antagonists on neutrophil chemotaxis induced by PAF and conditioned medium of leucocytes. Leucocytes in the pouch fluid at 4 h were collected and combined from 10 immunized rats, and incubated at 37°C for 4 h in the medium at a concentration of 4×10^7 cells per ml. The conditioned medium was diluted eight fold with fresh medium containing various concentrations of each PAF antagonist, and measured neutrophil chemotactic activity (open symbols). Migration index by eight fold diluted conditioned medium without PAF antagonist is expressed as 100%. Chemotactic activity of the medium containing 10 nM PAF and indicated concentrations of each PAF antagonist also was determined (solid symbols): Y-24,180 (○, ●); L-652,731 (△, ▲); CV-3988 (□, ■). Migration index for 10 nM PAF is taken as 100%. Values are the means from 4 samples; s.e.means are not shown since they are less than 3% of the mean value.

Effects of cycloheximide on chemotactic factor production

When leucocytes in the pouch fluid at 4 h were incubated at 37°C for 4 h in the medium containing various concentrations of cycloheximide, chemotactic factor production was inhibited in a concentration-dependent manner both in immunized and non-immmunized rats (Figure 5). The inhibitory effect of cycloheximide was maximum at 1.78 μM.

Effects of cycloheximide on uptake of [^{3}H]-leucine into acid insoluble fraction of leucocytes

Uptake of [^{3}H]-leucine into the 1 M perchloric acid-insoluble fraction of leucocytes was also inhibited by cycloheximide in a concentration-dependent manner (Figure 6). The time course of the inhibition of chemotactic factor production by cycloheximide at a concentration of 1.78 μM is shown in Figure 7. Almost complete inhibition of neutrophil chemotactic factor production was observed at 0.5 to 4 h of incubation, both in immunized and non-immunized groups.

Separation of chemotactic activity of neutrophils in the conditioned medium by isoelectric focusing

Chemotactic activity of neutrophils in the conditioned medium was separated into two peaks (Figure 8). One is a sharp peak of which the pI value is around 5.5, and the other is rather broad and the pI value is around 9.0. The first one is named leucocyte-derived neutrophil chemotactic factor-1 (LDNCF-1) and the second one is LDNCF-2. pI values of each factor varied from experiment to experiment, ranging 4 to 6 for LDNCF-1 and above 8 for LDNCF-2. When leucocytes were incubated in the presence of cycloheximide

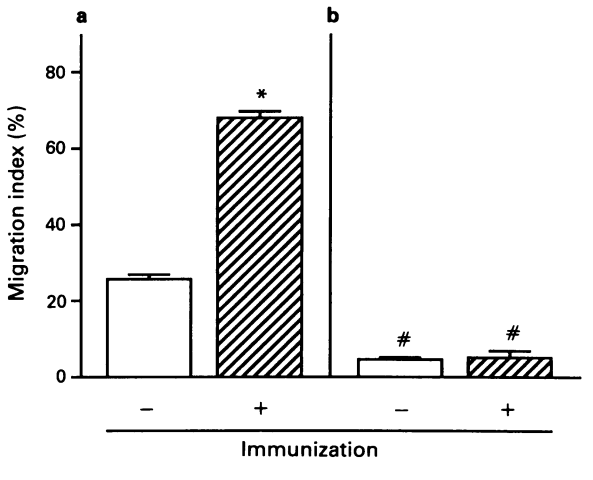

Figure 4 Chemotactic activity of the lipid fraction in the conditioned medium. Leucocytes in the pouch fluid at 4 h were collected and combined from 5 immunized rats (+) and 5 non-immunized rats (−), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium, and chemotactic activity was measured after eight fold dilution (a). Total lipids in the conditioned medium were extracted (Bligh & Dyer, 1959), reconstituted in the medium, finally diluted eight fold, and chemotactic activity was measured (b). Recoveries of [^{3}H]-leukotriene B_4 and [^{3}H]-PAF during the extraction procedure were more than 90% and 85%, respectively. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: $*P<0.001$ vs. corresponding non-immunized control. $\#P<0.001$ vs. corresponding conditioned medium.

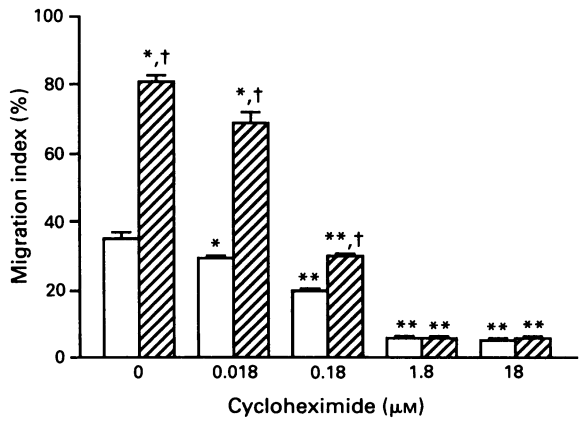

Figure 5 Effects of cycloheximide on chemotactic factor production by leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (hatched columns) and 7 non-immunized rats (open columns), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing indicated concentrations of cycloheximide. Chemotactic activity for neutrophils in the conditioned medium was determined after eight fold dilution. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: $*P<0.01$ and $**P<0.001$ vs. corresponding control. $\dagger P<0.001$ vs. corresponding non-immunized group.

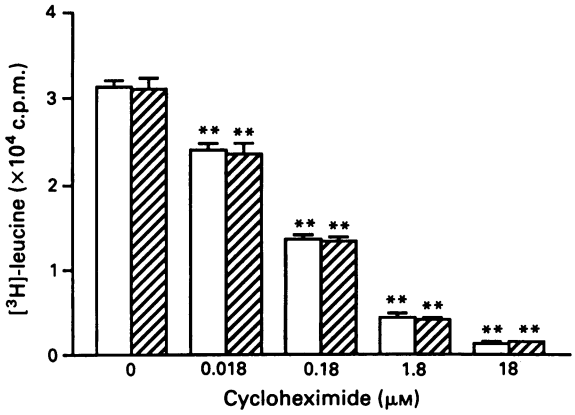

Figure 6 Effects of cycloheximide on [^{3}H]-leucine uptake into 1 M perchloric acid-insoluble fraction of leucocytes in the pouch fluid. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (hatched columns) and 7 non-immunized rats (open columns), respectively; 4×10^7 cells from each group were incubated at 37°C for 4 h in 4 ml of the medium containing 5 μCi of [^{3}H]-leucine, and indicated concentrations of cycloheximide. Values are the means with s.e.mean from 4 to 5 samples. Statistical significance: $^{**}P<0.001$ vs. corresponding control.

(1.78 μM) for 4 h, both peaks of chemotactic activity were diminished (not shown in Figure 8).

Discussion

Treatment with PAF antagonists suppresses neutrophil infiltration into the allergic inflammatory locus in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid (Table 1). Leucocytes that had infiltrated into the allergic inflammatory locus produced neutrophil chemotactic factors much more prominently than leucocytes that had infiltrated into the non-allergic inflammatory locus (Figure 1). These results suggested that leucocytes in the pouch fluid of immunized rats might have been stimulated to produce neutrophil chemotactic factors by a certain chemical mediator, possibly produced by the antigen challenge in the inflammatory locus. Since our previous work (Watanabe *et al.*, 1990) and the present work demonstrated that treatment with PAF antagonists suppresses neutrophil infiltration into the pouch fluid in the allergic inflammation, it was possible that PAF produced in the inflammatory locus might stimulate leucocytes to produce neutrophil chemotactic factors. PAF levels in the pouch fluid collected 4 h after the antigen challenge in the immunized rats, however, were less than the amount detectable by aggregation of guinea-pig platelets (Watanabe *et al.*, 1987; 1988). This result need not imply that PAF is not synthesized at the inflammatory locus, since PAF is easily metabolized into lyso-PAF in the pouch fluid (Watanabe *et al.*, 1987). However, when leucocytes in the pouch fluid from non-immunized rats were preincubated in the medium containing 10 or 50 nM PAF, neutrophil chemotactic factor production was not stimulated at all (Table 3). Pretreatment of leucocytes from the non-immunized rats with PAF at a concentration of 100 nM for 1 h also failed to stimulate chemotactic factor production (migration index (%), 30.2 ± 2.7 for control, 31.5 ± 2.1 for PAF-treated group, means ± s.e.mean from 4 samples). Consequently, it is concluded that the PAF that might be produced in the inflammatory locus does not participate in stimulation of leucocytes to produce neutrophil chemotactic factors.

As to the characteristics of neutrophil chemotactic factors produced by infiltrated leucocytes, although leukotriene B_4 is a potent chemotactic substance derived from arachidonic

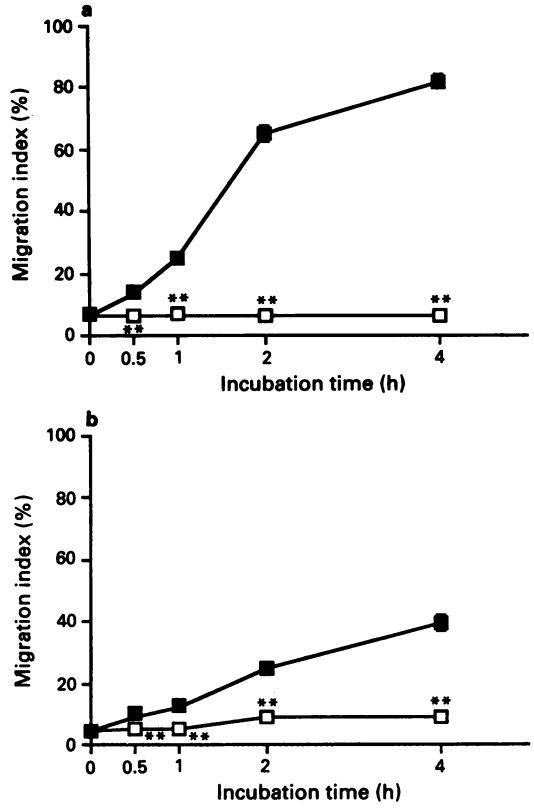

Figure 7 Time course of chemotactic factor production by leucocytes in the pouch fluid and effects of cycloheximide. Leucocytes in the pouch fluid at 4 h were collected and combined from 7 immunized rats (a) and 7 non-immunized rats (b), respectively; 4×10^7 cells were incubated at 37°C for indicated times in 4 ml of the medium with (open symbols) or without (solid symbols) cycloheximide (1.78 μM). Chemotactic activity was determined after eight fold dilution. Values are the means from 3 to 4 samples; s.e.means are not shown since they are within the symbols. Statistical significance: $^{**}P<0.001$ vs. corresponding control.

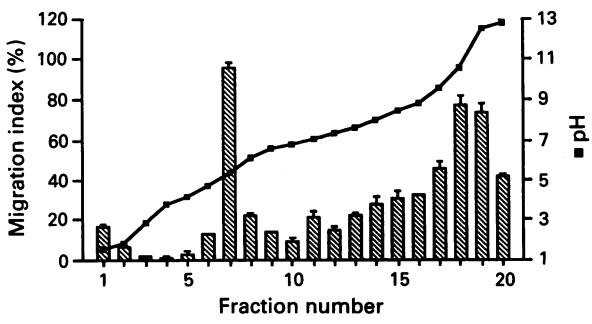

Figure 8 Separation of chemotactic activity for neutrophils in the conditioned medium by isoelectric focusing. Migration index in each fraction was measured after focusing proteins in the conditioned medium of infiltrated leucocytes from immunized rats. Values are the means with s.e.mean from 4 assays. The results were confirmed by four separate experiments.

acid (Ford-Hutchinson *et al.*, 1980; Smith *et al.*, 1980), it was demonstrated that the factor is unlikely to be leukotriene B_4 since neutrophil chemotactic factor production was not inhibited by AA861, a 5-lipoxygenase inhibitor (Figure 2). PAF is also reported to be a potent lipophilic chemoattractant for neutrophils (Shaw *et al.*, 1981). However, neutrophil chemotactic activity in the conditioned medium was not suppressed by the PAF antagonists, CV-3988, L-652,731 and

Y-24,180 (Figure 3). Consequently, neutrophil chemotactic activity in the conditioned medium of infiltrated leucocytes is probably not attributable to PAF. In fact, preliminary experiments revealed that levels of PAF in the conditioned medium measured by bioassay were less than the levels that induce neutrophil chemotaxis *in vitro* (0.1 nM). Furthermore, the finding that the lipophilic fraction obtained from the conditioned medium showed little chemotactic activity (Figure 4) also indicates the minor significance of leukotriene B_4 and PAF as chemotactic factors produced by infiltrated leucocytes. In summary, the present investigation demonstrated that (1) the infiltrated leucocytes are not activated by PAF to produce neutrophil chemotactic factors, and (2) chemoattractants produced by leucocytes are not PAF or leukotriene B_4. Consequently, it still remains to be clarified why PAF antagonists inhibit neutrophil infiltration into the inflammatory locus *in vivo* (Watanabe *et al.*, 1990). In the air pouch type allergic inflammation in rats, no significant infiltration of eosinophils into the pouch fluid is induced. This might be due to the effect of Freund's complete adjuvant used for the sensitization of rats. Consequently, this model is not suitable for determining the effect of PAF antagonists on eosinophil infiltration. The most plausible explanation for the inhibition by PAF antagonists of leucocyte infiltration might be as follows: PAF antagonists would inhibit leucocyte adherence to microvascular endothelial cells in the inflammatory locus, thus inhibiting the succeeding processes such as diapedesis and chemotaxis of leucocytes. This explanantion is based on our findings (Watanabe *et al.*, 1991) that the PAF antagonists, CV-3988, L-651,731 and Y-24,180 inhibit neutrophil adherence to cultured vascular endothelial cells from human umbilical vein stimulated by histamine or thrombin.

When pouch fluid collected 8 h after the antigen challenge from the immunized rats was fractionated into lipophilic and hydrophilic components, chemotactic activity for neutrophils in the lipophilic component was very small. Almost all the chemotactic activity in the pouch fluid was attributable to the hydrophilic component (Omata *et al.*, 1990). These results suggest that neutrophil infiltration into the pouch fluid at 8 h is not mediated by lipophilic substances such as PAF and leukotriene B_4 but by hydrophilic substances that might be produced by infiltrated leucocytes. When the number of peripheral leucocytes was decreased by treatment with cyclophosphamide, neutrophil infiltration into the pouch fluid in immunized rats was lowered in parallel with the decrease in neutrophil chemotactic activity in the pouch fluid (Omata *et al.*, 1991). These observations also suggest that infiltrated leucocytes are responsible for neutrophil chemotactic factor production. *In vivo* treatment with the protein synthesis inhibitor, cycloheximide, inhibits neutrophil infiltration in the air pouch type allergic inflammation in rats (Ohuchi *et al.*, 1982), and eosinophil accumulation in compound 48/80-induced rat pleurisy (Martins *et al.*, 1990) and in PAF-induced rat pleurisy (e Silva *et al.*, 1991). When the infiltrated leucocytes were incubated in the medium containing cycloheximide, neutrophil chemotactic factor production was inhibited (Figure 5) in parallel with the decrease in protein synthesis (Figure 6). These results strongly suggest that infiltrated leucocytes produce neutrophil chemoattractants through protein synthesis mechanisms. It is possible that the chemoattractants in the conditioned medium might be of the interleukin-8 (IL-8) family, since IL-8 is a polypeptide produced by mononuclear phagocytes (Schröder *et al.*, 1987; Walz *et al.*, 1987; Yoshimura *et al.*, 1987; Peveri *et al.*, 1988), macrophages (Rankin *et al.*, 1990), polymorphonuclear leucocytes (Bazzoni *et al.*, 1991) and lymphocytes (Gregory *et al.*, 1988; Schröder *et al.*, 1988). As to the proteinaceous neutrophil chemotactic factor, Watanabe *et al.* (1989) reported that rat kidney epithelioid cell line, NRK-52E, produces a cytokine-induced neutrophil chemoattractant (CINC), a superfamily of IL-8, of which the pI value is above 8. Analysis of chemotactic activity in the conditioned medium by isoelectric focusing revealed that leucocytes infiltrated into the pouch fluid produce two different factors viz. leucocyte-derived chemotactic factor-1 (LDNCF-1) and LDNCF-2 of which the pI values are 4–5 and above 8, respectively (Figure 8). Since pI values of CINC and other proteinaceous chemoattractant, murine macrophage inflammatory protein 2 (MIP-2) (Wolpe *et al.*, 1989), are more than 8, LDNCF-2 might contain CINC, MIP-2 or both. It should be stressed, however, that LDNCF-1 might be a new proteinaceous chemoattractant produced by leucocytes infiltrated into inflammatory lesions.

The mechanism of leucocyte activation in allergic inflammation and the types of leucocytes that produce neutrophil chemotactic factors remain to be elucidated. Further purification and characterization of LDNCF-1 and LDNCF-2 produced by infiltrated leucocytes are under investigation in our laboratories.

This work was supported in part by a Grant-in-Aid for General Scientific Research (02807198 and 05807205 to M.W. and K.O.) and by a Grant-in-Aid for Scientific Research on Priority Areas (05256204 to K.O. and M.W.) from The Ministry of Education, Science and Culture of Japan.

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(Received July 2, 1993
Revised August 23, 1993
Accepted September 17, 1993)

Br. J. Pharmacol. (1994), **111**, 131–136

A study of the mechanism of MDMA ('Ecstasy')-induced neurotoxicity of 5-HT neurones using chlormethiazole, dizocilpine and other protective compounds

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1 An investigation has been made in rats into the neurotoxic effect of the relatively selective 5-hydroxytryptamine (5-HT) neurotoxin, 3,4-methylenedioxymethamphetamine (MDMA or 'Ecstasy') using chlormethiazole and dizocilpine, both known neuroprotective compounds and also γ-butyrolactone, ondansetron and pentobarbitone.

2 Administration of MDMA (20 mg kg^{-1}, i.p.) resulted in a 50% loss of cortical and hippocampal 5-HT and 5-hydroxyindole acetic acid (5-HIAA) 4 days later. This reflects the long term neurotoxic loss of 5-HT that occurs. Injection of γ-butyrolactone (GBL; 400 mg kg^{-1}, i.p.) 5 min before and 55 min after the MDMA provided substantial protection. Pentobarbitone (25 mg kg^{-1}, i.p.) using the same dose regime was also protective, but ondansetron (0.5 mg kg^{-1} or 0.1 mg kg^{-1}, i.p.) was without effect.

3 MDMA (20 mg kg^{-1}) had no significant effect on striatal dopamine concentration 4 days later but did produce a small decrease in 3,4-dihydroxyphenylacetic acid (DOPAC) content. There were few significant changes in rats given MDMA plus GBL, ondansetron or pentobarbitone.

4 A single injection of MDMA (20 mg kg^{-1}, i.p.) resulted in a greater than 80% depletion of 5-HT in hippocampus and cortex 4 h later, reflecting the initial rapid release that had occurred. None of the neuroprotective compounds (chlormethiazole, 50 mg kg^{-1}; dizocilpine, 1 mg kg^{-1}; GBL, 400 mg kg^{-1}; pentobarbitone, 25 mg kg^{-1}) given 5 min before and 55 min after the MDMA injection, altered the degree of 5-HT loss.

5 Acute MDMA injection increased striatal dopamine content (28%) and decreased the DOPAC content. In general, administration of the drugs under investigation did not significantly alter these MDMA-induced changes. Both chlormethiazole and GBL produced a greater increase in dopamine than MDMA alone, but this was apparently an additive effect to the action of either drug alone.

6 The 5-HT loss 4 h following administration of the neurotoxin p-chloroamphetamine (2.5 mg kg^{-1}, i.p.) was not affected by chlormethiazole or dizocilpine. *p*-Chloroamphetamine did not appear to alter striatal dopamine metabolism.

7 None of the protective drugs inhibited the initial 5-HT loss following MDMA, rendering unlikely any proposal that they are protective because they inhibit 5-HT release and the subsequent formation of a toxic indole derivative. All the protective compounds (unlike ondansetron) probably inhibit dopamine release in the striatum. Since the neurotoxic action of some substituted amphetamines is dependent on the integrity of nigro-striatal neurones, this fact may go some way to explain the protective action of this diverse group of compounds.

Keywords: 3,4-Methylenedioxymethamphetamine; chlormethiazole; dizocilpine; γ-butyrolactone; pentobarbitone; neurotoxicity; brain monoamines; neuroprotection; 'Ecstasy'

Introduction

We recently reported that the neurotoxic effects of 3,4 methylenedioxymethamphetamine (MDMA or 'Ecstasy') on 5-hydroxytryptamine (5-HT) terminals in the rat cortex and hippocampus could be prevented by administration of chlormethiazole or dizocilpine (Colado *et al.*, 1993). Both drugs prevented the long term loss of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) which occurs in both brain regions following a single dose of MDMA. In contrast, neither chlormethiazole nor dizocilpine protected against the effect of two other 5-HT neurotoxins, namely *p*-chloroamphetamine (PCA) and fenfluramine (Colado *et al.*, 1993). Others have also recently reported on the failure of dizocilpine to protect against the toxic effect of fenfluramine (Sabol *et al.*, 1992) and PCA (Henderson *et al.*, 1992).

The neurochemical actions of chlormethiazole and dizocilpine in the brain are recognised to be very different. Chlormethiazole, a sedative, hypnotic and anticonvulsant agent (see Evans *et al.*, 1986), potentiates γ-aminobutyric acid (GABA) function (Harrison & Simmonds, 1983; Ogren, 1986; Cross *et al.*, 1989). Dizocilpine, in contrast, is a non-competitive N-methyl-D-aspartate (NMDA) antagonist (Wong *et al.*, 1986). While both biochemical (Cross *et al.*, 1993a) and electrophysiological (Addae & Stone, 1988) studies have indicated that chlormethiazole does not interact with the NMDA receptor complex, it nevertheless antagonizes various NMDA receptor-mediated events such as NMDLA-induced seizures (Cross *et al.*, 1993a), the harmaline-induced increase in cerebellar guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Cross *et al.*, 1993b) and NMDA-induced derangement of sensory evoked potentials (Thorén & Sjölander, 1993).

MDMA administration produces a marked and rapid (3–4 h) depletion of 5-HT in several brain regions (Schmidt, 1987; Gibb *et al.*, 1990). There is a recovery of brain 5-HT concentration by 24 h but this is followed by a long term decrease which is unequivocal by 3–4 days and which lasts for several months (Schmidt, 1987; see also McKenna & Peroutka, 1990). This second phase of monoamine loss reflects neurodegenerative changes that have occurred and

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which have been demonstrated by immunocytochemical and visualisation studies (see Molliver *et al.*, 1990). Similar acute and long term changes in 5-HT occur after administration of both PCA and fenfluramine (Sanders-Bush *et al.*, 1972; Fuller *et al.*, 1975; Harvey & McMaster, 1975; Neckers *et al.*, 1976).

The neurotoxic action of methamphetamine on 5-HT neurones in hippocampus and cortex and dopamine neurones in striatum is dependent on the integrity of nigrostriatal neurones (Schmidt *et al.*, 1985; and see McKenna & Peroutka, 1990). This also seems to be true of MDMA toxicity (Stone *et al.*, 1988; Johnson *et al.*, 1991) even though MDMA is a relatively specific 5-HT neurotoxin (Schmidt *et al.*, 1986; Stone *et al.*, 1986; Schmidt, 1987). Furthermore the short term changes in the concentration of dopamine and its metabolites which occur following MDMA administration have been shown to be blocked by certain neuroprotective agents (Nash, 1990; Schmidt *et al.*, 1990; Callaway *et al.*, 1991).

Since dopamine neurones in the substantia nigra are modulated by both glutamate and GABA (see Dray, 1979) it seemed possible that the neuroprotective action of chlormethiazole and dizocilpine against MDMA-induced toxicity that we observed (Colado *et al.*, 1993) might be due to an inhibition of dopamine release as we have previously suggested to be the case in the investigation of methamphetamine-induced neurotoxicity (Green *et al.*, 1992).

We have now extended our earlier findings (Colado *et al.*, 1993) to examine whether another GABA potentiating drug (pentobarbitone) will also confer protection and also investigated the effect of two compounds known to interfere with dopamine release in the brain, namely γ-butyrolactone (Gessa *et al.*, 1966; Anden *et al.*, 1973) and ondansetron (Butler *et al.*, 1988). The former inhibits dopamine release in the striatum (see for example Chrapusta *et al.*, 1992), while the latter is a 5-HT_3 antagonist (Butler *et al.*, 1988) and inhibits dopamine release in the nucleus accumbens (Kilpatrick *et al.*, 1987; Costall *et al.*, 1987).

A study has also been made of the effects of chlormethiazole, dizocilpine, pentobarbitone, γ-butyrolactone (GBL) and ondansetron on the acute release of 5-HT by MDMA (as measured by the 5-HT and 5-HIAA content in hippocampus and cortex 4 h after MDMA) to determine whether any of the drugs prevented neurotoxicity by inhibiting the release of 5-HT, since it has been proposed that indolic neurotoxins might be formed as a result of the massive 5-HT release which follows administration of substituted amphetamines (Commins *et al.*, 1987a,b; Wrona & Dryhurst, 1991; Wrona *et al.*, 1992) and Azmitia *et al.* (1990) have proposed that toxicity is related to 5-HT release.

Methods

Animals and drug administration

Adult male Lister Hooded rats (Harlan Olac, Bicester) weighing 220–300 g were used. They were housed in groups, in conditions of constant temperature (21°C) and a 12 h light/dark cycle (lights on 07 h 00 min) and given free access to food and water.

The effects of MDMA (20 mg kg^{-1}, i.p.) on cerebral indoleamine content were examined either 4 h or 4 days later. The putative protective agents were always given 5 min before and 55 min after the MDMA injection. The following agents were examined: di-chlormethiazole ethanedisulphonate (50 mg kg^{-1}, i.p.); dizocilpine HCl (1 mg kg^{-1}, i.p.); pentobarbitone Na (25 mg kg^{-1}, i.p.); γ-butyrolactone (400 mg kg^{-1}, i.p.); ondansetron (0.5 and 0.1 mg kg^{-1}, i.p.).

Measurement of monoamines and their metabolites

Rats were killed by cervical dislocation and decapitation, the brains removed and cortex, hippocampus and striatum dissected out. Tissue was homogenized and 5-HT, 5-HIAA, dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) measured by high performance liquid chromatography (h.p.l.c.) with electrochemical detection by the method previously reported in detail elsewhere (Green *et al.*, 1992; Colado *et al.*, 1993).

Drugs

Drugs were obtained from the following sources (in parenthesis): *p*-Chloroamphetamine, γ-butyrolactone, 3,4-methylenedioxymethamphetamine, pentobarbitone Na (Sigma Chemical Co, Poole); dizocilpine HCl (Semat Technical (U.K.) Ltd, St Albans) di-chlormethiazole ethanedisulphonate (Astra Arcus, Södertälje, Sweden), ondansetron (gift from Glaxo Group Research, Ware). All drugs were dissolved in saline (0.9% w/v NaCl) and all doses refer to the concentration of the base.

Statistics

All biochemical data were analysed by analysis of variance (1 way), followed by *post-hoc* 2-tailed *t* tests. Because of slight variation in monamine levels from experiment to experiment, some values have been presented as a percentage change from the control group in the experiment. All statistics were performed on original data, not following percentage transformation.

Results

Effect of pentobarbitone on MDMA-induced neurotoxicity

Administration of MDMA (20 mg kg^{-1}) resulted in a substantial depletion of 5-HT and 5-HIAA in the hippocampus and cortex 4 days later (Figure 1a).

Two injections of pentobarbitone (25 mg kg^{-1}) 60 min apart did not alter cerebral 5-HT or 5-HIAA 4 days later (data not shown) but did attenuate the neurotoxic effect of MDMA on the indole concentrations (Figure 1a), other than the 5-HIAA content in cortex.

Effect of γ-butyrolactone or ondansetron on MDMA-induced neurotoxicity

Neither GBL (400 mg kg^{-1}) nor ondansetron (0.5 mg kg^{-1}) administration altered cerebral indole concentrations in hippocampus or cortex 4 days later (data not shown). However, GBL (400 mg kg^{-1}) given before and after MDMA (20 mg kg^{-1}) provided substantial protection against the neurotoxic depletion of 5-HT and 5-HIAA (Figure 1b).

In contrast, ondansetron either at a dose of 0.5 mg kg^{-1} (Figure 1c) or 0.1 mg kg^{-1} (data not shown) failed to provide any protection.

Effect of MDMA on striatal dopamine and DOPAC content 4 days later and influence of pentobarbitone, GBL and ondansetron

MDMA (20 mg kg^{-1}) injection had no significant effect on striatal dopamine content 4 days later. Pentobarbitone ondansetron and GBL did not alter dopamine content while pentobarbitone produced a modest increase (10%; $P<0.01$). There were no significant changes in animals given these compounds in combination with MDMA (data not shown).

MDMA did produce a decrease in DOPAC content (saline: 2979 ± 220 ng g^{-1} tissue ($n=6$); MDMA: $1882 \pm 99(6)$; $P<0.01$). This change was less pronounced in rats also given pentobarbitone, GBL and ondansetron (data not shown).

Acute effect of MDMA on cerebral 5-HT content

A single injection of MDMA (20 mg kg^{-1}) resulted in a greater than 80% depletion of 5-HT in the hippocampus and cortex 4 h later (Figure 2). The percentage decrease in 5-HIAA content was somewhat smaller in both regions (Figure 2).

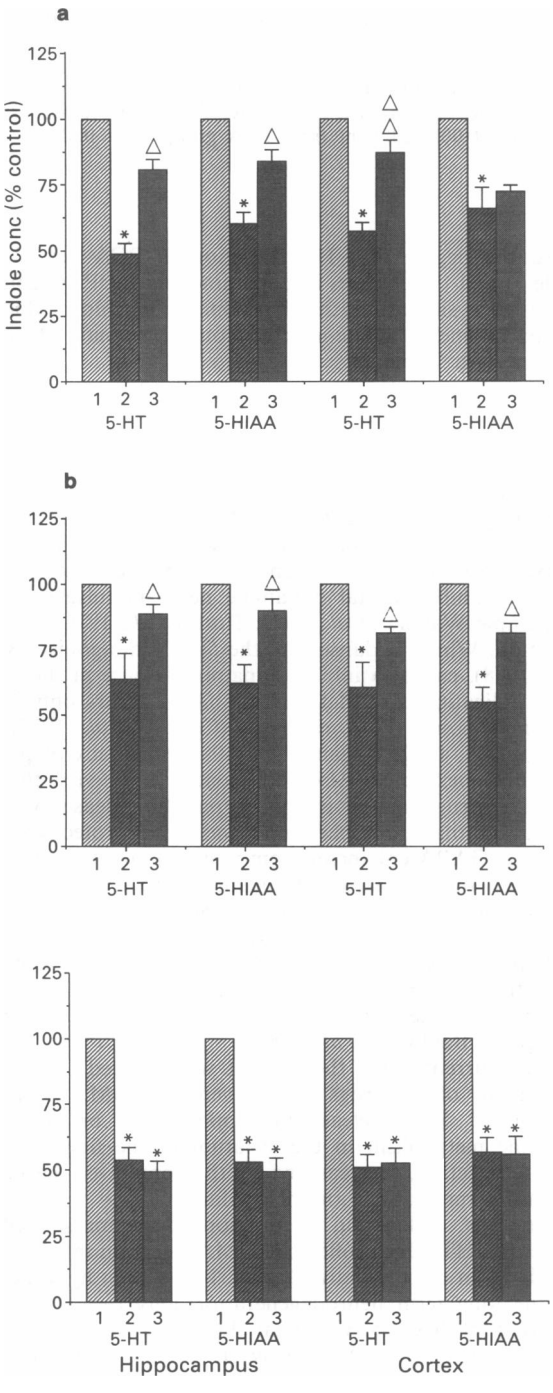

Figure 1 The effect of pentobarbitone, γ-butyrolactone and ondansetron on the 3,4-methylenediomethyoxyamphetamine (MDMA)-induced decrease in hippocampal and cortical indole concentration. Results show % change in brain 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentration compared to saline-injected control animals; shown as mean ± s.e.mean ($n = 5-6$). Group (1) saline injected; group (2) MDMA injected; group (3) MDMA + experimental drug injected. (a) Effect of pentobarbitone (25 mg kg^{-1}); (b) effect of γ-butyrolactone (400 mg kg^{-1}) and (c) effect of ondansetron (0.5 mg kg^{-1}) given 5 min before and 55 min after MDMA (20 mg kg^{-1}). Different from saline: *$P<0.001$; different from MDMA: $^{\Delta}P<0.05$; $^{\Delta\Delta}P<0.01$.

Effect of the chlormethiazole, dizocilpine, pentobarbitone, GBL and ondansetron on the MDMA-induced decrease of 5-HT in the brain

None of the compounds under investigation altered the 5-HT or 5-HIAA content of the hippocampus or cortex 4 h later with the exception of chlormethiazole which produced an increase in 5-HT content of hippocampus and cortex of approximately 20% (data not shown) and γ-butyrolactone which produced a similar increase in 5-HIAA content in both these regions. Furthermore none of the compounds influenced the acute decrease of 5-HT and 5-HIAA content induced by MDMA injection in either brain region (Figure 2) with the exception of GBL which produced a modest attenuation of the fall in 5-HIAA content (Figure 2).

Effect of MDMA on striatal dopamine and DOPAC concentrations and the action of chlormethiazole, dizocilpine, pentobarbitone, GBL and ondansetron

Administration of chlormethiazole, dizocilpine and GBL increased striatal dopamine concentrations 4 h later (Table 1).

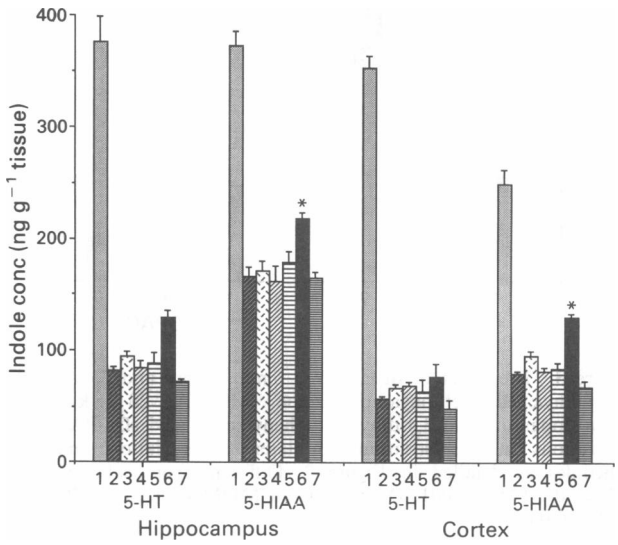

Figure 2 Effect of drugs given 5 min before and 55 min after 3,4-methylenediomethyoxyamphetamine (MDMA, 20 mg kg^{-1}) on brain indole concentration 4 h later. Column (1) saline injected; (2) MDMA; (3) MDMA + chlormethiazole (50 mg kg^{-1}); (4) MDMA + dizocilpine (1 mg kg^{-1}); (5) MDMA + pentobarbitone (25 mg kg^{-1}); (6) MDMA + γ-butyrolactone (400 mg kg^{-1}); (7) MDMA + ondansetron (0.5 mg kg^{-1}). Decrease in brain indole concentration different from control ($P<0.001$) after all treatments. *Different from MDMA alone: $P<0.05$. Results shown as mean ± s.e.mean ($n = 5-6$).

Table 1 Effect of various compounds on striatal dopamine and 3,4-dihydroxyphenylacetic acid DOPAC concentrations 4 h later

	Striatum	
	Dopamine	*DOPAC*
Saline	7161 ± 240 (5)	3212 ± 143 (5)
Chlormethiazole	9539 ± 436 (5)**	1247 ± 94 (5)**
Dizocilpine	8987 ± 80 (5)*	2100 ± 158 (5)**
Pentobarbitone	7794 ± 348 (6)	2569 ± 114 (6)*
γ-Butyrolactone	10495 ± 732 (4)*	3294 ± 391 (5)
Ondansetron	6749 ± 318 (6)	2947 ± 103 (6)

Rats were injected with the compounds under investigation 5 min before and 55 min after a saline injection and striatal dopamine and DOPAC concentration measured 4 h after the saline injection. Different from saline: *$P<0.05$; **$P<0.01$.

Table 2 Monoamine and metabolite levels in rat brain 4 h after *p*-chloroamphetamine (PCA) and effect of chlormethiazole and dizocilpine

		Hippocampus		*Cortex*		*Striatum*	
Treatment	n	*5-HT*	*5-HIAA*	*5-HT*	*5-HIAA*	*Dopamine*	*DOPAC*
Saline	4	363 ± 15	378 ± 8	369 ± 22	220 ± 9	7882 ± 330	2073 ± 43
PCA	5	100 ± 5**	172 ± 10**	63 ± 3**	82 ± 3**	8613 ± 227	2269 ± 155
PCA + chlormethiazole	5	125 ± 4**	220 ± **†	75 ± 3**	116 ± 4**†	11223 ± 403**†	1011 ± 25†
PCA + dizocilpine	5	105 ± 7**	172 ± 9**	62 ± 5**	89 ± 3**	10160 ± 285*	1920 ± 108

Rats were injected with saline or PCA (2.5 mg kg^{-1}) and monoamine levels measured 4 h later. Rats treated with chlormethiazole (50 mg kg^{-1}) or dizocilpine (1 mg kg^{-1}) were given the drug 5 min before and 55 min after the PCA. Results expressed as mean ± s.e.mean in ng g^{-1} tissue. Different from saline group: *$P<0.01$; **$P<0.001$; different from PCA group: †$P<0.05$.

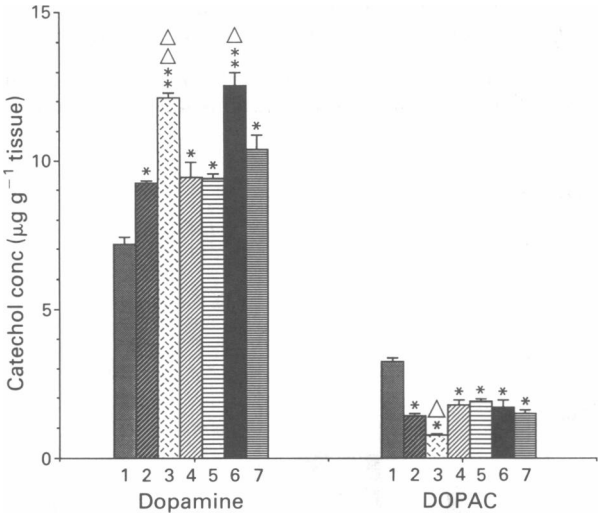

Figure 3 The effect of drugs given 5 min before and 55 min after 3,4-methylenediomethoxyamphetamine (MDMA, 20 mg kg^{-1}) on brain dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in the striatum 4 h later. Abbreviation and dose schedules as detailed in Figure 2. Different from saline-injected: *$P<0.01$; **$P<0.001$. Different from MDMA-injected, $^{\Delta}P<0.01$, $^{\Delta\Delta}P<0.001$.

Dizocilpine and pentobarbitone both decreased the DOPAC concentration, while chlormethiazole administration resulted in a substantial decrease in the concentration of that metabolite (Table 1).

The administration of MDMA (20 mg kg^{-1}) produced a rise in striatal dopamine content and a decrease in DOPAC concentration (Figure 3). In general, administration of the drugs under investigation in the current study did not significantly alter these changes. Both chlormethiazole and GBL administration to the MDMA-treated animals produced a greater increase than MDMA alone (Figure 3), while the DOPAC concentration in the MDMA plus chlormethiazole-treated rats was lowered by a greater amount than that in rats given MDMA alone (Figure 3). However these changes appeared to be additive (see Table 1 and Figure 3).

The effect of chlormethiazole and dizocilpine on the acute changes in brain monamine content following p-*chloroamphetamine administration*

Four hours after injection of PCA (2.5 mg kg^{-1}, i.p.) the 5-HT concentration in cortex and hippocampus had decreased substantially while dopamine metabolism appeared unaltered (Table 2). Neither chlormethiazole nor dizocilpine altered the degree of 5-HT depletion in PCA-treated rats (Table 2) and the changes in dopamine and DOPAC concentration were similar to those produced by chlormethiazole and dizocilpine in saline-injected control animals (Table 2, compare with Table 1).

Discussion

Consistent with the proposal that chlormethiazole and dizocilpine might protect against MDMA-induced neurotoxicity by inhibiting striatal dopamine release has been the current observation that γ-butyrolactone, a compound known for its ability to inhibit striatal dopamine nerve impulse flow (see for example Walters *et al.*, 1973; Roth *et al.*, 1976) afforded protection. While, ondansetron, the 5-HT$_3$ antagonist (Butler *et al.*, 1988), can also inhibit dopamine release in the brain, all the indications are that this inhibition occurs only in the n. accumbens not the n. caudatus (Costall *et al.*, 1987; Kilpatrick *et al.*, 1987; Hagan *et al.*, 1990) so a lack of protection is not unexpected. Also consistent with the view that the protective drugs might inhibit dopamine release was the observation that pentobarbitone was neuroprotective. This drug is a GABA-enhancing compound thought to act at a site near, but perhaps not identical to that at which chlormethiazole is active (Ogren, 1986; Cross *et al.*, 1989).

However a major problem with the proposals above is the fact that using *in vivo* microdialysis we found only a modest attenuation of dopamine release in the striatum in rats given methamphetamine and chlormethiazole and no effect at all of dizocilpine (Baldwin *et al.*, 1993).

In the current study therefore it was decided to examine whether any of the drugs that prevented MDMA neurotoxicity altered 5-HT release in hippocampus or cortex or dopamine metabolism in the striatum in the first 4 h after MDMA injection, as measured by the content of the neurotransmitter monoamine or metabolite.

There was, in fact, no attenuation of the massive (80%) release of 5-HT which follows MDMA injection in any of the animals given the protective agents. It has been hypothesized that the long term neurodegeneration may result from the formation of toxic 5-HT metabolites such as 5,6- or 5,7-dihydroxytryptamine (Wrona & Dryhurst, 1991) or 5,5-dihydroxy 4,4′ bitryptamine (Wrona *et al.*, 1992) following the substantial release of 5-HT by substituted amphetamines and Commins *et al.* (1987a,b) detected 5,6-dihydroxytryptamine in rat brain after substituted amphetamine administration. However, none of the drugs in the current study appear to be preventing neurotoxicity by blocking 5-HT release.

It does appear that some of the compounds do have acute effects on striatal dopamine metabolism. Chlormethiazole, in agreement with an earlier report (Ogren, 1986), produced both a rise in dopamine and a decrease in DOPAC, probably indicative of inhibition of dopamine release. γ-Butyrolactone

also produced a rise in striatal dopamine, and while it did not appear to decrease striatal DOPAC content, it does have very time-dependent effects, Chrapusta *et al.* (1992) reporting a decrease at 30 min and return by 4 h. Thus chlormethiazole and GBL share distinct similarities. Pentobarbitone and ondansetron however appeared to have little effect. The effect of dizocilpine was small but this is not surprising given that blockade of NMDA receptors can initially enhance dopamine tone (Yoshida *et al.*, 1991; Moore *et al.*, 1993) which would then presumably result in subsequent decrease in dopamine synthesis. The problem with ascribing importance to this change in dopamine biochemistry, at least as produced by chlormethiazole and GBL administration, is that MDMA also increased striatal dopamine content and decreased DOPAC concentration, in agreement with the findings of others (Schmidt *et al.*, 1986; Johnson *et al.*, 1991). Therefore far from opposing the neurochemical actions of MDMA in the striatum, both chlormethiazole and GBL appear to have enhanced them.

While measures of dopamine metabolites do not allow accurate interpretation in terms of dopamine release, it seems likely that all the neuroprotective compounds examined are probably inhibiting dopamine function which may go some way towards explaining the protective action of the diverse group of compounds in the current study. However, they are clearly not reversing the acute effect of MDMA on dopamine and DOPAC content as has been shown to occur following administration of other protective drugs such as 5-HT_2 antagonists (Schmidt *et al.*, 1990) and fluoxetine (Callaway *et al.*, 1991).

We previously reported that neither dizocilpine nor chlormethiazole were able to prevent PCA-mediated neurotoxicity (Colado *et al.*, 1993). The current study has further demonstrated that neither drug altered the marked release of 5-HT induced by PCA injection. Interestingly PCA did not appear to alter dopamine metabolism in the striatum. One wonders therefore if this may go some way to providing an explanation for the failure of chlormethiazole and dizocilpine to provide protection from the neurotoxicity since there may not be a perturbation to this system following PCA.

After many years of research there have been few clues as to the mechanisms by which substituted amphetamines produce their neurotoxic effects. There is however a substantial body of evidence as to what drugs afford protection. In the case of MDMA neurotoxicity the current study strengthens the view that the same group of compounds protect against both methamphetamine and MDMA neurotoxicity while PCA- and fenfluramine-induced neurotoxicity are different.

What is also clear is that in the case of MDMA-induced toxicity, the protective effect of chlormethiazole, barbiturates, GBL and dizocilpine is unlikely to have been due to an inhibition of the initial marked release of 5-HT.

M.C. thanks Astra Ifesa (Spain) for a grant to defray her travel costs.

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(Received August 9, 1993
Revised September 17, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 137–144

Cardiovascular effects of GR117289, a novel angiotensin AT_1 receptor antagonist

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1 The effect of GR117289, an angiotensin AT_1 receptor antagonist, on diastolic blood pressure (DBP) was determined in angiotensin-dependent and angiotensin-independent models of hypertension in rats. In addition, the antagonist profile of GR117289 at angiotensin AT_1 receptors was determined in conscious renal hypertensive rats and conscious normotensive rats, dogs and marmosets.

2 Intra-arterial and oral administration of GR117289 (0.3–3 mg kg^{-1}, i.a.; 1–10 mg kg^{-1}, p.o.) to 6-day left renal artery ligated hypertensive (RALH) rats (DBP > 140 mmHg) produced significant, dose-related reductions in DBP with little apparent effect on heart rate (< 15%). The antihypertensive effect of GR117289 developed progressively over several hours and with some doses persisted for 24–48 h after administration.

3 Administration of GR117289 (1 mg kg^{-1}, i.a.) on 5 consecutive days to RALH rats reduced DBP on each day. The antihypertensive effect of GR117289 was not cumulative as DBP had almost returned to base-line values, 24 h after administration of each dose.

4 A dose of GR117289 (3 mg kg^{-1}, i.a.), which produced a substantial reduction in DBP (about 70 mmHg) in RALH rats, was administered to rats in which blood pressure was elevated either by unilateral renal artery clipping, sustained infusion of angiotensin II (AII), DOCA-salt administration or genetic inbreeding. GR117289 reduced DBP in rats in which the renin-angiotensin system was activated by renal artery clipping or AII infusion but had little effect in normotensive rats, DOCA-salt rats and SHR.

5 Systemic administration of AII to RALH rats and to normotensive rats, dogs and marmosets elicited reproducible pressor responses in all species. Systemic or oral administration of GR117289 (3 mg kg^{-1}) inhibited the pressor responses produced by AII, resulting in parallel, rightward displacements of AII dose-response curves.

6 Maximal displacements of AII dose-response curves occurred 1 h and 1–7 h after systemic and oral administration, respectively. GR117289 produced a 32–246 fold displacement after systemic administration and a 4–12 fold displacement after oral administration. The effect in dogs was short lasting after systemic administration but the effect of GR117289 lasted for up to 24 h in rats and marmosets and for up to 24 h after oral administration in all species. The antagonist activity appeared specific for angiotensin receptors as GR117289 did not inhibit pressor responses to phenylephrine or vasopressin.

7 These experiments demonstrate that GR117289 reduces blood pressure in conscious hypertensive rats after both systemic and oral administration, and is an effective antagonist at angiotensin AT_1 receptors in conscious rats, dogs and marmosets.

Keywords: Conscious rat; conscious dog; conscious marmoset; blood pressure; hypertensive rat models; angiotensin AT_1 receptors; angiotensin AT_1 receptor antagonist; angiotensin pressor responses; GR117289

Introduction

GR117289 (1-[[3-bromo-2-[2-(1*H*-tetrazol-5-yl)phenyl]-5-benzofuranyl]methyl]-2-butyl-4-chloro-1*H*-imidazole-5-carboxylic acid; Figure 1) is a potent, selective, specific, non-peptide antagonist of angiotensin II (AII) at angiotensin AT_1 receptors in the rabbit isolated aorta (Middlemiss *et al.*, 1991; Robertson *et al.*, 1992). In view of the well established profile of action of the angiotensin converting enzyme inhibitors, a compound with the characteristics of GR117289 would be expected to exert significant haemodynamic effects *in vivo*, particularly under conditions in which the renin-angiotensin system is activated. Here we describe the general cardiovascular effects of GR117289 in several animal species, under different experimental conditions. Some of these findings have been reported to the British Pharmacological Society (Hilditch *et al.*, 1991; Hunt *et al.*, 1991).

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Methods

Antihypertensive activity of GR117289

The effect of GR117289 on diastolic blood pressure (DBP) was investigated in a variety of rat models of hypertension.

Angiotensin-dependent models of hypertension

In order to activate the renal renin-angiotensin system and induce hypertension, rats (male, Glaxo AH/A; 215–400 g) were anaesthetized briefly (isoflurane) and the left renal artery was exposed and either ligated close to its junction with the aorta (Cangiano *et al.*, 1979) or partially occluded (Wilson & Byrom, 1939) by the application of a silver clip (0.25 mm gap width). Renal artery ligated rats and renal artery clipped rats were left for 6 days and 21 days, respectively, before use.

Hypertension was also induced in rats (male, Glaxo, AH/A, 280–330 g) by the subcutaneous infusion of AII. Minioosmotic pumps (Alzet) containing AII (8 mg ml^{-1} in 0.9% NaCl solution) were implanted subcutaneously, in anaesthetized rats (isoflurane), in the subscapular region. The

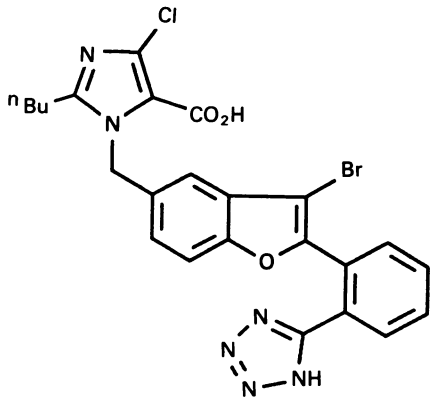

Figure 1 Chemical structure of GR117289.

pump capacity was 235 μl and the infusion rate of AII was 0.48 μl h^{-1}; thus the pumps delivered approximately 200 ng kg^{-1} min^{-1} AII to each rat. Griffin *et al.* (1991) have shown that the hypertension evoked by this technique develops progressively over a period of 5–7 days (slow pressor response) and is sustained.

Angiotensin-independent models of hypertension

Mineralocorticoid hypertension was induced by subcutaneous implantation of a deoxycorticosterone acetate (DOCA) tablet (75 mg) in anaesthetized rats (isoflurane; male, Glaxo AH/A, 70–100 g). The rats were given saline (1% w/v) to drink *ad libitum* for at least 4 weeks after implantation. Spontaneously hypertensive rats (SHR, male; 250–350 g; 9–14 weeks old; Charles River), originating as hypertensive mutants of a Wistar strain (Okamoto & Aoki, 1963), were also investigated.

Measurement of blood pressure

On the day before experimentation, rats were anaesthetized (isoflurane) and a polythene catheter (Portex, o.d. 0.8 mm) was inserted, retrogradely, into the left common carotid artery (Popovic & Popovic, 1960) for subsequent blood pressure recording and, in some cases, drug administration. The cannula was filled with heparinized saline (50 iu ml^{-1}) and was advanced so that its tip lay close to, or within, the aortic arch. The rats were allowed to recover from surgery for use on the following day. Rats were deprived of food for at least 18 h overnight. On the test day, the cannula was connected to a pressure transducer filled with heparinized saline (50 iu ml^{-1}). Arterial blood pressure was recorded in conscious, unrestrained rats and heart rate was derived electronically from the pressure pulse. Control measurements of blood pressure were taken 1 h prior to, and immediately before dosing with GR117289. GR117289 was administered intra-arterially (0.1 ml 100 g^{-1}), via the indwelling arterial cannula and flushed in with saline (0.2 ml). Oral administration of GR117289 was carried out by gavage (1 ml 100 g^{-1}). Subsequent recordings were made 1, 3, 5, 7, 24 and 48 h after dosing.

In a separate series of experiments using renal artery ligated hypertensive (RALH) rats, vehicle or GR117289 (1 mg kg^{-1}, i.a.) was administered daily on 5 consecutive days to rats allowed free access to food. Diastolic blood pressure was measured at about 10 h 00 min on each day, after which the rats were dosed and DBP measured 1, 6 and 24 h later.

Antagonist activity of GR117289

The ability of GR117289 to block angiotensin receptors was examined in RALH rats and in normotensive rats, dogs and marmosets. RALH rats or normotensive rats (male, Glaxo AH/A, 250–350 g) were anaesthetized (isoflurane) and the left common carotid artery cannulated for blood pressure measurement and drug administration as described above. Experiments were carried out the following day. Male beagle dogs (11–15 kg) were anaesthetized (halothane) and surgically prepared with an exteriorized loop of the left common carotid artery for direct measurement of arterial blood pressure (O'Brien *et al.*, 1971). Several weeks later, the dogs were trained to lie quietly in the prone position in a dog hammock. At the start of each experiment a jugular vein was cannulated, by venepuncture, with a saline-filled polythene cannula (Portex, o.d. 0.8 mm) for administration of AII and other drugs. In addition, the carotid loop was cannulated by arterial puncture for blood pressure measurement, with a cannula (Portex, o.d. 0.8 mm) advanced retrogradely into the artery for about 12 cm, such that its tip lay close to, or within, the aortic arch. The lower diastolic pressures of the blood pressure waveforms were measured (Drew *et al.*, 1983).

Marmosets (290–390 g) were anaesthetized (althesin) and polythene cannulae (Portex, o.d. 0.96 mm) containing heparinized saline (250 iu ml^{-1}) were implanted in the right femoral artery and left femoral vein. The animals were allowed to recover from the surgical procedures for at least 48 h before use. On the day of an experiment, marmosets were restrained in horizontal, tubular plastic chambers in a sound-proof box and blood pressure was recorded from the femoral arterial cannula. Three hours after drug administration and measurement of the effects of GR117289, each marmoset was disconnected from the pressure transducer and returned to the holding cage with access to food and water. Two hours later, the marmosets were replaced in the restraining tubes and reconnected to the pressure transducer for a further determination of the effects of GR117289 on blood pressure.

Prior to administration of GR117289, AII was administered by bolus, intra-arterial (rat) or intravenous (dog and marmoset) injection (0.25–3 ml, dependent on species) to produce submaximal, dose-related increases in blood pressure. After each dose, the cannula was flushed with saline (0.2–2 ml). Doses of AII were administered at the start of each experiment to generate a 3–4 point dose-response curve which was repeated 1 h later to generate a second control AII dose-response curve. The doses of AII were administered every 3–10 min; this interval allowed blood pressure to recover to base-line values. In control studies, the reproducibility of AII pressor responses was established by comparing dose-response curves obtained before and after administration of vehicle. Angiotensin II dose-response curves were highly reproducible when constructed 1, 3, 5, 7, 24 and 48 h after administration of vehicle to rats or 1, 3, 5 and 24 h after administration to other species; they varied less than 2 fold from initial dose-response curves.

After establishing control dose-response curves to AII, GR117289 (3 mg kg^{-1}) was administered intra-arterially (rat), intravenously (dog and marmoset) or by gavage (all species). Oral studies were performed in animals deprived of food for about 18 h. Dose-response curves to AII were then re-established, after administration of GR117289, at the same time points used in the vehicle studies. The antagonist activity of GR117289, at each time point in each animal, was expressed in terms of the AII dose-ratio; that is, the ratio of equi-pressor doses of AII in the presence and absence of GR117289. Angiotensin II dose-ratios were calculated from linear portions of the dose-response curves at a response level between 20–50 mmHg. In all studies, geometric mean dose-ratios were calculated from the values obtained from individual animals.

In some studies using RALH rats and normotensive rats, phenylephrine or vasopressin was used in place of AII to produce pressor responses of similar magnitude to AII, in order to determine the specificity of the antagonist effect of GR117289. The experimental protocol was the same as that

adopted when AII was used. Dose-response curves to phenylephrine and vasopressin varied less than 2 fold over the time course of a typical experiment in vehicle-treated rats ($n = 4-5$).

Drugs used

GR117289 was synthesized in the Department of Medicinal Chemistry, Glaxo Group Research Ltd. and dissolved in 2 M sodium hydroxide (0.1 ml per 5–10 mg GR117289) and diluted with saline to volume. Human AII (Calbiochem), phenylephrine hydrochloride (Sigma) and vasopressin (Sigma) were dissolved in distilled water and diluted with saline.

Statistics

Results are expressed as arithmetic mean ± s.e.mean (DBP and heart rate) or geometric mean and 95% confidence limits (dose-ratios) for (n) experiments. Student's paired and unpaired t tests or one-way (blood pressure, dose-range studies) and two-way (blood pressure, repeat administration studies) analysis of variance incorporating Dunnett's test or linear regression analysis (dose-related DBP effect and comparison of dose-ratio with DBP) was used, as appropriate, to determine the significant difference between means. A P value of <0.05 was taken as a significant difference between the means.

Results

Blood pressure studies

Diastolic blood pressure of a sample population of normotensive rats (from which others were selected either for renal artery ligation, renal artery clipping or AII infusion) was 119 ± 2 mmHg ($n = 19$). Blood pressure was not measured in rats prior to implantation of DOCA tablets.

Angiotensin-dependent models of hypertension

Six days after renal artery ligation, many of the animals had a DBP in excess of 140 mmHg and were considered sufficiently hypertensive for experimentation. Rats in which DBP was <140 mmHg were not used. The average group mean DBP of rats used in the study was approximately 160 mmHg (range 140–200 mmHg). Resting heart rate in RALH rats was 480 beats min^{-1} (range 375–550 beats min^{-1}).

Administration of the vehicle (0.1 ml 100 g^{-1}; i.a. or 1 ml 100 g^{-1}, p.o.) used to dissolve GR117289 had no significant effect ($P>0.05$) on DBP (<4%; Figures 2 and 3) or heart rate (<16%) over a 48 h period in RALH rats. Intra-arterial administration of GR117289 (0.3–3 mg kg^{-1}) to RALH rats produced statistically significant ($P<0.05$), dose-related reductions in DBP (Figure 2). Diastolic blood pressure was reduced to, or below, normotensive levels by the two highest doses of GR117289 administered. The peak reduction in DBP occurred 5–7 h after administration and DBP was still significantly reduced ($P<0.05$) by the highest dose used in the study, 24 h after administration. Diastolic blood pressure was not significantly reduced ($P>0.05$), 48 h after administration. Oral administration of GR117289 (1–10 mg kg^{-1}) also produced statistically significant ($P<0.05$), dose-related reductions in DBP (Figure 3). The peak effect of GR117289 on DBP produced after oral administration also occurred 5–7 h after dosing and DBP was still significantly reduced ($P<0.05$), 24 h after administration of GR117289 (3 and 10 mg kg^{-1}) but not at 48 h after administration ($P>0.05$). Lower doses of GR117289 (0.1 mg kg^{-1}, i.a., 0.3 mg kg^{-1}, p.o.) did not significantly reduce DBP ($P>0.05$). Heart rate was not significantly changed ($P>0.05$) by administration of GR117289 by either route of administration (Table 1).

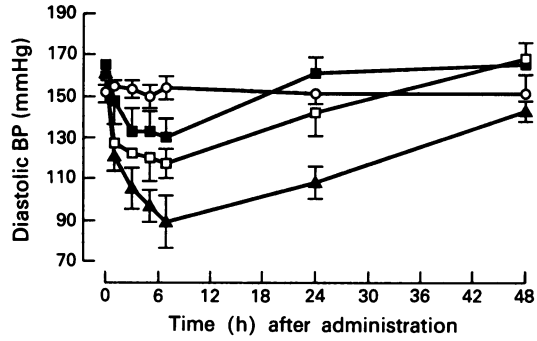

Figure 2 The effect of intra-arterial administration of vehicle, 0.1 ml 100 g^{-1} (○) or GR117289, 0.3 (■), 1 (□) and 3 (▲) mg kg^{-1} on diastolic blood pressure in the conscious renal artery ligated hypertensive rat ($n = 5-6$). Results shown are group means with s.e.mean. Some s.e.mean omitted for clarity.

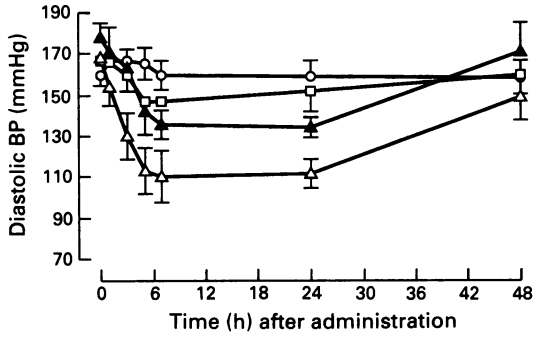

Figure 3 The effect of oral administration of vehicle, 1 ml 100 g^{-1} (○) or GR117289, 1 (□), 3 (▲) and 10 (△) mg kg^{-1} on diastolic blood pressure in the conscious renal artery ligated hypertensive rat ($n = 5-6$). Results shown are group means with s.e.mean. Some s.e.mean omitted for clarity.

The effects of repeated administration of vehicle or GR117289 (1 mg kg^{-1}, i.a.), on 5 consecutive days, were investigated in separate experiments. Vehicle had no significant effect ($P>0.05$) on DBP whereas administration of GR117289 significantly reduced DBP each day ($P<0.05$; Figure 4). Diastolic blood pressure was reduced 1 h after dosing on each occasion but a greater reduction was noted when DBP was measured 6 h after dosing. In addition, although the antihypertensive response obtained on the first day of dosing with GR117289 was larger than that previously observed in the dose-range study (cf. Figure 2) and DBP had not fully recovered before the second dose was administered, subsequent reductions in DBP were much the same as each other. Statistical analysis showed that although the antihypertensive effect of GR117289 on the fifth day of administration was significantly less ($P<0.05$) than on the first day of administration, the antihypertensive effect of GR117289 on the third, fourth and fifth days of administration were not significantly different ($P>0.05$) from each other. Consequently, the effect of GR117289 was not cumulative.

A dose of GR117289 (3 mg kg^{-1}), which produced a reduction in DBP of approximately 70 mmHg after intra-arterial administration to RALH rats, was subsequently chosen to compare the ability of GR117289 to reduce blood pressure in other angiotensin-dependent models and angiotensin-independent models of hypertension. Renal artery clipped rats or rats infused with AII, in which DBP was ⩾ 140 mmHg, were selected and treated with GR117289

Table 1 Effect of administration of GR117289 (10 mg kg^{-1}, p.o. and 3 mg kg^{-1}, i.a.) on heart rate in hypertensive rats

			Hours after administration						
	Route	n	*0*	*1*	*3*	*5*	*7*	*24*	*48*
Renal artery ligated rats	p.o.	6	491 ± 7	471 ± 19	473 ± 14	457 ± 17	448 ± 16	419 ± 19	448 ± 23
	i.a.	6	477 ± 29	523 ± 17	530 ± 16	522 ± 19	507 ± 15	498 ± 18	475 ± 11
Renal artery clipped rats	i.a.	8	469 ± 17	501 ± 14	489 ± 18	491 ± 14	499 ± 11	436 ± 20	421 ± 16
Osmotic mini-pump rats	i.a.	8	555 ± 7	514 ± 12	510 ± 13	501 ± 10	508 ± 7	471 ± 2	495 ± 11
DOCA rats	i.a.	4	433 ± 31	433 ± 31	395 ± 41	418 ± 35	413 ± 41	390 ± 25	388 ± 29
SHR	i.a.	5	466 ± 15	480 ± 21	488 ± 21	480 ± 21	480 ± 18	464 ± 20	426 ± 12

Values in table are group mean values (beats min^{-1}) ± s.e.mean, where n = number of rats in each group.

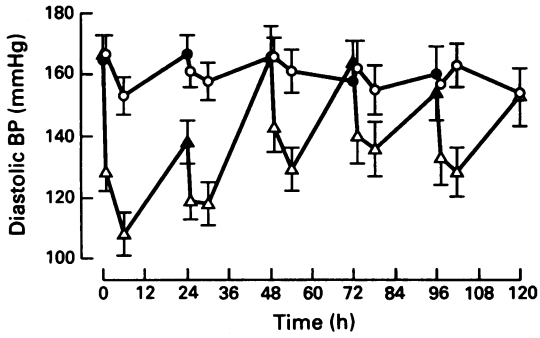

Figure 4 The effect of daily intra-arterial administration of vehicle (○) or GR117289, 1 mg kg^{-1} (△) on diastolic blood pressure in the conscious renal artery ligated hypertensive rat (n = 8–9). Results shown are group means with s.e.mean. Some s.e.mean omitted for clarity. Measurements obtained immediately prior to daily administration shown with filled symbols.

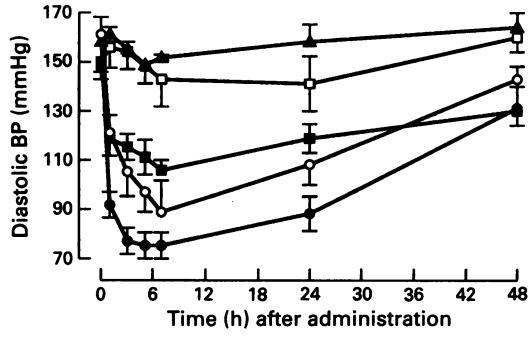

Figure 5 The effect of intra-arterial administration of GR117289 (3 mg kg^{-1}) on diastolic blood pressure in conscious renal artery ligated hypertensive rats (○), angiotensin II (AII)-infused hypertensive rats (●), renal artery clipped hypertensive rats (■), DOCA-salt hypertensive rats (□) and spontaneously hypertensive rats (▲) (n = 4–8). Results shown are group means with s.e.mean. Some s.e.mean omitted for clarity.

(3 mg kg^{-1}, i.a.). GR117289 reduced DBP (Figure 5) and the time of onset of the reduction in blood pressure and the duration of the antihypertensive effect were similar to that which occurred in RALH rats. The magnitude of the reduction in DBP produced by administration of GR117289 to both renal artery clipped rats and AII infused rats was not significantly different ($P > 0.05$) from that seen following administration of GR117289 to RALH rats. GR117289 had no significant effect ($P > 0.05$) on heart rate (Table 1).

Angiotensin-independent models of hypertension

In contrast to its effect in angiotensin-dependent models of hypertension, GR117289 (3 mg kg^{-1}, i.a.) had much less effect on resting DBP in both DOCA-salt rats and SHR (Figure 5) in which mean basal DBP was 150 ± 4 and 158 ± 3 mmHg, respectively. In both DOCA-salt rats and SHR the reduction in DBP produced by GR117289 was not statistically significant ($P > 0.05$). GR117289 had no significant effect ($P > 0.05$) on heart rate (Table 1).

Antagonist activity of GR117289

Administration of AII (10–3000 ng kg^{-1}, i.a., RALH rats; 10–100 ng kg^{-1}, i.a., normotensive rats; 10–300 ng kg^{-1}, i.v., dogs and 15–150 ng kg^{-1}, i.v., marmosets) elicited reproducible, dose-dependent pressor responses (Table 2). Increases in DBP ranged between 0–43 mmHg in RALH rats, 10–50 mmHg in normotensive rats, 12–88 mmHg in dogs and 16–55 mmHg in marmosets. A dose of GR117289 (3 mg kg^{-1}), which produced marked falls in DBP after intra-arterial and oral administration to RALH rats, was administered, in further studies, to RALH rats and to normotensive rats, dogs and marmosets to determine the extent and time course of angiotensin receptor blockade.

Systemic or oral administration of GR117289 reduced resting DBP in RALH rats, to a similar extent as before but produced little effect on resting DBP (< 15 mmHg) over the duration of the experiments in normotensive rats, dogs and marmosets. However, GR117289 reduced the pressor responses to systemically administered AII; dose-response curves to AII were displaced rightwards, in a parallel manner in RALH rats, normotensive rats (Figure 6), dogs and marmosets. The maximal displacements occurred 1 h and 1–7 h after systemic and oral administration, respectively. GR117289 produced maximal 32, 42, 111 and 246 fold displacements in AII dose-response curves in dogs, RALH rats, normotensive rats and marmosets, respectively, after systemic administration but only 4–12 fold displacements after oral administration. Little antagonist activity was apparent 3 h after systemic administration to dogs but antagonist activity was present for up to 24 h after systemic administration to rats and marmosets. Some degree of antagonism was evident for up to 24 h after oral administration to all species. Results obtained in RALH rats are shown in Figure 7. The relationship between DBP and inhibition of AII pressor responses was not linear and there was no significant correlation between these parameters ($P > 0.05$) after either oral or systemic administration. Results from normotensive rats, dogs and marmosets are shown in Figure 8.

Experiments were carried out to establish the specificity of the blocking action of GR117289 in rats. Pressor responses to phenylephrine (1–30 μg kg^{-1}, i.a.) in RALH rats and phenylephrine (1–10 μg kg^{-1}, i.a.) and vasopressin (1–10 ng kg^{-1}, i.a.) in normotensive rats were obtained before and after administration of GR117289 (3 mg kg^{-1}, p.o.). GR117289 did not influence pressor responses to either phenylephrine (Figure 9) or vasopressin (n = 4) in normotensive rats. However, the sensitivity of RALH rats (n = 5) to phenylephrine was significantly increased ($P < 0.05$); dose-

Table 2 Reproducibility of angiotensin II (AII) pressor responses in conscious rats, dog and marmosets

			Hours after administration					
	Route	n	*0*	*1*	*3*	*5*	*7*	*24*	*48*
RALH rats	i.a.	5	1.0	1.2 (0.8–1.9)	1.3 (0.4–5.2)	1.8 (1.0–3.5)	0.8 (0.3–1.9)	0.8 (0.6–1.0)	0.6 (0.1–1.3)
	p.o.	5	1.0	1.4 (0.8–2.3)	1.2 (0.7–1.9)	1.3 (0.9–1.9)	1.3 (0.5–3.4)	1.3 (0.5–3.8)	1.0 (0.4–2.5)
Normotensive rats	i.a.	4	1.0	1.0 (0.5–2.2)	1.2 (0.4–3.4)	1.1 (0.3–5.1)	1.0 (0.5–2.0)	1.4 (0.6–3.1)	1.3 (0.6–2.2)
Dogs	i.v.	4	1.0	0.7 (0.5–1.1)	0.7 (0.5–1.1)	0.7 (0.4–1.0)		0.6 (0.3–1.5)	
Marmosets	i.v.	4	1.0	1.0 (0.6–1.2)	0.8 (0.3–1.9)	1.2 (0.9–3.3)		1.4 (0.6–2.2)	

Values shown are geometric mean values (95% confidence limits) of the displacement of AII dose-response curves calculated from linear portions of the dose-response curves at a response level between 20–50 mmHg following administration of vehicle, where n = number of animals in each group.

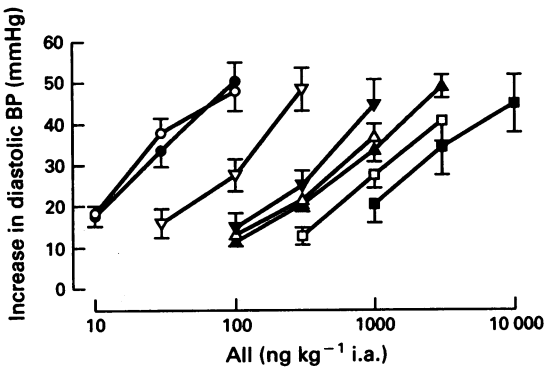

Figure 6 The effect of systemic administration of GR117289 (3 mg kg^{-1}) on pressor responses to angiotensin II (AII) prior to (1 h before ●, and immediately before ○) and 1 (■), 3 (□), 5 (▲), 7 (△), 24 (▼) and 48 h (▽) after administration to conscious normotensive rats. Results shown are group means (n = 5) with s.e.mean. Some s.e.mean omitted for clarity.

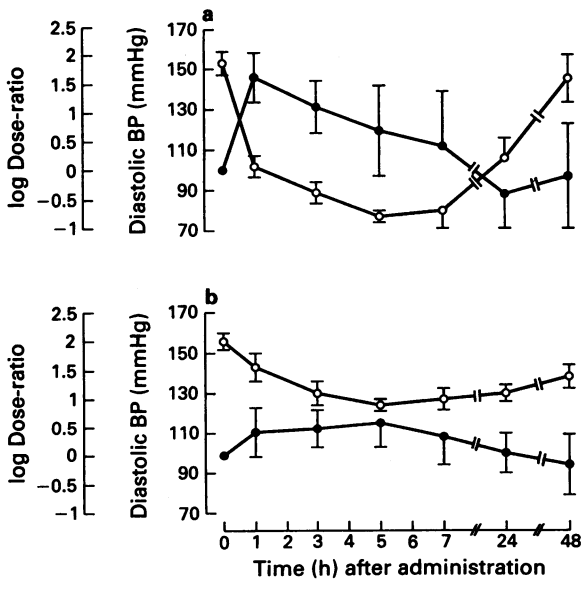

Figure 7 The effect of (a) systemic and (b) oral administration of GR117289 (3 mg kg^{-1}) on angiotensin II (AII) pressor responses (●) and diastolic blood pressure (○) in conscious RALH rats. Results shown are group geometric means (n = 5–6) and represent the displacements of AII dose-response curves (Dose-ratio) or the arithmetic means of diastolic blood pressure. Confidence limits of the geometric mean and s.e. of the arithmetic mean are shown. Some confidence limits omitted for clarity.

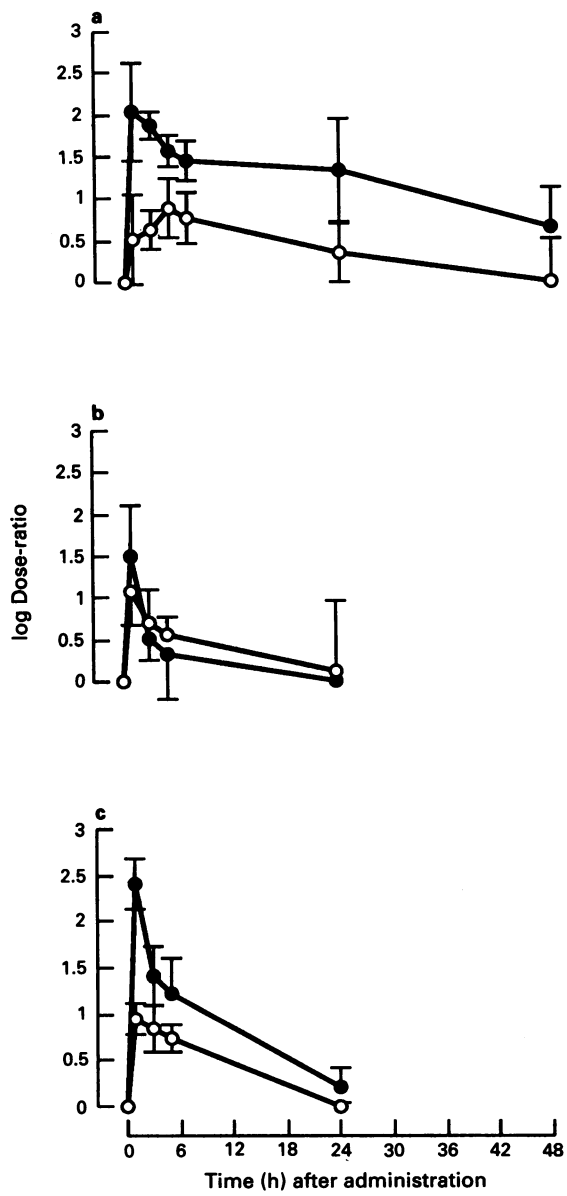

Figure 8 Antagonist effects of systemic (●) and oral (○) administration of GR117289 (3 mg kg^{-1}) to conscious, normotensive (a) rats, (b) dogs and (c) marmosets. Results shown are group geometric means (n = 5–6) with 95% confidence limits and represent the displacements of angiotensin II (AII) dose-response curves with time. Confidence limits of the geometric mean shown by vertical bars. Some confidence limits omitted for clarity.

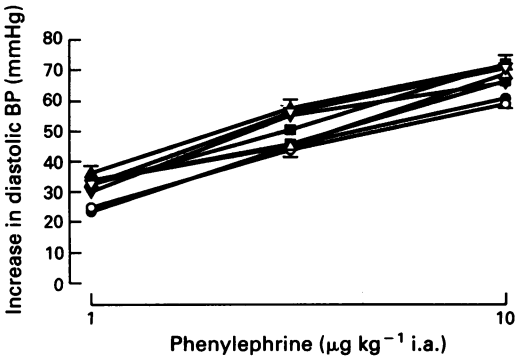

Figure 9 Pressor responses to phenylephrine prior to (1 h before ● and immediately before ○) and 1 (■), 3 (□), 5 (▲), 7 (△), 24 (▼) and 48 h (▽) after oral administration of GR117289 (3 mg kg^{-1}) to conscious normotensive rats. Results shown are group means ($n = 4$) with s.e.mean. Some s.e.mean omitted for clarity.

response curves were displaced about 5 fold to the left, 5–24 h after administration of GR117289, which coincided with the period when its antihypertensive effect was most marked.

Discussion

GR117289 is a potent, selective and specific non-peptide antagonist at angiotensin AT_1 receptors *in vitro* (Marshall *et al.*, 1991; Middlemiss *et al.*, 1991; Robertson *et al.*, 1992). Thus, in keeping with its pharmacological profile, GR117289 produced dose-dependent decreases in blood pressure in rats in which the renal renin-angiotensin system had been activated by unilateral renal artery ligation. The decreases in blood pressure produced by administration of GR117289 were characteristically slow in onset, reaching their peak approximately 5–7 h after either intra-arterial or oral administration. Furthermore, blood pressure was slow to recover, taking up to 48 h to return to pretreatment levels after administration of high doses (3 mg kg^{-1}, i.a. and 10 mg kg^{-1}, p.o.). In addition, a single dose of GR117289 (3 mg kg^{-1}, i.a.) reduced blood pressure in rats in which hypertension had been induced by unilateral renal artery clipping or by the sustained infusion of AII with osmotic mini-pumps. The profile of activity of GR117289, both quantitatively and qualitatively, was similar in both models of renal hypertension and in rats made hypertensive by sustained infusion of AII. In contrast, GR117289 produced much smaller, statistically insignificant decreases in blood pressure in SHR and DOCA-salt hypertensive rats.

It is well established that the hypertension that develops soon after renal artery ligation (Cangiano *et al.*, 1979) or renal artery clipping with the contra-lateral kidney left intact (see Zandberg, 1984; Leenen & Myers, 1984) is a result of renal ischaemia leading to increased plasma renin activity and presumably elevated levels of circulating AII. The hypertension induced by AII infusion is also likely to be the result of stimulation of angiotensin receptors. Thus it seems probable that the marked antihypertensive effect of GR117289 in these models of hypertension reflects blockade of angiotensin receptors. In contrast, little effect of GR117289 was observed in normotensive rats, SHR and DOCA-salt hypertensive rats where plasma renin activity and, usually, tissue renin activity is normal or suppressed (Swales, 1979; Thurston *et al.*, 1981; Asaad & Antonaccio, 1982; Marks *et al.*, 1982; Yamori, 1983). These findings support the conclusion that the antihypertensive effect of GR117289 in RALH rats, renal artery clipped rats and AII infused rats is largely, if not exclusively, attributable to angiotensin receptor blockade. In addition, when GR117289 was administered to RALH rats daily over 5 days, DBP was reduced daily and its effect was not cumulative. Moreover, despite the prolonged and often extensive blood pressure lowering activity, GR117289 appeared to have no effect on resting heart rate in RALH rats. In this respect, the profile of GR117289 is similar to that reported during clinical use of other agents that inhibit the activity of the renin-angiotensin system (Cody, 1984). It should be noted, however, that resting heart rate in the RALH rats and AII infused rats used in these studies was high and, as such, it may not be possible to increase it further. Heart rate appears to be elevated, partially as a consequence of the handling and recording techniques employed and, in addition, as a consequence of the elevation in plasma AII occurring after renal artery ligation or AII infusion. Resting heart rate has been shown to increase following AII infusion in rats by other workers (Guo & Abboud, 1984; Vari *et al.*, 1987).

As it seemed reasonable to attribute the antihypertensive activity of GR117289 primarily to blockade of vascular angiotensin AT_1 receptors, experiments were performed to evaluate the relationship between these parameters. RALH rats were used for these studies but, in addition, normotensive rats were used to measure the angiotensin receptor blocking activity of GR117289, in order to minimize any influence that a reduction in resting blood pressure might have on pressor responses to AII after administration of GR117289. A dose of 3 mg kg^{-1} of GR117289 was used in these experiments because it produced extensive and prolonged falls in blood pressure in RALH rats. GR117289 (3 mg kg^{-1}), administered intra-arterially or orally to RALH rats and to normotensive rats, inhibited the pressor effects of AII, resulting in parallel, rightward displacements of dose-response curves to intra-arterially administered AII. Systemic or oral administration of GR117289 (3 mg kg^{-1}) also pro duced qualitatively similar effects in conscious normotensive dogs and marmosets. In this respect, the profile of action of GR117289 *in vivo* differed from that seen *in vitro* in the rabbit aorta, where GR117289 caused a progressive and extensive suppression of the maximum contractile response to AII, with little rightward displacement (Robertson *et al.*, 1992). This phenomenon, observed *in vitro*, is also displayed by several other angiotensin AT_1 receptor antagonists, including EXP 3174, the potent and active metabolite of losartan (DuP 753; Wong *et al.*, 1990), and is generally attributed to slow kinetics of these compounds at angiotensin AT_1 receptors in a tissue which is believed to have little or no receptor reserve for AII (Liu *et al.*, 1992). Although pressor dose-response curves to AII *in vivo* before and after administration of GR117289 were incomplete, by necessity, there was no sign of suppression of the maximum response, despite extensive displacements of the AII dose-response curves. This was particularly evident in rats. It seems likely that the difference in the profile of GR117289 *in vivo* and *in vitro* is attributable to a much greater receptor reserve for AII in the microvasculature than in the rabbit isolated aorta. Although the degree of AII antagonism was similar at corresponding times after oral administration of GR117289 in all species examined, the duration of the angiotensin receptor blockade after systemic administration was shorter in dogs and marmosets than in rats. The different route of administration (i.a. rats; i.v. dogs and marmosets) could have influenced the duration of activity of GR117289 but this seems unlikely. A more likely explanation is that the pharmacokinetic profiles are different in the various species. Preliminary results confirm that GR117289 is cleared more rapidly by dogs than rats (G.R. Manchee, personal communication).

The findings described above confirm that, *in vivo* as *in vitro*, GR117289 exerts angiotensin AT_1 receptor blocking activity. The question that arises from these findings is whether this property of GR117289 is responsible for its antihypertensive activity. The onset and offset of the antihypertensive activity of GR117289 administered orally or

systemically to RALH rats are both slow; phenomena that are consistent with the slow rates of association of GR117289 with, and dissociation from, angiotensin AT_1 receptors occurring *in vitro* (Robertson *et al.*, 1992). However, close inspection of the angiotensin receptor antagonist activity of GR117289 *in vivo* suggests that the receptor kinetics of GR117289 do not fully explain its antihypertensive activity. For example antagonist activity of GR117289 in RALH rats and normotensive rats was seen for up to 7 h after its oral administration but had almost disappeared after 24 h, despite the well sustained decrease in blood pressure. In addition, the degree of receptor blockade was modest at all times, particularly so when it was estimated in RALH rats (see below). Furthermore, following systemic administration of GR117289 to both conscious RALH rats and normotensive rats, the degree of AII blockade was maximal 1 h after administration despite the fact that the reduction in blood pressure in RALH rats, at this time, was only about half of that achieved at its peak. Over the next 6 h, the degree of AII antagonist activity of GR117289 in RALH rats and normotensive rats declined progressively, whereas the blood pressure in RALH rats continued to decrease. As was found after oral administration, GR117289 appeared to exert less blockade of angiotensin receptors, at each recording interval, in RALH rats than in normotensive rats. Thus, 7 h or more after systemic administration of GR117289 to RALH rats, there was little or no apparent AII antagonism despite a marked reduction in DBP. Consequently it appears that there is no simple temporal relationship between the ability of GR117289 to antagonize AII and reduce DBP. It is unlikely that the explanation for the different time courses described above is a consequence of a non-specific effect of GR117289. The antagonism exerted by GR117289 against AII appears to be mediated specifically at angiotensin receptors, because GR117289 did not reduce vasopressor responses to phenylephrine in RALH rats or normotensive rats. On the contrary, GR117289 appeared to displace dose-response curves to phenylephrine to the left by as much as 5 fold, 5–24 h after administration to RALH rats. A likely explanation for this observation is that the magnitude of the pressor response to phenylephrine is dependent upon the level of the resting blood pressure; as this falls, pressor responses to phenylephrine increase. The same phenomenon could account for why GR117289 appeared to exert a lesser degree of antagonism towards AII in RALH rats than in normotensive rats. That is, the overall position of the dose-response curve to AII is dependent upon the degree of blockade exerted by GR117289 and by the change in resting blood pressure. For this reason, the extent of AII receptor blockade exerted by GR117289 is underestimated in RALH rats and may be measured better in normotensive rats than in hypertensive rats. Pharmacokinetic studies have revealed that in normotensive rats, the plasma concentration of GR117289 parallels its ability to antagonize AII, rather than its capacity to reduce blood pressure in hypertensive rats (G.R. Manchee, personal communication).

The failure of GR117289 to reduce responses to both phenylephrine and vasopressin in normotensive rats supports the view that it is a specific antagonist at angiotensin receptors. A similar specificity of action has been noted *in vitro* (Robertson *et al.*, 1992). It is difficult, at present, to account for the apparent temporal dissociation between blockade of angiotensin receptors *in vivo* and antihypertensive activity. However, this phenomenon is not unique to GR117289. Akers *et al.* (1991) reported a similar situation with losartan. It might be argued that progressive metabolism of losartan to its active metabolite (EXP 3174) is in some way responsible for this effect. This seems unlikely, however, because the metabolite of losartan is also slow to exert its maximum antihypertensive effect (Hodges *et al.*, 1992). Furthermore, such an explanation cannot account for the profile of activity of GR117289, which is excreted largely unchanged in rats (G.R. Manchee, personal communication). In addition, the maximum inhibition of pressor responses to AII in normotensive rats seems to precede, appreciably, the peak antihypertensive effects of EXP 3174 (Wong *et al.*, 1990) and L-158,809 (Siegl *et al.*, 1992). Interestingly, DuP 532 (0.3 mg kg^{-1}) produced a marked reduction in blood pressure (~40 mmHg, 4–6 h after administration) in renal hypertensive rats, but had little effect on pressor responses to intravenous administration of AII in normotensive rats over the same time-course (Wong *et al.*, 1991). In contrast, the antihypertensive effect of SK&F 108566 (10 mg kg^{-1}, i.d.) in renal hypertensive rats began to wane progressively 45–90 min after administration (Brooks *et al.*, 1992), despite sustained inhibition of pressor responses to AII in normotensive rats (Edwards *et al.*, 1992).

Taken together, these data seem to question whether the antihypertensive activity of GR117289 (and other related compounds) is simply attributable to its ability to block angiotensin AT_1 receptors, at least those in vascular smooth muscle. Recently it was suggested (Ohlstein *et al.*, 1992) that this might not be the case for losartan, although no specific alternative property of the molecule was identified that might be responsible for its antihypertensive effect. Since the only known pharmacological property of GR117289 is angiotensin AT_1 receptor blockade, its slow, progressive antihypertensive activity may reflect its accumulation at angiotensin AT_1 receptors at some, as yet, unidentified site(s). At present, we can only speculate on their location. For example, the initial antihypertensive effect of GR117289 may reflect blockade of angiotensin AT_1 receptors in smooth muscle cells close to the vascular lumen. It is probably at these receptors that systemically administered AII acts to evoke vasoconstriction. The more progressively developing antihypertensive effect may reflect the slow accumulation of GR117289 deeper within the vascular wall, where it may antagonize the influence of the local vascular renin-angiotensin system. An alternative possibility is that GR117289 may act at a central site; it clearly gains access to the central nervous system after peripheral administration (Marshall *et al.*, 1992) and it has been reported that sustained systemic administration of AII, to rats, can lead to a maintained hypertension, only if the area postrema is intact (Fink *et al.*, 1987). Thus, the slowly developing antihypertensive effect of GR117289 may reflect progressive accumulation within the central nervous system and subsequent inhibition of AII-induced modulation of central sympathetic outflow. Angiotensin AT_1 receptors are known to be present in those regions of the hind-brain intimately associated with the control of blood pressure (Song *et al.*,1991). These and other possible mechanisms whereby GR117289 may exert its antihypertensive activity will be the subject of further investigation.

In conclusion, GR117289 is a potent, long-lasting, orally active antihypertensive agent, particularly in animals in which blood pressure is elevated as a result of activation of the renin-angiotensin system or by sustained infusion of AII. Its antihypertensive effect is probably attributable to its ability to block angiotensin AT_1 receptors, and such a compound should be valuable in the therapy of hypertension and heart failure.

We would like to thank Jane Wheable and Averil Wythe for their excellent technical assistance and Katherine Shawley for statistical analysis.

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(Received July 26, 1993
Revised September 13, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 145–150

A pharmacodynamic study of SR 47436, a selective AT_1 receptor antagonist, on blood pressure in conscious cynomolgus monkeys

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1 Conscious normotensive cynomolgus monkeys were chronically instrumented for the measurement of arterial blood pressure and heart rate to investigate the relationships between the plasma concentration, suppression of the pressor response to angiotensin II (AII), compensatory increase in plasma AII, and hypotensive effect obtained after a single oral dose of SR 47436, a potent and specific nonpeptide AT_1 receptor antagonist. As blood sampling could influence the hypotensive effect of SR 47436 through activation of the renin angiotensin system (RAS), drug effects were studied in groups of animals with or without blood samplings.

2 SR 47436 at 10 mg kg^{-1} induced a hypotensive effect which was not greater following a second dose of 30 mg kg^{-1}, indicating that a maximal hypotensive effect had already been obtained.

3 A single oral dose of SR 47436 (10 mg kg^{-1}) caused a sustained hypotension and a marked inhibition of the AII-induced pressor response, lasting for up to 28 h. These effects of SR 47436 are consistent with good oral bioavailability and a slow elimination of the drug ($t_{1/2} \approx 20$ h), and were accompanied by a sustained increase in plasma AII concentration. Taken together, both the hypotensive response and the compensatory increase in AII indicated that vascular and juxtaglomerular AII receptors were blocked.

4 Although a fair correlation between individual plasma drug concentrations and inhibition of AII-induced pressor response was observed, neither the hypotensive effect nor the compensatory increase in AII correlated with the plasma drug levels.

5 Basal arterial pressure and AII-induced pressor response were not affected by blood samplings.

6 These results suggest that SR 47436 is an effective and long lasting AT_1 receptor antagonist with a potent hypotensive action in normotensive cynomolgus monkeys. It may be an efficacious blocker of the RAS in man and suitable for once-a-day dosing.

Keywords: Renin angiotensin system; SR 47436; AT_1 receptor antagonist; conscious cynomolgus monkey

Introduction

The renin angiotensin system (RAS) is recognized as playing a pivotal role in cardiovascular regulation by virtue of its action on blood pressure and fluid volume homeostasis (Valloton, 1987; Ferrario, 1990). The discovery of potent angiotensin converting enzyme inhibitors, such as captopril and enalapril, has clearly demonstrated the widespread benefit of drugs targeted as the RAS, especially for the treatment of hypertension and congestive heart failure (Cody, 1986). However, the angiotensin converting enzyme (ACE) is not specific for its substrate, angiotensin I (AI), since it also cleaves kinins and other endogenous peptides such as substance P (Skidgel & Erdos, 1987; Levens *et al.*, 1992). The side effects observed with ACE inhibitors (e.g. cough and angioedema) might be related to this lack of selectivity (Gavras & Gavras, 1988; Levens *et al.*, 1992). These observations have led to the search for drugs which would interfere with RAS at levels other than ACE, such as renin inhibitors and angiotensin II (AII) receptor antagonists.

Recently, a number of nonpeptide AII receptor antagonists has been described (Chiu *et al.*, 1990; Weinstock *et al.*, 1991). One such compound is SR 47436 (2-n-butyl-3-[(2'-(1H-tetrazol-5-yl)biphenyl-4)methyl]-1,3-diaza-spiro[4,4]non-1-en-4-one). It is a potent and highly selective AT_1 receptor antagonist which lacks agonistic activity (Nisato *et al.*, 1992) and is about 10 times more potent than DuP 753 (losartan), both *in vitro* (inhibition of the binding of radiolabelled AII to rat liver membranes and AII-induced contraction of rabbit isolated aorta) and *in vivo* (lowering of arterial pressure and reduction of the pressor response to exogenous AII in conscious cynomolgus monkeys) (Cazaubon *et al.*, 1993).

Since a long duration of action for a once-a-day treatment of hypertension is the ultimate prerequisite for the development of this class of antihypertensive agents, in the present investigation we have followed the time course of the hypotensive effect and the plasma drug levels of SR 47436 for 28 h following a single oral dose in conscious normotensive cynomolgus monkeys. At the same time, the degree of inhibition of blood pressure responses induced by exogenous AII and plasma concentrations of immunoreactive AII were also measured.

Some of the results of this investigation were recently communicated to the British Pharmacological Society (Nisato *et al.*, 1993).

Methods

Preparation of the animals

The experiments were performed in 6 female and 2 male conscious sodium-replete cynomolgus monkeys (*Macaca fascicularis*: body weights, 2.8 to 4.6 kg), obtained from the Centre de Recherches Primatologiques (CRP Le Vallon, Port Louis, Mauritius). Between experimental sessions, the monkeys were kept in individual stainless steel cages equipped with automatic water dispensers. They were maintained on a diet of normal monkey chow (UAR 107C) and fruit and

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housed under constant temperature and lighting conditions (12 h light cycle, 07 h 00 min to 19 h 00 min).

After overnight deprivation of food, the animals were anaesthetized with an intramuscular injection of a combination of acepromazine (0.5 mg kg^{-1}) and ketamine (20 mg kg^{-1}). Under aseptic conditions a silicone catheter (i.d. = 0.64 mm; o.d. = 1.19 mm) filled with an aqueous solution of 40% polyvinylpyrrolidone and 20% heparin (5000 u ml^{-1}) was implanted into a carotid artery. A patch of silicone was sewn around the artery to maintain the catheter which was routed subcutaneously and exteriorized at the top of the head into a stainless steel connector. The connector was fixed to the skull with screws and dental cement and closed with a screw cap to protect it from damage. Intramuscular injections of penicillin (30,000 u kg^{-1}) and dihydrostreptomycin (0.05 g kg^{-1}) were given once daily for 5 days post operatively. After a recovery period of at least 3 weeks, the animals were trained to sit quietly in primate restraining chairs on several different occasions of gradually increasing duration to accustom them to this study.

Arterial blood pressure was recorded by connecting the catheter to a Beckman (R611) polygraph via a P23 ID Gould Statham pressure transducer. Heart rate was determined from the pulsatile arterial pressure signals triggering a cardiotachometer.

The time period between two experiments performed in the same monkey was at least 3 weeks which allowed sufficient time for washout of any residual drug effect and recovery of the animals. Animals involved in the study were cared for and used in accordance with the guide-lines of an internal committee of animal experimentation.

Experimental protocols

Cumulative oral doses of SR 47436 After a resting period of about 40 min to establish steady baseline mean arterial pressure (MAP) and heart rate (HR), 10 mg kg^{-1} of SR 47436 was given orally to 5 monkeys by gavage via a gastric cannula in a volume of 3 ml kg^{-1}. MAP and HR were monitored over 2 h post drug until the animals received an additional 30 mg kg^{-1} of SR 47436. The haemodynamic changes following this last dose were recorded over 1 h.

Single oral dose of SR 47436 After a 40 min equilibration period, SR 47436 was given orally at 10 mg kg^{-1} by gavage with a gastric cannula in a volume of 3 ml kg^{-1}. Control animals received the vehicle only (3 ml kg^{-1}). MAP and HR were monitored 30 min before and for 3 h after the administration of the compound (or vehicle). The monkeys were then returned to their cages. MAP and HR were measured again for 30 min at 6, 9 and 28 h post drug.

The response of blood pressure to a 100 ng kg^{-1} bolus dose of exogenous AII was measured before the treatment and throughout the study period. This dose was chosen to produce a transient blood pressure increase of about 40 mmHg. AII was injected in a volume of 0.25 ml through a catheter inserted acutely into a saphenous vein. This challenge dose was given at 30 min and immediately before and then 0.5, 1, 2, 3, 6, 9 and 28 h after drug administration. The MAP responses to the first two injections of the test dose were averaged and the resulting mean was taken to be the baseline response to AII.

As blood samplings may influence the hypotensive effect of SR 47436 through activation of the RAS, the animals were randomly assigned to the following four experimental groups: group I ($n = 5$): the animals were given the vehicle; group II ($n = 5$): the animals were given the vehicle; arterial blood samples were collected for the measurement of AII plasma levels; group III ($n = 5$): the animals were given SR 47436; no blood samples were taken, and group IV ($n = 5$): the animals were given SR 47436; arterial blood samples were collected for the measurement of AII and drug plasma levels.

Analytical methods Blood samples (3 ml) were drawn via the arterial catheter immediately before and at 0.5, 1.5, 3, 6, 9 and 28 h after oral administration of SR 47436. Blood samplings were always performed immediately before the following AII challenge. The samples were collected in chilled tubes containing Na_2 EDTA (0.1 mg ml^{-1}). These tubes were then placed in melting ice before being centrifuged at 4°C for 15 min at 2500 *g*. The plasma thus obtained was divided into aliquots of 500 µl and quickly frozen at −20°C.

Plasma levels of SR 47436 were determined by a high performance liquid chromatography (h.p.l.c.) method with fluorometric detection (excitation: 254 nm and emission: 371 nm). In brief, 0.5 ml of plasma sample, 0.5 ml of buffer titrisol (pH = 5) and 100 ng internal standard were extracted with 10 ml of ethyl acetate and methylene chloride (50/50; v/v). After 15 min shaking and 8 min centrifugation at 1000 *g* the aqueous phase was discarded. The organic phase was dried with 3 g of anhydrous sodium sulphate and evaporated under a stream of nitrogen at 37°C. The dry residue was reconstituted with 0.15 ml of mobile phase and an aliquot (0.02 ml) was injected on to µBondapak C18 3.9 mm × 300 mm, 10 µm (Interchim). The mobile phase consisted of acetonitrile-buffer, pH = 3.5 (50/50; v/v) with a flow rate of 1 ml min^{-1}. The quantifiable limit was 20 ng ml^{-1} when 0.5 ml plasma sample was used. The precision based on back-calculated concentrations ranged from 1% to 6% and the corresponding accuracies based on the percentage found were 99% to 102%.

Immunoreactive AII was quantitated by a radioimmunoassay using a monoclonal antibody against AII after extraction of plasma (Simon *et al.*, 1992).

Drugs

Angiotensin II was purchased from Sigma Chimie (Saint Quentin Fallavier, France). SR 47436 (mol.wt. = 428.5) was synthesized as free base in the Chemistry Department at Sanofi-Recherche (Montpellier, France). SR 47436 was administered orally in suspension in a 5% gum arabic solution.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (s.e.mean) and the percentages calculated with reference to baseline values.

The following sequence of statistical tests was used: Bartlett's test was applied to assess the heterogeneity of variance. Then an analysis of variance on repeated measures was carried out, followed by a comparison of means by the Dunnett or Duncan test. In the present study a logarithmic transformation according to Tukey (1977) was used for the plasma AII in order to stabilize the variances. A probability value of less than 0.05 was considered statistically significant.

Results

Cumulative oral doses of SR 47436

The effects on MAP and HR are presented in Figure 1. Baseline MAP and HR averaged 115 ± 5 mmHg and 224 ± 7 beats min^{-1}, respectively. Oral administration of SR 47436 at a dose of 10 mg kg^{-1} produced a marked hypotensive effect. A 17% ($P < 0.05$) fall in MAP was still apparent 2 h after dosing. This effect was accompanied by an 8% ($P < 0.05$) decrease in HR. The administration of the additional dose of 30 mg kg^{-1} did not produce any further decrease in blood pressure and the hypotension persisted throughout the observation period (Figure 1). A 14% decrease in HR was observed 30 min after the administration of SR 47436 at 30 mg kg^{-1}; this effect was not significantly different from that observed 2 h after the first dose.

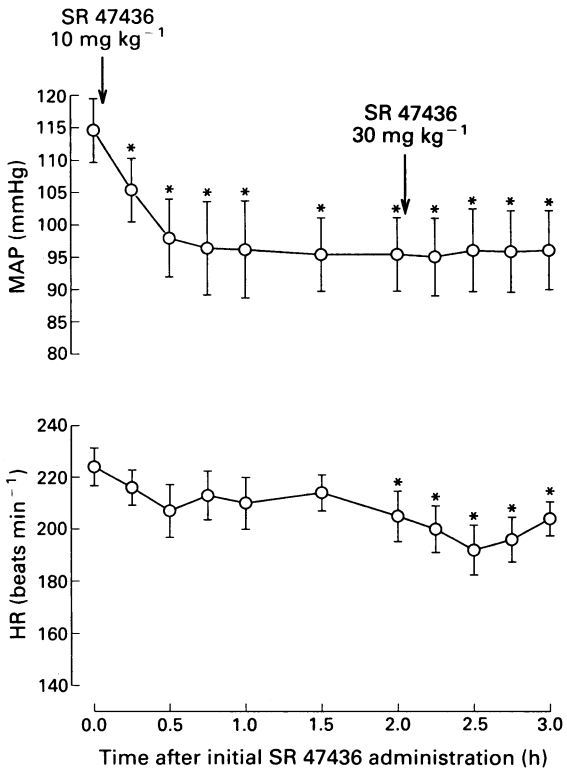

Figure 1 Effects of cumulative oral doses of SR 47436 (10 and 30 mg kg^{-1}) on mean arterial pressure (MAP) and heart rate (HR) in conscious cynomolgus monkeys. Values are mean with s.e.mean ($n = 5$). Significant difference from the baseline values is shown as $^{*}P < 0.05$ (analysis of variance followed by Dunnett's test).

Single oral dose of SR 47436

Response to exogenous AII challenge Baseline MAP responses to exogenous AII ranged from 35 ± 1 to 37 ± 2 mmHg in the four groups and were not statistically different (Table 1). The responses to AII challenge remained remarkably stable during vehicle treatment (groups I and II, Table 1), but they were significantly ($P < 0.05$ compared with the respective baseline and vehicle-control group) inhibited after oral treatment with 10 mg kg^{-1} of SR 47436 (groups III and IV, Table 1). The inhibition reached 56 to 70% between 0.5 to 6 h post drug and was still present 28 h post drug (38 and 44% in groups III and IV, respectively) (Figure 2). It should be noted that blood samplings did not modify the responses to AII challenge, in either treated or control groups (Table 1, Figure 2).

Effects of SR 47436 on blood pressure and heart rate Baseline MAP ranged from 105 ± 3 to 112 ± 3 mmHg in the four groups and these values were not statistically different (Table 1). HR ranged from 186 ± 4 to 205 ± 7 beats min^{-1}. As a significant difference was noted in baseline HR between groups I and II (205 ± 7 versus 186 ± 4 beats min^{-1}) no statistical comparison was performed between these two groups in the course of the study with regard to this variable (Table 1).

SR 47436 administered orally produced a rapid and sustained fall in MAP which remained significantly different from the corresponding baselines up to 28 h post drug, whereas no statistical changes were noted in the two control groups. The maximal hypotension occurred about 2 h post drug in both groups: 19 to 22% ($P < 0.05$ compared with the two respective vehicle-control groups). The hypotensive response to SR 47436 showed a tendency to decline slightly throughout the observation period but after 28 h the animals

Table 1 Time course of the effects of SR 47436 (10 mg kg^{-1} oral; groups III and IV) and placebo (groups I and II) on the pressor response to angiotensin II (AIIR; mmHg), mean arterial pressure (MAP; mmHg) and heart rate (HR; beats min^{-1}) in conscious cynomolgus monkeys; blood samples were withdrawn for the determination of plasma concentrations of SR 47436 (SR; ng ml^{-1}; group IV) and angiotensin II (AII; pg ml^{-1}; groups II and IV)

	Time (h)	*0*	*0.5*	*1*	*1.5*	*2*	*3*	*6*	*9*	*28*
Group I	AIIR	35 ± 1	34 ± 3	35 ± 3	–	35 ± 2	34 ± 2	35 ± 2	34 ± 2	35 ± 1
	MAP	106 ± 4	108 ± 3	106 ± 3	108 ± 4	106 ± 2	107 ± 3	104 ± 4	105 ± 3	105 ± 3
	HR	205 ± 7	207 ± 7	206 ± 7	207 ± 9	213 ± 5	212 ± 5	212 ± 5	213 ± 3	211 ± 7
Group II	AIIR	35 ± 2	36 ± 2	39 ± 2	–	38 ± 2	36 ± 2	38 ± 2	35 ± 2	35 ± 2
	MAP	108 ± 4	108 ± 4	108 ± 4	105 ± 4	105 ± 3	104 ± 4	102 ± 4	103 ± 4	106 ± 5
	HR	186 ± 4[b]	188 ± 4	190 ± 4	185 ± 4	193 ± 5	193 ± 3	194 ± 2	195 ± 5	201 ± 3
	AII	33 ± 7	61 ± 33	–	28 ± 11	–	49 ± 18	25 ± 10	28 ± 14	21 ± 4
Group III	AIIR	37 ± 2	14 ± 2[ab]	11 ± 1[ab]	–	15 ± 2[ab]	16 ± 2[ab]	15 ± 3[ab]	20 ± 3[ab]	23 ± 2[ab]
	MAP	112 ± 3	102 ± 3[a]	97 ± 3[a]	92 ± 3[ab]	93 ± 3[ab]	93 ± 2[ab]	96 ± 5[a]	100 ± 6[a]	99 ± 5[a]
	HR	192 ± 6	176 ± 10[ab]	172 ± 10[ab]	175 ± 12[ab]	173 ± 9[ab]	168 ± 9[ab]	177 ± 9[b]	191 ± 7	192 ± 5
Group IV	AIIR	36 ± 2	12 ± 2[ac]	12 ± 6[ac]	–	14 ± 5[ac]	16 ± 4[ac]	14 ± 2[ac]	14 ± 1[ac]	20 ± 2[ac]
	MAP	105 ± 3	91 ± 4[ac]	86 ± 6[ac]	84 ± 4[ac]	83 ± 2[ac]	87 ± 3[ac]	88 ± 2[a]	92 ± 2[a]	93 ± 3[a]
	HR	195 ± 6	179 ± 9[a]	185 ± 10	180 ± 6[a]	184 ± 7	190 ± 6	188 ± 5	195 ± 6	203 ± 2
	SR	0	1848 ± 574	–	800 ± 255	–	471 ± 114	509 ± 210	427 ± 122	185 ± 37
	AII	66 ± 16	231 ± 107[c]	–	205 ± 63[c]	–	170 ± 51[c]	207 ± 34[c]	163 ± 37[c]	201 ± 37[c]

Values are means ± s.e.mean ($n = 5$) in each group; –, not investigated. [a]$P < 0.05$ versus baseline values (0); [b]$P < 0.05$ versus group I; [c]$P < 0.05$ versus group II; [d]$P < 0.05$ versus group III; analysis of variance followed by the Dunnett's or Duncan's test

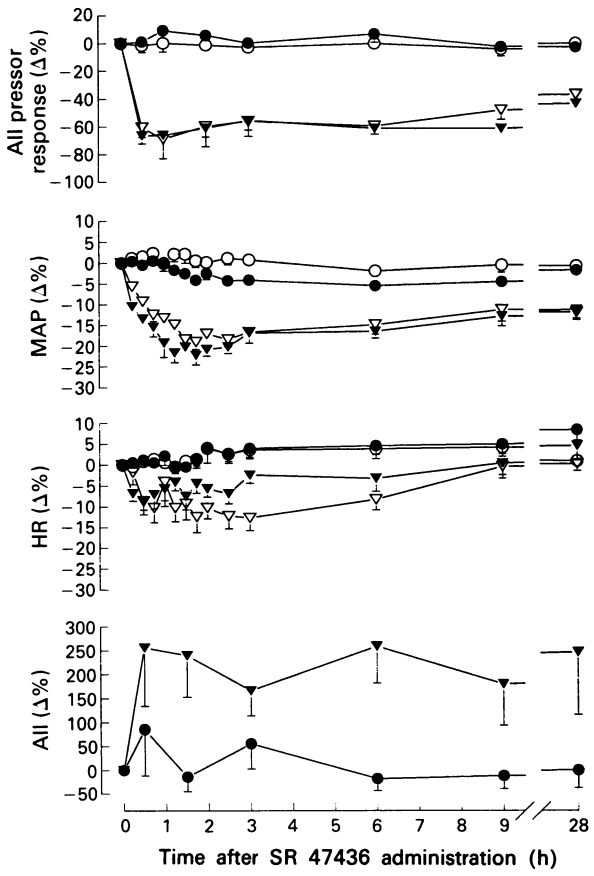

Figure 2 Effects of oral SR 47436 (10 mg kg^{-1}) and vehicle on the pressor response to angiotensin II (AII), mean arterial pressure (MAP), heart rate (HR) and plasma AII concentrations in conscious cynomolgus monkeys; (○) vehicle (group I); (●) vehicle + blood samplings (group II); (▽) SR 47436 (group III); (▼) SR 47436 + blood samplings (group IV). Values are mean with s.e.mean ($n = 5$ in each group).

had not recovered their baseline arterial pressure and the hypotension was still 11 and 12% in groups III and IV respectively. Blood samplings did not modify the hypotensive responses of SR 47436 since no significant difference was observed between these two groups (Table 1, Figure 2).

HR was not significantly changed over the course of the experiment in the two control groups. However, the hypotension induced by SR 47436 was accompanied by a slight bradycardia which occurred mainly in group III (without blood sampling). The maximal decrease in HR in this group was 13% and it was statistically different from baseline as well as the vehicle-control group between 0.5 and 3 h post drug. Bradycardia was also observed in group IV but was less pronounced: 4 to 8% from 0.25 to 2.5 h post drug (NS compared with groups II and III) (Table 1, Figure 2).

Plasma concentrations of AII

Baseline plasma concentrations of AII averaged 33 ± 7 and 66 ± 16 pg ml^{-1} in group II and IV, respectively and were not stastistically different (Table 1). The data showed that plasma AII levels were markedly elevated after oral treatment of SR 47436 and were statistically different from the corresponding values in the vehicle-treated group throughout the study. Plasma AII concentration was maximally increased (250%) at the first post drug blood collection (30 min) and remained elevated up to 28 h (205%) (Figure 2).

Plasma concentrations of SR 47436

The mean concentrations of SR 47436 after 10 mg kg^{-1} oral administration are represented versus time in Table 1. A considerable variability was observed between plasma drug levels in individual monkeys (data not shown), but SR 47436 was readily absorbed with a peak level (1848 ± 574 ng ml^{-1}) already attained at the first post drug blood collection (30 min) in all but one monkey. The drug concentration decreased rapidly (distribution process) to 471 ± 114 ng ml^{-1} until 3 h post drug and then a steady-state was observed for the next 6 h. The drug was still detectable 28 h post drug and the mean plasma concentration was 185 ± 37 ng ml^{-1} which is probably related to a slow elimination process.

The limited number of samplings allowed only a rough assessment of the pharmacokinetic parameters: the apparent terminal half-life was estimated between 11.6 and 40.6 h (20.6 ± 5.2 h), the oral plasma clearance between 0.4 and 1.8 l h^{-1} kg^{-1} (0.80 ± 0.26 l h^{-1} kg^{-1}) and the distribution volume/bioavailability factor between 7.5 and 34.8 l kg^{-1} (21.2 ± 5.3 l kg^{-1}).

To investigate the relationships between SR 47436 plasma levels and both inhibition of AII response and decrease in MAP, scatterplots representing each individual animal were constructed (Figures 3a and b). An overview of these plots suggested a correlation (not tested) between drug levels and inhibition of the AII response whereas no correlation was observed between the drug levels and the decrease in MAP.

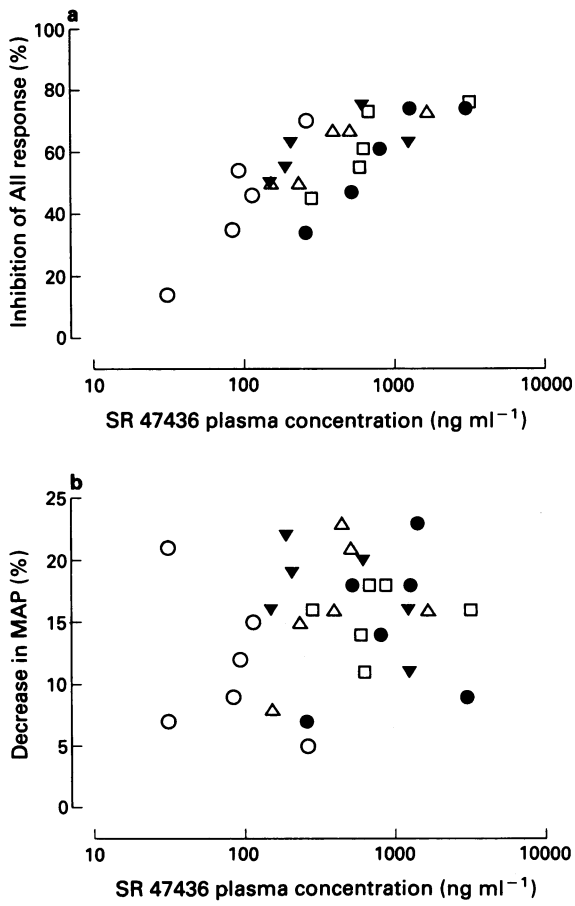

Figure 3 Relationships between drug plasma concentrations and inhibition of the pressor response to angiotensin II (AII) (a) and decrease in mean arterial pressure (MAP) (b) after oral administration of SR 47436 (10 mg kg^{-1}) in conscious cynomolgus monkeys. Individual data are shown and each symbol represents a separate monkey.

Discussion

In normotensive sodium-replete monkeys, the control of the basal arterial pressure is more dependent upon the RAS than in other species, since agents interfering with the RAS such as ACE and renin inhibitors induce hypotensive effects (Michel *et al.*, 1984; Kleinert *et al.*, 1988; Lacour *et al.*, 1989; Mann *et al.*, 1991). More recently, normotensive primates have been used for the investigation of AII receptor antagonists. Although losartan (DuP 753) did not elicit any effect on blood pressure and heart rate in normotensive rhesus monkeys (Gibson *et al.*, 1991), DeGraaf *et al.* (1993) showed that in sodium-replete as well as in sodium-depleted cynomolgus monkeys pretreated with dexamethasone, losartan caused a significant decrease in blood pressure associated with hyper-reninaemia.

In the present investigation, oral administration of SR 47436 (10 mg kg^{-1}) to cynomolgus monkeys induced a hypotensive effect which could not be enhanced by an additional threefold higher dose, indicating that the first dose had already produced a maximal hypotensive response. Furthermore, we had previously shown that lower doses (1 and 3 mg kg^{-1}) of SR 47436 induced weaker hypotensive effects (Cazaubon *et al.*, 1993). The maximal hypotensive response (about 20 mmHg) observed in the present investigation is in accordance with our previous observations concerning ACE or renin inhibitors (Lacour *et al.*, 1989).

The effectiveness of the vascular AII (AT_1) receptor blockade was evaluated by the inhibition of the pressor response to exogenous AII. At the peak drug level a 70% reduction of the pressor response was observed, and at 3 h post drug, when the plasma drug concentration was only a quarter of that at the peak, the blocking effect remained almost unchanged. A more potent inhibitory effect of AII pressor responses was expected at 30 min post-drug since in a previous study (Cazaubon *et al.*, 1993) we had shown that SR 47436, administered at a threefold lower dose, induced an 85% inhibition at the same time. We do not have any reasonable explanation for this discrepancy. In spite of this, a fairly close relationship between individual plasma drug levels and inhibition of the AII pressor response was observed despite the limited number of animals used in this study.

The AT_1 receptor antagonist, SR 47436, induced a significant and sustained decrease in basal arterial pressure, whereas, no change was observed in the vehicle-control groups. The onset of the hypotension was within 15 min of administration, which indicated a rapid absorption in the intestinal tract as confirmed by a high drug level at the first post-drug blood collection (30 min). The long-lasting hypotensive effect and a relatively long biological half-life (about 20 h) seem to be related to a slow elimination of the drug. Furthermore, the hypotensive effect observed after 28 h which corresponds to 50% of the maximal response is also probably due to persistent blockade of the vascular AII receptors, since we observed a significant inhibition of the pressor response to exogenous AII at the same time. However, it should be noted that the maximal hypotensive effect did not occur concomitantly with the peak drug level. Moreover, a lack of relationship between the onset of the AII blockade and the occurrence of the maximal fall in blood pressure was also noted. This last observation is not unique to SR 47436 since Wong *et al.* (1990) showed similar results with losartan in spontaneously hypertensive rats. As suggested by these authors, it may be that the AT_1 receptor antagonists require some time to penetrate completely into the vascular compartment to exert a maximal blockade of endogenous AII-induced vasoconstriction. Nonetheless, other mechanisms involved in the control of blood pressure could delay the maximal decrease in blood pressure. The present data, however, suggest that the blockade of the vasoconstrictor effects of AII on AT_1 receptors is likely to be the primary mechanism of the hypotensive effect of SR 47436.

We clearly showed that the hypotension induced by SR 47436 was accompanied by a marked increase in plasma AII levels which lasted up to 28 h, indicating the inhibition of AT_1 receptors on renal juxtaglomerular cells. It should be noted that the baseline values were about three times higher than those commonly observed in this experimental model; the reason for this discrepancy is unexplained. When the individual plasma drug levels were related to the concomitant plasma AII (not shown) or the hypotensive effect, there were no significant correlations. Moreover, at 28 h post-dose, the plasma concentration of SR 47436 was 430 nM which is about 100 times higher than the 50% inhibitory concentration of the AII (10^{-8} M)-induced vasoconstriction in rabbit isolated aorta (Nisato *et al.*, 1992). Although a direct correlation between functional *in vitro* data and *in vivo* results is open to criticism, the quantitative measurement of the compound in the plasma at 28 h strongly suggests that both the long lasting hypotensive effect and the compensatory increase in plasma AII concentrations are related to the blockade of the vascular and juxtaglomerular AT_1 receptors. On the other hand, the lack of correlation between plasma drug concentrations and circulating levels of AII was also observed with losartan and its active metabolite, EXP 3174, in normal volunteers (Munafo *et al.*, 1992). Nonetheless, the activation of the RAS cascade after treatment with an AT_1 receptor antagonist seems to reflect the acute interruption of the permanent negative feedback of AII on renin release (Brunner *et al.*, 1990). These results are also in agreement with findings obtained both in man and animals with other AT_1 receptor antagonists (Christen *et al.*, 1991; Munafo *et al.*, 1992; Wood *et al.*, 1992).

The hypotension was accompanied at its peak by a slight decrease in HR. A bradycardia is not commonly described after an AT_1 receptor antagonist (neither after ACE nor renin inhibitor) whatever the species studied. However, the use of primate restraining chairs is considered to impose a severe form of restraint which is associated with an increase in heart rate (at least 50 beats min^{-1}) that appears to be the result of persistent sympathetic nervous system stimulation (Adams *et al.*, 1988). In this respect, in unrestrained cynomolgus monkeys, HR values range from 140 to 150 beats min^{-1} (Adams *et al.*, 1988; Mann *et al.*, 1991). Thus, the slight bradycardia observed after SR 47436 might be due to the blunting effect of the AT_1 receptor antagonist on the AII-induced presynaptic facilitation of sympathetic neurotransmission (Zimmerman *et al.*, 1984; Story & Ziogas, 1987; Saxena, 1992). However, other mechanisms, such as an increase of vagal tone or a central effect, cannot be entirely excluded.

Frequent blood samplings could be considered as a mild haemorrhage which may further activate RAS, thus enhancing the hypotensive response to SR 47436. However, blood samplings affected neither basal blood pressure and hypotension nor the blood pressure response to AII in both SR 47436- and vehicle-treated groups.

In conclusion, our results show that after a single oral dose, SR 47436 is an effective and long-acting AT_1 receptor antagonist with a potent hypotensive action in conscious normotensive cynomolgus monkeys. Although a correlation between individual plasma drug concentrations and inhibition of AII pressor response was observed, neither the compensatory increase of AII plasma levels nor the hypotensive effect correlated with plasma drug concentrations. Furthermore, the results of this study suggest that SR 47436 may be an efficacious blocker of the RAS in human patients and suitable for once-a-day dosing.

We would like to thank Professor Pramod R. Saxena for valuable and helpful advice in the preparation of this manuscript.

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(Received May 16, 1993
Revised September 14, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 151–158

α_1-Adrenoceptors and calcium sources in adrenergic neurogenic contractions of rat vas deferens

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1 The involvement of α_1-adrenoceptor subtypes in adrenergic neurogenic contractions of different type was studied in epididymal and prostatic portions of the rat vas deferens.

2 The adrenergic component of neurogenic contractions was isolated by suramin (300 μM). Twitch-like and tonic contractions were elicited by appropriate pulse patterns of electrical field stimulation, and contractions relying on intracellular calcium mobilization and calcium entry were isolated by means of nifedipine (10 μM) and ryanodine (20 μM), respectively. Increasing concentrations of 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane (WB 4101), α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile (HV 723), prazosin and 5-methylurapidil progressively, monophasically and with potency decreasing in that order reduced and finally abolished all types of contraction, with one exception: concentration-effect curves of 5-methylurapidil in epididymal segments in the presence of ryanodine levelled off at about 75% inhibition. In the presence of both nifedipine (10 μM) and ryanodine (20 μM), contractions were abolished.

3 Contractions elicited by exogenous noradrenaline were also studied in the presence of either nifedipine 10 μM (prostatic segments) or ryanodine 20 μM (epididymal segments). Increasing concentrations of tamsulosin, WB 4101, benoxathian, HV 723, prazosin, 5-methylurapidil and urapidil progressively, monophasically and with potency decreasing in that order reduced and eventually abolished both kinds of contraction, with two exceptions: in epididymal segments in the presence of ryanodine, the concentration-effect curve of 5-methylurapidil was biphasic and the curve of urapidil levelled off at only partial inhibition.

4 In slices prepared from the prostatic end and preincubated with [^{3}H]-noradrenaline, WB 4101, HV 723, prazosin and 5-methylurapidil, at the highest concentrations tested against neurogenic contractions, increased only slightly the overflow of tritium elicited by trains of 50 pulses at 5 Hz.

5 It is concluded that two α_1-adrenoceptor subtypes mediate adrenergic neurogenic contractions of rat vas deferens. The main one, pharmacologically α_{1A}, activates both calcium mobilization and entry. In addition there is a second receptor, not previously detected in the vas deferens and not corresponding to any named α_1 subtype, characterized by high and similar affinity for tamsulosin, WB 4101, benoxathian, HV 723 and prazosin and very low affinity for 5-methylurapidil and urapidil, and linked exclusively to calcium entry. Both subtypes and their respective transduction pathways also contribute to contractions elicited by exogenous noradrenaline. An α_{1B}-adrenoceptor-mediated contraction was not found under any experimental conditions.

Keywords: Rat vas deferens; α_1-adrenoceptor subtypes; α_{1A}-adrenoceptor; α_{1B}-adrenoceptor; Ca^{2+} entry; intracellular Ca^{2+} mobilization; postganglionic sympathetic neurotransmission

Introduction

Two sources of calcium contribute to adrenergic, α_1-adrenoceptor-mediated, neurogenic contractions of the rat vas deferens: mobilization inside the smooth muscle cells and entry into these cells from the extracellular space. Calcium mobilization predominates in the twitch contractions elicited by single electric pulses as well as in the initial twitch phase of contractions elicited by pulse trains; this component is resistant to the dihydropyridine derivative, nifedipine, which blocks L-type calcium channels (reviewed by Porzig, 1990), but suppressed by ryanodine, which depletes the intracellular calcium stores mobilized upon α_1-adrenoceptor activation (Iino *et al.*, 1988; Itoh *et al.*, 1992). Calcium entry predominates in the late tonic contractions elicited by pulse trains; this component is blocked by nifedipine but is resistant to ryanodine (Blakeley *et al.*, 1981; Brown *et al.*, 1983; Amobi & Smith, 1987; Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b).

The discovery of α_1-adrenoceptor subtypes (reviewed by Minneman, 1988; Ruffolo *et al.*, 1991) opened up the possibility that different calcium sources might be linked to different subtypes. Experiments with exogenous noradrenaline in rat vas deferens led to the view that the α_{1A} subtype activates calcium influx through dihydropyridine-sensitive channels whereas the α_{1B} subtype activates phosphatidylinositol breakdown followed by calcium mobilization from intracellular stores (Han *et al.*, 1987). The same relationship has recently been postulated for the effect of released endogenous noradrenaline in rat vas deferens (Mallard *et al.*, 1992a). The conclusion was based on experiments with chloroethylclonidine, a compound that blocks (irreversibly) α_{1B}- but not α_{1A}-adrenoceptors (Minneman *et al.*, 1988). Chloroethylclonidine did not change the tonic phase of adrenergic neurogenic contractions but abolished single pulse-evoked adrenergic twitches as well as the twitch phase of contractions elicited by pulse trains, in accordance with the view that released noradrenaline triggers calcium entry (tonic contraction) via the α_{1A} and calcium mobilization (twitches) via the α_{1B} subtype (Mallard *et al.*, 1992a).

However, this conclusion seems questionable. It has been shown that 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane (WB 4101), a selective antagonist at α_{1A}-adrenoceptors, blocks adrenergic twitches and tonic contractions with the same potency (Mallard *et al.*, 1992b; Ohmura *et al.*, 1992). Moreover, WB 4101 blocks adrenergic twitches with higher potency than prazosin (Figure 1 of McGrath,

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1984; Ohmura *et al.*, 1992; Aboud *et al.*, 1993), which is the opposite order of potency to that at α_{1B}-adrenoceptors (e.g. Hanft & Gross, 1989). Chloroethylclonidine, the tool used by Mallard *et al.* (1992a), irreversibly activates prejunctional α_2-autoreceptors in rat vas deferens, and evidence has been presented that prejunctional inhibition of noradrenaline release is responsible for the suppression of neurogenic twitches (Bültmann & Starke, 1993).

We re-investigated the coupling of calcium sources to specific α_1-adrenoceptor subtypes in neurogenic contractions of rat vas deferens by means of four competitive antagonists, WB 4101, α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile (HV 723), prazosin and 5-methylurapidil. They distinguish between α_{1A} and α_{1B} subtypes (Morrow & Creese, 1986; Gross *et al.*, 1988) as well as between the α_{1H}, α_{1L} and α_{1N} subtypes of another recent subclassification system (Muramatsu *et al.*, 1990). Both epididymal and prostatic segments were studied because they differ in their α_1-adrenoceptor mechanisms (Vardolov & Pennefather, 1976; Hay & Wadsworth, 1983). Adrenergic contractions relying on intracellular calcium mobilization were isolated by nifedipine and contractions relying on calcium entry were isolated by ryanodine (see Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b). The interpretation of the results will be based on the accepted specificity of action of these two compounds. Some of these results have been communicated to the German Society for Pharmacology and Toxicology (Kurz *et al.*, 1993).

Methods

Male Wistar rats (240–360 g) were decapitated and the vasa deferentia removed, cleaned of adherent tissue and cut into three pieces equal in length of which the two outer ones were used as the epididymal and prostatic portion, respectively. The medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Contraction

In neural stimulation experiments, the medium contained suramin (300 μM) in order to suppress the purinergic contraction component (Mallard *et al.*, 1992b; Bültmann *et al.*, 1993a,b) and either nifedipine (10 μM) or ryanodine (20 μM) in order to isolate adrenergic contractions relying on calcium mobilization and calcium influx, respectively (Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b). Prostatic or epididymal segments were suspended vertically in an organ bath, equilibrated and electrically stimulated as described (Bültmann *et al.*, 1993a,b; 0.3 ms pulse width, current strength 100 mA). There were up to 8 periods of electrical stimulation at 60 min intervals. Each stimulation period consisted of a single pulse, followed 10 s later by 3 pulses/100 Hz (3 p/100 Hz) and 5 min later by 50 p/5 Hz (Figure 1). Cumulative concentration-effect curves for α_1-adrenoceptor antagonists were determined by stepwise concentration increases immediately after the first and each of the following stimulation periods.

When contractions induced by exogenous noradrenaline were examined, the medium contained nifedipine (10 μM) or ryanodine (20 μM) but no suramin. A fixed concentration of noradrenaline was added 8 to 11 times at 60 min intervals and left in the bath until the contraction had reached its maximum. Cumulative concentration-effect curves for α_1-adrenoceptor antagonists were determined by concentration increases immediately after the first and each of the following responses to noradrenaline.

Contractions were measured at their maxima. They were expressed as a percentage of the first corresponding contraction in the same tissue and corrected for the mean corresponding percentage obtained in solvent-treated control tissues. For evaluation of concentration-effect relationships, logistic curves were fitted to the weighted mean percentage contractions using equation No. 25 of Waud (1976) and non-linear regression analysis, with the minimal drug effect fixed to zero (contraction 100%). The calculation yielded the maximal inhibition, the IC_{50}, i.e. the concentration of antagonist producing 50% of the maximal inhibition, and a slope value. In the case of 5-methylurapidil, the sum of two logistic functions was also fitted to the data, and the two fits (one logistic function *versus* the sum of two) were compared (p. 371 of Motulsky & Ransnas, 1987). Curves in Figures 2 to 5 are the fitted curves.

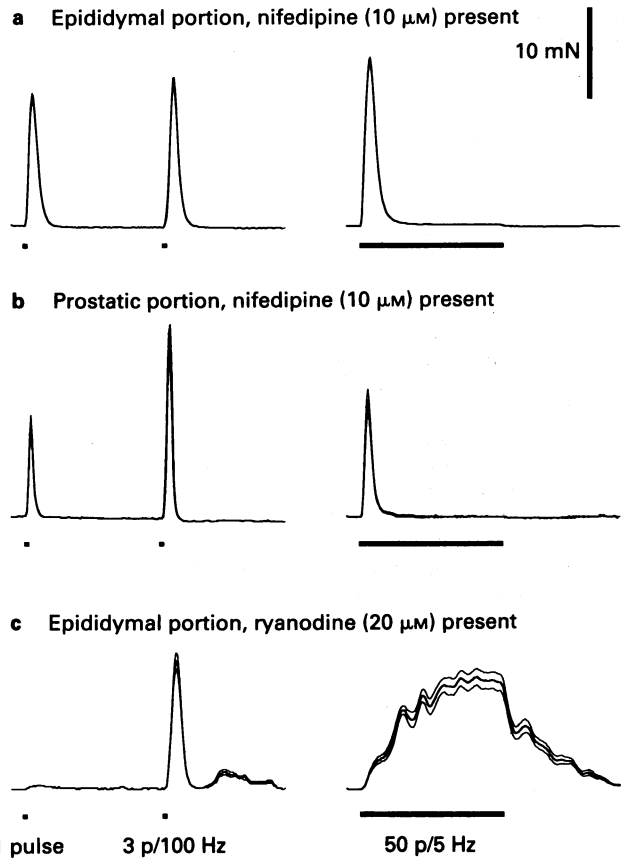

Figure 1 Adrenergic neurogenic contractions of epididymal (a,c) and prostatic (b) segments. The medium contained suramin 300 μM and either nifedipine 10 μM (a,b) or ryanodine 20 μM (c) from the beginning. Shown are responses to the first stimulation period which consisted of a single pulse followed after 10 s by 3 p/100 Hz and after 5 min (break in tracings) by 50 p/5 Hz. Tissue tension was digitized at 100 Hz. In each experiment, the initial baseline tension was subtracted from all subsequent values. The tension values of the individual tissues thus obtained were then averaged at each time point sampled. Curves are the mean with the s.e.mean envelope from all control experiments ($n = 11$, 7 and 11 for a, b and c, respectively). In the presence of ryanodine, spontaneous contractions followed the neurogenic contractions proper.

Tritium overflow

Experiments with [^{3}H]-noradrenaline were carried out as described by Bültmann *et al.* (1993b). Briefly, slices of the prostatic portion were preincubated with [^{3}H]-noradrenaline and then superfused. The medium did not contain suramin. The stimulation periods (S_1 to S_6) consisted of either 6 p/100 Hz or 50 p/5 Hz. Drugs or solvent were added after S_3. For evaluation of their effects on basal tritium efflux, ratios were calculated as efflux in the 2 min before S_4 over efflux in the 2 min before S_1. For evaluation of effects on

stimulation-evoked tritium overflow, the sum of the overflow elicited by S_1 to S_3 ('S_{1-3}') and the sum of the overflow elicited by S_4 to S_6 ('S_{4-6}') were obtained and the ratio S_{4-6}/S_{1-3} was then calculated.

Materials

Suramin hexasodium salt was a gift from Bayer (Wuppertal, Germany); (−)-tamsulosin HCl ((−)-YM-12617) from Dr C. Korstanje (Brocades, Delft, The Netherlands); α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile fumarate (HV 723) from Dr I. Muramatsu (Fukui, Japan); prazosin HCl from Pfizer (Karlsruhe, Germany); 5-methylurapidil from Dr K.H. Sanders (Byk Gulden, Konstanz, Germany). The following compounds were purchased: (±)-benoxathian HCl, ryanodine, (±)-2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane HCl (WB 4101), urapidil HCl (Biotrend, Köln, Germany); rauwolscine HCl (Roth, Karlsruhe, Germany); nifedipine, (−)-noradrenaline di-(+)-tartrate, tetrodotoxin (Sigma, Deisenhofen, Germany); (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 56.9 Ci mmol^{-1} (Du Pont, Dreieich, Germany). Nifedipine and ryanodine were dissolved in absolute ethanol (maximal final bath concentration below 0.1%), WB 4101 and 5-methylurapidil in 1 mM HCl, tetrodotoxin in 0.1 M sodium acetate buffer pH 4.85 and other drugs in distilled water. Experiments with nifedipine were carried out in near darkness or under sodium light.

Statistics

The arithmetic mean and s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. Differences with error probabilities <0.05 were taken to be statistically significant.

Results

Adrenergic neurogenic contractions

Neurogenic contractions were elicited in medium containing suramin 300 μM in order to isolate the adrenergic component.

In the presence of nifedipine (10 μM) to block calcium entry, all responses of the epididymal segment were twitch-like, even in the case of 50 p/5 Hz (i.e., the tonic contraction normally following the twitch during the 50 p/5 Hz train was suppressed by nifedipine; averaged responses to first stimulation period in Figure 1a). The contractions remained approximately constant or declined slightly when stimulation periods were repeated at 60 min intervals in controls: in the last (7th) stimulation period they were 85.6 ± 2.6%, 98.4 ± 1.6% and 103.2 ± 1.7% of those in the first period for single pulses, 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 11$). Cumulative addition of increasing concentrations of WB 4101, HV 723, prazosin and 5-methylurapidil progressively and monophasically reduced and finally abolished contractions elicited by all three modes of stimulation (Figure 2; IC_{50} values in Table 1). Rauwolscine 100 nM, when added after the first stimulation period for one further period, did not alter the twitch elicited by a single pulse but slightly increased the twitch elicited by the 3 p/100 Hz train 10 s later and also that elicited by 50 p/5 Hz (by 8 ± 1 and 17 ± 1%, respectively; $n = 6$).

Contractions also of the prostatic end in the presence of nifedipine (10 μM) were all twitch-like (Figure 1b). In control experiments amplitudes in the last (8th) stimulation period were 75.4 ± 6.6%, 88.7 ± 3.4% and 84.1 ± 4.4% of those in

Table 1 IC_{50} values (nM) for inhibition by α-adrenoceptor antagonists of adrenergic neurogenic contractions

	Contractions elicited by		
Antagonist	*1 pulse*	*3 p/100 Hz*	*50 p/5 Hz*
Epididymal segments, nifedipine (10 μM) present			
WB 4101	1.4 ± 0.1	6.0 ± 0.5	2.9 ± 0.1 (5)
HV 723	4.1 ± 0.3	16.8 ± 1.4	8.0 ± 0.2 (5)
Prazosin	9.0 ± 0.7	68.2 ± 4.3	18.1 ± 0.8 (6)
5-Methylurapidil	14.6 ± 0.1	44.2 ± 1.8	26.9 ± 1.4 (6)
Prostatic segments, nifedipine (10 μM) present			
WB 4101	0.5 ± 0.1	1.7 ± 0.3	0.8 ± 0.1 (4)
HV 723	2.6 ± 0.5	20.9 ± 3.5	4.2 ± 0.9 (4)
Prazosin	2.2 ± 0.1	22.0 ± 1.2	3.2 ± 0.4 (4)
5-Methylurapidil	16.0 ± 0.1	39.3 ± 4.1	25.9 ± 2.0 (3)
Epididymal segments, ryanodine (20 μM) present			
WB 4101		0.7 ± 0.1	0.8 ± 0.3 (5)
HV 723		2.7 ± 0.7	5.5 ± 0.3 (6)
Prazosin		2.7 ± 0.5	4.6 ± 0.3 (6)
5-Methylurapidil		20.4 ± 3.3	26.1 ± 4.4 (6)

IC_{50} values were estimated from experiments shown in Figures 2, 3 and 4. Means ± s.e.mean from (n) experiments.

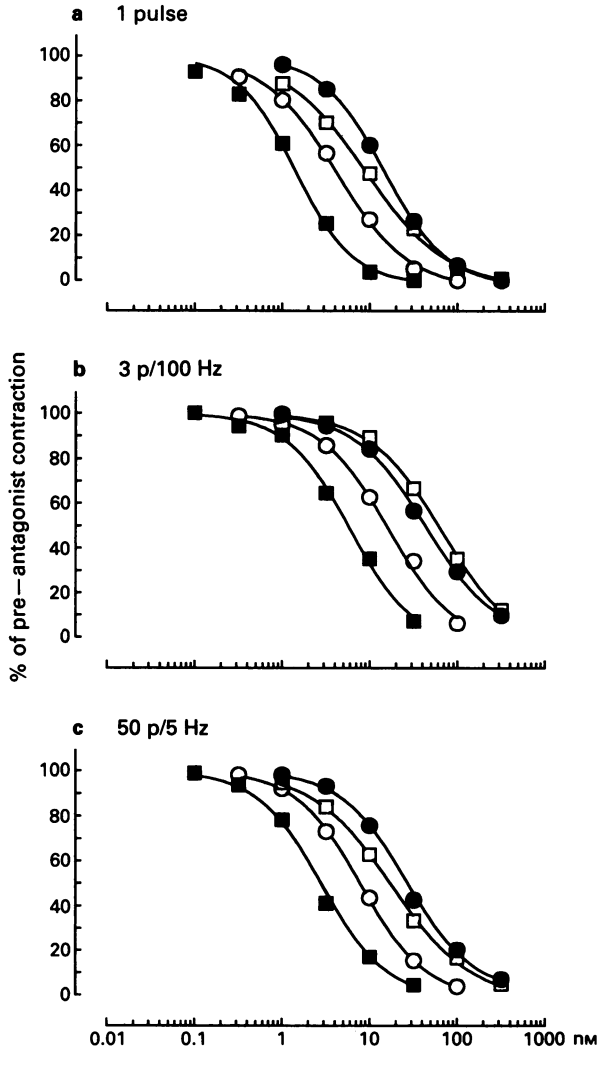

Figure 2 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in epididymal segments in the presence of suramin (300 μM) and nifedipine (10 μM). Shown is the inhibition of the twitch-like contraction elicited by single pulses (a), 3 p/100 Hz (b) and 50 p/5 Hz (c). Abscissae, α-adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 or 6 experiments. Standard errors of means did not exceed 6% of pre-antagonist contractions.

the first period for single pulses, 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 7$). Responses were progressively and monophasically reduced and eventually abolished by increasing concentrations of WB 4101, HV 723, prazosin and 5-methylurapidil, for all three modes of stimulation (Figure 3; IC_{50} values in Table 1). Rauwolscine 100 nM, when added as mentioned above, did not alter the twitch elicited by a single pulse or 3 p/100 Hz but slightly increased the twitch elicited by 50 p/5 Hz (by 25 ± 1%; $n = 5$).

In the presence of ryanodine (20 μM) to deplete intracellular calcium stores in epididymal portions, the only large responses were the twitch elicited by 3 p/100 Hz 10 s after the single pulse and a tonic response to 50 p/5 Hz (i.e., the twitches normally produced by the single pulse and initially during the 50 p/5 Hz train were suppressed by ryanodine; Figure 1c). In control experiments, amplitudes in the last (7th) stimulation period in the presence of solvent were 105.3 ± 9.9% and 80.7 ± 13.6% of those in the first for 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 11$). Increasing concentrations of WB 4101, HV 723, prazosin and 5-methylurapidil again caused progressive inhibition. However, whereas WB 4101, HV 723 and prazosin at sufficient concentrations produced complete blockade, only about 84% of the twitches elicited by 3 p/100 Hz and 69% of the tonic contractions elicited by 50 p/5 Hz were sensitive to 5-methylurapidil (Figure 4; percentages from curve fitting; IC_{50} values in Table 1). Rauwolscine (100 nM), when added as mentioned, did not significantly alter the twitch elicited by 3 p/100 Hz but slightly increased the tonic contractions at 50 p/5 Hz (by 26 ± 7%; $n = 6$).

The only contraction in prostatic segments in the presence of ryanodine (20 μM) was a twitch at 3 p/100 Hz (not shown). Its inhibition by α-adrenoceptor antagonists was not investigated.

When ryanodine (20 μM) was added to tissue pre-exposed to nifedipine (10 μM) or *vice versa*, contractions were abolished ($n = 2$ for either sequence and for both epididymal and prostatic portions). Tetrodotoxin (0.5 μM) abolished all contractions ($n = 2$).

Contractions elicited by exogenous noradrenaline

Experiments using exogenous noradrenaline were conducted in suramin-free medium. Three additional α-adrenoceptor antagonists were tested, tamsulosin, benoxathian and urapidil. The former two were chosen for their ability to distinguish between α_{1A} and α_{1B} subtypes (Han *et al.*, 1987; Michel *et al.*, 1989; Büscher *et al.*, 1992), the latter for its structural similarity to 5-methylurapidil.

Noradrenaline failed to contract the epididymal portion in the presence of nifedipine 10 μM (cf. Hay & Wadsworth, 1983). In the prostatic portion, noradrenaline 30 μM elicited rapid and transient contractions of 3.8 ± 0.2 mN in the

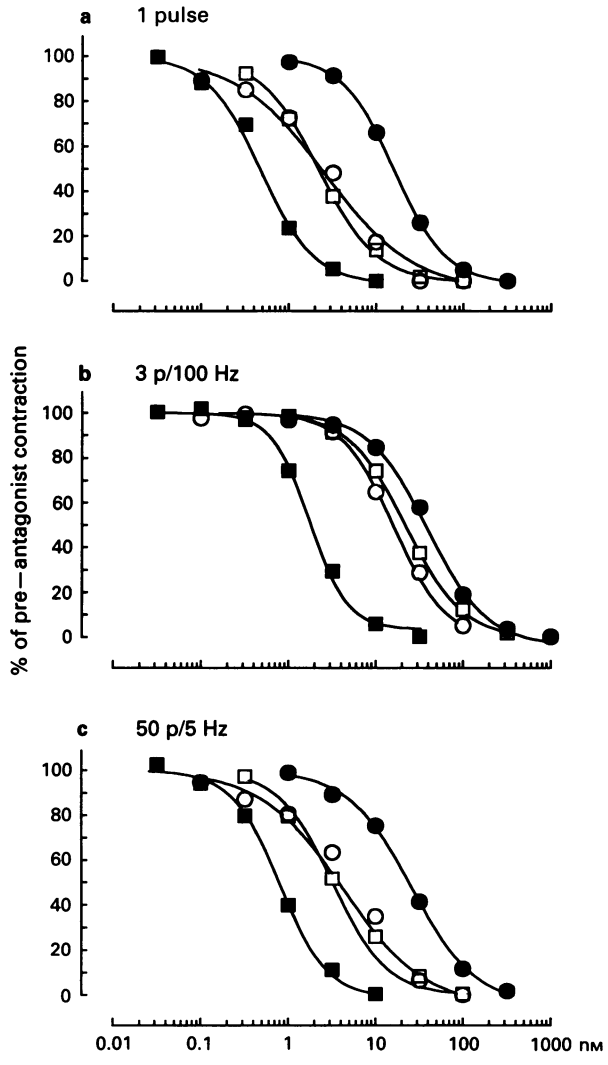

Figure 3 Inhibition of WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in prostatic segments in the presence suramin (300 μM) and nifedipine (10 μM). Shown is the inhibition of the twitch-like contraction elicited by single pulses (a), 3 p/100 Hz (b) and 50 p/5 Hz (c). Abscissae, α-adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 3 or 4 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

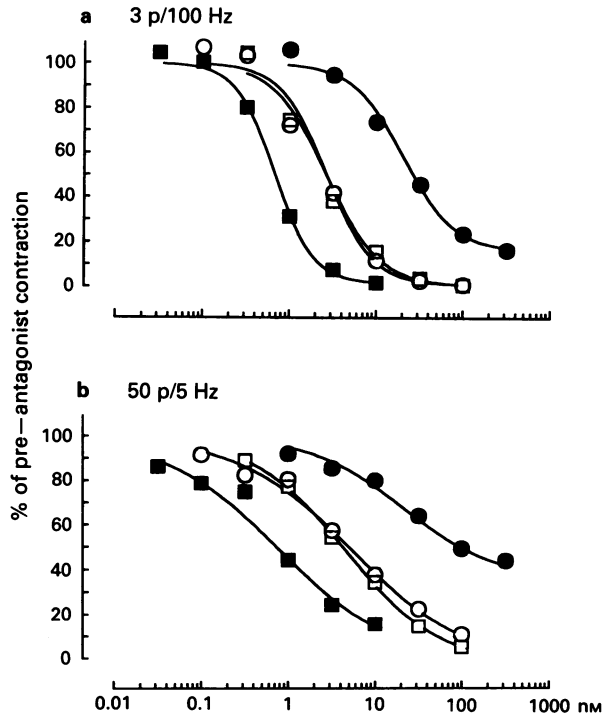

Figure 4 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in epididymal segments in the presence of suramin (300 μM) and ryanodine (20 μM). Shown is the inhibition of the twitch-like contraction elicited by 3 p/100 Hz (a) and the tonic contraction elicited by 50 p/5 Hz (b). Abscissae, α-adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 or 6 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

Table 2 IC_{50} values (nM) for inhibition by α-adrenoceptor antagonists of contractions elicited by exogenous noradrenaline

Antagonist	*Prostatic segments, nifedipine (10 μM) present*	*Epididymal segments, ryanodine (20 μM) present*
Tamsulosin	0.7 ± 0.1 (5)	1.0 ± 0.1 (5)
WB 4101	1.6 ± 0.2 (6)	1.3 ± 0.4 (5)
Benoxathian	6.6 ± 1.3 (5)	2.4 ± 0.4 (5)
HV 723	7.4 ± 0.9 (6)	5.6 ± 0.5 (5)
Prazosin	7.4 ± 2.1 (6)	7.0 ± 1.9 (5)
5-Methylurapidil	20.9 ± 3.2 (5)	26.9 ± 8.6[a] (7)
Urapidil	1583 ± 283 (4)	602 ± 144 (4)

IC_{50} values for WB 4101, HV 723, prazosin and 5-methylurapidil were estimated from experiments shown in Figure 5. Means ± s.e.mean from (*n*) experiments.
[a]IC_{50} for high affinity site; IC_{50} for low affinity site was 9.1 ± 1.4 μM.

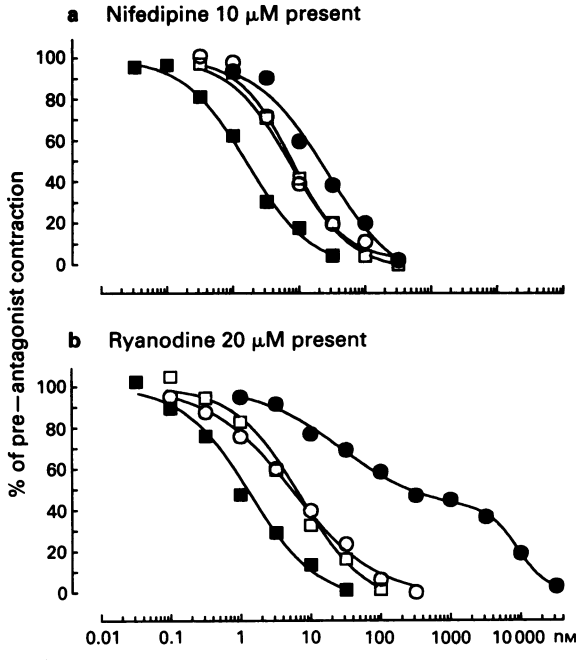

Figure 5 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of contractions elicited by exogenous noradrenaline. (a) Contractions of prostatic segments in the presence of nifedipine (10 μM); noradrenaline concentration 30 μM. (b) Contractions of epididymal segments in the presence of ryanodine (20 μM); noradrenaline concentration 3 μM. Abscissae, α-adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 to 7 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

presence of nifedipine 10 μM (*n* = 46); noradrenaline 30 μM was approximately an EC_{50} under these conditions (Bültmann, unpublished data). The contractions remained approximately constant when noradrenaline was added every 60 min in controls: upon the last (8th) addition they were 96.0 ± 4.9% of those obtained upon the first addition (*n* = 9). WB 4101, HV 723, prazosin and 5-methylurapidil progressively and monophasically reduced and finally abolished the contractions (Figure 5a). The same was true for tamsulosin, benoxathian and urapidil. IC_{50} values are in Table 2.

Epididymal segments were chosen for experiments with ryanodine, since it was in this portion that the 5-methylurapidil resistance of neurogenic contractions had been observed (Figure 4). In the presence of ryanodine (20 μM), exogenous noradrenaline 3 μM caused tonic contractions similar in height (9.2 ± 0.5 mN; *n* = 45) and shape to

Table 3 Effect of α-adrenoceptor antagonists on evoked tritium overflow from vas deferens slices preincubated with [^{3}H]-noradrenaline

Drug present from S_4 to S_6 (μM)	*S_{4-6}/S_{1-3} ratio 6 p/100 Hz*	*50 p/5 Hz*
Solvent	0.99 ± 0.04 (12)	1.08 ± 0.04 (14)
	0.86 ± 0.04 (5)[a]	
Tetrodotoxin 0.5	0.10 ± 0.02* (4)[a]	0.06 ± 0.02* (3)
WB 4101 0.032	0.94 ± 0.08 (4)	1.52 ± 0.13* (4)
HV 723 0.32	0.90 ± 0.09 (4)	1.74 ± 0.06* (4)
Prazosin 0.32	0.99 ± 0.09 (4)	1.66 ± 0.06* (4)
5-Methylurapidil 1	1.07 ± 0.08 (4)	1.67 ± 0.07* (4)
Rauwolscine 0.1	0.94 ± 0.09 (4)[a]	2.60 ± 0.19* (4)

Stimulation periods (S_1–S_6) consisted either of 6 p/100 Hz or of 50 p/5 Hz. Solvent or drug was added after S_3, 64 min before S_4, and kept for the remainder of the experiment. The fractional rate of tritium efflux immediately before S_1 was 0.00192 ± 0.00004 min^{-1} (*n* = 65). The overflow evoked by S_1 to S_3 (sum of S_1, S_2 and S_3) was 0.129 ± 0.005% of tissue tritium for 6 p/100 Hz (*n* = 28) and 0.245 ± 0.087% for 50 p/5 Hz (*n* = 37). S_{4-6}/S_{1-3} values are ratios of overflow elicited by S_4 to S_6 over overflow elicited by S_1 to S_3. Means and s.e.mean from (*n*) slices.
*Significantly different from solvent controls ($P < 0.05$).
[a]From Bültmann *et al.* (1993b).

contractions elicited by 50 p/5 Hz in the presence of ryanodine (Figure 1c). Noradrenaline is a more potent agonist in epididymal than prostatic portions (Vardolov & Pennefather, 1976; Sallés & Badia, 1991), and 3 μM was approximately an EC_{50} (Bültmann, unpublished data). Contractions produced by the last (11th) exposure to noradrenaline were 105.3 ± 7.6% of first contractions in controls (*n* = 8). WB 4101, HV 723 and prazosin progressively and monophasically reduced and finally abolished noradrenaline-induced contractions (Figure 5b), as did tamsulosin and benoxathian. The concentration-effect relationship of 5-methylurapidil, in contrast, was significantly ($P < 0.05$) better fit by a biphasic curve. Only about 60% of noradrenaline-induced contractions were sensitive to low concentrations of 5-methylurapidil (percentage from curve fitting), and the two IC_{50} values differed by a factor of about 340 (Figure 5b; IC_{50} values in Table 2). The concentration-effect curve of urapidil resembled the left-hand part of the curve of 5-methylurapidil: it levelled off at about 78% inhibition (percentage from curve fitting; Table 2).

Tritium overflow

Experiments on vas deferens slices preincubated with [^{3}H]-noradrenaline were carried out in order to detect possible presynaptic effects of the α-adrenoceptor antagonists. Release of [^{3}H]-noradrenaline was measured as overflow of total

tritiated compounds. In solvent controls, the evoked overflow of tritium remained approximately constant (Table 3). Tetrodotoxin practically abolished the evoked overflow. WB 4101, HV 723, prazosin and 5-methylurapidil, when given at the highest concentrations used in neurogenic contraction experiments, did not change the overflow of tritium elicited by 6 p/100 Hz, and the same was true for rauwolscine (100 nM). All antagonists, however, increased the overflow elicited by 50 p/5 Hz. The effect was marked for rauwolscine and smaller for the other antagonists. HV 723, prazosin and 5-methylurapidil accelerated the basal outflow of tritium.

Discussion

Nature of contractions

The neurogenic contractions elicited in this study were adrenergic since they were abolished by α-adrenoceptor antagonists. The postjunctional receptors involved were only α_1 since the α_2-adrenoceptor antagonist, rauwolscine, did not cause any inhibition of the responses (in contrast to mouse vas deferens: Bültmann *et al.*, 1991). Contractions remaining in the presence of nifedipine were abolished by ryanodine, and contractions remaining in the presence of ryanodine were abolished by nifedipine. Under the premise stated in the Introduction, concerning the specificity of action of nifedipine and ryanodine, this observation shows that contractions remaining in the presence of nifedipine were due to intracellular calcium mobilization, whereas contractions remaining in the presence of ryanodine were due to calcium entry.

The complex stimulation protocol needs a brief comment. One aim was to study both twitch-like and tonic contractions, another to obtain calcium mobilization-mediated and calcium entry-mediated contractions of similar size and shape. Calcium mobilization and entry usually lead to responses of different shape, twitch-like and tonic, respectively (see Introduction). However, application of a 3 p/100 Hz train 10 s after a single pulse elicited a sizeable twitch in the prescence of ryanodine (i.e. calcium entry-mediated), possibly due to the increase in calcium entry that has been suggested to occur when a second neurogenic contraction follows a priming contraction within a few seconds (Bültmann *et al.*, 1993b).

Rauwolscine did not change contractions elicited by single pulses but occasionally increased contractions elicited 10 s later by 3 p/100 Hz and always increased the response to 50 p/5 Hz. Moreover, rauwolscine did not change the release of [^{3}H]-noradrenaline elicited by 6 p/100 Hz but greatly increased the release elicited by 50 p/5 Hz. This is the pattern of effect expected from the operation of prejunctional α_2-autoreceptors (see Starke *et al.*, 1989) and indicates, moreover, that postjunctional consequences of prejunctional α_2-autoinhibition were small under the conditions used. The increase in [^{3}H]-noradrenaline release (50 p/5 Hz) produced by WB 4101, HV 723, prazosin and 5-methylurapidil was similar and less than in the case of rauwolscine. Prejunctional α_2-autoreceptor blockade by the four drugs, therefore, did not interfere with the comparison of their effects at postjunctional α_1-adrenoceptors.

α_1-Adrenoceptors and calcium source-specific contractions

Our results indicate that two types of α_1-adrenoceptor are involved in adrenergic neurogenic contractions of rat vas deferens. The first, main one mediates all calcium mobilization-dependent contractions (isolated by nifedipine) and the major part of calcium entry-dependent contractions (isolated by ryanodine); it corresponds to the α_{1A} subtype. The second receptor mediates a minor part of calcium entry-dependent contractions; it does not correspond to any named α_1 subtype.

The common receptor for calcium mobilization and entry

In support of one common receptor for calcium mobilization and entry, four antagonists displayed a similar rank order of potency against twitches elicited by single pulses in the presence of nifedipine (Figure 2a, 3a); twitches elicited by 3 p/100 Hz in the presence of nifedipine (Figure 2b, 3b); twitches that developed initially in response to 50 p/5 Hz in the presence of nifedipine (Figure 2c, 3c); twitches elicited by 3 p/100 Hz in the presence of ryanodine (Figure 4a); and tonic contractions in response to 50 p/5 Hz in the presence of ryanodine (Figure 4b). The pulse patterns applied created different extracellular concentrations of noradrenaline, and this probably accounts for the fact that the antagonists usually were very effective against single pulse responses, less effective against responses to 50 p/5 Hz and least effective against 3 p/100 Hz (Table 1). The rank order of potency, however, was common to all pulse patterns. It has been suggested that twitches are mediated by junctional receptors and tonic contractions by extrajunctional receptors (Brown *et al.*, 1983), and these have been equated with α_{1B} and α_{1A}, respectively (Mallard *et al.*, 1992a). However, we infer from our experiments that junctional and extrajunctional receptors are pharmacologically alike.

In radioligand binding studies in rat tissues, α_{1A} binding sites are characterized by affinity ratios prazosin/WB 4101 = 0.4–2.5 and prazosin/5-methylurapidil = 2.5–16, whereas at α_{1B} sites these affinity ratios are prazosin/WB 4101 = 10–50 and prazosin/5-methylurapidil = 100–1200 (Morrow & Creese, 1986; p. 110 of Minneman, 1988; Grosss *et al.*, 1988; Hanft & Gross, 1989; Michel *et al.*, 1989; Michel *et al.*, 1990). The corresponding ratios for the main α_1-adrenoceptor mediating neurogenic contractions in rat vas deferens were prazosin/WB 4101 = 0.1–0.25 and prazosin/5-methylurapidil = 0.6–8.1 (calculated from the IC_{50} values of Table 1). Both ratios are close to, or overlapping with, the corresponding α_{1A} but far from the corresponding α_{1B} ratios. The relatively low prazosin/5-methylurapidil affinity ratio of 0.6–8.1 also distinguishes the vas deferens receptor from cloned α_1 sites (Perez *et al.*, 1991; Schwinn & Lomasney, 1992). In terms of the $\alpha_{1A/B}$ classification system, the common receptor involved in all neurogenic contraction types, calcium mobilization– as well as calcium entry-mediated, corresponds best to α_{1A}. α_{1A}-Adrenoceptors have previously been suggested to mediate the tonic phase of adrenergic neurogenic contractions (Mallard *et al.*, 1992a), in accord with the present results. The twitch-like phase was suggested to be α_{1B}-mediated (Mallard *et al.*, 1992a). Here we disagree: the rank order of antagonist potency, in accordance with previous observations (see Introduction), indicates an α_{1A} character; the inhibition of the twitches by chloroethylclonidine (Mallard *et al.*, 1992a) presumably was due to activation of prejunctional α_2- rather than blockade of postjunctional α_{1B}-adrenoceptors (Bültmann & Starke, 1993). Since this paper was submitted, Aboud *et al.* (1993) suggested that adrenergic twitches of rat vas deferens were mediated by a non-α_{1A}-, non-α_{1B}-adrenoceptors because they were (i) reduced by chloroethylclonidine (arguing against α_{1A}) and (ii) blocked by competitive antagonists such as WB 4101 with high potency (arguing against α_{1B}); if one assumes a prejunctional explanation for the effect of chloroethylclonidine, the results of these authors are compatible with our α_{1A} postulate.

The experiments discussed so far rule out a selective coupling of α_{1A}-adrenoceptors to calcium entry and α_{1B}-adrenoceptors to calcium mobilization in neurogenic contractions. Does the differential link hypothesis hold true for exogenous noradrenaline, as originally suggested (Han *et al.*, 1987)? This seems also unlikely. All seven antagonists attenuated the effect of exogenous noradrenaline with the same potency

rank order in the presence of nifedipine (Figure 5a) and in the presence of ryanodine (Figure 5b); even the two absolute IC_{50} values for any given antagonist agreed well (Table 2). For WB 4101, HV 723, prazosin and 5-methylurapidil the rank order was identical with that against neurogenic contractions, indicating an α_{1A}-adrenoceptor, as discussed, a classification confirmed by the high potency of tamsulosin and benoxathian (Han *et al.*, 1987; Michel *et al.*, 1989; Büscher *et al.*, 1992). Our results do not exclude the possibility that noradrenaline, acting at α_{1B}-adrenoceptors, can elicit calcium mobilization and ensuing contractions in rat vas deferens (Han *et al.*, 1987; Martinotti *et al.*, 1991; Aboud *et al.*, 1993). However, no indication for an α_{1B} component was found under the present conditions.

Effects of WB 4101, HV 723 and prazosin on adrenergic neurogenic contractions of rat vas deferens have also been reported by Ohmura *et al.* (1992). The authors used one pulse pattern only and did not isolate calcium entry-dependent responses. However, their antagonist affinity rank order and their conclusion – one main receptor for twitches and tonic contractions and for calcium mobilization and entry – are identical to ours. Ohmura *et al.* (1992) classified the receptor as α_{1L} in terms of the $\alpha_{1H/L/N}$ proposal (Muramatsu *et al.*, 1990). Given the excellent agreement with our values, the receptor characterized in the present study also fits the α_{1L} type. Decision between the α_{1A} and α_{1L} designations has to await clarification of the relationship between the $\alpha_{1A/B}$ and $\alpha_{1H/L/N}$ nomenclature (but see the criticism of the latter scheme by Esbenshade *et al.*, 1993).

The receptor selective for calcium entry

Unfortunately, the conclusion from the present study is not a 'one physiological receptor' concept. Concentration-effect curves in epididymal portions, in the presence of ryanodine to isolate calcium entry, showed that part of the effect of both endogenous (Figure 4) and exogenous noradrenaline (Figure 5b) was resistant to moderate concentrations of 5-methylurapidil. The same was true for urapidil (tested against exogenous noradrenaline only). The remaining contractions were α_1-adrenoceptor-mediated since no resistant component was observed with any other antagonist. Hence, there is a second α_1-adrenoceptor. It is characterized by similar and high affinity for tamsulosin, WB 4101, benoxathian, HV 723 and prazosin but very low affinity for 5-methylurapidil and urapidil, with a prazosin/WB 4101 affinity ratio of 0.2 (identical to the α_{1A} receptor) but a prazosin/5-methylurapidil affinity ratio of 1,300 (from IC_{50} values in Table 2). Obviously, it is neither α_{1A} nor α_{1B}. It also differs from the cloned α_1 sites mentioned. It resembles, however, a [^{3}H]-prazosin binding site in rat lung that resists inactivation by chloroethylclonidine and possesses a prazosin/WB 4101 affinity ratio of 0.8 and a prazosin/5-methylurapidil ratio of 140 (Hiramatsu *et al.*, 1992; see Han & Minneman, 1991, for possibly similar sites in rat heart and hippocampus). No 5-methylurapidil or urapidil resistance was observed in the presence of nifedipine, suggesting that the second α_1-adrenoceptor in rat vas deferens operates exclusively through activation of calcium entry.

Conclusion

The study suggests some revision of the current view of α_1-adrenoceptor subtypes and calcium sources in adrenergic neurogenic contractions of the rat vas deferens. Two α_1-adrenoceptor subtypes mediate these contractions. The main one, pharmacologically α_{1A} (or α_{1L}), activates both calcium entry and mobilization. A second receptor, not previously detected in the vas deferens and not corresponding to any named α_1 subtype, is linked exclusively to calcium entry. Both subtypes and their respective transduction pathways similarly contribute to contractions elicited by exogenous noradrenaline. α_{1B}-Adrenoceptors did not mediate contraction under the present experimental conditions, irrespective of whether the contractions were twitch-like or tonic, neurogenic or caused by exogenous noradrenaline, due to calcium entry or due to intracellular calcium mobilization.

This study was supported by the Jung-Stiftung, Hamburg, and the Deutsche Forschungsgemeinschaft (SFB 325). We thank Drs C. Korstanje, I. Muramatsu and K.H. Sanders for tamsulosin, HV 723 and 5-methylurapidil, respectively, U. Grosse for the analog-digital converter, Dr N. Limberger for curve-fitting and Bayer and Pfizer for gifts of other drugs.

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(Received July 8, 1993
Revised September 16, 1993
Accepted September 22, 1993)

Br. J. Pharmacol. (1994), **111**, 159–164

Cutaneous permeability responses to bradykinin and histamine in the guinea-pig: possible differences in their mechanism of action

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1 Plasma protein extravasation (PPE) responses in guinea-pig skin have been measured using accumulation of intravenously injected ^{125}I-labelled human serum albumin (^{125}I-HSA).

2 The nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 μmol/site) significantly reduced responses to bradykinin (BK; 0.5 nmol/site) or histamine (4.5 nmol/site) when co-injected with the inflammatory mediators. D-NAME (0.1 μmol/site) had no significant effect.

3 L-NAME (0.01–0.1 μmol/site) appeared to produce greater shifts of the dose-response curve to BK (0.1–3 nmol/site) than of that to histamine (2.3–27 nmol/site). Both 0.01 and 0.1 μmol L-NAME/site significantly reduced the response to BK (0.5 nmol/site) whereas only the higher dose of L-NAME produced a significant reduction in the response to histamine (4.5 nmol/site).

4 The inhibitory effect of L-NAME (0.1 μmol/site) on the response to BK but not on that to histamine was significantly reversed by L-arginine (L-Arg; 10 μmol/site). D-arginine (D-Arg; 10 μmol/site) had no significant effect in either case.

5 L-Arg (10 μmol/site) significantly enhanced the response to BK but inhibited that to histamine. D-Arg (10 μmol/site) had no significant effect on BK but significantly inhibited histamine. L-Lysine (L-Lys: 10 μmol/site) had no significant effect on the response to either BK or histamine.

6 L-Arg (100 mM) had a significant inhibitory effect on isometric contractions to histamine, but not BK in guinea-pig ileum *in vitro*. D-Arg (100 mM) also significantly inhibited histamine responses whereas L-Lys (100 mM) had no effect.

7 The α-adrenoceptor agonist, phenylephrine (0.3 or 6 nmol/site) inhibited matched responses to BK (0.5 nmol/site) or histamine (5.4 nmol/site) to comparable degrees, but gave significant inhibition only at the higher dose.

8 The β-adrenoceptor agonist, isoprenaline (0.5 or 10 nmol/site) had a significant inhibitory effect on the response to histamine (5.4 nmol/site) whereas a comparable response to BK (0.5 nmol/site) was significantly reduced by the higher dose only.

9 Our results with L-NAME suggest that local production of NO is involved in the modulation of mediator-induced vascular permeability. It is possible that NO may play a greater role in the extravasation response to BK than to that induced by histamine.

10 The differential effects of L-NAME and isoprenaline on BK- and histamine-induced PPE raise the possibility that BK and histamine may induce vascular permeability via different mechanisms in guinea-pig skin.

Keywords: N^G-nitro-L-arginine methyl ester (L-NAME); arginine; vascular permeability; bradykinin; histamine; adrenoceptor agonists

Introduction

Under normal conditions, the vascular endothelium is relatively impermeable to macromolecules. There is now general agreement that increased vascular permeability observed in response to various endogenous and exogenous mediators is via transient, reversible formation of junctional gaps between endothelial cells in postcapillary venules (Majno & Palade, 1961; Schachter, 1963; Majno *et al.*, 1969; Arfors *et al.*, 1979; Fox *et al.*, 1980). Suggestions of the involvement of a contractile mechanism, based on observed shape changes in endothelial cells (Majno *et al.*, 1969), have been strengthened by evidence of the presence of contractile proteins in these cells (Becker & Nachman, 1973; Drenckhahn, 1983). Unlike bradykinin (BK) or histamine, prostaglandins are poor at eliciting plasma exudation when injected into guinea-pig (Williams & Morley, 1973) or rabbit (Williams, 1976) skin. However, exogenous prostaglandins, notably prostacyclin (PGI_2) and E-type prostaglandins, increase skin blood flow and potentiate exudation produced by other mediators (Williams & Morley, 1973; Williams, 1976; 1979). Moreover, a range of α-adrenoceptor agonists which reduce cutaneous blood flow inhibit BK-induced exudation responses (Beets & Paul, 1980). Thus, the magnitude of an acute inflammatory response may be modulated by changes in local blood flow interacting with alterations in microvessel permeability.

Endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) has been identified as nitric oxide (NO) or a closely related molecule (Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987) synthesized from L-arginine (L-Arg; Palmer *et al.*, 1988). NO may play an important role as an intrinsic modulator of blood flow in various tissues (Ignarro, 1989) and alterations in its local production could therefore modify acute inflammatory responses. In support of this concept, inhibitors of NO production such as the arginine analogues N^G-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990) and N^G-monomethyl-L-arginine (L-NMMA; Rees *et al.*, 1989) reduce blood flow (Hughes *et al.*, 1990) and inhibit plasma exudation induced by substance P (Hughes *et al.*, 1990) or carrageenin (Ialenti *et al.*, 1992) in rat skin and oedema in response to carrageenin or dextran in rat paw (Ialenti *et al.*, 1992).

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We have examined the effects of L-NAME and arginine on vascular permeability responses to BK and histamine in guinea-pig skin. These effects have been compared to those of phenylephrine and isoprenaline which act by reducing local blood flow (Beets & Paul, 1980) and by an anti-permeability effect (O'Donnell & Persson, 1978; Beets & Paul, 1980) respectively. These data have been presented in part to the British Pharmacological Society (Paul *et al.*, 1992b).

Methods

Animals

Experiments were performed on conscious, male guinea-pigs (400–500 g) (OLAC). The hair on the skin of the back and flanks was closely clipped at least 2 h before each experiment.

Intravenous injections

Guinea-pigs were injected via a hind paw dorsal vein with 0.5 ml Evans blue dye (2.5% w/v in sterile phosphate buffered saline, PBS) containing approximately 0.07 MBq ^{125}I-labelled human serum albumin ([^{125}I]-HSA).

Intradermal injections

Test solutions were prepared in sterile PBS immediately prior to use. Intradermal injections (0.1 ml/site) were made according to a balanced Latin Square design to allow for inter-site variation. Each animal received up to 6 treatments in duplicate. PBS and drug controls were included in each experiment.

Preparation of skin samples

Forty minutes after the last i.d. injection, each animal was killed with an overdose of pentobarbitone sodium, a blood sample was taken into heparin by cardiac puncture and the skin was removed. Discs of skin containing the whole of each lesion were removed with a 17 mm diameter metal wad punch, placed in 5 ml polycarbonate tubes, and counted, together with two 100 µl samples of plasma from each animal, in a gamma spectrometer.

Guinea-pig ileum in vitro

Guinea-pig terminal ileum (GPI) was suspended in gassed (95% O_2; 5% CO_2) Krebs solution at 34°C under 1 g tension for isometric recording. Dose-response curves were constructed in Krebs in the absence and presence of D-Arg, L-Arg or L-lysine (L-Lys), all 100 mM. Any one piece of ileum was exposed to only one amino acid. Responses were expressed as a % of the response to supramaximal effective concentrations of histamine (25 µM) or BK (1 µM). EC_{50} values were calculated from individual dose-response curves.

Calculation of results

Extravasation in each skin lesion was expressed as µl plasma (^{125}I counts in lesion/^{125}I counts in 1 µl plasma). These values were corrected by subtracting the control response for PBS or test drug alone, as appropriate. Results are expressed as mean ± s.e.mean values for the stated number of animals. Data were analysed by analysis of variance followed by Tukey's test to allow for multiple comparisons.

EC_{50} values obtained in guinea-pig ileum were expressed as geometric means (95% confidence limits) for $n = 4–6$ replicates.

Materials

Histamine diphosphate, bradykinin acetate, Evans blue dye, pentobarbitone sodium, sterile phosphate buffered saline, L-lysine HCl, L-NAME, L-arginine HCl, D-arginine HCl, (−)-phenylephrine HCl, (−)-isoprenaline sulphate and pentobarbitone sodium were obtained from Sigma Chemical Co., Poole; D-NAME from Bachem, Switzerland, and heparin (Multiparin) from C.P. Pharmaceuticals Ltd., Wrexham. [^{125}I]-HSA (1.85 MBq ml^{-1}; 20 mg albumin ml^{-1}) was supplied by Amersham International plc., Amersham.

Results

Vascular permeability responses induced by test compounds

At concentrations up to 0.1 µmol/site, neither D- nor L-NAME produced any noteworthy leakage of plasma protein but higher concentrations caused significant extravasation responses in some animals and were not studied further (Table 1). L-Arg or L-Lys at concentrations up to 10 µmol/site had no pronounced effects whereas 10 µmol D-Arg produced a modest extravasation (Table 1). Neither isoprenaline (up to 10 nmol/site) nor phenylephrine (up to 6 nmol/site) had any significant direct effect on plasma exudation (data not shown). All mediator-induced responses have been corrected by subtracting the appropriate control response.

Effects of D- and L-NAME on responses to BK and histamine

Responses to BK (0.5 nmol/site; 76.3 ± 7.4 µl; $n = 5$) were significantly ($P < 0.01$) reduced by concomitant treatment with L-NAME (0.1 µmol/site; 21.5 ± 2.3 µl) but not with D-NAME (0.1 µmol/site; 79.7 ± 10.7 µl). Plasma protein exudation in response to histamine (4.5 nmol/site; 138.2 ± 15.2 µl; $n = 6$) was also significantly ($P < 0.01$) inhibited by 0.1 µmol L-NAME (51.6 ± 11.5 µl) but not by the same concentration of D-NAME (118.0 ± 13.6 µl). Maximum inhibition with L-NAME was between 54–62%. The dose-response curves for plasma protein extravasation responses to BK (0.1–3 nmol/site) and histamine (2.3–27 nmol/site) were shifted by local

Table 1 Skin plasma protein exudation (PPE) in sites injected with PBS or drugs alone

Treatment	*PPE*	*Treatment*	*PPE*
PBS	13.7 ± 2.1	PBS	17.9 ± 1.0
L-Arg, 1 µmol	15.4 ± 3.7	L-NAME, 0.01 µmol	17.7 ± 0.9
L-Arg, 10 µmol	16.8 ± 3.6	L-NAME, 0.1 µmol	17.9 ± 1.3
D-Arg, 1 µmol	18.3 ± 4.2	L-NAME, 1.0 µmol	36.4 ± 4.4
D-Arg, 10 µmol	29.1 ± 5.1	D-NAME, 0.1 µmol	15.9 ± 0.9
L-Lys, 10 µmol	19.8 ± 1.2	D-NAME, 1.0 µmol	47.2 ± 6.0

Skin plasma volumes measured over 40 min after i.d. injection of 0.1 ml PBS alone or containing the specified amounts of test compounds. Values are mean ± s.e.mean for duplicate sites in $n = 4$ animals. L-Arg, D-Arg = L- and D-arginine; L-Lys = L-lysine; L-NAME = N^G-nitro-L-arginine methyl ester.

administration of L-NAME at 0.01 and 0.1 μmol/site (Figure 1). The apparently greater effect of L-NAME on responses to BK than on those to histamine was confirmed by a within animals ($n = 6$) comparison which showed that the lower dose of L-NAME (0.01 μmol/site) significantly ($P < 0.01$) inhibited the response to BK (0.5 nmol/site), but had no significant effect on a comparable response to histamine (4.5 nmol/site) (Figure 2).

Effects of arginine

L-Arg (1–10 μmol/site) reversed the inhibitory effect of L-NAME (0.1 μmol/site) on the response to BK (0.5 nmol/site; $n = 5$) (Figure 3a) with significant ($P < 0.05$) reversal occurring with the higher dose. D-Arg (10 μmol/site) produced a partial reversal which was not statistically significant (Figure 3a). In the same dose range, neither D- nor L-Arg produced any reversal of the inhibitory effect of L-NAME (0.1 μmol/site) on the response to histamine (4.5 nmol/site; $n = 5$) (Figure 3b). In the absence of L-NAME, L-Arg (1–10 μmol/site) increased the response to BK (0.5 nmol/site; $n = 6$) but, in contrast, had an inhibitory effect on histamine (4.5 nmol/site; $n = 6$), both effects being significant ($P < 0.05$ and $P < 0.01$ respectively) at the higher dose (Figure 4). D-Arg (10 μmol/site) had no significant effect on the response to BK but inhibited ($P < 0.05$) that to histamine (Figure 4). The opposite effects of L-Arg (10 μmol/site) on responses to BK (0.5 nmol/site) and histamine (4.5 nmol/site) were confirmed by a within animals ($n = 5$) comparison (Figure 5a) whereas L-lysine (10 μmol/site) had no significant effect ($n = 6$) on responses to either BK or histamine (Figure 5b).

Effects of adrenoceptor agonists on responses to BK and histamine

Phenylephrine (0.3 nmol and 6 nmol/site) reduced the response to BK (0.5 nmol/site) by 14% and 57% respectively and reduced an equivalent response to histamine (5.4 nmol/site) by 19% and 65% (Figure 6a). Inhibition of exudation induced by either mediator was significant ($P < 0.05$; $n = 6$)

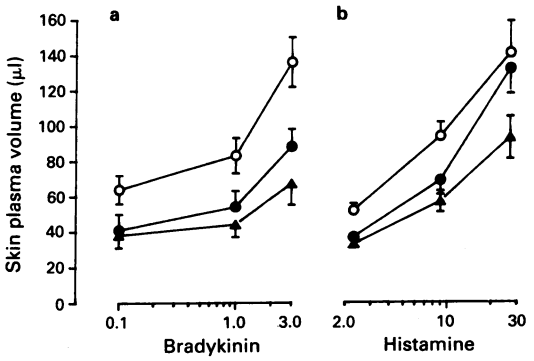

Figure 1 The effects of co-injection of N^G-nitro-L-arginine methyl ester (L-NAME) 0.01 or 0.1 μmol/site on skin plasma exudation responses to (a) bradykinin (0.1–3.0 nmol/site; log scale) or (b) histamine (2.3–27 nmol/site; log scale). Responses to the mediators alone (○; $n = 8$) or in the presence of 0.01 μmol (●) or 0.1 μmol (▲) L-NAME/site ($n = 4$ in each case) are expressed as μl plasma (corrected values) and mean ± s.e.mean values are shown.

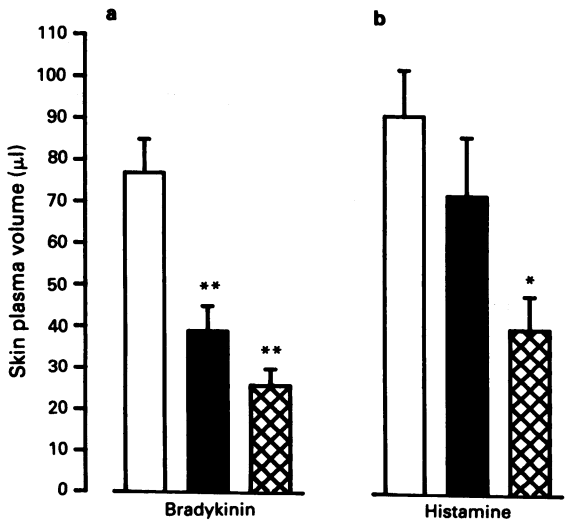

Figure 2 Within animals ($n = 6$) comparison of co-injection of 0.01 μmol (solid column) or 0.1 μmol (cross-hatched columns) N^G-nitro-L-arginine methyl ester (L-NAME) per site on skin plasma protein exudation responses to bradykinin (0.5 nmol/site) or histamine (4.5 nmol/site). Responses to the mediators alone (open column) or in the presence of L-NAME are expressed as μl plasma (corrected values) and mean ± s.e.mean values are shown. $^*P < 0.05$, $^{**}P < 0.01$ compared with mediator alone.

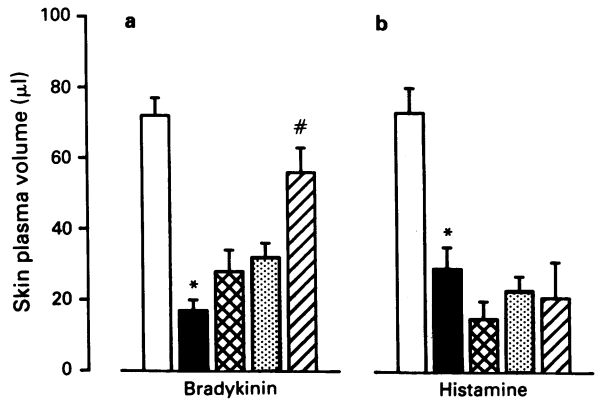

Figure 3 The effects of D- and L-arginine on N^G-nitro-L-arginine methyl ester (L-NAME, 0.1 μmol/site) inhibition of skin plasma protein exudation responses to (a) bradykinin, 0.5 nmol/site, $n = 5$ or (b) histamine, 4.5 nmol/site, $n = 5$. Responses to the mediators alone (open columns); with 0.1 μmol L-NAME (solid columns) or with L-NAME plus 10 μmol D-arginine (cross-hatched columns); or 1 μmol (stippled columns) or 10 μmol (hatched columns) L-arginine are expressed as μl plasma (corrected values) and mean ± s.e.mean values are shown. $^*P < 0.01$ compared with mediator alone; $^\#P < 0.05$ compared with mediator plus L-NAME.

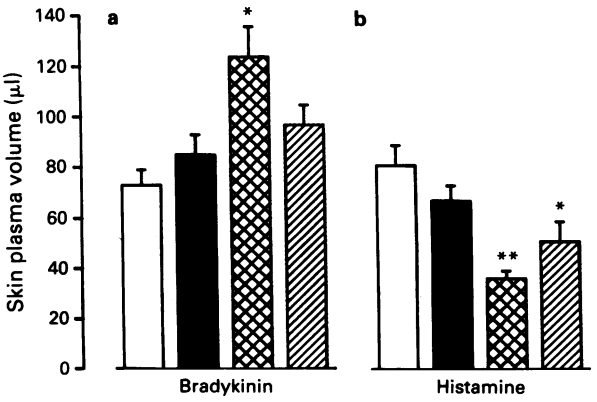

Figure 4 The effects of D- and L-arginine on skin plasma protein exudation responses to (a) bradykinin, 0.5 nmol/site, $n = 6$ or (b) histamine, 4.5 nmol/site, $n = 6$. Responses to the mediators alone (open columns) or in the presence of 1 μmol (solid columns) or 10 μmol (cross-hatched columns) L-Arg/site or 10 μmol D-Arg/site (hatched columns) are expressed as μl plasma (corrected values) and mean ± s.e.mean values are shown. $^*P < 0.05$; $^{**}P < 0.01$ compared with mediator alone.

with the higher dose of phenylephrine. Matched responses to BK (0.5 nmol/site) and histamine (5.4 nmol/site) were inhibited by isoprenaline (0.5 nmol and 10 nmol/site) by 24% and 60% and 59% and 86% respectively (Figure 6b). Inhibition of the histamine response was significant with both doses of isoprenaline ($P<0.05$ and $P<0.01$) whereas the BK response was significantly reduced ($P<0.05$; $n=6$) by the high dose of isoprenaline only.

Effects on guinea-pig ileum in vitro

L-Arg (100 mM) significantly ($P<0.05$) increased the EC_{50} value for histamine but had no significant effect on responses to BK (Table 2). There was no marked decrease in the maximum response obtainable with either agonist in the presence of L-Arg. D-Arg (100 mM) significantly ($P<0.05$) increased the EC_{50} for histamine whereas L-Lys (100 mM) had no significant effect (Table 2). D-Arg depressed the maximum response to 70 ± 3.5% ($n=6$) and L-Lys produced a marked reduction of the maximum response (50%) in 1 out of 4 experiments.

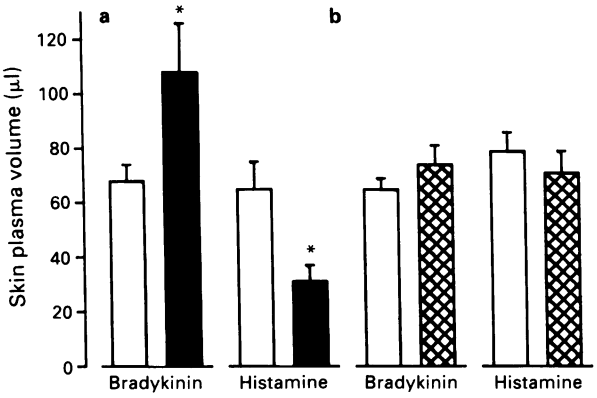

Figure 5 Within animals comparison of the effects of (a) L-arginine (10 µmol/site; solid columns; $n=5$) or (b) L-lysine (10 µmol/site; cross-hatched columns; $n=6$) on skin plasma protein exudation responses to bradykinin (0.5 nmol/site) or histamine (4.5 nmol/site). Responses to the mediators alone (open columns) or in the presence of L-arginine or L-lysine are expressed as µl plasma (corrected values) and mean ± s.e.mean values are shown. *$P<0.05$, compared with mediator alone.

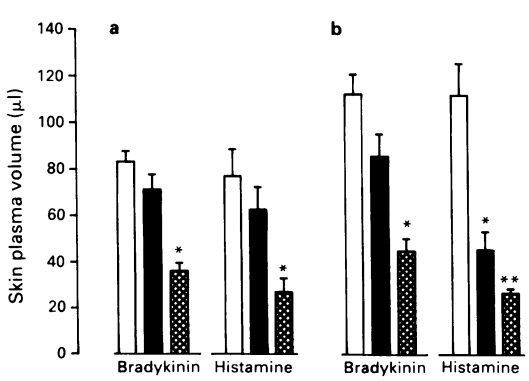

Figure 6 Within animals comparison of the effects of (a) phenylephrine (0.3 or 6 nmol/site; $n=6$) or (b) isoprenaline (0.5 or 10 nmol/site; $n=6$) on skin plasma protein exudation responses to bradykinin (0.5 nmol/site) or histamine (5.4 nmol/site). Responses to the mediators alone (open columns) or in the presence of (a) 0.3 nmol (solid column) or 6 nmol (cross-hatched column) phenylephrine per site or (b) 0.5 nmol (solid column) or 10 nmol (cross-hatched column) isoprenaline per site are expressed as µl plasma (corrected values) and mean ± s.e.mean values are shown. *$P<0.05$; **$P<0.01$ compared with mediator alone.

Table 2 Effects of arginine (L- and D-Arg) and lysine (L-Lys) on contractile responses in guinea-pig ileum *in vitro*

Treatment	EC_{50}
(a) Histamine alone	0.4 (0.1–1.5) µM
+ L-Arg 100 mM	*68.3 (8.3–563.2) µM
Histamine alone	113 (60–211) nM
+ D-Arg 100 mM	*3800 (2000–7400) nM
Histamine alone	71 (12–426) nM
+ L-Lys 100 mM	188 (63–560) nM
(b) Bradykinin alone	32.4 (16.7–62.5) nM
+ L-Arg 100 mM	53.7 (37.2–77.4) nM

Values are geometric means (95% confidence limits) of the EC_{50} values for (a) histamine and (b) bradykinin determined from $n=4$–6 replicate experiments in guinea-pig ileum. Isometric contractions were measured in Krebs solution alone or containing the specified concentration of test compound. *$P<0.05$ compared with mediator alone.

Discussion

The ability of L- but not D-NAME to inhibit BK- and histamine-induced cutaneous permeability responses in the guinea-pig extends previous studies in the rat (Hughes *et al.*, 1990; Ialenti *et al.*, 1992). Taken along with our observations of the ability of L-NAME to inhibit polycation- and antigen-induced exudation in rabbit skin (Paul *et al.*, 1992a), it is evident that L-NAME can inhibit oedema responses induced by a wide range of inflammatory stimuli. In the rat, L-NAME reduces skin blood flow at doses which inhibit the exudation response to substance P (Hughes *et al.*, 1990). Such data are compatible with the concept that L-NAME may reduce cutaneous permeability responses by inhibiting basal and/or mediator-induced NO production, thereby reducing local blood flow. Since we have not measured blood flow in the experiments described here, the observed effect of L-NAME could be a result of either a reduction in blood flow or an anti-permeability action. L-NAME has a greater effect on the exudation response to BK than on that to histamine. In contrast, isoprenaline, an anti-permeability agent acting via β-adrenoceptors, has a greater inhibitory effect on the exudation response to histamine than on that to BK. There is now ample persuasive evidence that isoprenaline (and other β-adrenoceptor agonists) acts by opposing the effects of inflammatory mediators on endothelial cell contraction and thus inhibits junctional gap formation (Persson & Svensjo, 1985; Grega, 1986). The fact that isoprenaline and L-NAME have opposite quantitative selectivities in their ability to reduce exudation responses to BK and histamine suggests that L-NAME may be acting by a different mechanism from isoprenaline. The more pronounced effect of L-NAME on the exudation response to BK than on that to histamine also contrasts with the effect of phenylephrine, a vasoconstrictor acting via α-adrenoceptors, which has comparable effects on responses to both mediators. Since phenylephrine does not exhibit a mediator selective effect, the results obtained with L-NAME could imply that NO production plays a more significant role in control of the leakage response to BK than in that to histamine. It remains possible that L-NAME may be acting by a mechanism other than reduction in blood flow. For example, endothelin-1 (ET-1), which has been shown to be a potent vasoconstrictor in the microcirculation (Brain *et al.*, 1988; 1989), has recently been reported to inhibit oedema induced by platelet activating factor (PAF) at a dose which has no effect on responses induced by BK, 5-hydroxytryptamine or histamine in the mouse paw suggesting that it may not be acting via vasoconstriction alone (Henriques *et al.*, 1992).

The ability of L-Arg to potentiate the response to BK could be a result of increased substrate leading to enhanced production of NO, although intracellular levels of L-Arg have

been reported to be relatively high (Mitchell *et al.*, 1990). L-, but not D-arginine, has previously been found to increase plasma extravasation in rat skin in response to carrageenin and in rat paw oedema in response to carrageenin or dextran (Ialenti *et al.*, 1992). The apparent paradox that L- and, to a lesser extent, D-Arg inhibit the histamine response in guinea-pig skin becomes explicable in light of the data with guinea-pig ileum. This confirms previous reports that Arg inhibits responses to histamine more effectively than those to kallidin in guinea-pig ileum and uterus (see Frey *et al.*, 1977). It is not clear whether Arg exerts its effect on histamine at a post-receptor level or acts directly on the H_1 receptor. Although the concentration of Arg is high, the same concentration of L-Lys, which possesses a similar charge, has no significant effect on the exudation response to BK or histamine or on the contractile response to histamine. The fact that L-Arg enhances the BK response in the absence of L-NAME makes interpretation of its ability to reverse the effect of L-NAME somewhat problematical. Similar considerations apply to analysis of the data of Ialenti *et al.* (1992), and Hughes *et al.* (1990) do not report whether L-Arg has a direct effect on the skin exudation response to substance P in the rat.

Taken together, our observations that, in a quantitative manner, L-NAME selectively inhibits BK responses, isoprenaline selectively inhibits histamine responses and phenylephrine has equivalent effects on vascular permeability induced by the two mediators imply that, in guinea-pig skin, the mode of action of L-NAME remains to be resolved. The different quantitative selectivities of isoprenaline and L-NAME could possibly be related to previously reported differences in dye leakage responses produced by BK and histamine in guinea-pig skin. Schachter (1960) noted that high doses of BK produced lesions which were more intensely coloured, though of smaller diameter, than those produced by high doses of histamine when viewed from the outer aspect of the skin. This observation was confirmed by Carr & Wilhelm (1964) who, in addition, showed differences between BK and histamine in terms of the layer of skin showing maximal exudation of dye. BK induced leakage mainly in the superficial dermis whereas histamine produced intense staining in the deep dermis. These morphological findings raise the possibility that BK and histamine may act preferentially on microvessels at different levels in the skin and these vessels may also differ in their responsiveness to other agents.

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(Received February 19, 1993
Revised September 14, 1993
Accepted September 24, 1993)

Br. J. Pharmacol. (1994), **111**, 165–172

Effects of a newly synthesized K^+ channel opener, Y-26763, on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of the rabbit mesenteric artery

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1 The mechanisms underlying the vasodilatation induced by (−)-(3S,4R)-4-(N-acetyl-*N*-hydroxyamino)-6-cyano-3,4-dihydro-2, 2-dimethyl-2H-1-benzopyran-3-ol (Y-26763) were investigated by measuring membrane potential, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and isometric force in smooth muscle cells of the rabbit mesenteric artery.

2 Y-26763 (0.03–1 μM) concentration-dependently hyperpolarized the membrane and glibenclamide (1–10 μM) inhibited this hyperpolarization. Noradrenaline (NA, 10 μM) depolarized the membrane and generated spike potentials. Y-26763 (1 μM) inhibited these NA-induced electrical responses.

3 In thin smooth muscle strips in 2.6 mM Ca^{2+} containing (Krebs) solution, 10 μM NA produced a large phasic, followed by a small tonic increase in $[Ca^{2+}]_i$ and force with associated oscillations. In Ca^{2+}-free solution (containing 2 mM EGTA), NA produced only phasic increases in $[Ca^{2+}]_i$ and force. In ryanodine-treated strips, NA could not produce the phasic increases in $[Ca^{2+}]_i$ and force even in the presence of 2.6 mM Ca^{2+}, suggesting that ryanodine functionally removes the NA-sensitive intracellular storage sites.

4 Nicardipine (1 μM) partly inhibited the NA-induced tonic increases in $[Ca^{2+}]_i$ and force but had no effect on either the resting $[Ca^{2+}]_i$ or the NA-activated phasic increases in $[Ca^{2+}]_i$ and force. By contrast, Y-26763 (10 μM) lowered the resting $[Ca^{2+}]_i$ and also inhibited both the phasic and the tonic increases in $[Ca^{2+}]_i$ and force induced by NA. All these actions of Y-26763 were inhibited by glibenclamide (10 μM).

5 In ryanodine-treated strips, nicardipine partly, but Y-26763 completely inhibited the NA-induced increases in $[Ca^{2+}]_i$, suggesting that Y-26763 inhibits both the nicardipine-sensitive and -insensitive Ca^{2+} influxes activated by NA. Y-26763 attenuated the phasic increase in $[Ca^{2+}]_i$ and force in a Ca^{2+}-free solution containing 5.9 mM K^+, but not in one containing 50 mM K^+, suggesting that Y-26763 inhibits NA-induced Ca^{2+} release, probably as a result of its membrane hyperpolarizing action.

6 In β-escin-skinned strips, Y-26763 (10 μM) had no effect on either the NA-induced Ca^{2+} release or the Ca^{2+}-tension relationship in the presence and absence of NA (10 μM) with guanosine 5′-triphosphate (GTP, 10 μM), suggesting that Y-26763 has no direct action on either NA-induced Ca^{2+} release or the contractile proteins.

7 It is concluded that Y-26763 inhibits NA-activated Ca^{2+} release and Ca^{2+} influx and thus inhibits the NA-contraction. Y-26763 also lowers the resting $[Ca^{2+}]_i$ through an inhibition of the nicardipine-insensitive Ca^{2+} influx. These actions of Y-26763 may be linked with the membrane hyperpolarization it produces by activation of the ATP-sensitive K^+ channels.

Keywords: Newly synthesized K^+ channel opener; Y-26763; noradrenaline-induced Ca^{2+} mobilization; membrane hyperpolarization; vascular smooth muscle pharmacology

Introduction

(+)-(3S,4R)-4- (N-acetyl-*N*-benzyloxyamino)-6-cyano-3,4-dihydro-2, 2-dimethyl-2H-1-1-benzopyran-3-ol (Y-27152) is a newly synthesized K^+ channel opener which has a long duration of anti-hypertensive action with little tachycardia (Nakajima *et al.*, 1992). This compound is itself pharmacologically inert and after oral administration it is converted to an active desbenzyl form Y-26763 by cytochrome P450. Y-26763 has been purported to be a representative of a new class of vasodilator which hyperpolarize the membrane by opening K^+ channels in vascular smooth muscle cells (Hamilton & Weston, 1989; Quast & Cook, 1989; Standen *et al.*, 1989; Nakajima *et al.*, 1992). These K^+ channel openers inhibit the activation of the voltage-dependent L-type Ca^{2+} channels which are sensitive to organic Ca^{2+}-channel blockers, by hyperpolarizing the membrane (Standen *et al.*, 1989). In smooth muscle cells, Ca^{2+} influx can be increased by agonists through activation of both the L-type Ca^{2+} channel and the receptor-operated non-selective cation channel (Benham & Tsien, 1987; Nelson *et al.*, 1988; Inoue & Isenberg, 1990; Pacaud & Bolton, 1990). These channels are voltage-sensitive, but the latter channel is insensitive to organic Ca^{2+}-channel blockers in longitudinal smooth muscle of the guinea-pig ileum (Inoue & Isenberg, 1990). We previously suggested that, in the rabbit mesenteric artery, noradrenaline (NA) enhances both nifedipine-sensitive and -insensitive Ca^{2+}-influxes, possibly due to depolarization of the membrane (Kanmura *et al.*, 1983). Therefore, in the rabbit mesenteric artery, an agent that hyperpolarizes the smooth muscle membrane would be likely to inibit the NA-induced contraction more than an organic Ca^{2+}-channel blocker.

In the rabbit mesenteric artery, NA produces a large phasic, followed by a tonic increase in both the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and force (Itoh *et al.*, 1992a, b). The phasic response is related to the NA-induced Ca^{2+} release from the intracellular storage sites whereas the tonic response may be provoked by an interplay between Ca^{2+} influx through the plasmamembrane and Ca^{2+}-uptake into, or Ca^{2+} release from the storage sites (Itoh *et al.*, 1983). We recently found that K^+ channel openers such as pinacidil or

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lemakalim inhibit the NA-induced synthesis of inositol 1,4,5-triphosphate ($InsP_3$) with an associated membrane hyperpolarization which results in a decrease in the release of Ca^{2+} from the storage sites. It is of interest to know whether this is the case for other types of drugs which are classified as K^+ channel openers.

The present study was carried out to clarify the mechanism underlying the vasodilatation induced by Y-26763 in the face of the NA-induced contraction. To this end, we studied the effects of Y-26763 on the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-containing and in Ca^{2+}-free solution under membrane-polarized and -depolarized conditions. The effects of Y-26763 on the NA-induced increase in $[Ca^{2+}]_i$ were also studied in ryanodine-treated smooth muscle strips which lack functional NA-sensitive Ca^{2+} storage sites (Itoh *et al.*, 1992a) and these effects were compared with those of nicardipine (an L-type Ca^{2+}-channel blocker). Furthermore, the direct actions of Y-26763 on NA-induced Ca^{2+} release and on contractile proteins were also studied in β-escin-skinned smooth muscle strips.

Methods

Male albino rabbits, weighing 1.9–2.5 kg were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then exsanguinated. A segment of the third branch of the mesenteric artery distributing to the ileum was excised immediately and cleaned by removal of connective tissue in Krebs solution at room temperature.

Membrane potential measurement

Glass microelectrodes filled with 3 M KCl were made from borosilicate glass tube (o.d. 1.2 mm with a core inside, Hilgenberg, Germany), the resistance of the electrodes being 40–80 MΩ. The electrode was inserted into smooth muscle cells from the adventitial side and responses displayed on a cathode-ray oscilloscope (VC-10, Nihon-Kohden) and on a pen-writing recorder (Recticorder RJG-4024, Nihon-Kohden).

$[Ca^{2+}]_i$ and force measurement

To enable recording of $[Ca^{2+}]_i$ and isometric force simultaneously, fine circularly-cut strips (0.3–0.5 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) were prepared as previously described (Itoh *et al.*, 1983). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The absence of endothelial cells was confirmed by the inability of acetylcholine (1 μM) to cause relaxation during contractions induced by NA, as described previously (Itoh *et al.*, 1992a,b). The strip was transferred into a chamber of 0.2 ml volume and mounted horizontally on an invert-microscope (Diaphoto TMD with special optics for epifluorescence, Nikon). The resting force was adjusted to obtain a maximal contraction in 128 mM K^+.

To enable loading of Fura 2 into smooth muscle cells of the strip, 1 μM acetoxy methyl ester of Fura 2 (Fura 2AM) dissolved in dimethyl sulphoxide (1 mM stock solution) was applied for 1 h in Krebs solution at room temperature (20°C), as reported previously (Itoh *et al.*, 1992a,b). Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm) were applied by a spectro-fluorimeter (Spex, NJ, U.S.A.) and the data analyzed using customized software provided by Spex (DM-3000CM). The ratio of the Fura 2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) as excited by 340 and 380 nm u.v. light was measured following application of a solution containing 50 μM ionomycin, 20 mM $MnCl_2$, 110 mM KCl and 10 mM 3-(N-morpholino) propanesulphonic acid (MOPS) (pH 4.8) after the experiment. Under these conditions, the background fluorescence intensity was 10–15% of the Fura 2 signals in smooth muscle strips at either excitation wavelength. Cytosolic Ca^{2+} concentrations were calculated by the formula described by Grynkiewicz *et al.* (1985) and using *in vitro* calibration (Poenie *et al.*, 1986), as reported previously (Itoh *et al.*, 1992a,b).

NA (10 μM) was applied for 2–2.5 min at 30 min intervals so as to obtain reproducible responses and then Y-26763 was applied for 10 min before and during subsequent applications of NA. Ryanodine (50 μM) was used to remove functionally the NA-sensitive Ca^{2+} storage sites (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987; Itoh *et al.*, 1992a). In this type of experiment, NA was applied at 30 min intervals in Krebs solution in the absence of ryanodine and 50 μM ryanodine with 10 mM caffeine was then applied for 10 min. Twenty min after removal of caffeine, NA was again applied in the presence of 50 μM ryanodine in Krebs solution.

To investigate the effect of Y-26763 on NA-induced Ca^{2+} release in intact smooth muscle strips, effects of Y-26763 on NA-induced increase in $[Ca^{2+}]_i$ and force were studied in Ca^{2+}-free solution containing 2 mM EGTA. NA was applied for 2 min in the Ca^{2+}-free solution every 30 min. After 2 min in Ca^{2+}-free solution, the strips were stimulated by NA for 2 min and then brought back to the Ca^{2+}-containing Krebs solution (Ca^{2+} = 2.6 mM) for 25 min.

Experiments on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were produced by use of β-escin (Kobayashi *et al.*, 1989; Itoh *et al.*, 1991; 1992a). The methods used to produce skinned muscles and the composition of the solutions have been described elsewhere (Itoh *et al.*, 1986a). When the Ca^{2+}-tension relationship was to be determined, the concentration of EGTA in the solution was 4 mM EGTA and 1 μM ionomycin was applied to avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle strips. To prevent deterioration of the Ca^{2+}-induced contraction, 0.1 μM calmodulin was applied throughout the experiments, as described previously (Itoh *et al.*, 1986a). Various concentrations of Ca^{2+} were applied cumulatively at 10 min intervals from low to high concentration. The amplitude of the contractions induced by various concentrations of Ca^{2+} were normalized with respect to that induced by 10 μM Ca^{2+} in the same strip.

To enable measurement of NA-induced Ca^{2+} release from the storage sites, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 3 min (to load Ca^{2+} into the store sites) and Ca^{2+} then removed from the solution by application of Ca^{2+}-free solution containing 4 mM EGTA for 0.5 min. Then, a solution containing 50 μM EGTA, 10 μM GTP, 1 μM propranolol and 2 μM Fura 2 was applied for 2 min. Finally 10 μM NA plus 1 μM propranolol with 10 μM GTP was applied for 2 min in a solution containing 50 μM EGTA and 2 μM Fura 2.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The concentration of K^+ was modified by replacing NaCl with KCl, isosmotically. To prevent both NA outflow from sympathetic nerve terminals and β-adrenoceptor stimulation by exogenously applied NA, guanethidine (3 μM) and propranolol (1 μM) were present in the Krebs solution throughout the experiment. Ca^{2+}-free Krebs solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ and adding 2 mM EGTA. The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH maintained at 7.3–7.4.

The calibration solution for Ca^{2+} measurement in intact strips contained 11 mM EGTA, 110 mM KCl, 1 mM $MgCl_2$, 2 μM Fura 2 and 20 mM N-2-hydroxyethylpiperazine-N'-2-

ethanesulphonic acid (HEPES) (pH 7.1) with or without 11 mM $CaCl_2$.

For experiments on skinned muscle, the composition of the relaxing solution was (mM): potassium methanesulphonate (KMS 87), piperazine-*N*-*N*'-*bis*-(2-ethanesulphonic acid) (PIPES 20), $Mg(MS)_2$ 5.1, ATP 5.2, phosphocreatine 5, ethyleneglycol-*bis*-(β-aminoethyl)-*N*,*N*,*N*'*N*'-tetraacetic acid (EGTA4) and propranolol (1 μM). To enable measurement of Ca^{2+} release from skinned strips, the concentration of EGTA was reduced to 50 μM and 2 μM Fura 2 with 10 μM GTP added. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(MS)_2$ to 4 mM EGTA, based on the calculation reported previously (Itoh *et al.*, 1986a). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were: Fura 2, Fura 2AM, EGTA, PIPES, HEPES and MOPS (Dojin, Japan), NA, GTP, β-escin, nicardipine and glibenclamide (Sigma), ryanodine (Agri-system), guanethidine (Tokyo Kasei, Japan), ATP (Na salt; Kojin, Japan), propranolol (Nacalai, Japan) and ionomycin (free acid; Calbiochem). (−)-(3S,4R)-4- (N-acetyl-*N*-hydroxyamino)-6-cyano-3, 4-dihydro-2, 2-dimethyl-2H-1-benzopyran-3-ol (Y-26763) was kindly provided by Yoshitomi Pharmaceutical Ind., Ltd. (Japan).

Statistics

The values recorded were expressed as mean ± s.d., and statistical significance determined by a paired or unpaired Student's *t* test. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effects of Y-26763 on membrane potential

In smooth muscle cells of the rabbit mesenteric artery, the resting membrane potential was −65 to −75 mV (mean −70.2 ± 1.8 mV, $n = 26$). Application of Y-26763 (> 10 nM) hyperpolarized the membrane. In our system in which change of solutions occupied about 30 s, the delay before the start of the hyperpolarization induced by 1 μM Y-26763 was 51.3 ± 7.0 s ($n = 16$) and a steady amplitude was reached at 134.4 ± 27.3 s ($n = 14$) (Figure 1a). The sustained hyperpolarization produced by Y-26763 lasted for up to 15 min. On removal of Y-26763 from the superfusate, the membrane potential returned to the resting level with a very slow time course: complete recovery required 25–30 min when 1 μM Y-26763 was applied. Similar hyperpolarization of the membrane was produced by lemakalim, in concentrations over 1 nM (data not shown).

Glibenclamide (1 μM) depolarized the membrane by 4–7 mV and changed the membrane hyperpolarization produced by 1 μM Y-26763 as follows: (1) the time to the start of the hyperpolarization and that for the hyperpolarization to reach peak amplitude were both greatly prolonged (Table 1), (2) the amplitude of the hyperpolarization was slightly reduced and (3) the time required for recovery from the hyperpolarization was shortened to 7–10 min (Figure 1b). In the presence of 10 μM glibenclamide, the membrane potential was about −68 mV, not significantly different from that in the presence of 1 μM glibenclamide. Application of 1 μM Y-26763 in the presence of 10 μM glibenclamide produced only a small amplitude hyperpolarization after a very long delay (about 2 min, Figure 1c).

Figure 1d summarizes the effects of Y-26763 on the membrane potential of smooth muscle cells in the rabbit mesenteric artery. Y-26763 (over 30 nM) hyperpolarized the membrane, in a concentration-dependent manner, and 0.3 μM was required to produce the maximum hyperpolarization of about 10 mV from the resting membrane potential. Glibenclamide (1 μM) shifted the concentration-response relationship to the right and increased to 10 μM the concentration of Y-26763 needed to produce the maximum hyperpolarization. In the presence of 10 μM glibenclamide, the hyperpolarization produced by 10 μM Y-26763 was greatly reduced.

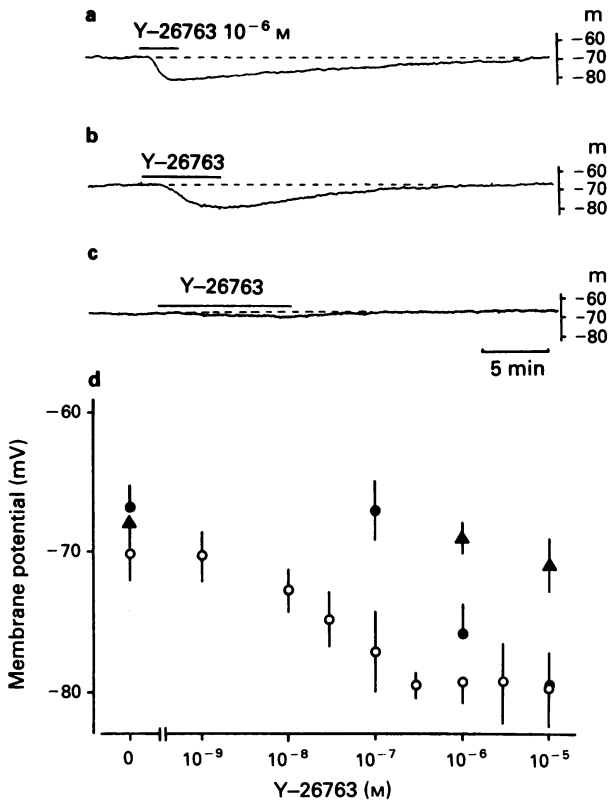

Figure 1 Effects of glibenclamide on membrane hyperpolarization produced by Y-26763 in smooth muscle cells of the rabbit mesenteric artery. Y-26763 (1 μM) was applied before (a) and after application of glibenclamide (b, 1 μM; c, 10 μM), for 3 min, 5 min and 10 min, respectively, at the bar as indicated in each record. The membrane potentials before application of Y-26763 were: (a) −71 mV; (b) −67 mV; (c) −67 mV. All the responses were recorded from the same tissue. (d) The concentration-response relationship for the hyperpolarization produced by Y-26763 in smooth muscle cells of rabbit mesenteric artery. Membrane potentials were measured by impalement of different cells by the microelectrode, in the presence of Y-26763 (1 nM–10 μM) for 30 min, before (○) and after application of glibenclamide (1 μM, ●; 10 μM, ▲). Mean ± s.d. collected from 3–5 tissues ($n = 10–35$).

Effects of Y-26763 on NA-induced depolarization

Figure 2 shows electrical responses produced by NA (10 μM) in a smooth muscle cell of the rabbit mesenteric artery. Application of NA depolarized the membrane and generated spike potentials; these properties are similar to those reported previously (Itoh *et al.*, 1983). The mean amplitude of the NA-induced depolarization was 10.6 ± 2.7 mV ($n = 10$). In the presence of 1 μM Y-26763, the membrane was hyperpolarized by about 10 mV and the amplitude of the NA-induced depolarization was reduced to 1.6 ± 1.2 mV ($n = 5$), with no generation of spike potentials (Figure 2b).

Effects of Y-26763 on contraction induced by NA in Ca^{2+}-containing solution

The effects of a wide range of concentrations of Y-26763 on contraction induced by 10 μM NA were investigated in thin

Table 1 The time required to start and to reach peak amplitude of hyperpolarizations produced by 1 μM Y-26763 (A) and 1 μM lemakalim (B) and their modulation by 1 μM glibenclamide in smooth muscle of the rabbit mesenteric artery

A *Y-26763*	*Control*†	*Glibenclamide*
Time to start hyperpolarization	51.3 ± 7.0 s ($n = 16$)	104.3 ± 23.7 s ($n = 9$)*
Time to reach peak amplitude	134.4 ± 27.3 s ($n = 14$)	323.3 ± 98.4 s ($n = 9$)*
B *Lemakalim*	*Control*†	*Glibenclamide*
Time to start hyperpolarization	50.2 ± 6.4 s ($n = 6$)	114.0 ± 43.5 s ($n = 3$)*
Time to reach peak amplitude	135.6 ± 19.2 s ($n = 5$)	278.0 ± 44.5 s ($n = 3$)*

†The value obtained before application of glibenclamide. All values include time spent changing solutions contained in the tubing system (the approximate time was about 30 s).
*Represents statistical difference from the corresponding control ($P < 0.05$).

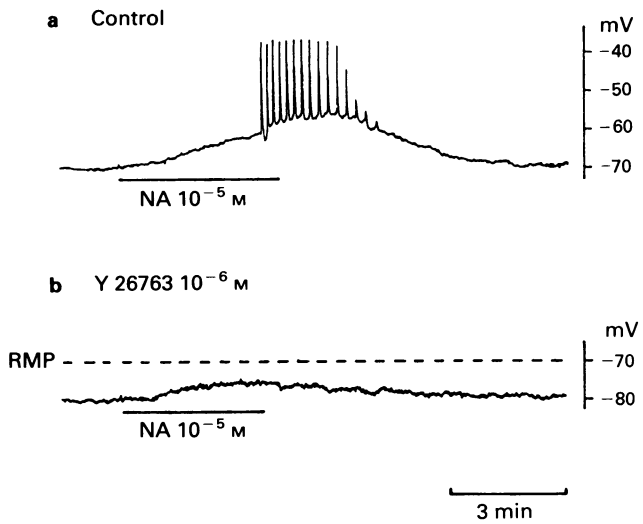

Figure 2 Effects of Y-26763 on electrical responses produced by noradrenaline (NA) in smooth muscle of rabbit mesenteric artery. NA (1 μM) was applied before (a) and during (b) application of 1 μM Y-26763.

smooth muscle strips of the rabbit mesenteric artery (Figure 3). NA (10 μM) produced a phasic, followed by a tonic contraction. In the NA-contraction, an oscillation in tension was often observed during the tonic phase. In the presence of increasing concentrations of Y-26763, the oscillatory components disappeared first, then the amplitude of the initial phase component was successively decreased. After pretreatment with 10 μM Y-26763, NA produced no oscillatory contractions and the initial phasic and subsequent tonic contractions were both greatly reduced (Figure 3a(iv)). These effects of Y-26763 were reversible: removal of Y-26763 from the superfusate restored the responses to NA within 40–50 min.

The inhibitory actions of Y-26763 (0.03–1 μM) on the NA-contraction were greatly attenuated when glibenclamide (1 μM) was given as pretreatment (Figure 3b). Increasing the concentration of glibenclamide to 10 μM abolished the effect of 10 μM Y-26763 on the NA-contraction. The concentration-response relationship for the effects of glibenclamide on the inhibition by Y-26763 of the NA-induced contraction is summarized in Figure 3c, in which effects on the relative amplitude of the initital phasic tension are shown. The figure shows that the inhibitory action of Y-26763 is antagonized by glibenclamide, in a concentration-dependent manner.

Effects of Y-26763 on changes in $[Ca^{2+}]_i$ and force induced by NA in Ca^{2+}-containing solution

It is known that K^+-channel openers hyperpolarize the membrane and then inhibit voltage-dependent L-type Ca^{2+} chan-

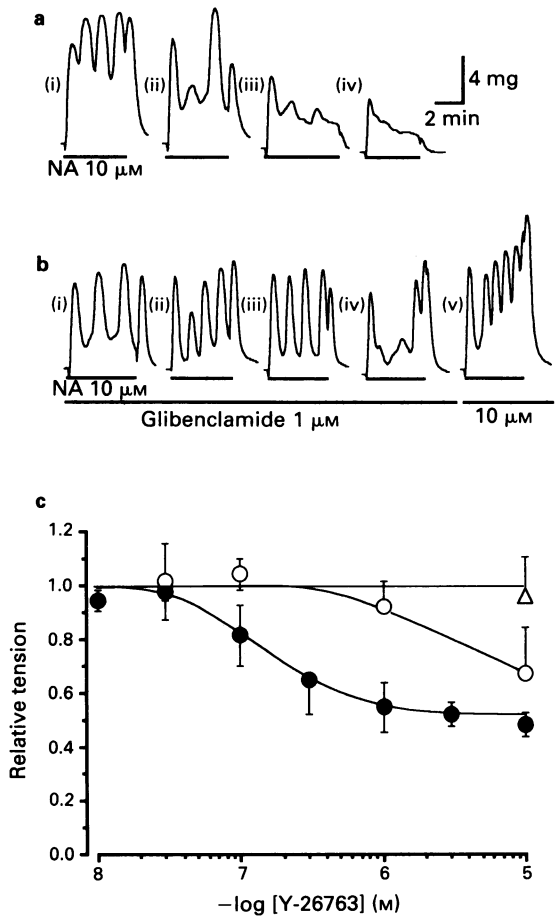

Figure 3 Concentration-dependent effects of Y-26763 on contraction induced by noradrenaline (NA) in the presence and absence of glibenclamide in Ca^{2+}-containing solution. Actual traces of the effect of Y-26763 in the absence (a) and presence (b) of glibenclamide. NA (10 μM) was applied for 4 min at 10 min intervals in the absence of (a(i) and b(i)) or presence of Y-26763 without (a) or with (b) glibenclamide. After the recording of the control response (a(i)), Y-26763 (0.03 μM) was pre-treated for 10 min and was present during application of 10 μM NA (a(ii)). Concentration-dependent effects of Y-26763 were obtained by increasing its concentration from low to high in a stepwise manner: (a(iii)), in the presence of 1 μM Y-26763; (a(iv)), in 10 μM. Time interval between traces (a) and (b) was 60 min. In (b), Y-26763 was applied in the presence of 1 μM (b(i)–b(iv)) or 10 μM (b(v)) glibenclamide. The concentrations of Y-26763 were 0.03 μM (b(ii)), 1 μM (b(iii)) and 10 μM (b(iv) and b(v)). (c) Effects of Y-26763 on the maximum amplitude of contraction induced by 10 μM NA in the presence and absence of glibenclamide: (●) control; (○) in the presence of 1 μM glibenclamide; (△), in 10 μM glibenclamide. The maximum amplitude of contraction induced by 10 μM NA in the absence of both Y-26763 and glibenclamide was normalized as a relative tension of 1.0 for each strip. Each value represents mean ± s.d. ($n = 4$).

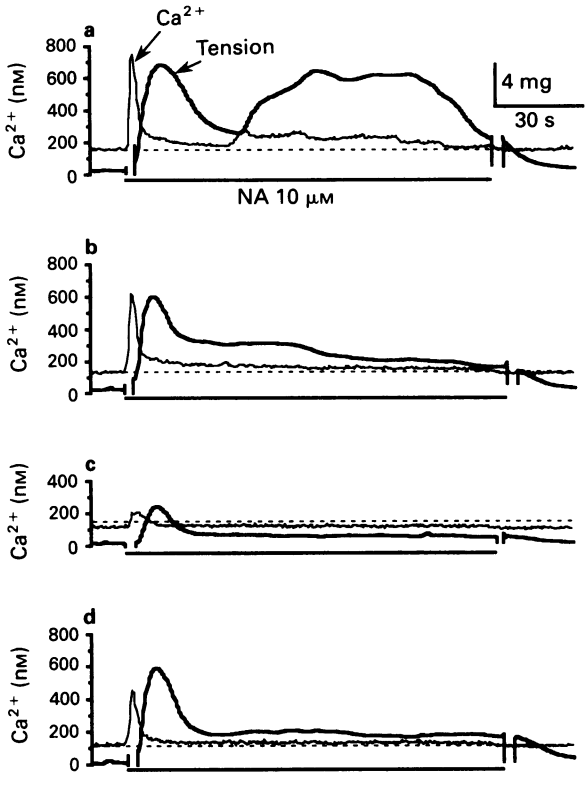

Figure 4 Effects of 10 μM Y-26763 on changes in $[Ca^{2+}]_i$ and force induced by 10 μM noradrenaline (NA) in the presence and absence of 1 μM nicardipine in Ca^{2+}-containing solution. Broken lines indicate resting $[Ca^{2+}]_i$ under control conditions. $[Ca^{2+}]_i$, thinner line; force, thicker line. NA was applied for 2.5 min at 30 min intervals in Krebs solution. (a) Control; (b) nicardipine (1 μM) was given as pretreatment for 5 min and was present throughout the experiment; (c) Y-26763 was given as pretreatment for 10 min in the presence of nicardipine and was present during application of NA: (d) Glibenclamide (10 μM) was given as pretreatment for 5 min in the presence of Y-26763 with nicardipine and was present during application of NA. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

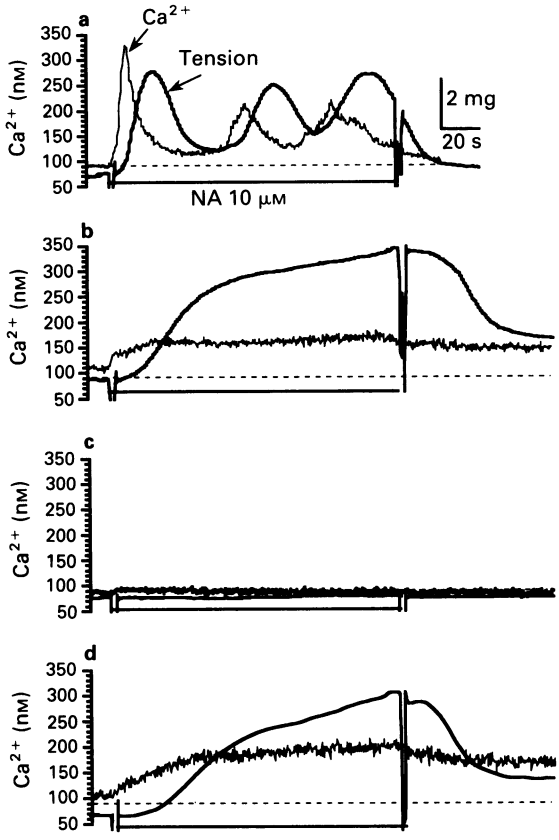

Figure 5 Effects of 10 μM Y-26763 on changes in $[Ca^{2+}]_i$ and force induced by 10 μM noradrenaline (NA) in Ca^{2+} containing solution in a ryanodine-treated smooth muscle strip. NA was applied for 2.5 min at 30 min intervals in Krebs solution. (a) Control; between traces (a) and (b) 50 μM ryanodine with 10 mM caffeine was given for 10 min, followed by washout of caffeine for 20 min and then 10 μM NA was applied. (b) In the presence of ryanodine; (c) Y-26763 was given as pretreatment for 10 min and was present throughout the experiment. (d) Glibenclamide (10 μM) was applied for 5 min before and throughout the experiment. Broken lines indicate resting $[Ca^{2+}]_i$ under control conditions. The results shown were obtained from a single smooth muscle strip and were reproducible in another 2 strips.

nels causing the inhibition of NA-induced contraction (Standen *et al.*, 1989). To test whether or not Y-26763 attenuates the NA-induced contraction solely due to an inhibition of L-type Ca^{2+} channels, the effects of Y-26763 on the changes in $[Ca^{2+}]_i$ and force induced by 10 μM NA were studied in the presence of nicardipine. The resting $[Ca^{2+}]_i$ and force were 109 ± 25 nM and 0.8 ± 0.3 mg, respectively ($n = 4$). NA produced a transient phasic, followed by a oscillatory increase in both $[Ca^{2+}]_i$ and force in the absence of nicardipine (Figure 4a). The maximum increase in $[Ca^{2+}]_i$ induced by NA was 545 ± 247 nM in these muscle strips ($n = 4$). Nicardipine (1 μM) reduced the NA-induced increases in $[Ca^{2+}]_i$ and force, and the effects were more marked in the oscillatory phase of the response than in the phasic component (Figure 4b). This concentration of nicardipine completely inhibited the contraction induced by 128 mM K^+ (data not shown). Y-26763 (10 μM) greatly attenuated both the phasic and the small tonic increases in $[Ca^{2+}]_i$ and force induced by NA in the presence of 1 μM nicardipine (Figure 4c). Glibenclamide (10 μM) restored the increases in $[Ca^{2+}]_i$ and force induced by NA to levels similar to those seen in the presence of nicardipine alone (Figure 4d, compare with Figure 4b). The extent of the inhibition induced by 10 μM Y-26763 on NA-responses was similar to that observed in the absence of nicardipine (data not shown). These results suggest that Y-26763 inhibits both nicardipine-sensitive and -insensitive Ca^{2+} influxes activated by NA. However, since NA-induced increase in $[Ca^{2+}]_i$ is provoked by an interplay between the NA-induced Ca^{2+} influx and Ca^{2+} release from the storage sites (Itoh *et al.*, 1983) and K^+-channel openers, such as lemakalim and pinacidil inhibit the NA-induced Ca^{2+} release (Ito *et al.*, 1991; Itoh *et al.*, 1992b), this hypothesis is obscure. To test this hypothesis more precisely, the effect of Y-26763 on the changes in $[Ca^{2+}]_i$ and force induced by 10 μM NA was studied in ryanodine-treated muscle strips which lack functional NA-sensitive Ca^{2+} storage sites (Itoh *et al.*, 1992a).

Following the application of ryanodine, the resting $[Ca^{2+}]_i$ was slightly increased (from 199 ± 10 nM to 158 ± 18 nM, $n = 4$) and additional application of NA failed to induce either the phasic or the subsequent oscillatory increases in $[Ca^{2+}]_i$ and force (Figure 5b). In ryanodine-treated strips, $[Ca^{2+}]_i$ and force were induced by NA to increase slowly and the time to peak was delayed. Y-26763 (10 μM) lowered the resting $[Ca^{2+}]_i$ (to 117 ± 10 nM, $n = 4$) and almost completely inhibited the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA in ryanodine-treated strips (Figure 5c), and glibenclamide greatly attenuated the inhibitory action of Y-26763 (Figure 5d). In contrast, nicardipine (1 μM) partly inhibited the increase in $[Ca^{2+}]_i$ induced by NA with no significant change in the resting $[Ca^{2+}]_i$ (data not shown).

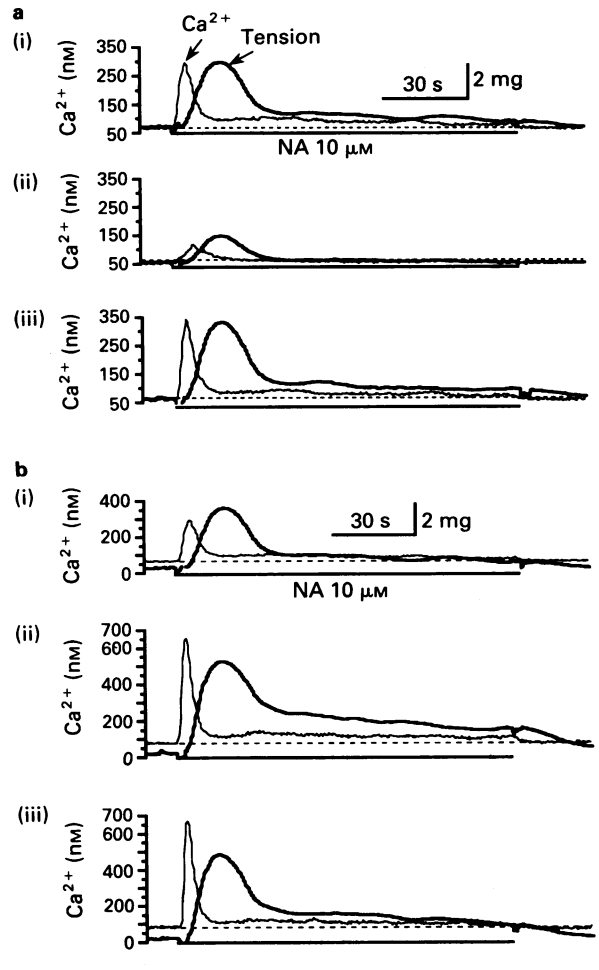

Figure 6 Effects of Y-26763 on changes in $[Ca^{2+}]_i$ and force induced by 10 μM noradrenaline (NA) in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ (a) or 50 mM K^+ (b). NA was applied for 2 min after a 2 min period of Ca^{2+} removal, then Ca^{2+}-free solution was applied for 3 min to wash out NA. This protocol was repeated at 30 min intervals with the strips being kept in Krebs solution (containing 2.6 mM Ca^{2+}) for the 25 min between tests. When used, Y-26763 was applied for 10 min before and was present throughout the experiment. Broken lines indicate resting $[Ca^{2+}]_i$ in Ca^{2+}-free solution in the absence of 10 μM Y-26763. In (a): (i), control; (ii), in the presence of Y-26763, (iii), in the presence of 10 μM Y-26763 with 10 μM glibenclamide. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips. N.B. the resting $[Ca^{2+}]_i$ before application of NA was lower in the presence of Y-26763 than in control. In (b): (i) NA-response in Ca^{2+}-free solution containing 5.9 mM K^+; (ii) NA-response in Ca^{2+}-free solution containing 50 mM. K^+. Ca^{2+}-free solution containing 50 mM K^+ was applied for 1 min after a 1 min removal of Ca^{2+} by Ca^{2+}-free solution containing 5.9 mM K^+. NA (10 μM) was then applied for 2 min in Ca^{2+}-free solution containing 50 mM K^+ and finally Krebs solution (containing 2.6 mM Ca^{2+}) was applied for 25 min. This protocol was repeated at 30 min intervals. (b(iii)) In the presence of 10 μM Y-26763. N.B. the resting $[Ca^{2+}]_i$ before application of NA in the presence of Y-26763 was the same as that in the corresponding control. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 2 strips.

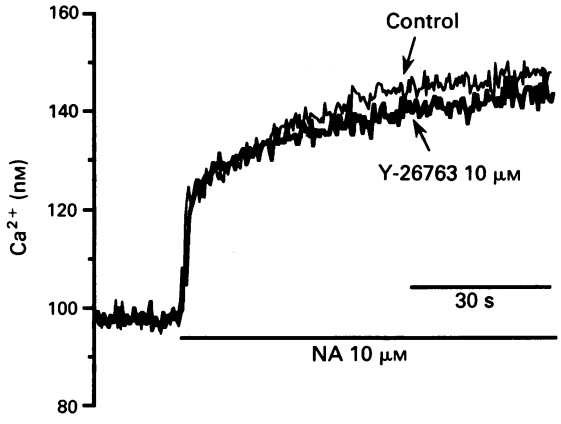

Figure 7 Effects of Y-26763 on increase in Ca^{2+} induced by 10 μM noradrenaline (NA) in a β-escin-skinned smooth muscle strip. After the strip was skinned by application of 25 μM β-escin for 25 min, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 3 min and Ca^{2+}-free solution containing 4 mM EGTA then applied for 0.5 min to remove Ca^{2+}. Subsequently, 10 μM NA was applied for 2 min in Ca^{2+}-free solution containing 50 μM EGTA, 10 μM GTP and 2 μM Fura 2 following a 2 min application of Ca^{2+}-free solution containing 50 μM EGTA, 10 μM GTP and 2 μM Fura 2. Y-26763 (10 μM) was applied for 10 min before and was present throughout application of NA. These results were reproducible in another 2 strips.

Effects of Y-26763 on changes in $[Ca^{2+}]_i$ and force induced by NA in Ca^{2+}-free solution

The effects of Y-26763 on NA-induced Ca^{2+} release were examined using as control the increase in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. In Ca^{2+}-free solution for over 15 s, no increase in $[Ca^{2+}]_i$ or force was induced by 128 mM K^+. Following the application of the Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ rapidly decreased from 118 ± 25 to 56 ± 13 nM ($n = 4$) within 1 min and then remained at a new steady level. NA transiently increased $[Ca^{2+}]_i$ (to 313 ± 94 nM, $n = 4$) and force (to 8.3 ± 1.5 mg, $n = 4$) in Ca^{2+}-free solution. Y-26763 (10 μM) slightly lowered the resting $[Ca^{2+}]_i$ and inhibited the NA-evoked increases in $[Ca^{2+}]_i$ and force (Figure 6a(ii)). The resting $[Ca^{2+}]_i$ was 56 ± 13 nM or 48 ± 8 nM in the absence or presence of Y-26763, respectively ($P < 0.05$ using a paired t test, $n = 4$). Glibenclamide (10 μM) blocked the action of Y-26763 on the resting $[Ca^{2+}]_i$ (54 ± 12 nM in the presence of Y-26763 with glibenclamide, $n = 4$) and also attenuated the inhibitory action of Y-26763 on the NA-evoked responses (Figure 6a(iii)). In contrast, Y-26763 had no effect on the increase in $[Ca^{2+}]_i$ and force induced by 10 mM caffeine in Ca^{2+}-free solution containing 5.9 mM K^+ (data not shown).

Figure 6b shows the effects of Y-26763 on the increases in $[Ca^{2+}]_i$ and force induced by NA in Ca^{2+}-free solution containing 50 mM K^+. Following an application of Ca^{2+}-free solution containing 5.9 mM K^+ for 1 min, the solution containing 50 mM K^+ failed to increase $[Ca^{2+}]_i$ but enhanced the increase in $[Ca^{2+}]_i$ and force induced by subsequently applied NA (Figure 6b(ii)). In Ca^{2+}-free solution containing 50 mM K^+, Y-26763 had no effect on either the resting $[Ca^{2+}]_i$ or the NA-induced increases in $[Ca^{2+}]_i$ and force (Figure 6b(iii)).

Actions of Y-26763 in β-escin-skinned smooth muscle

The direct effects of Y-26763 on the NA-induced Ca^{2+} release were observed in β-escin-treated skinned smooth muscle strips which cannot generate a membrane potential. NA (10 μM) increased $[Ca^{2+}]_i$ in Ca^{2+}-free solution after brief application of Ca^{2+} (Figure 7). Y-26763 (10 μM) had no significant effect on this NA-induced Ca^{2+} release.

The direct actions of Y-26763 on contractile proteins were also estimated from the effects of this drug on Ca^{2+}-force relationships in the presence and absence of NA in β-escin-treated skinned strips. The minimum concentration of Ca^{2+} that produced contraction was 0.1 μM and the maximum contraction was obtained at 10 μM. The concentrations of Ca^{2+} required for the half maximal contraction were 0.49 ± 0.05 μM. Y-26763 (10 μM) had no effect on the Ca^{2+}-force relationship.

In the skinned muscles, NA (10 μM), added with GTP (10 μM), shifted the Ca^{2+}-force relationship to the left with a slight increase in the amplitude of the maximum Ca^{2+}-induced contraction. The concentration of Ca^{2+} required for the half maximal contraction in the presence of NA with GTP was 0.28 ± 0.03 μM and the maximum amplitude of contraction induced by 10 μM Ca^{2+} was 1.13 ± 0.03 times the corresponding control ($n = 4$). Y-26763 (10 μM) had no effect on either the Ca^{2+}-force relationship or the maximum Ca^{2+}-induced contraction in the presence or absence of NA with GTP.

Discussion

In many types of smooth muscle, agents that activate the ATP-sensitive K^+ channel, such as pinacidil, cromakalim and lemakalim induce membrane hyperpolarization and muscle relaxation (Hamilton & Weston, 1989; Quast & Cook, 1989; Standen *et al.*, 1989; Kajioka *et al.*, 1991; Itoh *et al.*, 1992b). The hyperpolarization can be selectively inhibited by a sulphonylurea, glibenclamide (Standen *et al.*, 1989; Itoh *et al.*, 1992b). In the present experiments, Y-26763 (0.1–10 μM) concentration-dependently hyperpolarized the membrane and this effect was antagonized by glibenclamide. These results suggest that Y-26763 hyperpolarizes the smooth muscle cell membrane of the rabbit mesenteric artery by activating the glibenclamide-sensitive (probably ATP-sensitive) K^+ channel.

The present experiments showed that Y-26763 lowers the resting $[Ca^{2+}]_i$ in both Ca^{2+}-containing and Ca^{2+}-free solutions, by a glibenclamide-sensitive mechanism. These actions of Y-26763 are comparable with those of both pinacidil and lemakalim in the same tissue (Ito *et al.*, 1991; 1992b). In Ca^{2+}-free solution containing 2 mM EGTA, the extracellular Ca^{2+} concentration may be below 10 nM (Itoh *et al.*, 1986a) and the intracellular Ca^{2+} concentration might be 50–80 nM. Under such conditions, when influxes of Ca^{2+} may be minimized, Y-26763 still reduced the $[Ca^{2+}]_i$, as reported for both pinacidil and lemakalim. Thus, the membrane hyperpolarization induced by an activation of the ATP-sensitive K^+ channel may negatively control the resting $[Ca^{2+}]_i$.

The resting concentration of $[Ca^{2+}]_i$ was slightly higher in ryanodine-treated strips than in non-treated strips. Y-26763, but not nicardipine (an L-type Ca^{2+} channel blocker), lowered the resting $[Ca^{2+}]_i$ in ryanodine-treated strips. Since, in Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ in ryanodine-treated arterial smooth muscle cells is almost the same as that in non-treated cells (Katsuyama *et al.*, 1990), the increase in the resting $[Ca^{2+}]_i$ in ryanodine-treated strips may be caused by Ca^{2+} influxes which is insensitive to the L-type Ca^{2+} channel blocker under conditions where the function of the Ca^{2+} storage sites is lost. These results suggest that resting $[Ca^{2+}]_i$ may be regulated by both L-type Ca^{2+} channel-sensitive and -insensitive Ca^{2+} influxes, and both are sensitive to membrane hyperpolarization.

In the rabbit mesenteric artery, NA (10 μM) depolarizes the membrane and produces a contraction with two phases: the transient phasic increase in both $[Ca^{2+}]_i$ and force is possibly induced by release of Ca^{2+} from its storage sites, while the subsequent tonic phase and the associated oscillations may be due to activation of Ca^{2+}-influx (Itoh *et al.*, 1983; Kanmura *et al.*, 1983). Y-26763 (1 and 10 μM) inhibited all of these NA-induced responses. The tonic increases in $[Ca^{2+}]_i$ and force induced by NA were abolished by Y-26763 (10 μM), while they were inhibited only partly by nicardipine. Glibenclamide attenuated the inhibitory actions of Y-26763 on the membrane depolarization and the increases in $[Ca^{2+}]_i$ and force induced by NA. In ryanodine-treated strips, Y-26763 (10 μM) completely, and nicardipine (1 μM) partly, inhibited the NA-induced increase in $[Ca^{2+}]_i$. At this concentration, nicardipine completely blocked the contraction induced by 128 mM K^+. These results again suggest that the membrane hyperpolarization induced by Y-26763 inhibits the NA-induced increases in $[Ca^{2+}]_i$ and force through an inhibition of L-type Ca^{2+} channel-sensitive and -insensitive Ca^{2+} influxes.

NA binds to α-receptors and synthesizes inositol 1,4,5-triphosphate ($InsP_3$) which releases Ca^{2+} from the intracellular storage sites in smooth muscle of the rabbit mesenteric artery (Hashimoto *et al.*, 1986; Itoh *et al.*, 1992b). In intact smooth muscle strips, Y-26763 inhibited the increase in $[Ca^{2+}]_i$ induced by NA, but not that induced by caffeine in Ca^{2+}-free solution containing 5.9 mM K^+. This inhibitory action of Y-26763 was prevented by glibenclamide and was not seen in Ca^{2+}-free solution containing 50 mM K^+. Skinned smooth muscle strips made using β-escin retain the α-receptor-phospholipase C coupling mechanism (Kobayashi *et al.*, 1989; Ito *et al.*, 1991; Itoh *et al.*, 1992b). In the present experiments, NA increased Ca^{2+} in Ca^{2+}-free solution containing a low concentration of EGTA after a brief application of Ca^{2+} in β-escin skinned strips. Such NA-induced increases in Ca^{2+} are inhibited by prazosin (an α-receptor blocker) or heparin (a blocker of $InsP_3$ receptor) (Kobayashi *et al.*, 1989; Itoh *et al.*, 1992b). These results suggest that in the β-escin-skinned smooth muscle, NA releases Ca^{2+} from intracellular storage sites through the action of synthesized $InsP_3$ via α-receptor-phospholipase C coupling. Y-26763 did not modify this NA-induced Ca^{2+} release, indicating that Y-26763 has no direct effect on either $InsP_3$-production through α-receptor-phospholipase C coupling or the $InsP_3$-induced Ca^{2+} release mechanism. These results suggest that Y-26763 has no direct action on NA-induced Ca^{2+} release and therefore that the membrane hyperpolarization may be the essential factor for the inhibitory action of Y-26763 on the NA-induced Ca^{2+} release.

Y-26763 had no effect on the Ca^{2+}-force relationship whether applied in the presence or absence of NA with GTP in β-escin-skinned smooth muscle strips. This result indicates that Y-26763 has no direct effects on the contractile machinery in the smooth muscle of the rabbit mesenteric artery. However, activation of protein kinase C (PKC) by 1,2-diacylglycerol (DAG), which is synthesized as a result of agonist-receptor activation through hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), increases the sensitivity of the contractile proteins to Ca^{2+} in various types of smooth muscle (Rasmussen *et al.*, 1987; Itoh *et al.*, 1986b; 1988). We previously suggested that, in smooth muscle of the rabbit mesenteric artery, pinacidil and lemakalim each hyperpolarize the membrane and inhibit the NA-induced hydrolysis of PIP_2, causing a reduction of $InsP_3$ and DAG production (Ito *et al.*, 1991; Itoh *et al.*, 1992b). If this is the case for Y-26763, there is the possibility that, in addition to its inhibition of NA-induced Ca^{2+} mobilization, this drug may also inhibit the NA-induced tonic contraction through an attenuation of PKC-activation via inhibition of DAG production.

We thank Dr R.J. Timms for the language editing. This work was partly supported by a Grant-in-Aid from the Ministry of Education of Japan. Y-26763 was a gift from Yoshitomi Pharmaceutical Ind., Ltd. (Japan).

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(Received June 4, 1993
Revised September 6, 1993
Accepted September 24, 1993)

Br. J. Pharmacol. (1994), **111**, 173–178

Mechanisms of the hyperkalaemia caused by nafamostat mesilate: effects of its two metabolites on Na^+ and K^+ transport properties in the rabbit cortical collecting duct

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1 The present experiments were undertaken to determine the mechanism(s) of hyperkalaemia caused by nafamostat mesilate (NM), a serine-protease inhibitor.

2 We investigated the effects of luminal addition of two metabolites of NM, *p*-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN), on Na^+ and K^+ transport properties of the collecting duct (CD) cell in the isolated perfused cortical collecting duct (CCD) from rabbit kidneys, because these metabolites, but not NM, were mainly excreted into the urine.

3 Addition of PGBA at 10^{-5} and 10^{-4} M in the lumen resulted in a hyperpolarization of V_A in parallel with increases in transepithelial resistance (R_T) and fractional apical membrane resistance (fR_A). PGBA added to the luminal perfusate at 10^{-5} and 10^{-4} M changed V_A, R_T and fR_A in a dose-dependent manner. These effects were completely inhibited by pretreatment with luminal amiloride (50 μM). PGBA at 10^{-6} M in the lumen had no effect on the electrical parameters.

4 Luminal addition of AN at 10^{-4} M also caused the apical membrane to hyperpolarize in parallel with increases in R_T and fR_A. These effects were also completely inhibited by pretreatment with luminal amiloride (50 μM). AN at 10^{-5} M in the lumen had no effect on the electrical parameters.

5 We conclude that two metabolites of NM, PGBA and AN, act on the apical membrane of the CD cell and inhibit the amiloride-sensitive Na^+ conductance, resulting in an inhibition of K^+ secretion. This direct action of these metabolites, rather than NM, on the CCD might contribute to the NM-induced hyperkalaemia.

Keywords: Nafamostat mesilate; *p*-guanidinobenzoic acid; 6-amidino-2-naphthol; electrophysiology; collecting duct cell; Na^+ conductance

Introduction

Nafamostat mesilate, 6-amidino-2-naphthyl *p*-guanidino-benzoate dimethanesulphonate (NM, Torii Pharmaceutical Co., Japan), is a novel protease-inhibiting agent (Aoyama *et al.*, 1984) used for treatment of pancreatitis (Iwaki *et al.*, 1986) and disseminated intravascular coagulation (DIC) (Yoshikawa *et al.*, 1983; Hitomi *et al.*, 1985; Takahashi *et al.*, 1989). It has also been widely used in haemodialysis as an anticoagulant in patients with bleeding tendencies (Akizawa *et al.*, 1985; Yamazaki *et al.*, 1989; Ohtake *et al.*, 1991).

Recently, it has been reported that continuous intravenous infusion of NM to the patients with pancreatitis or DIC occasionally causes hyperkalaemia due to reduced urinary excretion of K^+ (Okamoto *et al.*, 1990; 1992). However, the exact mechanism(s) underlying this disorder are not fully understood. Very recently, Muto *et al.* (1993) examined the effects of NM on Na^+ and K^+ transport properties in the isolated perfused cortical collecting duct (CCD), which is one of the main sites of K^+ secretion in the kidney (Wright & Giebisch, 1992), and demonstrated the direct action of NM on the CCD. They found that NM mainly acted on the apical membrane of the collecting duct (CD) cell, which plays an important role in Na^+ and K^+ transport in the CCD (Koeppen *et al.*, 1983; Muto *et al.*, 1987a,b; 1988), and that it inhibited the amiloride-sensitive Na^+ conductance. These findings suggest that the direct inhibitory action of NM on the apical membrane Na^+ conductance might contribute, at least in part, to its hyperkalaemic effect. Although the renal handling of NM has not yet been elucidated, it has been reported that NM is metabolized to *p*-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN) (Figure 1), which are inactive forms as protease inhibitors (Aoyama *et al.*, 1985; Yang *et al.*, 1990). In addition, these two metabolites, but not NM, are known to be excreted into the urine in rats (Esumi *et al.*, 1984; Shibuya *et al.*, 1984). These findings strongly suggest the possibility that PGBA and/or AN might directly act from the apical membrane of the CD cell and modulate Na^+ and K^+ transport in the CCD. The purpose of the present study was therefore to determine the effects of luminal addition of PGBA and AN on Na^+ and K^+ transport properties in the isolated perfused CCD from rabbit kidneys.

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Methods

Isolation and perfusion of tubules

Japanese female white rabbits weighing 1.5 to 2.5 kg were fed a standard laboratory chow and had free access to tap water. They were anaesthetized with intravenous administration of pentobarbitone (35 mg kg^{-1}). Both kidneys were removed and placed in a dish containing a cold intracellular-fluid-like solution of the following composition (mM): KCl 14, K_2HPO_4 44, KH_2PO_4 14, $NaHCO_3$ 9 and sucrose 160.

Segments of the CCD were dissected from the cortex, and transferred to a bath fixed on an inverted microscope (Diaphot: Nikon, Tokyo, Japan). Each tubule was perfused *in vitro* according to the techniques developed by Burg *et al.* (1966) as modified in this laboratory for use of intracellular microelectrodes (Muto *et al.*, 1990; 1991; 1993). Since the details of the technique have been published previously (Muto *et al.*, 1990; 1991; 1993), they will be presented here briefly. Tubules were suspended between two pipettes. The perfusion rate exceeded 20 nl min^{-1} in all tubules. The distal end of the tubule was held in the collecting pipette with unpolymerized Sylgard 184 (Dow Corning, Midland, MI,

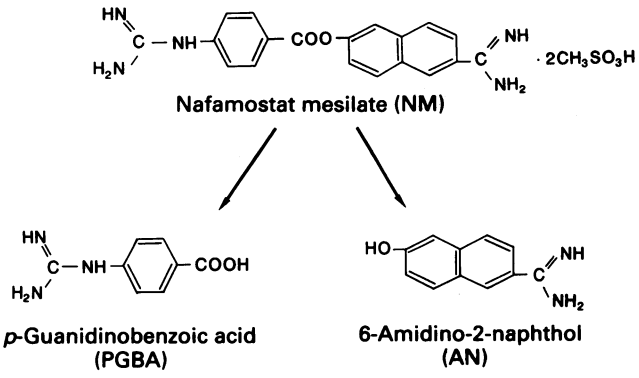

Figure 1 Structural formulae of nafamostat mesilate (NM), *p*-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN).

U.S.A.). The tubule was perfused in the bathing chamber of ~100 μl which permits rapid exchange of the bathing solution within 5 s. The bathing solution flowed at 5–15 ml min^{-1} from the reservoirs by gravity through a water jacket to permit the bath temperature to be regulated at 37°C.

Microelectrode studies

The transepithelial and cellular electrical properties of the tubule were measured by techniques used previously in this laboratory and described by Muto *et al.* (1990,1991,1993). The transepithelial voltage (V_T) was measured through the perfusion pipette, which was connected to one channel of a dual electrometer (Duo 773; W-P Instruments, Inc., Sarasota, FLA, U.S.A.) with a 3 M KCl-3% agar bridge and a calomel half-cell electrode. The basolateral membrane voltage (V_B) was measured with 0.5 M KCl-filled microelectrodes, which were fabricated from borosilicate glass capillaries (GD-1.5; 1.5 mm o.d., 1.0 mm i.d.; Narishige Scientific Laboratory, Tokyo, Japan) by using a vertical puller (PE-2, Narishige Scientific Laboratory). Both voltages were referenced to the bath and were recorded on a four-pen chart recorder (R64; Rikadenki, Tokyo, Japan). The electrical potential difference across the apical membrane (V_A) was calculated by the following equation: $V_A = V_T - V_B$.

As previously described (Muto *et al.*, 1990; 1991; 1993), cable analysis was used to calculate the transepithelial resistance (R_T), and the fractional apical membrane resistance (fR_A) [$= R_A/(R_A + R_B)$], where R_A and R_B are the resistances of the apical and basolateral membranes, respectively.

Identification of the CD cell

Electrical identification of the CD cell was performed according to the criteria described previously by Muto *et al.* (1987a, 1990, 1991, 1993). The CD cell has a relatively low fR_A; high V_B; apical Na^+ and K^+ conductances; and basolateral K^+ and Cl^- conductances.

Solutions and materials

The composition of the control bathing and perfusing solution contained (mM): NaCl 10, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, $NaHCO_3$ 25, Na acetate 10, Na_2HPO_4 0.8, NaH_2PO_4 0.2, L-alanine 5 and D-glucose 8.3. This control solution had an osmolality between 285 and 295 mOsm kgH_2O^{-1}, and was equilibrated with 95% O_2/5% O_2 and adjusted to pH 7.4 at 37°C.

Amiloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the luminal perfusate to achieve a final concentration of 50 μM. PGBA and AN were supplied by Torii & Co., Ltd. (Tokyo, Japan).

Statistics

Experimental values in the text, tables and figures are presented as means ± s.e.mean. Differences between two groups

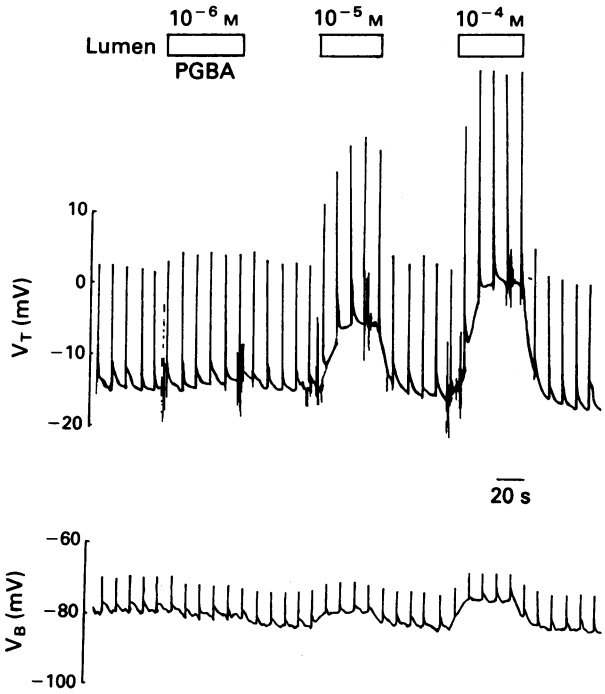

Figure 2 Typical tracings showing effects of *p*-guanidinobenzoic acid (PGBA) in the lumen on V_T and V_B of the collecting duct cell. Voltage spikes are due to 50 nA constant-current pulses at 10 s intervals.

Table 1 Effects of *p*-guanidinobenzoic acid (PGBA) in the lumen on the electrical properties of the collecting duct cell in the rabbit cortical collecting duct

Condition	V_T (mV)	V_B (mV)	V_A (mV)	R_T (Ω·cm²)	fR_A
Control	− 10.2 ± 1.1 (18)	− 77.3 ± 1.6 (18)	67.1 ± 1.4 (18)	106.0 ± 8.1 (15)	0.48 ± 0.03 (15)
PGBA 10^{-4} M (lumen)	− 4.0 ± 0.9* (18)	− 67.5 ± 1.6* (18)	71.6 ± 1.3* (18)	132.8 ± 7.5* (15)	0.65 ± 0.03* (15)
Control	− 10.8 ± 2.0 (9)	− 76.9 ± 1.8 (9)	66.0 ± 1.4 (9)	103.8 ± 9.8 (9)	0.49 ± 0.05 (9)
PGBA 10^{-5} M (lumen)	− 4.8 ± 1.3** (9)	− 72.3 ± 1.4** (9)	67.5 ± 1.7† (9)	113.2 ± 10.0* (9)	0.57 ± 0.05* (9)
Control	− 10.9 ± 2.1 (9)	− 78.2 ± 2.1 (9)	67.3 ± 1.4 (9)	105.3 ± 10.1 (9)	0.49 ± 0.03 (9)
PGBA 10^{-6} M (lumen)	− 10.7 ± 2.0 (9)	− 77.4 ± 2.0 (9)	66.6 ± 1.4 (9)	106.4 ± 10.6 (9)	0.49 ± 0.03 (9)

Values are mean ± s.e.mean; no. of experiments in parentheses. *$P < 0.001$; **$P < 0.005$; †$P < 0.05$ compared with preceding period.

were evaluated with Student's paired t test. Values of $P<0.05$ were considered significant.

Results

Effect of luminal addition of PGBA on electrical properties of the CD cell

To examine whether or not PGBA acts directly on the apical membrane of the CD cell, we added PGBA at 10^{-6}–10^{-4} M to the luminal perfusate and observed the electrical parameters. Figure 2 shows a typical tracing of the changes in V_T and V_B of the CD cell before and after addition of the drug. Table 1 summarizes the effects of PGBA in the lumen on barrier voltages and resistances. PGBA at 10^{-6} M in the lumen had no effect on the barrier voltages or resistances. Significant effects of PGBA on the CCD were observed when the concentrations of PGBA were 10^{-5} and 10^{-4} M. Upon luminal addition of PGBA at 10^{-5} and 10^{-4} M, the lumen-negative V_T was rapidly decreased and the $-V_B$ was slightly decreased, resulting in a significant hyperpolarization of V_A. At that time, both the R_T and the fR_A were significantly increased. However, the changes in V_A, R_T and fR_A upon luminal addition of PGBA were greater at 10^{-4} M than those observed at 10^{-5} M (Figure 3). These results suggest that PGBA at 10^{-5} and 10^{-4} M in the lumen may inhibit the Na^+ conductance at the apical membrane of the CD cell.

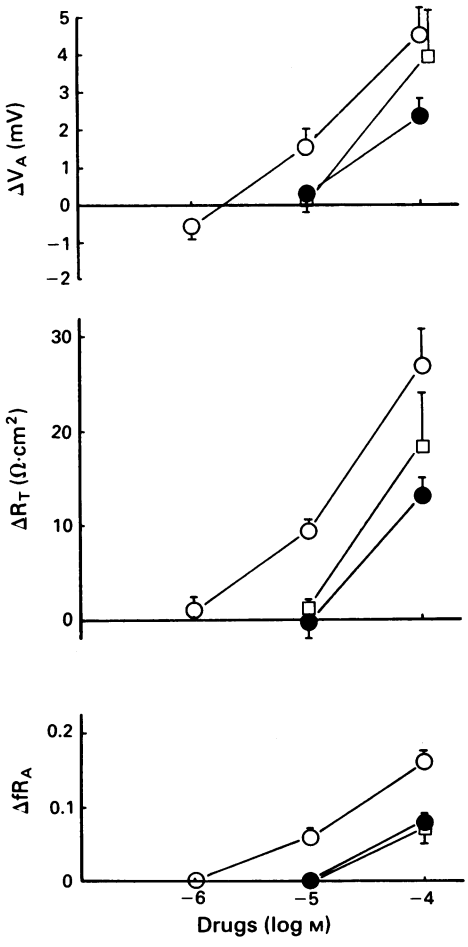

Figure 3 The concentration-response relationships for luminal addition of nafamostat mesilate (NM, □), *p*-guanidinobenzoic acid (○) and 6-amidino-2-naphthol (●) on the changes in V_A, R_T and fR_A. Each point represents the mean of 5–18 separate experiments ± s.e.mean. The data for NM were taken from the paper by Muto *et al.* (1993).

To examine whether this luminal action of PGBA is due to direct inhibition of the apical membrane Na^+ conductance of the CD cell, we added a Na^+ channel inhibitor, amiloride, to the luminal perfusate, and then examined the effect of PGBA on the CD cell. Figure 4 shows a typical tracing of the changes in V_T and V_B of the CD cell before and after addition of luminal PGBA (10^{-4} M) in the presence of luminal amiloride (50 μM). Table 2 summarizes the effects of PGBA in the lumen on the electrical properties of the CD cell in the presence of luminal amiloride. After addition of 50 μM amiloride to the perfusate, the lumen-negative V_T was significantly decreased by 18.1 mV, and the V_B was also significantly depolarized by 10.7 mV, resulting in a hyperpolarization of V_A by 7.3 mV. The R_T and fR_A were also significantly elevated by 26.7 Ω·cm² and 0.12, respectively. However, luminal addition of PGBA in the presence of luminal amiloride produced no further changes in barrier voltages or resistances. These results are consistent with the notion that PGBA inhibits the amiloride-sensitive Na^+ conductance at the apical membrane of the CD cell.

Effect of luminal addition of AN on electrical properties of the CD cell

To examine whether AN also acts on the apical membrane of the CD cell, we added AN at 10^{-5} or 10^{-4} M to the luminal perfusate, and observed the barrier voltages and resistances. Figure 5 shows a typical tracing of the changes in V_T and V_B of the CD cell before and after addition of the drug. Table 3 summarizes the effects of luminal addition of AN on barrier voltages and resistances. AN at 10^{-5} M in the lumen had no effect on the electrical parameters. When AN at 10^{-4} M was added in the lumen, the lumen-negative V_T was significantly decreased by 7.9 mV, and the V_B was also significantly depolarized by 5.7 mV, resulting in a significant hyperpolarization of V_A by 2.2 mV. At that time, both the R_T and the fR_A were significantly increased by 13.4 Ω·cm² and 0.09, respectively. These results suggest that AN in the lumen also

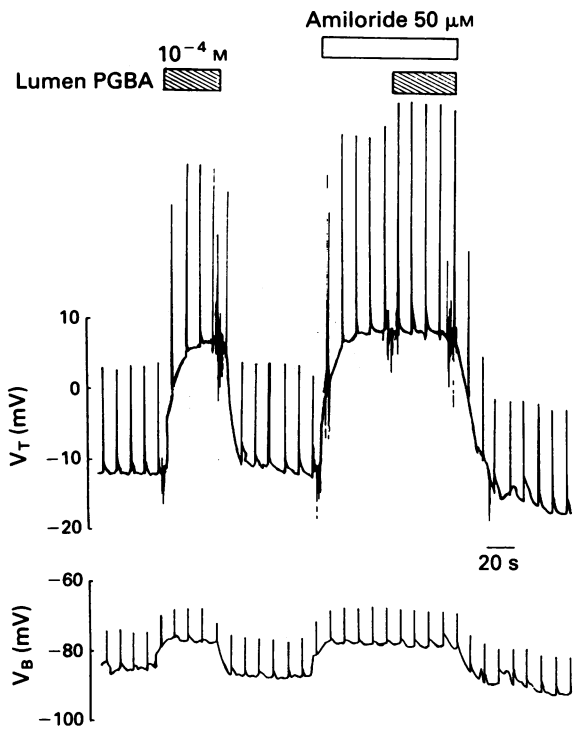

Figure 4 Typical tracings showing effects of *p*-guanidinobenzoic acid (PGBA) in the lumen on V_T and V_B of the collecting duct cell in the presence of luminal amiloride. Voltage spikes are due to 50 nA constant-current pulses at 10 s intervals.

Table 2 Effects of *p*-guanidinobenzoic acid (PGBA) in the lumen on the electrical properties of the collecting duct cell in the presence of luminal amiloride

Condition	V_T (mV)	V_B (mV)	V_A (mV)	R_T (Ω·cm²)	fR_A
Control	− 9.8 ± 1.0 (14)	− 78.9 ± 2.2 (14)	69.1 ± 1.8 (14)	112.0 ± 6.9 (12)	0.46 ± 0.04 (12)
Amiloride 50 μM (lumen)	8.3 ± 0.9* (14)	− 68.2 ± 2.3* (14)	76.4 ± 2.9* (14)	138.7 ± 6.0* (12)	0.58 ± 0.03* (12)
Amiloride 50 μM (lumen) + PGBA 10^{-4} M (lumen)	8.5 ± 0.9 (14)	− 68.3 ± 2.2 (14)	76.8 ± 2.1 (14)	139.1 ± 6.0 (12)	0.58 ± 0.03 (12)

Values are mean ± s.e.mean; no. of experiments in parentheses. *$P < 0.001$ compared with preceding period.

Table 3 Effects of 6-amidino-2-naphthol (AN) in the lumen on the electrical properties of the collecting duct cell in the rabbit cortical collecting duct

Condition	V_T (mV)	V_B (mV)	V_A (mV)	R_T (Ω·cm²)	fR_A
Control	− 7.4 ± 0.8 (16)	− 81.3 ± 2.5 (16)	73.9 ± 2.9 (16)	100.6 ± 6.0 (15)	0.50 ± 0.02 (15)
AN 10^{-4} M (lumen)	0.5 ± 1.4* (16)	− 75.6 ± 3.2* (16)	76.1 ± 2.9* (16)	114.0 ± 6.9* (15)	0.59 ± 0.02* (15)
Control	− 5.9 ± 0.9 (8)	− 80.8 ± 3.8 (8)	74.9 ± 3.7 (8)	119.1 ± 8.6 (7)	0.51 ± 0.04 (7)
AN 10^{-5} M (lumen)	− 5.8 ± 0.9 (8)	− 81.0 ± 3.7 (8)	75.2 ± 3.6 (8)	119.4 ± 9.0 (7)	0.50 ± 0.04 (7)

Values are mean ± s.e.mean; no. of experiments in parentheses. *$P < 0.001$ compared with preceding period.

Table 4 Effects of 6-amidino-2-naphthol (AN) in the lumen on the electrical properties of the collecting duct cell in the presence of luminal amiloride

Condition	V_T (mV)	V_B (mV)	V_A (mV)	R_T (Ω·cm²)	fR_A
Control	− 6.9 ± 2.4 (6)	− 85.6 ± 1.7 (6)	78.7 ± 2.4 (6)	114.8 ± 19.8 (6)	0.50 ± 0.03 (6)
Amiloride 50 μM (lumen)	9.2 ± 1.8* (6)	− 77.1 ± 2.5† (6)	86.4 ± 3.7** (6)	146.1 ± 20.3** (6)	0.60 ± 0.03* (6)
Amiloride 50 μM (lumen) + AN 10^{-4} M (lumen)	9.1 ± 1.7 (6)	− 76.5 ± 2.8 (6)	85.6 ± 3.8 (6)	145.3 ± 17.8 (6)	0.58 ± 0.03 (6)

Values are mean ± s.e.mean; no. of experiments in parentheses. *$P < 0.001$, **$P < 0.005$, †$P < 0.005$ compared with preceding period.

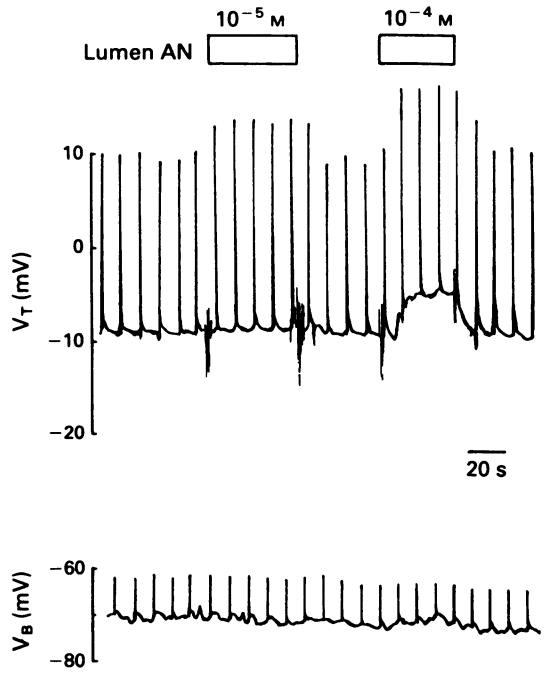

Figure 5 Typical tracings showing effects of 6-amidino-2-naphthol (AN) in the lumen on V_T and V_B of the collecting duct cell. Voltage spikes are due to 50 nA constant-current pulses at 10 s intervals.

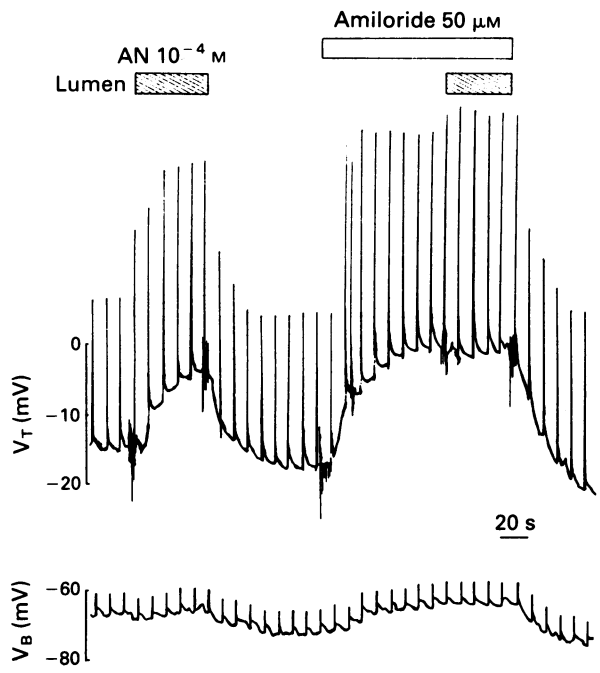

Figure 6 Typical tracings showing effects of 6-amidino-2-naphthol (AN) in the lumen on V_T and V_B of the collecting duct cell in the presence of luminal amiloride. Voltage spikes are due to 50 nA constant-current pulses at 10 s intervals.

inhibits the apical membrane Na^+ conductance of the CD cell.

To examine this possibility, we determined the luminal effects of AN on the barrier voltages and resistances of the CD cell in the presence of luminal amiloride. Figure 6 shows a typical tracing of the changes in V_T and V_B of the CD cell before and after addition of luminal AN (10^{-4} M) in the presence of luminal amiloride (50 μM). Table 4 summarizes the effects of AN in the lumen on the electrical properties of the CD cell in the presence of luminal amiloride. When amiloride was added to the luminal perfusate, the lumen-negative V_T was significantly decreased by 16.1 mV, and the V_B was also significantly depolarized by 8.5 mV, resulting in a significant hyperpolarization of V_A by 7.7 mV. However, luminal addition of AN in the presence of luminal amiloride had no effects on these electrical parameters. Taken together, these results indicate that AN also inhibits the amiloride-sensitive Na^+ conductance at the apical membrane of the CD cell.

Discussion

This study was designed to determine the mechanism(s) of hyperkalaemia caused by NM. The present study focused especially on the luminal action of two metabolites of NM, PGBA and AN, on Na^+ and K^+ transport properties in the CCD *in vitro*, because both metabolites are secreted into the urine and are in contact with the tubular cell apical membrane. We observed that luminal addition of PGBA or AN brings about changes in the electrophysiological behaviour of the CD cell quite similar to those of luminal addition of NM. Thus, the most important findings of our study were that both PGBA and AN act on the apical membrane of the CD cell to inhibit the amiloride-sensitive Na^+ conductance.

The mechanism for Na^+ reabsorption and K^+ secretion in the CCD is well established (Koeppen *et al.*, 1983; O'Neil & Sansom, 1984; Sansom & O'Neil, 1985 and 1986; Muto *et al.*, 1987a,b; Sansom *et al.*, 1989). For Na^+ reabsorption, the first step is passive diffusion of Na^+ from the tubular lumen into the cell via amiloride-sensitive Na^+ conductance across the apical membrane. Na^+ is then actively extruded from the cell across the basolateral membrane via the Na^+-K^+-ATPase pump, thereby maintaining a low intracellular Na^+ activity. On the other hand, the first step in K^+ secretion is active uptake across the basolateral membrane via the Na^+-K^+-ATPase pump, thereby maintaining a high intracellular K^+ activity. K^+ can then diffuse passively across the apical membrane via Ba^{2+}-sensitive K^+ conductance to bring about net K^+ secretion. In the present study, we found that both PGBA and AN acted from the apical membrane of the CD cell and inhibited the Na^+ conductance in the apical membrane. This notion is directly supported by the hyperpolarization of V_A and increases in R_T and fR_A observed after addition of these two drugs in the lumen. Furthermore, pretreatment with luminal amiloride produced no further effects of PGBA or AN in the lumen on the barrier voltages or resistances. Taken together, these results indicate that luminal addition of PGBA or AN results in an inhibition of the amiloride-sensitive Na^+ conductance in the apical membrane. Therefore, the major mechanism of action of both PGBA and AN is to prevent the Na^+ entry into the cell across the apical membrane; the ensuing reduction in the favourable electrical gradient for K^+ secretion from cell to lumen could explain the inhibition of K^+ transport.

Figure 2 shows the comparison of the concentration-response relationships for luminal addition of NM, PGBA and AN on the changes in V_A, R_T and fR_A. It should be noted that luminal application of PGBA at 10^{-5} M caused significant changes in V_A, R_T and fR_A, whereas luminal NM and AN at the same concentration had no significant effects on electrical parameters. These findings strongly suggest that the inhibitory action of PGBA on the apical membranes Na^+ conductance may contribute in a major way to the mechanism of hyperkalaemia caused by NM.

It is also noteworthy that all the electrical parameters returned to the control levels within 30 s after the drugs were eliminated. This would mean that hyperkalaemia induced by NM could be improved simply by interrupting the administration of the drug.

Although we found that PGBA at 10^{-5} and 10^{-4} M, and AN at 10^{-4} M act directly on the apical membrane, the question arises as to whether the concentration of PGBA and AN in the tubular lumen *in vivo* comes in the range of 10^{-5} to 10^{-4} M. At the present time, no clear answer is available on this point. However, when NM was given intravenously to rats at a dose of 1 mg kg^{-1}, the urinary concentration of PGBA and AN reached the level of 10^{-5} and 10^{-6} M respectively, whereas little NM was excreted into the urine (Torii Pharmaceutical Company, personal communication). These findings suggest the possibility that under certain conditions, the concentration of two metabolites of NM in the CCD could reach the level of 10^{-5} to 10^{-4} M, exerting natriuresis and antikaliuresis. Further studies will be required to determine whether PGBA and AN, in fact, cause the natriuresis and antikaliuresis.

In summary, two metabolites of NM, PGBA and AN, act mainly on the apical membrane of the CD cell and inhibit the amiloride-sensitive Na^+ conductance in the apical membrane, resulting in an inhibition of K^+ secretion. PGBA is most potent in this action. This direct action of these two metabolites, rather than NM, could contribute to the NM-induced hyperkalaemia.

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(Received August 24, 1993
Revised September 2, 1993
Accepted September 28, 1993)

Br. J. Pharmacol. (1994), **111**, 179–184

Non-specific actions of the non-peptide tachykinin receptor antagonists, CP-96,345, RP 67580 and SR 48968, on neurotransmission

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1 Three non-peptide tachykinin receptor antagonists, CP-96,345, RP 67580 and SR 48968, were found to inhibit the electrically-evoked, tachykinin-mediated contractile responses of the rabbit iris sphincter in a concentration-dependent fashion; the pIC_{50} values were 5.6 ± 0.01, 5.4 ± 0.07 and 4.8 ± 0.03, respectively.

2 These antagonists also inhibited the electrically-evoked, parasympathetic response of the rabbit iris sphincter and the sympathetic response of the guinea-pig vas deferens in a concentration-dependent manner; the pIC_{50} values were 0.3–1.2 log units lower than those recorded for the tachykinin-mediated responses.

3 Two local anaesthetics, bupivacaine and oxybuprocaine, were also found to inhibit the tachykinin-mediated, cholinergic and sympathetic contractile responses in these tissues in a concentration-dependent manner; the concentration ranges for producing the inhibition were similar to those of the non-peptide tachykinin receptor antagonists.

4 On the sciatic nerves of frogs, the tachykinin receptor antagonists inhibited action potentials in a concentration-dependent manner; the potency of the three drugs was similar to that of bupivacaine.

5 Our results suggest that, in addition to blocking tachykinin receptors, the non-peptide tachykinin receptor antagonists, CP-96,345, RP 67580 and SR 48968, may exert non-specific inhibitory effects on neurotransmission.

Keywords: Non-peptide tachykinin receptor antagonists; non-specific inhibition on neurotransmission; local anaesthetic-like actions; rabbit iris sphincter; guinea-pig vas deferens; frog sciatic nerve

Introduction

The mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B, act at receptors designated NK_1, NK_2 and NK_3 respectively (for reviews see Maggio, 1988; Guard & Watson, 1991; Maggi *et al.*, 1993; Otsuka & Yoshioka, 1993). Very recently, non-peptide tachykinin receptor antagonists such as CP-96,345, RP 67580 and SR 48968 have been described (Garret *et al.*, 1991; Snider *et al.*, 1991; Emonds-Alt *et al.*, 1992; Watling, 1992). Both CP-96,345 and RP 67580 possess similar chemical moieties and are claimed to be potent and selective NK_1 receptor antagonists. Meanwhile, SR 48968 has been described as a selective NK_2 receptor antagonist (Emonds-Alt *et al.*, 1992). Interestingly, SP and CP-96,345 bind to different epitopes on the NK_1 receptor (Fong *et al.*, 1993; Gether *et al.*, 1993).

However, CP-96,345 has been found to produce effects that are not related to tachykinin receptor antagonism (Boyle *et al.*, 1991; Lecci *et al.*, 1991; Hall *et al.*, 1992; Nagahisa *et al.*, 1992; Berge & Ståhlberg, 1993), including non-specific effects on neurotransmission (Wang & Håkanson, 1992a). Recent reports suggest that CP-96,345 might be a Ca^{2+}-channel blocker (Schmidt *et al.*, 1992; Guard *et al.*, 1993). Furthermore, the suppression by CP-96,345 of the excitatory response of myenteric neurones to SP appears not to be related to blockade of tachykinin receptors (Tamura *et al.*, 1993).

We have noted structural similarities between these three non-peptide antagonists and local anaesthetics, which have a lipophilic group (usually an aromatic ring) connected by an intermediate chain to an ionizable group (usually a tertiary amine) (Courtney & Strichartz, 1987). As shown in Figure 1, CP-96,345, RP 67580 and SR 48968 fulfil these requirements.

In the present study, we have investigated some apparently non-specific inhibitory effects of CP-96,345, RP 67580 and SR 48968 on neurotransmission. The 'non-specific' actions of these drugs have been compared with the actions of two local anaesthetics, bupivacaine and oxybuprocaine.

Methods

Effects on electrically-evoked contractile responses in the iris sphincter of rabbit and the vas deferens of guinea-pig

Adult pigmented rabbits of either sex (1.5–3.0 kg) and male guinea-pigs (200–250 g) were killed by a blow on the neck and exsanguinated. The iris sphincter muscle of the rabbit and the vas deferens of the guinea-pig were prepared and mounted vertically on a perspex holder in 8 ml modified Krebs solution as described elsewhere (Stjernquist *et al.*, 1983; Wahlestedt *et al.*, 1985). The preparation was stretched with a force of 1.5 mN (iris sphincter sphincter) or 5 mN (vas deferens). Before the start of each experiment, the preparation was allowed to equilibrate for 60–90 min.

Electrical stimulation with square wave pulses (25 V, voltage drop 14–17 V over the electrodes, 0.3–1 ms duration) (Stjernquist *et al.*, 1983; Fujiwara *et al.*, 1984; Wahlestedt *et al.*, 1985) was applied to both tissues by means of platinum electrodes connected to a Grass S4C stimulator. All electrically evoked responses were abolished by 1 μM tetrodotoxin (TTX) (Kao, 1966; Stjernquist *et al.*, 1983; Leander & Håkanson, 1985; Wahlestedt *et al.*, 1985). Drugs were given in a cumulative manner. Two pieces of iris sphincter from the same eye or two pieces of vas deferens from the same animal were mounted in separate baths, one segment being exposed to the drugs and the other being exposed to the correspon-

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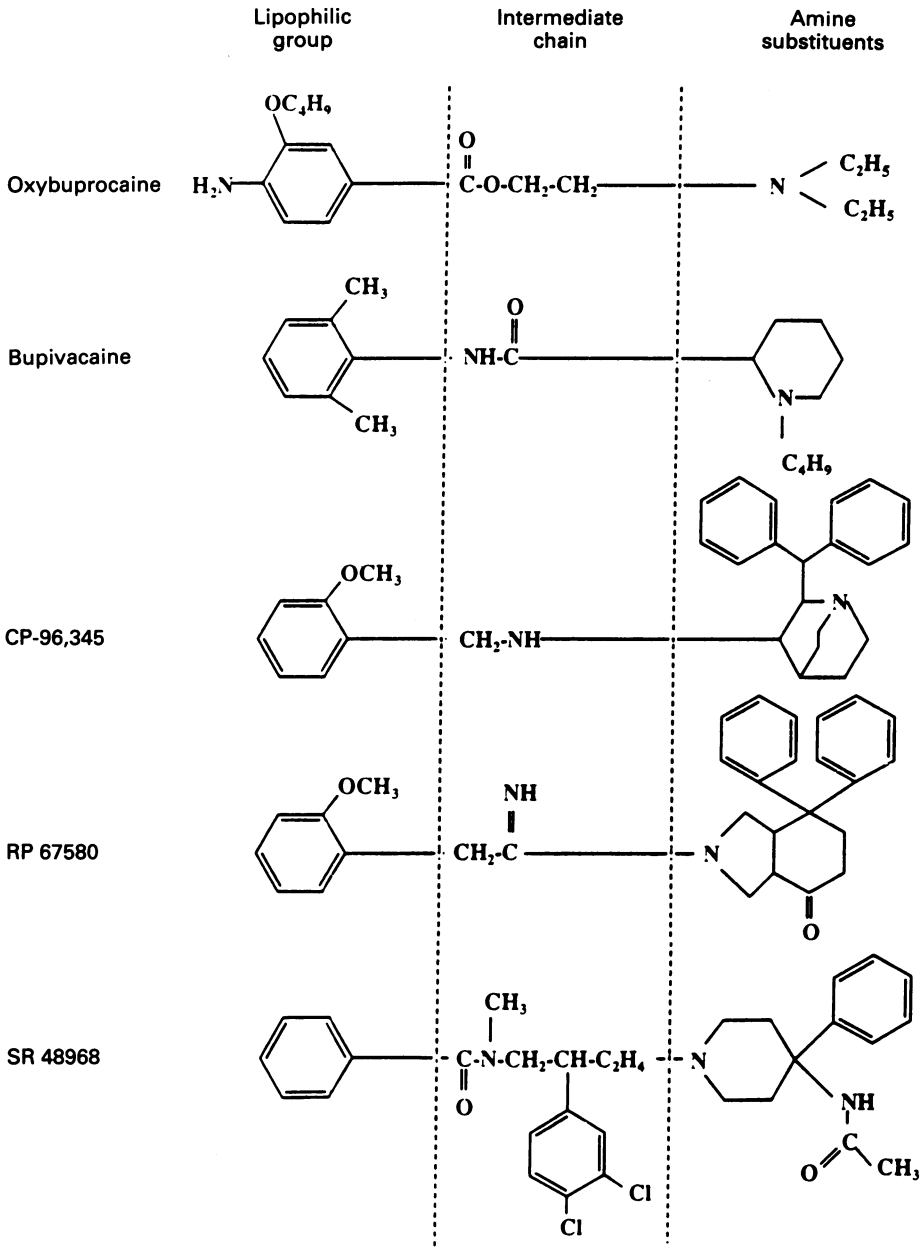

Figure 1 Structural similarities between non-peptide tachykinin receptor antagonists and local anaesthetics.

ding vehicle (control preparation). The preparations were exposed to each concentration of the drug or vehicle for 15 min before electrical stimulation.

Effects on action potentials in the sciatic nerves of frog

Bullfrogs (*Rana catesbeiana*) were killed by decapitation after hypothermic general anaesthesia (30 min at − 20°C). The sciatic nerves were excised, desheathed and split longitudinally into two bundles. Each preparation was mounted in a sucrose-gap chamber as previously described (Hahin & Strichartz, 1981; Strong *et al.*, 1978). One end of the nerve was stimulated in drug-free Ringer solution with Ag/AgCl or platinum electrodes, using isolated square-wave supermaximal current pulses of 0.05 ms duration (Grass Instrument Co., Braintree, MA, Model S44). Similar Ag/AgCl or platinum electrodes were used to record potential changes between two regions of the nerve separated by a sucrose gap. These changes in potential were amplified and recorded as compound action potential on an a.c.-coupled, dual-beam storage oscilloscope (Tektronix 5113). All sucrose-gap experiments were conducted at 21–24°C.

The nerve was first stimulated to determine a supramaximal stimulus. Next, the flow of sucrose was commenced, rapidly at first (20–30 ml min^{-1} for 30 s) to flush out the system, and then maintained at 2–3 ml min^{-1} for the duration of the experiment. The test chamber held 350 µl solution. During the stabilization period, the solution in the test chamber was changed twice with normal frog Ringer solution to eliminate potential 'solution switching artifacts'. This baseline period usually lasted 20–30 min. Afterwards, stimulation once each minute showed steady compound action potentials, changing by less than 5% over a 15 min period. Compound action potentials were measured in frog Ringer solution containing (mM): NaCl 110, KCl 2.5, $CaCl_2$ 2.0, morpholino-propanesulphonic acid 5.0, the pH was adjusted to 7.2 with NaOH and measured and adjusted to this pH after drug addition. One or two concentrations of drug were tested in each preparation. Percentage inhibition was expressed as a fraction of the initial control compound action potential amplitude.

Drugs

RP 67580, {(3a**R**, 7a**R**)-7, 7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one}, was a gift from Dr P.M. Laduron, Rhône-Poulenc, Vitry-sur-Seine, France. CP-96345, {[(2S, 3S)-*cis*-2-(diphenylmethyl)-*N*-(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine}, was provided by Pfizer Inc., Groton, CT, U.S.A. SR 48968, {(S)-N-methyl-*N*[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl) butyl] benzamide}, was a gift from Dr X. Emonds-Alt, Sanofi Recherche, Montpellier, France. CP-96,345 was dissolved in distilled water. RP 67580 was prepared in a manner recommended by the supplier. Briefly, the drug was dissolved in 0.1 M hydrochloric acid at a concentration of 0.1 M. This solution was then diluted with distilled water as required. SR 48968 was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 0.1 M, distilled water was added when further dilutions were required. Bupivacaine was from Astra, Södertälje, Sweden. Oxibuprocaine was from Apoteksbolaget, Stockholm, Sweden. Atropine was from Alcon, Fort Worth, TX, U.S.A. and guanethidine from CIBA-Geigy, Basel, Switzerland.

Analysis of results

Drug-exposed and vehicle-exposed preparations were stimulated in parallel. Repeated electrical stimulations caused reproducible cholinergic contractile responses of the iris sphincter and sympathetic contractile responses of the vas deferens throughout the duration of the experiment. However, repeated electrical stimulations led to a gradual reduction (fatigue) in tachykinin-mediated contractile responses of the iris sphincter (Håkanson *et al.*, 1987). Tachykinins are synthesized in the nerve body cell and transported to the nerve endings (see Gamse *et al.*, 1982) and it is to be expected that the tachykinin-mediated responses are more rapidly exhausted than responses that involve acetylcholine or sympathetic transmitters, which are being continuously resynthesized locally. Thus, in the present study, the contractile responses of drug-exposed preparations were expressed as % of those of parallel control preparations. Concentration-response curves were constructed and the pIC_{50} values (the negative logarithm of the molar concentration of the antagonist or local anaesthetic producing 50% inhibition of the electrically-evoked contraction) were estimated by linear regression analysis of the results in the 10–90% response interval. Statistical analysis was made by Student's unpaired *t* test. A probability level of $P<0.05$ was considered statistically significant.

Results

Effects on tachykinin-mediated responses of the electrically-stimulated rabbit iris sphincter

In the presence of 1 μM atropine and 5 μM guanethidine, the contractile response of the rabbit iris sphincter to electrical stimulation (20 Hz, 25 V, 10 s) is mediated by tachykinins released from nerve fibres in the preparation (Wahlestedt *et al.*, 1985; Wang & Håkanson, 1992b). Both the non-peptide antagonists and the local anaesthetics inhibited the tachykinin-mediated contraction in a concentration-related manner (0.1–100 μM concentration-range for the non-peptide antagonists and 0.1–300 μM for the local anaesthetics) (Figure 2). The pIC_{50} values are given in Table 1. CP-96,345 and RP 67580 were about 10 times more potent than the local anaesthetics. SR 48968 was 2–2.5 times more potent than the local anaesthetics. After extensive washing with fresh Krebs solution every 15 min for 90–120 min, the contractile responses to electrical stimulation in drug-exposed preparations were similar to those in control preparations, showing recovery (data not shown).

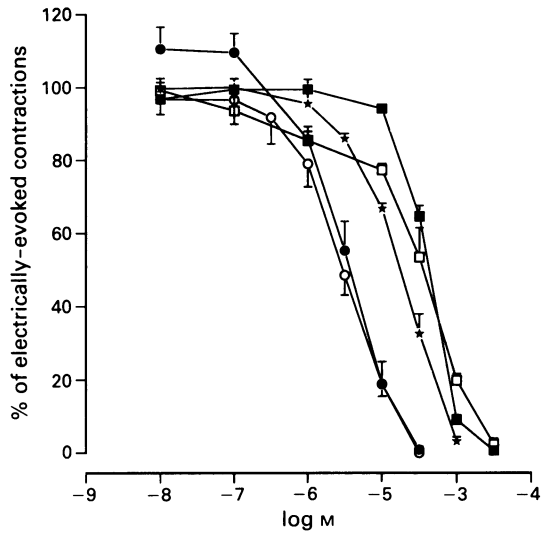

Figure 2 Inhibition of tachykinin-mediated contractile responses of the rabbit iris sphincter evoked by electrical stimulation by CP-96,345 (○), RP 67580 (●), SR 48968 (★), bupivacaine (□) and oxybuprocaine (■). Means ± s.e.mean of 4–6 experiments.

Effects on cholinergic responses of the electrically-stimulated rabbit iris sphincter

The iris sphincter muscle responds to single pulse stimulation (1 pulse/60 s, 25 V) with a twitch-like contraction. This contraction can be blocked by 1 μM atropine (Leander & Håkanson, 1985). Both the non-peptide antagonists and the local anaesthetics inhibited the cholinergic contractile response in a concentration-dependent manner (1–300 μM) (Figure 3). The pIC_{50} values are given in Table 1. CP-96,345 and RP 67580 were more potent than the local anaesthetics, while SR 48968 was slightly less potent. However, the pIC_{50} values of the three drugs in this test system were lower than the pIC_{50} values for the inhibition of the tachykinin-mediated response ($P<0.01$ for CP-96,345 and SR 48968, $P<0.05$ for RP 67580). After extensive washing with fresh Krebs solution every 15 min for 60–90 min, the contractile response to electrical stimulation showed complete recovery (data not shown).

Effects on sympathetic responses of the electrically-stimulated guinea-pig vas deferens

The vas deferens responds to low frequency stimulation (5 Hz, 25 V, 3 s) with a twitch-like contraction (Stjernquist *et al.*, 1983). This contraction was unaffected by 1 μM atropine, but abolished by 5 μM guanethidine (data not shown). As shown in Figure 4, RP 67580 and bupivacaine first enhanced (at low concentrations) and then inhibited (at high concentrations) the electrically-evoked response. CP-96,345, SR 48968 and oxybuprocaine inhibited the contraction in a concentration-dependent manner (0.1–300 μM) (Figure 4). The pIC_{50} values are given in Table 1. In this preparation, the rank order of potency of inhibition was oxybuprocaine > CP-96,345 and RP 67580 > SR 48968 > bupivacaine. After extensive washing with fresh Krebs solution every 15 min for 60 min, the contractile response to electrical stimulation showed complete recovery (data not shown).

Effects on compound action potentials of the electrically-stimulated frog sciatic nerve

Depolarization of nerves promptly opens sodium channels, thereby giving rise to transient action potentials. As shown in Figure 5, CP-96,345, RP-67580 and SR 48968 concentration-

Table 1 Inhibitory potency of non-peptide tachykinin receptor antagonists and local anaesthetics on responses to electrical stimulation

	pIC_{50}			
	Tachykinergic response (Rabbit iris sphincter)	*Cholinergic response* (Rabbit iris sphincter)	*Sympathetic response* (Guinea-pig vas deferens)	*Action potentials* (Frog sciatic nerve)
CP-96,345	5.6 ± 0.01	4.3 ± 0.02	4.7 ± 0.02	3.8
RP 67580	5.4 ± 0.07	5.0 ± 0.03	4.6 ± 0.03	4.1
SR 48968	4.8 ± 0.03	4.0 ± 0.04	4.2 ± 0.05	4.0
Oxybuprocaine	4.4 ± 0.01	4.1 ± 0.01	5.2 ± 0.04	ND
Bupivacaine	4.5 ± 0.06	4.1 ± 0.05	-	3.9

Mean ± s.e.mean. $n = 6-8$.
-: A reliable pIC_{50} could not be calculated. ND: not determined.

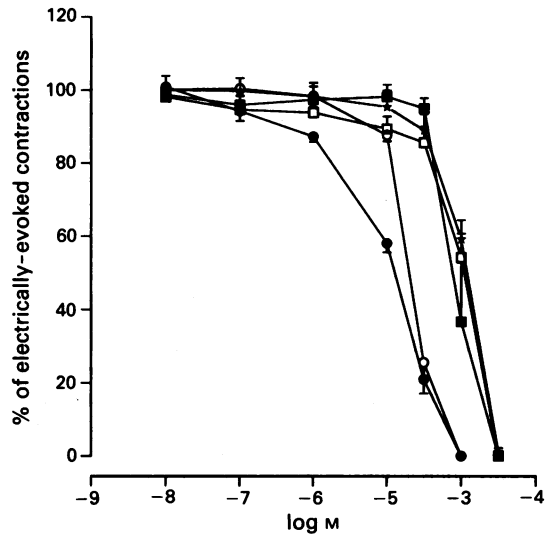

Figure 3 Inhibition of cholinergic-mediated contractile responses of the rabbit iris sphincter evoked by electrical stimulation by CP-96,345 (○), RP 67580 (●), SR 48968 (★), bupivacaine (□) and oxybuprocaine (■). Means ± s.e.mean of 4–6 experiments.

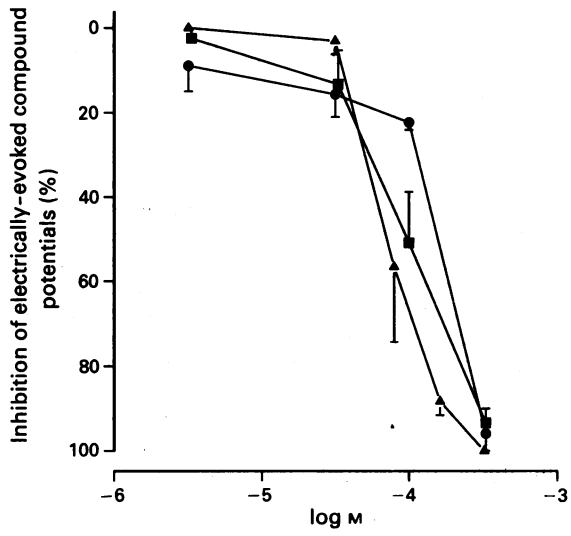

Figure 5 Inhibition of compound action potentials of the electrically-stimulated frog sciatic nerve by CP-96,345 (●), RP 67580 (▲) and SR 48968 (■). Means ± s.e.mean of 3–4 experiments.

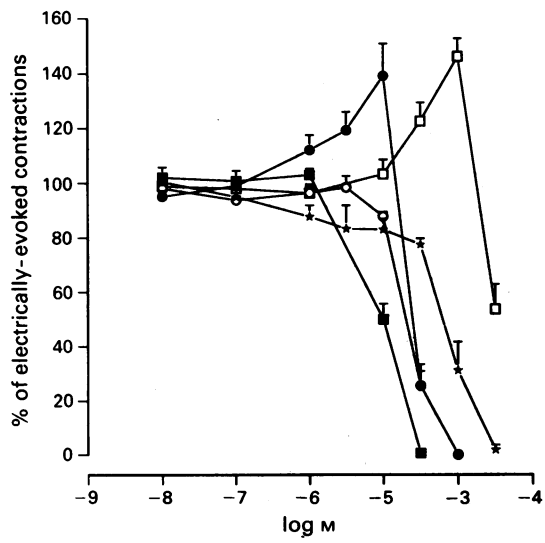

Figure 4 Inhibition of sympathetic-mediated contractile responses of the guinea-pig vas deferens evoked by electrical stimulation by CP-96,345 (○), SR 48968 (★) and oxybuprocaine (■). RP 67580 (●) and bupivacaine (□) first enhanced and then inhibited the contractile responses. Means ± s.e.mean of 4–6 experiments.

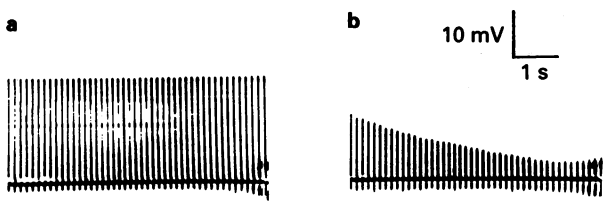

Figure 6 The response of the compound action potential to repetitive stimulation at 5 Hz in nerves incubated in Ringer solution (a) or exposed to 326 μM RP 67580 (b). The nerve had been incubated with the tachykinin antagonist for 12–14 min, yielding a non-equilibrium resting inhibition of 36%, when the 5 Hz train was applied. (The apparent increase in signal amplitude at the right hand edge of the figure is an artifact from the beam reflection in the storage oscilloscope). Similar observations were made with CP-96,345 and SR 48968.

dependently inhibited the action potentials with IC_{50} values of 157, 75 and 96 μM, respectively. Bupivacaine was also found to produce concentration-dependent inhibition of the action potentials in this preparation with an IC_{50} value of about 135 μM (Lee Son *et al.*, 1992). The inhibitory effects of the non-peptide antagonists were partly reversible upon washout of the drug (data not shown).

At frequencies of action potential firing greater than 2 Hz, the inhibition by all the antagonists showed a pronounced additional component, the 'use dependent' inhibition (Figure 6). This added component of inhibition was concentration-dependent and is a hallmark of the action of almost all local anaesthetics (Butterworth & Strichartz, 1990).

Discussion

In the present study, three non-peptide tachykinin receptor antagonists, CP-96,345, RP 67580 and SR 48968, were found to inhibit not only tachykinin-evoked neurotransmission (the response to electrical stimulation of the atropinized rabbit iris sphincter), but also non-tachykinin-evoked neurotransmission (the response to electrical stimulation of the non-atropinized iris sphincter muscle of the rabbit and of the vas deferens of the guinea-pig). The inhibition produced by CP-96,345, RP 67580 and SR 48968 resembled that produced by the two local anaesthetics, bupivacaine and oxybuprocaine. In all instances, the inhibitory effects of the three tachykinin receptor antagonists and the two local anaesthetics were concentration-dependent and reversible. The three non-peptide antagonists, like local anaesthetics, were found to inhibit the action potentials of the frog sciatic nerves, which is the most commonly used preparation for testing local anaesthetics (Courtney & Strichartz, 1987). This result supplies further evidence in support of a similar mechanism underlying the inhibitory effects of the non-peptide antagonists and local anaesthetics. The potency of each of the three non-peptide antagonists was similar to that of bupivacaine. In a previous study, CP-96,345 was found to be without effect on carbachol- and noradrenaline-evoked contractions of the iris sphincter (Wang & Håkanson, 1992a). Also, both local anaesthetics tested were without effect on neurokinin A (NKA)-induced contractions of the iris sphincter (Wang & Håkanson, unpublished observations). It is possible that the three non-peptide antagonists, as well as the local anaesthetics, suppress electrically-evoked contractile responses by acting at prejunctional sites, although the inhibition of tachykinin-mediated responses in the rabbit iris sphincter by the non-peptide antagonists may reflect a combination of both prejunctional and postjunctional effects.

The non-peptide tachykinin receptor antagonists and the local anaesthetics are structurally similar (Figure 1). Local anaesthetics block impulse conduction by binding to specific receptors on the sodium channel of the excitable membrane of nerve fibres (Courtney & Strichartz, 1987; Butterworth & Strichartz, 1990). A recent report has suggested that CP-96,345 binds to L-type calcium channels (Schmidt *et al.*, 1992; Guard *et al.*, 1993). Since the L-type calcium channel has an overall structure that is homologous to that of the sodium channel (Tanabe *et al.*, 1987; Montal, 1990), it cannot be excluded that CP-96,345, RP 67580 and SR 48968 may exert a non-specific inhibitory effect on neurotransmission by involving a mechanism similar to that of local anaesthetics (i.e. blockade of sodium channels). Hence, we thought it worthwhile to study the mode of action and the structure-activity relationship for the non-peptide antagonists in relation to local anaesthetics, which consist of a lipophilic group connected by an intermediate chain with a tertiary amine. It is possible that the amine group of local anaesthetics may play a key role in binding to the receptors on the sodium channels (Courtney & Strichartz, 1987). Similarly, the amine substituents of CP-96,345, RP 67580 and SR 48968 may play a key role in binding to tachykinin receptors (Fong *et al.*, 1993). Also the recently described non-peptide NK_1 receptor antagonists, WIN 51708 and WIN 62577 (Appell *et al.*, 1992; Lawrence *et al.*, 1992) have an amine substituent, being heterosteroid derivatives of imidazo[4,5-b] quinoxaline. After the first submission of this paper, a report appeared suggesting that CP-96,345 blocks sodium channels in neurones (Caeser *et al.*, 1993). The results were consistent with our IC_{50} values (typically 5–10 times the K_D for Na^+ conductance in frog nerve; Hahin & Strichartz, 1981) and our observation of use-dependent impulse blockade.

In many test systems, CP-96,345, RP-67580 and SR 48968 act as tachykinin receptor antagonists (Constantine *et al.*, 1991; Garret *et al.*, 1991; Snider *et al.*, 1991; Emonds-Alt *et al.*, 1992; Carruette *et al.*, 1992; Martin *et al.*, 1992; Patacchini *et al.*, 1992). In the rabbit iris sphincter, CP-96,345 was found to inhibit the contractile response to the selective NK_1 receptor agonist, [Sar^9, $Met(O_2)^{11}$]SP, yielding a pA_2 value of 5.5 (Wang & Håkanson, 1993). While CP-96,345 and RP-67580 act selectively on NK_1 receptors, SR-48968 is thought to be a highly selective NK_2 receptor antagonist. The reason SR 48968 acts to inhibit the electrically-evoked, tachykinin-mediated response in the rabbit iris sphincter (thought to be exclusively NK_1-receptor-mediated) (Hall *et al.*, 1991; 1993; Wang & Håkanson, 1993) may be because it interacts with the NK_1 receptors in this preparation. In fact, SR 48968 was recently found to inhibit NK_1 receptor-mediated responses of the rabbit caval vein with a pA_2 value of 6.1 (Advenier *et al.*, 1992). Alternatively, SR 48968 might affect the contractile response of the rabbit iris sphincter through the non-specific inhibitory effect on neurotransmission referred to above.

In conclusion, our results suggest that, in addition to blocking tachykinin receptors, the non-peptide tachykinin receptor antagonists, CP-96,345, RP 67580 and SR 48968, may exert non-specific inhibitory effects on neurotransmission.

This study was supported by grants from the Swedish Medical Research Council (04X-1007) and from the Medical Faculty of Lund, Sweden and by a USPHS grant GM 15904. S.R.T. is a fellow of the Harvard Anesthesia Center.

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(Received September 3, 1993
Revised September 27, 1993
Accepted September 28, 1993)

Br. J. Pharmacol. (1994), **111**, 185–190

A comparison of A_2 adenosine receptor-induced cyclic AMP generation in cerebral cortex and relaxation of pre-contracted aorta

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1 A comparative study was carried out between the adenosine receptor mediating a stimulation of cyclic AMP formation in guinea-pig cerebral cortical slices with the adenosine receptor mediating relaxation of phenylephrine precontracted guinea-pig aortic rings.

2 [^{3}H]-cyclic AMP accumulation in [^{3}H]-adenine-prelabelled guinea-pig cerebral cortical slices was stimulated by adenosine and its analogues with the following EC_{50} values (μM): 5'-N-ethylcarboxamidoadenosine (3.1 ± 0.3) > 2-chloroadenosine (10 ± 2) > adenosine (109 ± 15).

3 2-Chloroadenosine and adenosine elicited maximal responses for [^{3}H]-cyclic AMP accumulation that were 100 ± 7 and 71 ± 6% of the maximal response to 5'-N-ethylcarboxamidoadenosine, respectively. CGS 21680 (100 μM) and DPMA (100 μM) elicited − 2 ± 2 and 12 ± 3% of the response to 100 μM 5'-N-ethylcarboxamidoadenosine.

4 Estimation of antagonist potencies at the A_2 adenosine receptor of cerebral cortex showed a rank order of potency (K_i, nM): xanthine amino congener (35 ± 3) > 8-cyclopentyl-1,3-dipropylxanthine (130 ± 22) > PD 115,199 (407 ± 82) > 3,7-dimethyl-1-propargylxanthine (13 ± 2 μM).

5 Adenosine analogues produced long-lasting relaxation of phenylephrine-precontracted aortic rings with the following rank order of potency (EC_{50} values, μM): 5'-N-ethylcarboxamidoadenosine (0.68 ± 0.06) > 2-chloroadenosine (4.3 ± 0.6) > adenosine (104 ± 13). Maximal relaxations elicited by these agents were 71 ± 3, 98 ± 1, and 100 ± 1%, respectively. CGS 21680 and DPMA at 100 μM elicited smaller relaxations of the precontracted tissues (12 ± 2 and 43 ± 15%, respectively).

6 Antagonism by xanthine derivatives of the 5'-N-ethylcarboxamidoadenosine-induced relaxation of aortic rings showed the following rank order of potency (K_i, nM): xanthine amino congener (17 ± 4) > 8-cyclopentyl-1,3-dipropylxanthine (171 ± 36) > PD 115,199 (341 ± 64) > 3,7-dimethyl-1-propargylxanthine (5520 ± 820).

7 We conclude that the A_2 adenosine receptor mediating relaxation of phenylephrine-contracted aortic rings is an A_{2b} adenosine receptor which exhibits certain minor differences from the A_{2b} receptor which stimulates cyclic AMP accumulation in cerebral cortical slices.

Keywords: Adenosine receptors; xanthines; cyclic AMP; guinea-pig cerebral cortex; guinea-pig aorta

Introduction

Two classes of extracellular adenosine receptor were originally delineated from studies of the effects of adenosine in mouse primary astroglial culture, subserving an inhibition (A_1) and a stimulation (A_2) of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Van Calker *et al.*, 1979). Radioligand binding to the A_1 receptor has been demonstrated in brain (Schwabe & Trost, 1980; Bruns *et al.*, 1980), adipocyte (Ukena & Schwabe, 1985) and testes (Murphy *et al.*, 1983) to varying extents. A subtype of the A_2 receptor, the high affinity A_2 receptor (Daly *et al.*, 1983) which stimulates adenylyl cyclase activity in membranes from neostriatum, nucleus accumbens and olfactory tubercle (Fredholm, 1977; Wojcik & Neff, 1983) can also be visualised by radioligand binding techniques (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). A second subtype of A_2 receptor, the low affinity A_2 receptor, stimulates cyclic AMP production in most brain regions, but fails to stimulate significantly adenylyl cyclase activity in cell-free preparations (Daly *et al.*, 1983). This subtype has also been recognised in the VA13 human fibroblast cell line, also linked to a stimulation of adenylyl cylcase activity (Bruns *et al.*, 1986). As yet, no selective agonist, antagonist or radioligand binding assay exists for this subtype, making examination of the tissue distribution of this receptor problematic. These subtypes have been termed A_{2a} and A_{2b} receptors equivalent to the high and low affinity types of the A_2 receptor (Bruns *et al.*, 1986).

Molecular biological approaches to studying adenosine receptors have yielded sequences for A_1 and A_{2a} receptors from dog (Maenhaut *et al.*, 1990; Libert *et al.*, 1991), rat (Mahan *et al.*, 1991; Reppert *et al.*, 1991) and human tissues (Furlong *et al.*, 1992; Libert *et al.*, 1992; Townsend-Nicholson & Shine, 1992). In addition, there are published sequences for an 'A_{2b}' receptor from rat (Stehle *et al.*, 1992) and human tissues (Pierce *et al.*, 1992). Northern blot analysis of the regional distribution of the rat 'A_{2b}' receptor mRNA showed highest density of expression in large intestine, caecum, and urinary bladder, with lower density in the brain and spinal cord (Stehle *et al.*, 1992). Expression of mRNA in the aorta was not examined. Both the rat and human clones, when expressed in COS-6 and Chinese hamster ovary cells, respectively, led to stimulation of cyclic AMP formation upon incubation with the adenosine analogue 5'-N-ethylcarboxamidoadenosine (NECA). However, the former receptor expressed in *Xenopus* oocytes appeared to be coupled to phosphoinositide hydrolysis (Yakel *et al.*, 1993). As the native receptor has only been observed to be coupled to generation of cyclic AMP, there must be some doubt as to the exact nature of this receptor.

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A_1 and A_2 classes have been differentiated on the basis of rank order of agonist potency (Hamprecht & van Calker, 1985; Gurden *et al.*, 1993). Typically N^6-derivatives of adenosine are more potent than 5'-derivatives at A_1 receptors, and the reverse is true at A_2 receptors. Receptor classification using agonists may, however, be complicated by factors such as receptor reserve or partial activity of agonists, difficulties which the use of selective antagonists would be expected to surmount. The archetypal adenosine receptor antagonists, theophylline and caffeine, show little potential for differentiating between the three receptor types, however, and therefore are not useful as discriminatory tools (Daly *et al.*, 1983). We have used four xanthine-based adenosine antagonists with a range of affinities and reported A_1:A_2 selectivities in radioligand binding assays of rat CNS tissues to characterize the A_{2b} adenosine receptor of guinea-pig cerebral cortical slices linked to cyclic AMP generation. We have compared the pharmacological profile of the A_{2b} receptor with the relaxant A_2 receptor of guinea-pig aorta which has previously also been suggested to be an A_{2b} receptor (Collis *et al.*, 1987; Martin, 1992; Gurden *et al.*, 1993). A preliminary account of some of this work has been presented to the British Pharmacological Society (Losinski *et al.*, 1993).

Methods

Cyclic AMP production in cerebral cortex

Cyclic AMP production was assessed in guinea-pig brain (Hartley strain, 200–600 g, of either sex) as previously described (Alexander *et al.*, 1989). Briefly, cross-chopped slices (350 × 350 μm) of cerebral cortex were prelabelled with [^{3}H]-adenine (74 kBq ml^{-1}) in Krebs Henseleit medium at 37°C, before being washed and pre-incubated in the presence of antagonist in a total volume of 300 μl for 10 min. Except when the effects of adenosine were investigated, the incubation with agonist was carried out in the presence of adenosine deaminase (1 u ml^{-1}) to prevent potential interference from endogenous adenosine. Histamine (0.3 or 1 mM) was also included to amplify responses during the agonist incubation period of 10 min. The incubation was halted by the addition of 200 μl of 1 M HCl containing 25–30 Bq [^{14}C]-cyclic AMP to act as a recovery marker. [^{3}H]-cyclic AMP was isolated by sequential Dowex 50 – Alumina column chromatography as previously described (Alexander *et al.*, 1989). Analysis of [^{3}H]-cyclic AMP formation was carried out on triplicate samples.

Relaxation of precontracted aortic rings

Guinea-pig aorta was rapidly dissected and freed from connective tissue, cut into 4 mm cylinders and suspended horizontally on thin wires of an isometric apparatus fitted with a Washington Type D transducer (Searle, Kent) under a resting tension of 1 g. Contractile responses were recorded either on a Washington chart recorder (Searle, Kent) or using a Personal Computer equipped with Chart software (CED, Cambs.). The cylinders were allowed to equilibrate for a period of up to 60 min at 37°C in gassed (O_2:CO_2 95:5) Krebs Henseleit buffer containing indomethacin (10 μM), with occasional changes of medium. Rings were contracted with repeated applications of a submaximally-effective concentration of phenylephrine (4 μM) until a reproducible response was obtained. Cumulative concentration-relaxation curves for the adenosine analogues were constructed in the presence of phenylephrine, discontinuing the addition of agonist when an increase in agonist concentration failed to elicit further relaxation. Agonists were washed out and the tissue allowed to re-equilibrate for a further 60 min before repetition of the contraction-relaxation cycle in the presence of xanthine-based antagonist.

Data analysis and statistics

Data were fitted to a sigmoidal curve using the computer programme GraphPad InPlot (GraphPad, California, U.S.A.). Apparent inhibition constants for the antagonists (K_i) from functional assays were calculated as previously described (Alexander *et al.*, 1989) based on the antagonist-induced shift in agonist (unless otherwise indicated, NECA) concentration-response curves. Statistical analyses were carried out using the Student's *t* test.

Chemicals

[^{14}C]-cyclic AMP (1.6 GBq mmol^{-1}) and [^{3}H]-adenine (888 GBq mmol^{-1}) were obtained from NEN Dupont (Herts.) and Amersham International (Bucks.) respectively. N-[2-(dimethylamino) ethyl]*N*-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide (PD 115,199) was a gift from Warner-Lambert, U.S.A. Adenosine analogues (including 2-*p*-(2-carboxyethyl) phenethylamino -5'-N -ethylcarboxamidoadenosine (CGS 21680) and N^6- [2- (3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA)) and xanthine derivatives were obtained from Research Biochemicals Incorporated (Semat. Herts.). All other chemicals were from Fisons (Leics.) or Sigma Chemicals (Dorset).

Results

Adenosine receptor-stimulated cyclic AMP production in guinea-pig cerebral cortex

Adenosine analogues were observed to enhance the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-prelabelled guinea-pig cerebral cortical slice in a concentration-dependent manner. Of the analogues tested, NECA (5'-N-ethylcarboxamidoadenosine) was the most potent, followed by 2CA (2-chloroadenosine) and adenosine (Figure 1, Table 1). The response to adenosine was conducted in the absence of adenosine deaminase (NECA responses in the absence and presence of adenosine deaminase were not significantly different). The response to adenosine showed a reduced maximal effect when compared to NECA and 2CA. In the presence of a maximally-active concentration of adenosine (300 μM), the response to 100 μM NECA was reduced to approximately the same level as the response to adenosine alone (NECA response = 100%; 300 μM adenosine = 82 ± 6%; adenosine + NECA = 81 ± 7%; n = 3). The reportedly A_2-selective agents N^6- [2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl] adenosine (DPMA) and 2-*p*-(2-carboxyethyl)phenethylamino-5'-

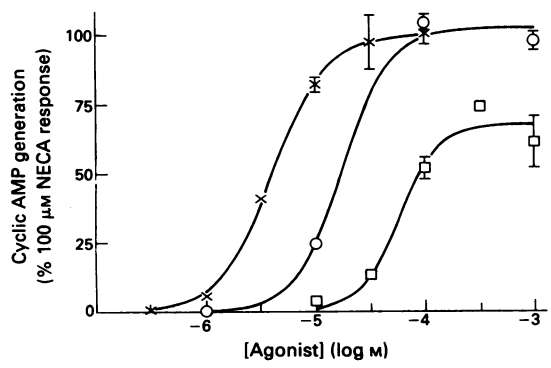

Figure 1 Stimulation of [^{3}H]-cyclic AMP accumulation in [^{3}H]-adenine-prelabelled guinea-pig cerebral cortical slices by 5'-N-ethylcarboxamidoadenosine (X), 2-chloroadenosine (O) and adenosine (□). Data are means and s.e.mean from single experiments representative of 5–14.

Table 1 Adenosine analogue potency at A_2 adenosine receptors of guinea-pig cerebral cortex and aorta

	Cerebral cortex			*Aorta*		
Agonist	*EC_{50} value* (μM)	*Intrinsic[a] activity* (%)	n	*EC_{50} value* (μM)	*Intrinsic[b] activity* (%)	n
NECA	3.1 ± 0.3	100	14	0.68 ± 0.06	71 ± 3	40
2CA	10 ± 2	97 ± 6	7	4.7 ± 0.6	98 ± 1	5
Adenosine[c]	109 ± 15	71 ± 6	5	104 ± 13	100 ± 1	4
DPMA[d]	–	12 ± 3	3	–	43 ± 15	3
CGS 21680[d]	–	−2 ± 2	3	–	12 ± 2[e]	2

[a]Intrinsic activity compares the maximal response to each agent (for DPMA and CGS 21680, the response at 100 μM) with that to 100 μM NECA. [b]Intrinsic activity compares the maximal relaxation elicited by each agent as a function of the contractile effect of phenylephrine. [c]The cyclic AMP response to adenosine was determined in the absence of adenosine deaminase. The response to 100 μM NECA was not significantly altered in the presence of adenosine deaminase. [d]Responses to DPMA and CGS 21680 were examined at a single agonist concentration of 100 μM. Data are means ± s.e.mean ([e]means ± range) of the indicated number of experiments. For abbreviations, see text.

N-ethylcarboxamidoadenosine (CGS 21680) showed little or no significant stimulatory effects of cyclic AMP formation at concentrations up to 100 μM.

In the presence of fixed concentrations of xanthine-based adenosine receptor antagonists, concentration-response curves to NECA were shifted rightward in an apparently competitive manner (see Alexander *et al.*, 1989), allowing estimation of the apparent inhibition constants for these antagonists (Table 2). A rank order of antagonist potency was observed of xanthine amino congener (XAC) > 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) > N- [2-(dimethylamino)ethyl]-*N*-methyl-4-(1,3-dipropylxanthine)-benzenesulphonamide (PD 115,199) > 3,7-dimethyl-l-propargylxanthine (DMPX). The concentration-response curve to adenosine was examined in the presence and absence of 40 nM DPCPX (data not shown). This concentration of DPCPX was chosen in order to occupy over 80% of A_1 adenosine receptors in this tissue (based on a K_i for DPCPX of 6 nM; Alexander *et al.*, 1992b) with less than 25% of the A_{2b} receptors occupied (based on a K_i of 130 nM, Table 1). However, this concentration of DPCPX failed to alter significantly either the EC_{50} value or maximal response of adenosine, indicating little contribution of A_1 receptors to the reduced maximal response of adenosine compared to NECA.

Adenosine receptor-mediated relaxation of guinea-pig aortic rings

We carried out a number of preliminary investigations to determine the optimal conditions for assaying adenosine receptor activity in guinea-pig aortic rings. Analysis of cumulative concentration-response curves for phenylephrine-induced contractions gave an EC_{50} value of 0.92 ± 0.03 μM with a maximal response of 1.36 ± 0.14 g ($n = 3$). Histamine showed a greater maximal response (2.25 ± 0.18 g; EC_{50} value 1.21 ± 0.22 μM; $n = 7$); however, the response to phenylephrine was better maintained. In further experiments designed to investigate the relaxant action of adenosine receptor agonists, 4 μM phenylephrine was used to elicit aortic contractions.

Addition of 3 μM 5'-N-ethylcarboxamidoadenosine (NECA) to precontracted aorta elicited a rapid, maintained relaxation of the aorta (Figure 2). Cumulative concentration-relaxation curves were constructed for the agonists NECA, 2-chloroadenosine (2CA) and adenosine (Figure 3, Table 1). Interestingly, 2CA and adenosine fully relaxed the tissue, while in

Table 2 Antagonist affinities at the A_2 adenosine receptors of cerebral cortex and aorta

	Cerebral cortex		*Aorta*	
Antagonist	*K_i* (nM)	n	*K_i* (nM)	n
XAC	35 ± 3	4	17 ± 4	4
DPCPX	123 ± 21	14	171 ± 36	8
PD 115,199	407 ± 82	5	341 ± 64	6
DMPX	12843 ± 1849	3	5521 ± 824	3

Data are means ± s.e.mean of apparent inhibition constants (calculated as described in Methods) estimated on the indicated number of occasions. For abbreviations, see text.

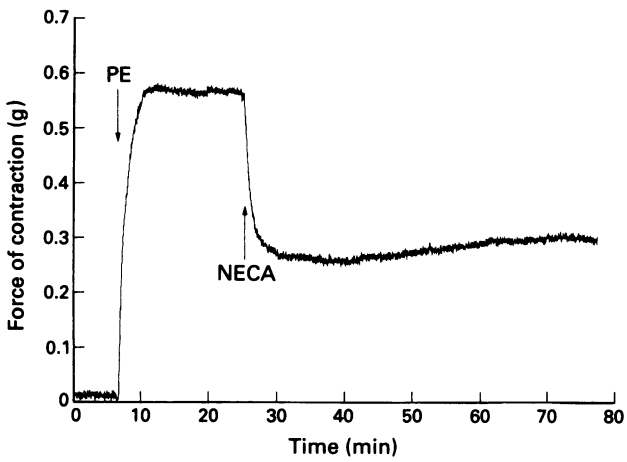

Figure 2 Long-lasting relaxation of a phenylephrine (PE)-contracted guinea-pig aortic cylinder by 300 nM 5'-N-ethylcarboxamidoadenosine (NECA).

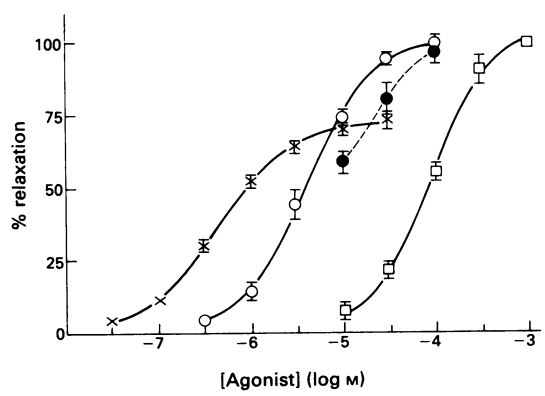

Figure 3 Relaxation of phenylephrine-precontracted guinea-pig aortic rings by 5'-N-ethylcarboxamidoadenosine (NECA) (X), 2-chloroadenosine (2CA, O) and adenosine (□). The relaxant response to 2CA in the presence of a maximally-active concentration of NECA is also shown (●). Data are means and s.e.mean of 4–11 experiments.

comparison, the NECA response relaxed the aorta to only 71%. In the presence of a maximally-active concentration of NECA (100 μM), addition of 2CA elicited a further relaxation of the tissue to completeness (EC_{50} 2CA in the presence of NECA 26 ± 4 μM, 97 ± 3% relaxation, $n = 4$). DPMA and CGS 21680 induced smaller responses at 100 μM (Table 1).

In the presence of fixed concentrations of xanthine-based adenosine receptor antagonists, concentration-response curves to NECA were shifted rightward in an apparently competitive manner (see Alexander *et al.*, 1989), allowing estimation of the apparent inhibition constants for these antagonists (Table 2). Thus, NECA concentration-response curves in the presence of 300 nM XAC, 1 μM DPCPX, 3 μM PD 115,199, or 30 μM DMPX were shifted rightward compared to control NECA responses 22, 14, 11 and 7 fold, respectively. Slopes of the NECA concentration-response curves in the presence of XAC (− 1.16 ± 0.08), DPCPX (− 1.19 ± 0.15), PD 115,199 (− 1.24 ± 0.15) and DMPX (− 0.98 ± 0.09) were not significantly different from the control response (− 1.06 ± 0.03, Student's *t* test). Thus, the adenosine receptor of guinea-pig aorta exhibits an antagonist rank order of potency such that XAC > DPCPX > PD 115,199 > DMPX. Preliminary experiments with 2CA as the relaxant agonist in place of NECA produced a similar affinity estimate for the antagonist XAC (100 nM XAC elicited an 11 fold shift in the 2CA concentration-response curve to generate a K_i estimate of 10 nM, $n = 2$, data not shown).

Discussion

Research into the characterisation of adenosine receptors has mainly concentrated on tissues of nervous system origin. In CNS preparations, three subclasses of adenosine receptor are demonstrable, based on changes in levels of the second messenger cyclic AMP (Daly *et al.*, 1983). Thus, A_1 receptors are linked to an inhibition of cyclic AMP formation in both cell-free and slice preparations (Ebersolt *et al.*, 1983; Fredholm *et al.*, 1986). A_2 receptors were subdivided into high and low affinity receptors on the basis of regional localization and demonstration in cell-free or intact slice preparations. These receptors were later renamed A_{2a} and A_{2b} receptors, respectively (Bruns *et al.*, 1986). A_{2a} receptors are almost exclusively located in the neostriatum, nucleus accumbens and olfactory tubercle and stimulate adenylyl cyclase activity in cell-free preparations, while A_{2b} receptors stimulate cyclic AMP formation in intact slice preparations from most brain regions (Daly *et al.*, 1983). Similar adenosine receptor subclasses have been suggested to exist in the periphery (Collis *et al.*, 1987; Martin *et al.*, 1992; Gurden *et al.*, 1993), but a direct comparison of CNS and peripheral adenosine receptors has not been carried out. In the present study, we compare the A_{2b} adenosine receptor of guinea-pig cerebral cortex with the suggested A_{2b} adenosine receptor of guinea-pig aorta. Our data suggest marked similarities in the pharmacological profile of the two receptors, but raise the possibility that the two represent variants of the A_{2b} receptor class.

The A_{2b} receptor of guinea-pig cerebral cortex

Stimulation by adenosine analogues of cyclic AMP formation in guinea-pig cerebral cortex shows a rank order of potency of NECA > 2CA > adenosine >> DPMA ≃ CGS 21680. This rank order of NECA, 2CA and adenosine concurs with that observed for stimulation of cyclic AMP generation in rat cerebral cortex (Bazil & Minneman, 1986). In the latter tissue, a reduced maximal response to adenosine was also observed in comparison with NECA and 2CA, raising the possibility that adenosine, the presumed endogenous ligand, is a partial agonist at the A_{2b} receptor. The lack of effect of DPCPX at a concentration chosen to block almost completely A_1 receptors (40 nM) suggests that A_1 receptor-induced inhibition of cyclic AMP generation is not involved in the reduced maximal response to this agent. The reduced response to 100 μM NECA observed in the presence of a maximally effective concentration of adenosine adds further evidence for the partial agonist nature of adenosine at the A_{2b} receptor.

DPMA and CGS 21680 have been proposed as A_2-selective agonists based on radioligand binding assays in rat brain membranes (Bridges *et al.*, 1988; Jarvis *et al.*, 1989); however, it is clear from the present study that they fail to stimulate significantly A_{2b} receptors, and thus may ultimately prove to be A_{2a}-selective.

Recent advances in the synthesis of adenosine receptor antagonists have produced compounds which exhibit high specificity for adenosine receptor subclasses, assessed mainly by use of radioligand binding assays in rat CNS preparations. Thus, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, PD 116,948) was shown to have a 740 fold selectivity for the A_1 against the A_{2a} receptor of rat brain in both radioligand binding and functional adenylate cyclase assays (Lee & Reddington, 1986; Bruns *et al.*, 1987a; Lohse *et al.*, 1987). PD 115,199, a benzenesulphonamide derivative of 1,3-dipropylxanthine was shown to have high affinity for rat CNS A_1 and A_{2a} receptors, with, however, little selectivity between the two classes (Bruns *et al.*, 1987b). Xanthine amino congener apparently exhibits high affinity for all three classes of adenosine receptor in the rat, with some A_1 selectivity (van Galen *et al.*, 1992). A further xanthine-based compound, 3,7-dimethyl-1-propargylxanthine has been suggested to be 4 fold A_2-selective in radioligand binding assays (Ukena *et al.*, 1986; Daly *et al.*, 1986). The rank order of antagonist potency at the A_{2b} adenosine receptor mediating cyclic AMP generation is XAC > DPCPX > PD 115,199 > DMPX. Our recent examination of A_1 and A_{2a} adenosine receptor binding in membranes from guinea-pig brain show antagonist rank order of potencies of DPCPX > XAC > PD 115,199 and PD 115,199 > DPCPX > XAC, respectively (Alexander *et al.*, unpublished observations). The behaviour of these xanthine antagonists at CNS adenosine receptors in the guinea-pig correlates well with data from the rat and may therefore prove to be a useful means of discriminating adenosine receptor subtypes.

A_2 receptor relaxation of guinea-pig aorta

Adenosine and its analogues elicited a long-lasting relaxation of phenylephrine-induced aortic cylinder contraction, with a rank order of potency NECA > 2CA > adenosine > DPMA > CGS 21680. The long-lasting relaxation induced by NECA in the guinea-pig aorta resembles the well-maintained NECA stimulation of cyclic AMP levels in guinea-pig cerebral cortex (Alexander *et al.*, 1992a). Whether the relaxation of guinea-pig aorta induced by NECA is mediated by a stimulation of cyclic AMP levels will be the subject of further investigations. In the aorta, NECA elicited a reduced maximal relaxant response compared to 2CA and adenosine, implying that it may well be a partial agonist in this tissue. In the presence of a maximally-active concentration of NECA, 2CA was able to relax the tissue further; however, the concentration-response curve to 2CA was shifted rightward. This is consistent with both these agents acting through the same receptor.

The xanthine derivatives induced rightward shifts in the NECA concentration-response curve. Calculating apparent inhibition constants from the antagonist-induced shifts indicated a rank order of antagonist potency of XAC > DPCPX > PD 115,199 > DMPX. The similar affinity of XAC for antagonism of NECA- and 2CA-elicited relaxation of phenylephrine-induced contractions is further evidence for an identical site of action of the two agonists.

Molecular biology of A_{2b} receptors

A human 'A_{2b}' adenosine receptor has been expressed in Chinese hamster ovary cells and exhibited positive coupling

to cyclic AMP generation when stimulated by NECA (EC_{50} value of 0.9 μM, c. 10 fold maximal stimulation) and adenosine (1 mM also elicited a c. 10 fold stimulation). CGS 21680, in contrast, failed to elicit a stimulation of cyclic AMP generation in transfected cells. Binding of adenosine receptor radioligands (2-chloro-N^6-cyclopentyl-[^{3}H]-adenosine, [^{3}H]-CGS 21680 or 5'-N-ethylcarboxamido-[^{3}H]-adenosine) was not supported in cells transfected with this clone. The rat 'A_{2b}' adenosine receptor expressed in COS-6 cells also exhibited a NECA-elicited stimulation of cyclic AMP generation. When this clone was expressed in *Xenopus* oocytes, adenosine evoked an inward current with an EC_{50} value of ~50 μM (Yakel *et al.*, 1993). NECA, 10 μM, elicited a response comparable with that induced by 100 μM adenosine, while 10 μM CGS 21680 was ineffective as a stimulus of inward current. The inward current induced by 100 μM adenosine was reversed by 100 nM (27 ± 3% inhibition) and 1 μM DPCPX (95 ± 4%) and 10 μM DMPX (75 ± 5%). The pharmacological profile (albeit very limited) from either of these clones is therefore consistent with the profile of the A_{2b} adenosine receptor from guinea-pig cerebral cortex and the A_2 receptor from the guinea-pig aorta. The potential localization of the cloned 'A_{2b}' receptors in aortic tissues has unfortunately not been examined. The relative intrinsic activity of NECA and adenosine has also not been examined in these clones.

Concluding remarks

The identical rank order of potency for the selected agonists and antagonists suggests that the A_2 adenosine receptor of guinea-pig aorta is analogous to the A_{2b} adenosine receptor of guinea-pig cerebral cortex. However, adenosine appears to be a partial agonist in the cerebral cortex, whilst behaving as an apparent full agonist in the aorta and the reverse is true for NECA. This is therefore a substantial inconsistency between the tissues. We must conclude that although these receptors exhibit very similar pharmacological profiles, they may well be variants of the A_{2b} adenosine receptor. This question may best be resolved through the application of molecular biological studies.

We thank the Wellcome Trust for financial support and Dr Vince Wilson for useful discussion.

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(Received July 28, 1993
Revised September 24, 1993
Accepted September 29, 1993)

Br. J. Pharmacol. (1994), **111**, 191–198

Adenosine A_{2B}-receptor-mediated cyclic AMP accumulation in primary rat astrocytes

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1 The effects of adenosine receptor agonists and antagonists on the accumulation of cyclic AMP have been investigated in primary cultures of rat astrocytes.

2 Adenosine A_2-receptor stimulation caused a concentration-dependent increase in the accumulation of [^{3}H]-cyclic AMP in cells prelabelled with [^{3}H]-adenine. The rank order of agonist potencies was 5'-N-ethylcarboxamidoadenosine (NECA; $EC_{50} = 1\ \mu M$) > adenosine ($EC_{50} = 5\ \mu M$) > 2-chloroadenosine ($EC_{50} = 20\ \mu M$) >> CGS 21680 ($EC_{50} > 10\ \mu M$). The presence of 0.5 μM dipyridamole, an adenosine uptake blocker, had no effect on the potency of adenosine.

3 The response to 10 μM NECA was antagonized in a concentration-dependent manner by the non-selective adenosine receptor antagonists, xanthine amine congener (apparent $K_D = 12$ nM), PD 115,199 (apparent $K_D = 134$ nM) and 8-phenyltheophylline (apparent $K_D = 126$ nM). However, the A_1-receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine, had no significant effect on the responses to NECA or 2-chloroadenosine at concentrations up to 1 μM.

4 Stimulation of A_1-receptors with the selective agonist, N^6-cyclopentyladenosine, did not alter the basal accumulation of [^{3}H]-cyclic AMP but inhibited a forskolin-mediated elevation of [^{3}H]-cyclic AMP accumulation by a maximal value of 42%. This inhibition was fully reversed in the presence of 0.1 μM, 8-cyclopentyl-1,3-dipropylxanthine.

5 The time course for NECA-mediated [^{3}H]-cyclic AMP accumulation was investigated. The results suggest that there is a substantial efflux of cyclic AMP from the cells in addition to the rapid and sustained elevation of intracellular cyclic AMP (5 fold over basal) which was also observed.

6 These data indicate that rat astrocytes in primary culture express an A_{2B}-adenosine receptor coupled positively to adenylyl cyclase. Furthermore, the presence of A_1-receptors negatively coupled to adenylyl cyclase appears to have no significant effect on the A_{2B}-receptor-mediated cyclic AMP responses to NECA and 2-chloroadenosine.

Keywords: Rat astrocytes; cyclic AMP accumulation; adenosine receptors; methylxanthines

Introduction

In 1970, Sattin & Rall were the first to demonstrate adenosine-mediated accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in slices of guinea-pig cerebral cortex. This result was subsequently confirmed in brain slices from rat, mouse and man (Rall & Sattin, 1970; Kodama *et al.*, 1973). Using primary cultures of glial cells from perinatal mouse brain, Van Calker *et al.* (1979) suggested that two different classes of adenosine receptor could mediate changes in intracellular cyclic AMP levels and introduced the terms 'A_1'- and 'A_2'-receptors to classify sites which were, respectively, negatively and positively coupled to adenylyl cyclase via different (G_i and G_s) guanosine 5'-triphosphate (GTP)-binding proteins.

Analysis of structure-activity relationships has now yielded agonists and antagonists that have selective actions at A_1- or A_2-receptors (for review see Jacobson *et al.*, 1992). Thus, the A_1-selective agonist, N^6-cyclopentyladenosine, exhibits 2,500 fold A_1-selectivity in radioligand binding studies (Lohse *et al.*, 1988). In contrast, the 5'-substituted adenosine analogue, 5'-N-ethylcarboxamidoadenosine (NECA), is equipotent at A_1- ($K_D = 6$ nM) and A_2-receptors ($K_D = 10$ nM) (Bruns *et al.*, 1986). Use of this latter compound in characterizing A_2-receptor-mediated tissue responses must, therefore, be accompanied by either demonstration of the lack of A_1-receptor involvement or removal of any A_1-component with a selective A_1-receptor antagonist. The prototypic adenosine receptor antagonist, theophylline, has only moderate receptor affinity and does not distinguish between A_1-($K_D = 9\ \mu M$) and A_2-subtypes ($K_D = 25\ \mu M$) (Bruns *et al.*, 1986). However, substitutions in positions 1, 3 and 8 have produced, 8-cyclopentyl-1,3-dipropylxanthine, which has K_D values of 0.5 nM and 340 nM at A_1- and A_2-receptors respectively (Bruns *et al.*, 1987a) and has been used to block A_1-components of binding during A_2-receptor studies (Bruns *et al.*, 1987b).

In 1983, Daly *et al.* observed that adenosine analogues were more potent at A_2-receptors in rat striatal membranes than at those in other membrane or slice preparations from rat brain. The selective targeting of striatal membrane A_2-receptors by the agonist CGS 21680 (Jarvis *et al.*, 1989) and the antagonist PD115,199 (Bruns *et al.*, 1987b) instigated a proposal for the subdivision of A_2-receptors into 'high affinity' A_{2A}-and 'low affinity' A_{2B}-subtypes (for review see Stiles, 1992). This theory has been supported by the characterization of peripheral A_2-receptors in guinea-pig tissues using a range of adenosine receptor agonists and antagonists. For example, receptors in the guinea-pig trachea appear to be of the A_{2A}-subtype (Alexander *et al.*, 1989a). The high affinity of PD115,199 for these receptors is in contrast to its low affinity for A_{2B}-receptors in guinea-pig aorta and cerebral cortex (Losinski *et al.*, 1993). Molecular cloning and expression of proteins which appear to correspond to A_1- (Mahan *et al.*, 1991; Libert *et al.*, 1991; 1992; Olah *et al.*, 1992), A_{2A}- (Maenhaut *et al.*, 1990; Furlong *et al.*, 1992) and A_{2B}-receptors (Pierce *et al.*, 1992; Rivkees & Reppert, 1992), has further substantiated this classification.

A number of groups have reported that adenosine and derivatives such as NECA, 2-chloroadenosine and phenylisopropyladenosine can stimulate cyclic AMP accumulation in rat, mouse and human glial preparations (Van Calker *et al.*,

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1979; Ebersolt *et al.*, 1983; Elfman *et al.*, 1984; Woods *et al.*, 1989; Murphy *et al.*, 1991). However, the adenosine A_2-receptor subtype involved in these cyclic AMP responses remains to be determined. The aim of this study was to use subtype-selective compounds to investigate the adenosine receptor subtype (A_{2A} or A_{2B}) positively coupled to cyclic AMP accumulation in primary cultures of rat astrocytes.

Methods

Cell culture

Primary cultures of rat astrocytes were prepared as reported previously (Ruck *et al.*, 1991) by a modification of the methods of McCarthy & de Vellis (1980) and Ebersolt *et al.* (1981a,b). Briefly, 2 day old Wistar rats were killed and the forebrains (whole brain minus brain stem and cerebellum) dissected out under sterile conditions. After removal of the meninges, the tissue was dissociated mechanically through nylon meshes (pore sizes 250 and 150 μm) to obtain a single cell suspension. The suspension was diluted to give 6.5×10^5 cells ml^{-1} and aliquoted into 24 well cluster dishes. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% foetal calf serum (FCS) at 37°C in a water-saturated atmosphere of 10% CO_2 in air. Penicillin 100 u ml^{-1}, streptomycin 100 μg ml^{-1} and amphotericin B 0.25 μg ml^{-1} were also present during the first 24 h of culture. The medium was changed after 1 and 3 days *in vitro* and thereafter every 5 days. Cells were used for cyclic AMP accumulation experiments after 13–17 days *in vitro*.

Immunocytochemistry

In order to characterize the prepared cultures, cells were plated (6.5×10^5 cells ml^{-1}) onto 10 mm glass coverslips in a 24 well cluster dish. At confluence, they were washed three times with phosphate buffered saline (PBS) (composition, mM: NaCl 137, KCl 2.68, KH_2PO_4 1.47, Na_2HPO_4 8.10) and fixed with 3.8% formaldehyde for 15 min. The cells were then washed twice more with PBS and incubated for 15 min with 1 mg ml^{-1} glycine/0.1% Triton X 100 in PBS to enhance antibody penetration. After three more PBS washes, 10% horse serum in PBS (blocking solution) was used for 30 min to block non-specific antibody binding. Monoclonal rabbit immunoglobulins to cow glial fibrillary acidic protein were diluted 1:20 in blocking solution. The blocking solution was then aspirated from the cover slips and the cells were incubated overnight at 4°C with 15 μl of the appropriate antibody solution. The following day, the coverslips were washed twice with PBS and then incubated for 60 min, at room temperature, in the dark with 15 μl of fluorescein-conjugated swine anti-rabbit anti-sera (1:20 dilution in blocking solution). The coverslips were then rinsed twice with PBS, once with distilled water and allowed to dry. They were mounted in 1:1, glycerol:PBS with 1 mg ml^{-1} phenylenediamine to prevent photobleaching. Fluorescent micrographs were taken with a Zeiss transmitted-light photomicroscope III.

Accumulation of [^{3}H]-cyclic AMP

Cyclic AMP accumulation was investigated by a modification of the method previously described by Donaldson *et al.* (1988). Cells were incubated for 2 h at 37°C in 1 ml/well Hanks' HEPES (N-[2-hydroxyethyl] piperazine-*N'*- [2-ethanesulphonic acid]) buffer (20 mM HEPES in Hanks' Balanced Salts Solution) containing 2 μCi/well (0.08 μM) 8-[^{3}H]-adenine. The labelled cell monolayers were then washed twice with Hanks' HEPES and incubated for 30 min in 1 ml/well buffer containing 100 μM rolipram, an inhibitor of the cyclic AMP selective (type IV) phosphodiesterase isoenzyme (Donaldson *et al.*, 1988). Where appropriate, adenosine deaminase, 1.2 u ml^{-1}, and antagonists in 10 μl buffer were also added at this stage. In experiments investigating the regulation of forskolin-elevated [^{3}H]-cyclic AMP levels, the receptor-selective agonist was added either 10 min before or 10 min after 1 μM forskolin. Cells were then incubated in the presence of both compounds for a further 10 min. In all other experiments, agonists were added, in 10 μl buffer, and incubated with the cells for 10 min. Incubations were terminated by the addition of 50 μl concentrated hydrochloric acid and the cells were frozen overnight. In experiments where intracellular and extracellular [^{3}H]-cyclic AMP were measured separately, the incubation medium in each well was collected for measurement of extracellular cyclic AMP, immediately prior to acid addition. This was replaced with 1 ml fresh Hanks' HEPES buffer and then 50 μl concentrated hydrochloric acid was immediately added to lyse the cells and release intracellular cyclic AMP. [^{3}H]-cyclic AMP was isolated by sequential Dowex-alumina chromatography as described by Donaldson *et al.* (1988). [^{14}C]-cyclic AMP (~2000 d.p.m.) was added to each sample prior to elution to correct for percentage recovery of the columns. Eluted samples were collected, 500 μl, 1 M hydrochloric acid and 10 ml scintillant were added and the levels of [^{3}H]- and [^{14}C]-cyclic AMP were determined by liquid scintillation counting.

Data analysis

Agonist concentration-response curves were fitted to a logistic equation by use of the non-linear regression programme, GraphPAD (ISI). The equation fitted was:

$$\text{Response} = \frac{E_{max} \times A^n}{(EC_{50})^n + A^n}$$

where E_{max} is the maximal response, A is the agonist concentration, EC_{50} is the concentration of agonist producing half maximal stimulation, and n is the Hill coefficient.

Antagonist dissociation constants (K_D) were estimated by a modification of the null method described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to NECA was generated and a concentration (C: 10 μM) of NECA was chosen which gave a response greater than 50% of the maximum agonist response. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of NECA by 50% was then determined. The NECA concentration-response curve was fitted to the logistic equation as above and a concentration of NECA (C') identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_d was then determined from the relationship:

$$C/C' = (IC_{50}/K_d) + 1$$

Statistical analysis was performed within single experiments by using an unpaired Student's *t* test and between multiple experiments by using 2 way analysis of variance (ANOVA) with *post-hoc* Newman-Keuls. In each experiment triplicate or quadruplicate determinations were made: however, unless otherwise stated, each value given in the text represents mean ± s.e.mean of *n* separate experiments.

Materials

Wistar rats were obtained from the Medical School Animal Unit, University of Nottingham. DMEM was purchased from Biological Industries (Cumbernauld), L-glutamate from ICN Flow Laboratories (Irvine), FCS from Advanced Protein Products (Brierley Hill), and antibiotic antimycotic solution (100x) from Gibco Laboratories (Paisley). The monoclonal rabbit immunoglobulins to cow glial fibrillary acidic protein,

and the corresponding fluorescent antibody, fluorescein-conjugated swine immunoglobulins to rabbit immunoglobulins were obtained from Dako patt (Denmark). 8-[^{3}H]-adenine (27 Ci mmol^{-1}) and 8-[^{14}C]-cyclic adenosine monophosphate (309 mCi mmol^{-1}) ammonium salt were supplied by Amersham International (Aylesbury). Rolipram was a gift from Schering (Berlin, Germany) and adenosine deaminase, dissolved in glycerol, was purchased from Boehringer Mannheim GmbH (Germany). Hanks' Balanced Salts Solution (10x) was purchased from Northumbria Biologicals (Northumberland) and HEPES from Sigma Chemical Co. (Poole). Adenosine, 2-chloroadenosine, N^6-cyclopentyladenosine, 8-phenyltheophylline and forskolin were all supplied by Sigma Chemical Co. (Poole). NECA, CGS 21680, (2-[[4-(2-carboxyethyl) phenethyl]-amino] adenosine-5'-*N*-ethylcarboxamide, 8-cyclopentyl-1,3-dipropylxanthine and 8-[4-[[[[(2-aminoethyl) amino] carbonyl] methyl] oxy]-phenyl]-1,3-dipropylxanthine (xanthine amine congener) were all supplied by Research Biochemicals Inc. Semat (St. Albans). N-[2-(dimethylamino)-ethyl]*N*-methyl-4-(1,3-dipropylxanthine) benzene sulphonamide (PD 115,199) was a generous gift from Warner Lambert (Ann Arbor, U.S.A.).

Results

Primary cultures of rat glia contained two predominant cell types. At confluence, process-bearing type-2 astrocytes formed on top of a monolayer of flat, epithelioid, type-1 astrocytes. Using indirect immunofluorescence, both cell types stained positively for the intermediate filament protein glial fibrillary acidic protein (Figure 1). The difference between the two astroglial morphologies is clearly evident in Figure 1a (type-1) and Figure 1b (type-2), which were taken from peripheral fields of view at low cell density. These results are consistent with previous findings for astrocytes (Ruck *et al.*, 1991) and confirm the astroglial nature of the cells.

Adenosine produced marked and significant accumulations of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes in a dose-dependent manner (Figure 2). After 10 min incubation with the agonist (100 μM), [^{3}H]-cyclic AMP levels had increased from a mean basal response of 3,503 ± 429 d.p.m. to 31,021 ± 2,711 d.p.m. ($P<0.01$, $EC_{50} = 5.1 \pm 0.8$ μM, $n = 7$). The response reached a maximum at 100 μM adenosine but was reduced when a higher concentration (1 mM) was used. The presence of 0.5 μM dipyridamole (Hill & Kendall, 1987) (Figure 2) had no significant effect on the potency of adenosine, $EC_{50} = 5.6 \pm 2.0$ μM ($n = 3$).

In the presence of 1.2 u ml^{-1} adenosine deaminase, the mixed adenosine A_1/A_2-receptor agonists, NECA (Figure 3) and 2-chloroadenosine (data not shown) also caused dose-dependent accumulations of [^{3}H]-cyclic AMP in these cultures. The maximal NECA-mediated (10 μM) response was 11.5 ± 1.5 fold over basal ($P<0.01$, $n = 6$), whereas the maximal response to 2-chloroadenosine (100 μM) was only 68.4 ± 3.9% ($P<0.01$, $n = 6$) of that produced by 10 μM NECA, which was measured in every experiment. Under the same conditions, however, the A_{2A}-selective compound, CGS 21680 (Hutchison *et al.*, 1989; Jarvis *et al.*, 1989) caused no significant accumulation at concentrations up to 10 μM ($n = 3$) (Figure 3, Table 1). The EC_{50} values calculated for NECA and adenosine were in close agreement with the values previously reported for stimulation of cyclic AMP accumulation in rat astrocytes (Murphy *et al.*, 1991) (Table 1).

Initial studies to investigate the time course of this response (Figure 4) indicated that accumulation of 'total' (intracellular plus extracellular) [^{3}H]-cyclic AMP, increased throughout the experiment and had not achieved steady-state conditions even after 40 min (27.2 ± 3.5 fold over basal, $n = 3$) incubation with NECA. In order to analyse this result more closely, we examined the accumulations of intra- and extracellular [^{3}H]-cyclic AMP individually. As shown in Figure 5a, the accumulation of intracellular [^{3}H]-cyclic AMP increased rapidly to a level which was then maintained for the remainder of the experiment (5.2 ± 0.7 fold over basal: $n = 3$). In contrast, Figure 5b shows that accumulation of extracellular [^{3}H]-cyclic AMP increased linearly throughout the experiment. There was apparently no lag time for this response and in three experiments, accumulation had risen by 10.4 ± 3.5 fold over basal after 40 min.

The response to 10 μM NECA was antagonized, in a concentration-dependent manner, by 8-phenyltheophylline,

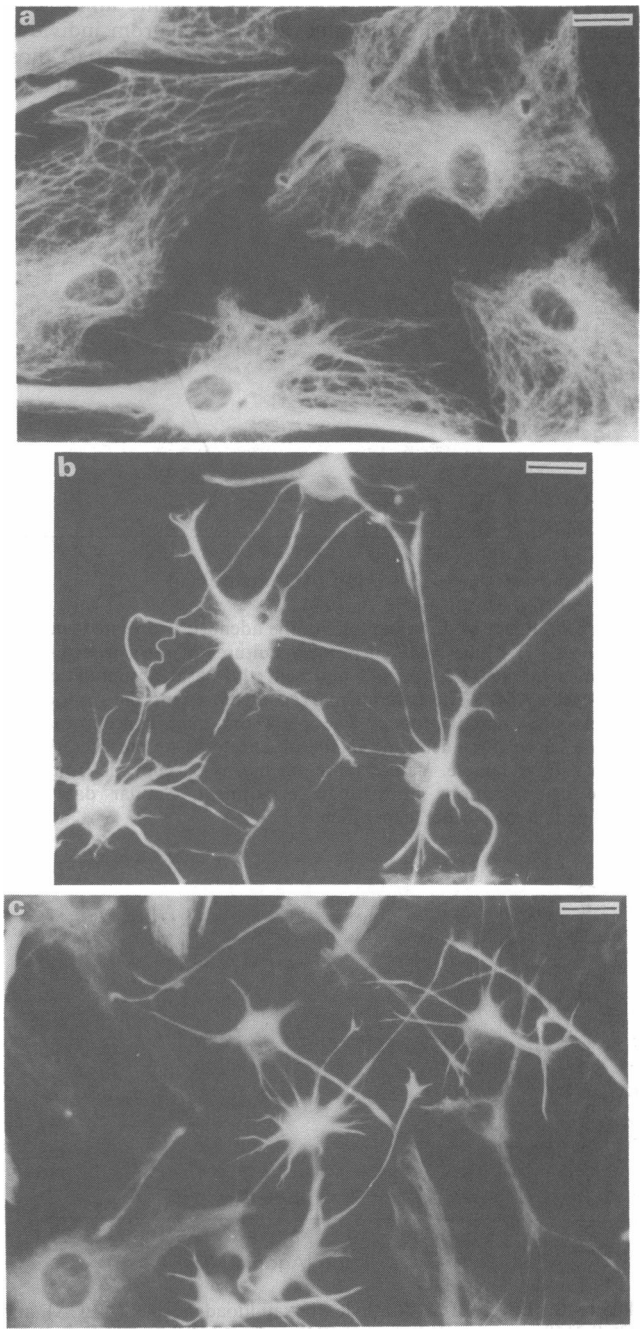

Figure 1 Micrographs of primary astrocyte cultures from 2-day-old rat forebrain. Pictures of either type-1 (a) or type-2 (b) astroglia stained with glial fibrillary acidic protein antiserum were taken from peripheral fields of view where the cell density was much lower than central fields. In central fields of view type-1 and type-2 astroglia, stained with glial fibrillary acidic protein antiserum, were in close proximity, (c). Bar = 20 μm.

PD 115,199 (Bruns *et al.*, 1987b) and xanthine amine congener (Figure 6). The K_D values determined from individual inhibition curves for these antagonists were similar to those previously reported at the A_{2B}-receptor site (Table 2). The selective A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (Bruns *et al.*, 1987a; Lohse *et al.*, 1987), however, had no significant effect on NECA-stimulated [^{3}H]-cyclic AMP accumulation (Figures 6, 7).

The presence of A_1-receptor subtypes was further investigated using the A_1-selective agonist, N^6-cyclopentyladenosine (Lohse *et al.*, 1988). At concentrations below micromolar, N^6-cyclopentyladenosine, had no effect on basal levels of [^{3}H]-cyclic AMP. Above this concentration, non-specific stimulation of [^{3}H]-cyclic AMP accumulation was observed (Figure 8). The use of forskolin (1 μM) to stimulate adenylyl cyclase directly, however, revealed an N^6-cyclopentyladenosine-mediated inhibition of [^{3}H]-cyclic AMP accumulation (Figure 9a). When N^6-cyclopentyladenosine was added to the cells 10 min before forskolin, the stimulation of [^{3}H]-cyclic AMP accumulation was reduced by a mean, maximal value of 41.7 ± 6.8% ($n = 5$, $P < 0.01$) at 0.1 μM, N^6-cyclopentyladenosine. This inhibition was decreased to a non significant value of 10.9 ± 4.2% ($n = 6$, $P > 0.05$, data not shown) when forskolin was added 10 min prior to N^6-cyclopentyladenosine. In all experiments, non-specific stimulation of [^{3}H]-cyclic AMP accumulation was observed at concentration above 0.1 μM N^6-cyclopentyladenosine. Increasing concentrations of 8-cyclopentyl-1,3-dipropylxanthine, reversed the A_1-receptor-mediated inhibition of [^{3}H]-cyclic AMP accumulation (Figure 9b). Full reversal of the response was achieved with 0.1 μM 8-cyclopentyl-1,3-dipropylxanthine.

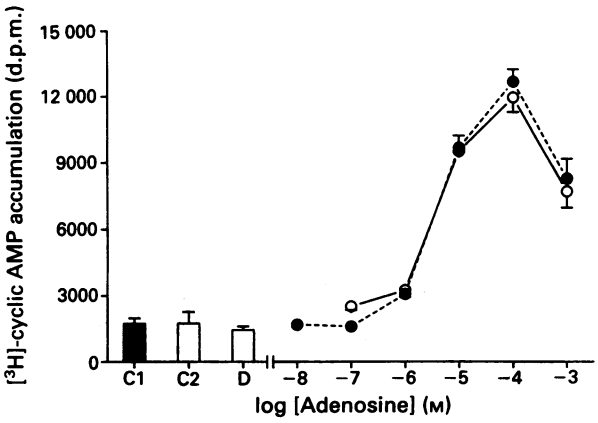

Figure 2 Effect of dipyridamole on the adenosine-mediated accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes. The columns marked C1 and C2 represent the basal accumulations of [^{3}H]-cyclic AMP. The column marked D represents the response to 0.5 μM dipyridamole alone. The plots represent concentration-response curves for adenosine in the absence (●) or presence (○) of 0.5 μM dipyridamole. Values represent means ± s.e.means from triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

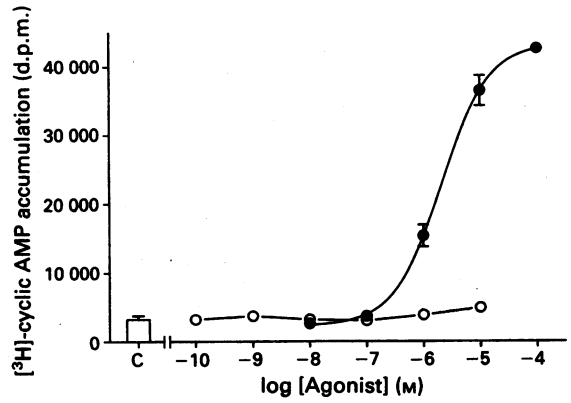

Figure 3 Effect of 5'-N-ethylcarboxamidoadenosine (NECA) and CGS 21680 on accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astroctyes. The column marked C represents the basal accumulation of [^{3}H]-cyclic AMP. Data represent concentration-response plots for NECA (●) and CGS 21680 (○). Values represent means ± s.e.means from triplicate (CGS 21680) or quadruplicate (NECA) determinations in a single experiment. Similar data were obtained in three other experiments.

Discussion

Recent functional investigation of the adenosine receptors present in a variety of isolated tissues and cell types has

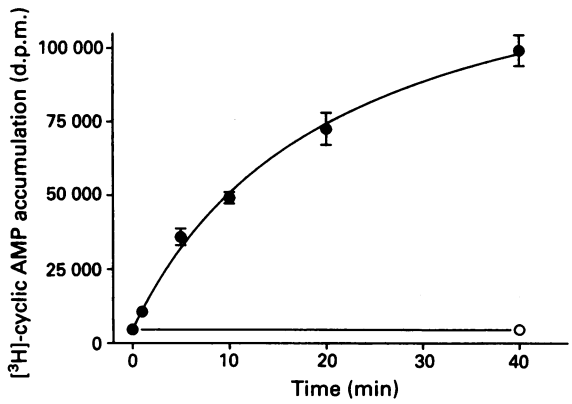

Figure 4 Time course of the accumulation of total [^{3}H]-cyclic AMP in response to 10 μM 5'-N-ethylcarboxamidoadenosine (NECA) in primary cultures of rat astrocytes. Agonist added at time zero (●); unstimulated control (○). Values represent means ± s.e.means from quadruplicate determinations in a single experiment. Similar data were obtained in two other experiments.

Table 1 Agonist potencies of adenosine analogues for stimulation of [^{3}H]-cyclic AMP accumulation in primary cultures of rat astrocytes

Agonist	*Maximal response expressed as mean % response to 10 μM NECA*	*[Agonist] which produces maximal response (μM)*	n	*Cyclic AMP accumulation EC_{50} (μM)* *Rat astrocytes*	*Rat cerebral cortex*
NECA	100	10	7	1.2 ± 0.2, (1.3[a])	(24.5[b])
Adenosine	77.2 ± 4.6	100	5	5.1 ± 0.8, (9.6[a])	(77.6[b])
2-CADO	68.4 ± 3.9	100	6	20.1 ± 5.3	(63.1[b])
CGS 21680	9.0	10	3	> 10.0	

The maximal response for each agonist is expressed as a mean percentage of the response to 10 μM NECA which was measured in every experiment. EC_{50} values are represented as means ± s.e.means for [^{3}H]-cyclic AMP accumulation in *n* separate experiments. Data taken from [a]Murphy *et al.*, 1991 and [b]Bazil & Minneman, 1986. Abbreviations: 2-CADO, 2-chloroadenosine; NECA, 5'-N-ethylcarboxamidoadenosine.

supported the existence of three receptor subtypes, A_1, A_{2A} and A_{2B} (Gurden *et al.*, 1993). The presence of A_1- and A_2-receptors in cultures of human foetal (Woods *et al.*, 1989), murine (Van Calker *et al.*, 1979; Ebersolt *et al.*, 1983) and rat astrocytes (Murphy *et al.*, 1991) has previously been suggested on the basis of pharmacological studies using, primarily adenosine receptor agonists and antagonists with little or no subtype selectivity. In the present study we have used

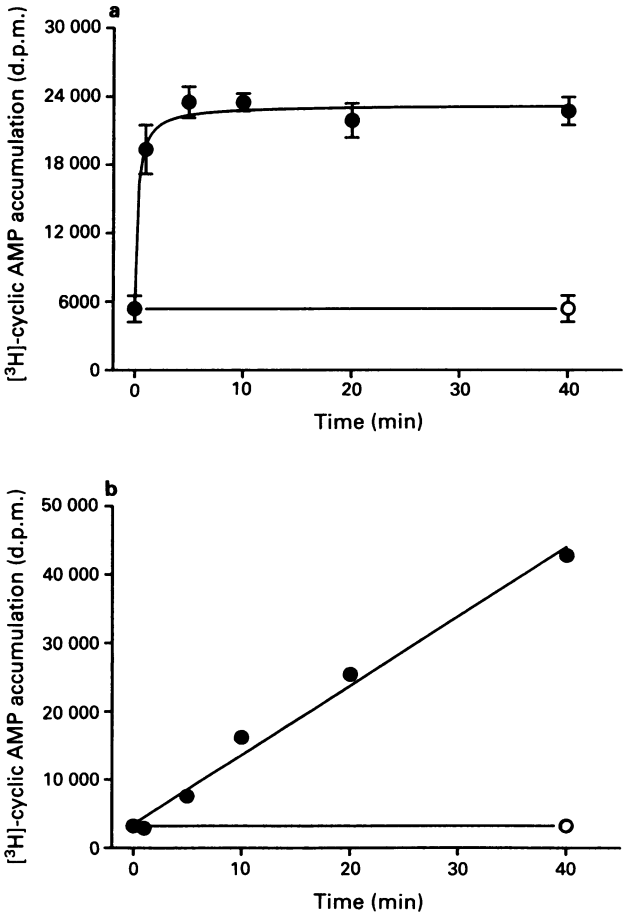

Figure 5 Time courses of the accumulation of (a) intracellular and (b) extracellular [^{3}H]-cyclic AMP in response to 10 μM 5'-N-ethylcarboxamidoadenosine (NECA) in primary cultures of rat astrocytes. Agonist added at time zero (●); unstimulated control (○). Values represent means ± s.e.means from quadruplicate determinations in a single experiment. Similar data were obtained in two other experiments.

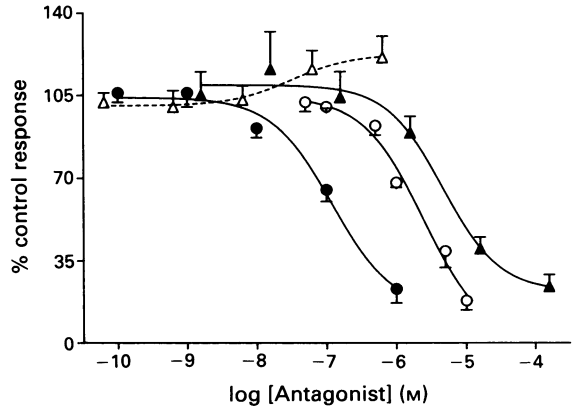

Figure 6 Effect of adenosine-receptor antagonists on 5'-N-ethylcarboxamidoadenosine (NECA)-stimulated accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes. Data produced by xanthine amine congener (●); PD 115,199 (○); 8-phenyltheophylline (▲) and 8-cyclopentyl-1,3-dipropylxanthine (△) are expressed as a percentage of the response to 10 μM NECA which was measured in every experiment. Values represent the combined means ± s.e.means from triplicate determinations in three separate experiments.

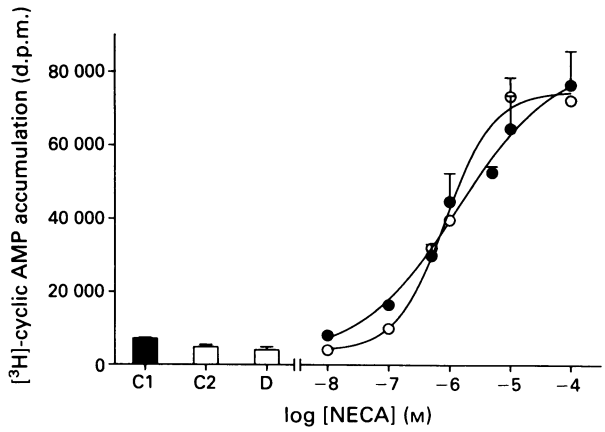

Figure 7 Effect of 8-cyclopentyl-1,3-dipropylxanthine on 5'-N-ethylcarboxamidoadenosine (NECA)-stimulated accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes. The columns marked C1 and C2 represent the basal accumulations of [^{3}H]-cyclic AMP. The column marked D represents the response to 100 nM 8-cyclopentyl-1,3-dipropylxanthine alone. The plots represent concentration-response curves for NECA in the absence (●) or presence (○) of 100 nM 8-cyclopentyl-1,3-dipropylxanthine. Values represent means ± s.e.means from triplicate determinations in a single experiment. Similar data were obtained in one other experiment.

Table 2 Antagonism of 5'-N-ethylcarboxamidoadenosine (NECA)-mediated [^{3}H]-cyclic AMP accumulation in primary cultures of rat astrocytes

	Cyclic AMP accumulation			*Adenosine receptor K_D (nM)*	
Antagonist	*IC_{50} (μM)*	*Apparent K_D (nM)*	n	*A_{2B}*	*A_{2A}*
XAC	0.2 ± 0.04	11.5 ± 2.1	4	35[a], 17[c]	24[f]
PD 115,199	2.2 ± 0.5	134.0 ± 68.5	3	407[a], 395[d]	16[g]
8-PT	3.0 ± 0.6	125.7 ± 33.7	3	400[b], 1200[e]	850[g]
DPCPX	> 1.0	> 97.3 ± 44.8	3	123[a], 163[d]	340[g]

Values for cyclic AMP accumulaton represent means ± s.e.means of the antagonist IC_{50} values obtained from inhibition of NECA-stimulated (10 μM) [^{3}H]-cyclic AMP accumulation in n separate experiments. Apparent K_D values were calculated from these IC_{50} values as described under methods. K_D values for A_{2B}-receptor obtained from antagonism of elevation of [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortex ([a]Losinski *et al.*, 1993; [b]Alexander *et al.*, 1989b; [d]Alexander *et al.*, 1989a), antagonism of A_{2B}-receptor-mediated relaxation of guinea-pig aorta ([c]Losinksi *et al.*, 1993) or antagonism of adenylyl cyclase stimulation in rat cerebral cortex ([e]Bazil & Minneman, 1986). K_D values for A_{2A}-receptor obtained from antagonism of binding of [^{3}H]-NECA to rat striatal membranes ([f]Lohse *et al.*, 1987; [g]Bruns *et al.*, 1987c). Abbreviations: XAC; xanthine amine congener; 8-PT, 8-phenyltheophylline; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

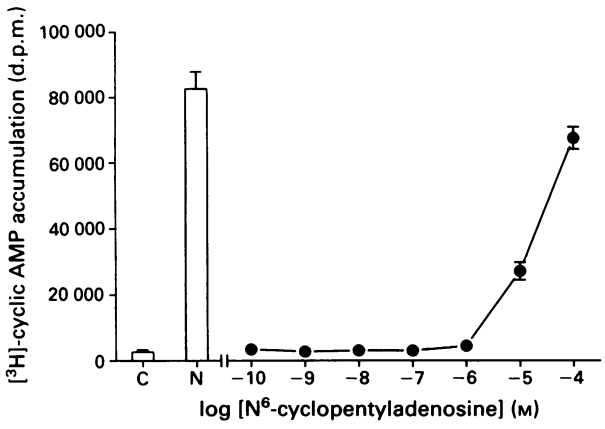

Figure 8 Effect of N^6-cyclopentyladenosine on the accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes. The column marked C represents the basal accumulation of [^{3}H]-cyclic AMP. The column marked N represents the response to 10 μM 5'-N-ethylcarboxamidoadenosine (NECA). Values represent means ± s.e.means from triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

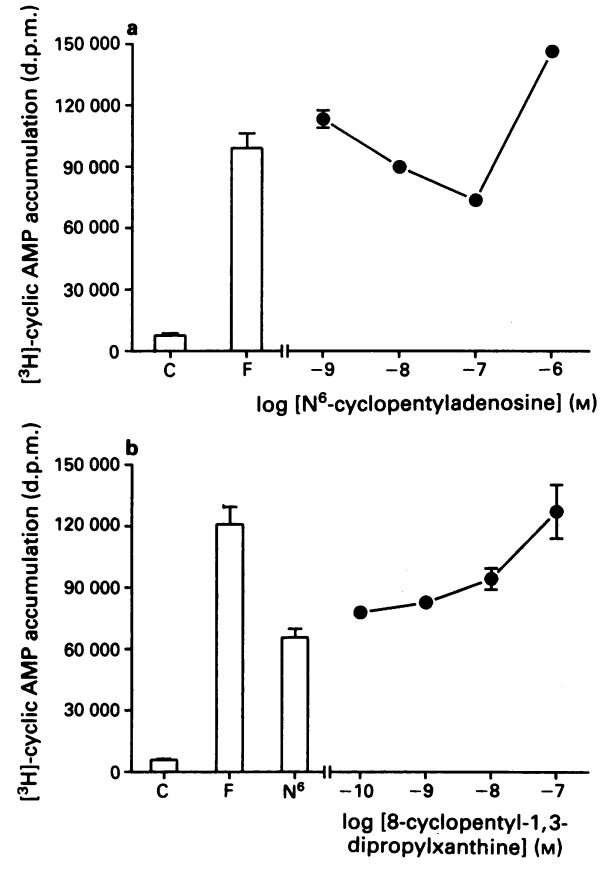

Figure 9 Effect of A_1-receptor selective compounds on the forskolin-mediated accumulation of [^{3}H]-cyclic AMP in primary cultures of rat astrocytes. The columns marked C represent the basal accumulations of [^{3}H]-cyclic AMP. The columns marked F represent the response to 1 μM forskolin alone. (a) Effect of increasing concentrations of N^6-cyclopentyladenosine on the response to 1 μM, forskolin. Values represent means ± s.e.means from quadruplicate determinations in a single experiment. Similar data were obtained in four other experiments. (b) Effect of increasing concentrations of 8-cyclopentyl-1,3-dipropylxanthine on the N^6-cyclopentyladenosine-mediated inhibition of [^{3}H]-cyclic AMP accumulation. The column marked N^6 represents the response to 1 μM, forskolin in the presence of 0.1 μM, N^6-cyclopentyladenosine. Values represent means ± s.e. means from triplicate determinations in a single experiment. Similar data were obtained in three other experiments.

subtype-selective compounds to delineate the A_2-receptor subtype present in primary cultures of rat astrocytes and to establish the extent of A_1-receptor involvement in the cyclic AMP responses to NECA and 2-chloroadenosine.

Adenosine, 2-chloroadenosine and NECA, but not CGS 21680, were able to elevate significantly [^{3}H]-cyclic AMP accumulation in primary cultures of rat astrocytes. From the agonist concentration-response curves, the rank order of agonist potencies was, NECA > adenosine > 2-chloroadenosine >> CGS 21680. The stimulation of adenylyl cyclase by adenosine and its analogues, and the order of agonist potencies are consistent with the presence of A_2-receptors in these cultures. The potency of NECA relative to CGS 21680 (Table 1, Figure 2) additionally suggests that these are of the A_{2B}-receptor subtype since CGS 21680 exhibits high affinity (K_D = 16 nM) for A_{2A}-receptors in rat striatal membranes and displays similar potency to NECA (K_D = 10 nM) at these sites (Bruns *et al.*, 1986). This is supported by the apparent lack of effect of CGS 21680, in this study at concentrations up to 10 μM, in contrast to an EC_{50} of 100 nM (Lupica *et al.*, 1990) at rat striatal A_{2A}-receptors for which it is reported to have 170 fold selectivity (Jarvis *et al.*, 1989).

The apparent EC_{50} values for NECA and adenosine were in close agreement with those published by Murphy *et al.* (1991) using cultures of rat astrocytes, although they were notably different from values obtained with rat cerebral cortical slices (Bazil & Minneman, 1986). However, unlike the situation in brain slices (Bazil & Minneman, 1986) the adenosine uptake inhibitor, dipyridamole (K_D = 1.3 nM; Geiger *et al.*, 1988) did not significantly affect the potency of adenosine in rat astrocytes. These data suggest that in monolayer cell cultures of astrocytes, uptake of adenosine does not significantly reduce the concentration of applied adenosine. The apparent low efficacy (relative to NECA; Table 1) of 2-chloroadenosine in these cultures may be due to its 7 fold selectivity for A_1-receptors, which could result in a depression of A_2-receptor-stimulated [^{3}H]-cyclic AMP accumulation. However, experiments to investigate this possibility, using 8-cyclopentyl-1,3-dipropylxanthine at concentrations up to 1 μM to block any A_1-component, did not reveal a modification of the A_2-receptor-mediated response to 2-chloroadenosine (data not shown; n = 3).

Further evidence for A_{2B}-receptor presence is provided by antagonist potencies. Xanthine amine congener has been reported to show a 7 fold selectivity for A_1-receptors (K_D = 4 nM) in binding studies using rat brain membranes (Lohse *et al.*, 1987), but does not discriminate between A_2-receptor subtypes. In primary rat astrocytes, it potenty inhibited NECA-stimulated [^{3}H]-cyclic AMP accumulation with a K_D which closely agreed with the values previously reported for A_{2A}-receptors in rat striatal membranes (Lohse *et al.*, 1987) and A_{2B}-receptors in the guinea-pig cerebral cortex and aorta (Losinski *et al.*, 1993) (Table 2). Likewise, the apparent K_D value observed for 8-phenyltheophylline-mediated inhibition was of the same order of magnitude as those recorded at A_2-receptors (Bazil & Minneman, 1986; Bruns *et al.*, 1987c; Alexander *et al.*, 1989b) whereas at A_1-receptors this compound is 10 fold more potent (K_D = 86 nM; Bruns *et al.*, 1986).

PD115,199 has been used to discriminate between A_2-receptor subtypes in guinea-pig tissues (Alexander *et al.*, 1989a). It exhibits a similar, high affinity at both A_1- (K_D = 14 nM) and A_{2A}-receptors (K_D = 16 nM) in rat whole brain and striatal membranes respectively (Bruns *et al.*, 1987c), which is comparable to the affinity of xanthine amine congener at A_2-receptors (see Table 2). In this study, however, PD115,199 displayed a 12 fold lower affinity than xanthine amine congener and an 8 fold lower affinity than at rat A_{2A}-receptor sites (Bruns *et al.*, 1987c). The apparent K_D value obtained is in agreement with previously recorded affinities at A_{2B}-receptors in the guinea-pig aorta (Hargreaves *et al.*, 1991; Losinski *et al.*, 1993) and cerebral cortex (Alexander *et al.*, 1989a; Losinski *et al.*, 1993). Taken together,

these data are indicative of the presence of A_{2B}-receptors in primary rat astrocyte cultures.

In order to investigate the role of adenosine A_1-receptors in the cyclic AMP response of these cultures, experiments were performed with the A_1-selective agonist, N^6-cyclopentyladenosine (Lohse *et al.*, 1988). Although no inhibition of basal [^{3}H]-cyclic AMP accumulation was seen at concentrations below micromolar, there was a stimulation of enzyme activity at higher concentrations, possibly via a non-specific activation of A_{2B}-receptors. When adenylyl cyclase was directly activated with forskolin (1 μM) following A_1-receptor stimulation with N^6-cyclopentyladenosine, an inhibition of [^{3}H]-cyclic AMP accumulation was observed which reached a maximal of 42% at 0.1 μM receptor agonist. Above this concentration there was again, non-specific elevation of [^{3}H]-cyclic AMP levels. A response of similar size and nature has been reported in human foetal astrocytes (Woods *et al.*, 1988).

The A_1-receptor selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (Bruns *et al.*, 1987a; Lohse *et al.*, 1987), abolished the inhibition of adenylyl cyclase activity caused by N^6-cyclopentyladenosine. This is in agreement with the reversal of the inhibition of isoprenaline (1 μM)-stimulated [^{3}H]-cyclic AMP accumulation caused by NECA and L-phenylisopropyladenosine in foetal astrocytes by 10 μM, 8-cyclopentyl-1,3-dipropylxanthine (Woods *et al.*, 1989). These results indicate the presence of adenosine A_1-receptors in primary cultures of rat astrocytes. However, although adenosine A_1-receptors are present in primary cultures of rat astrocytes, they do not appear to modify significantly the cyclic AMP responses of mixed A_1/A_2-receptor agonists such as NECA and 2-chloroadenosine.

A study of the time-course for NECA-stimulated [^{3}H]-cyclic AMP accumulation revealed that after an initial, rapid rise, intracellular levels of [^{3}H]-cyclic AMP reached a plateau which was then maintained for the remainder of the incubation period. This suggests that there is no rapid desensitization of the A_{2B}-receptor-mediated cyclic AMP response. A similar observation has been made in guinea-pig cerebral cortical slices (Donaldson *et al.*, 1988). However, in addition, levels of extracellular [^{3}H]-cyclic AMP increased linearly throughout the incubation, indicating the presence of an efflux mechanism in rat astrocytes. Efflux of cyclic AMP has previously been reported in both C6 glioma (Doore *et al.*, 1975; Rindler *et al.*, 1978) and B50 neuroblastoma (McCrea & Hill, 1993) cell lines from rat central nervous system and appears, in the former at least, to depend on a chemical energy source such as ATP (Rindler *et al.*, 1978). This mechanism may provide an additional means of regulating the levels of intracellular cyclic AMP (in addition to cyclic nucleotide phosphodiesterase enzymes).

In conclusion, the data from this study support earlier suggestions that rat astrocytes in primary culture express adenosine receptors coupled both positively and negatively to adenylyl cyclase. We have established that the stimulatory receptors present are of the low affinity, A_{2B}-subclass and, furthermore, have shown that the elevation of [^{3}H]-cyclic AMP accumulation caused by stimulation of these receptors with NECA and 2-chloroadenosine is not significantly affected by concurrent A_1-receptor activation. It remains to be established, however, whether both receptor subtypes are expressed by all cells in the cultures or if cell subpopulations (e.g. type-1 and type-2 astrocytes) differ in receptor expression.

We would like to thank S.P.H. Alexander for helpful advice and the Wellcome Trust for financial support. M.C.P. holds a Wellcome Trust Prize Studentship.

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(Received July 28, 1993
Revised September 28, 1993
Accepted September 29, 1993)

Br. J. Pharmacol. (1994), **111**, 199–204

Cardiovascular actions of dopexamine in anaesthetized and conscious dogs

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1 Arterial blood pressure, heart rate and cardiac contractility were measured in pentobarbitone-anaesthetized mongrel dogs and in conscious, instrumented dogs.

2 In anaesthetized dogs ($n = 5$), dose-response curves were obtained by intravenous infusion of increasing doses of dopexamine ($5-20\ \mu g\ kg^{-1}\ min^{-1}$). Infusions were administered three times to each animal to determine whether the responses were reproducible. Dopexamine increased heart rate and myocardial contractility and decreased blood pressure. The dose-response curves for dopexamine did not differ significantly over time.

3 In a second group of dogs ($n = 6$), dose-response curves ($5-20\ mg\ kg^{-1}\ min^{-1}$) were obtained as above and repeated after the administration of amitriptyline ($2\ mg\ kg^{-1}$, i.v.). Amitriptyline caused a non-significant reduction in the inotropic and chronotropic responses to dopexamine.

4 Control dose-response curves for dopexamine ($5-50\ \mu g\ kg^{-1}\ min^{-1}$) were similarly obtained in a third group of dogs ($n = 6$), and repeated after bilateral vagotomy and sympathetic denervation of the heart. In these animals, a third dose-response curve for dopexamine was obtained after the administration of ICI 118551 ($0.2\ mg\ kg^{-1}$, followed by $0.2\ mg\ kg^{-1}\ h^{-1}$). The chronotropic response to dopexamine was significantly reduced after cardiac denervation. There was a small, non-significant reduction in the inotropic and depressor responses after denervation. Administration of ICI 115881 significantly reduced both the inotropic and chronotropic response to dopexamine and caused a non-significant reduction in the depressor response.

5 The effect of raclopride ($0.2\ \mu mol\ kg^{-1}$, p.o.) was investigated by comparison of the dose-response curves for dopexamine in a control group of dogs ($n = 6$) to those obtained in dogs which had been pretreated with raclopride ($n = 5$). Raclopride had no significant effect on the cardiovascular responses to dopexamine.

6 In conscious, instrumented dogs ($n = 5$), pretreated with raclopride, dose-related positive inotropic and chronotropic and depressor responses to dopexamine infusions were recorded. The chronotropic responses in conscious animals were significantly greater than those in the anaesthetized animals.

7 The results of this study indicate that both the positive inotropic and chronotropic actions of dopamine are due to a combination of direct, β_2-adrenoceptor-mediated effects and the baroreceptor reflex response to the depressor action of the drug.

Keywords: Dopexamine; β_2-adrenoceptor agonists; amitriptyline; heart rate; ICI 115881; inotropic agents

Introduction

Dopexamine is a catecholamine which has been used in the acute treatment of low cardiac output states (Smith & Fileck, 1989). Responses to dopexamine in animals include a fall in blood pressure and increases in heart rate and myocardial contractility. The depressor effect is thought to result from the vasodilator response to stimulation of dopamine D_1 receptors in renal and mesenteric vascular beds and vascular β_2-adrenoceptors (Brown *et al.*, 1985a; Smith *et al.*, 1987). Reduced noradrenaline release from sympathetic nerves, due to stimulation of prejunctional D_2 receptors may also contribute to the fall in blood pressure (Brown *et al.*, 1985a). There is some disagreement about the mechanism of the positive inotropic and chronotropic effects of dopexamine.

The increased cardiac activity may be due to stimulation of β_1- and/or β_2-adrenoceptors (Bass *et al.*, 1987; Smith *et al.*, 1987; Brodde, 1991). Dopexamine has also been shown to inhibit noradrenaline $Uptake_1$ (Mitchell *et al.*, 1987) and it has been suggested (Bass *et al.*, 1987) that this may contribute to the positive inotropic and chronotropic response. These authors also showed that, in anaesthetized dogs, the cardiac response to dopexamine was markedly reduced after ganglion blockade and suggested that baroreceptor reflex activity in response to the depressor effect was an important component of the increased rate and contractility. In other studies (Smith *et al.*, 1987), only the responses to low doses of dopexamine were attenuated by ganglion blockade; those to higher doses were not reduced and some were enhanced. The assessment of the importance of the reflex component of the response to dopexamine is further complicated by the observation that stimulation of D_2-receptors by dopexamine inhibits the response to *cardiac accelerans* nerve stimulation (Brown *et al.*, 1985a). This action would tend to reduce the impact of baroreceptor reflex activation.

The positive inotropic and chronotropic actions of dopexamine have also been demonstrated in conscious dogs (Brown *et al.*, 1985b). In these studies, the maximum dose of dopexamine which was administered was $10^{-7}\ mol\ kg^{-1}\ min^{-1}$ (approximately $43\ \mu g\ kg^{-1}\ min^{-1}$). Dose limitation was imposed by dopexamine-induced stimulation of the D_2 receptors in the chemoreceptor trigger zone which led to panting, licking and emesis which was evident in some animals at doses of $3 \times 10^{-8}\ mol\ kg^{-1}\ min^{-1}$ ($13\ \mu g\ kg^{-1}\ min^{-1}$). It is possible that the cardiovascular responses to the lower doses of dopexamine were influenced by the sensations of nausea and most likely that responses to the higher doses were affected by vomiting.

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Thus the aims of the present experiments were to assess the importance of Uptake$_1$ blockade, baroreceptor reflex activity, D_2 receptor and β_2-adrenoceptor stimulation in the response to dopexamine by the use of selective antagonists and to compare the actions of a wide range of doses of dopexamine in anaesthetized and conscious animals.

Methods

Acute experiments in anaesthetized dogs

Mongrel dogs ($n = 26$) (12–28 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.); supplementary doses were administered as required. The animals were intubated and mechanically ventilated (Ugo Basile dog ventilator 5025). Both femoral veins were cannulated for drug administration. Arterial pressure was monitored from the femoral artery (Statham P23AC pressure transducer) and heart rate was obtained from a tachograph (Grass 7P4D5) triggered by the QRS complex of the Lead II ECG.

The chest was opened by either a mid-sternal incision (where subsequent cardiac denervation was performed) or at the 4th intercostal space. A miniature pressure manometer (Konigsberg P6.5) was inserted into the left ventricular cavity. The first derivative of left ventricular pressure (dP/dt) was derived by electronic differentiation of the ventricular pressure signal using an active circuit with a time constant of 50 ms. The index of myocardial contractility used in this study was obtained beat by beat, by an on-line digital computer (Digital Decgraphic 11/40). Under programme control, the R-wave of the Lead II ECG initiates the sampling of left ventricular pressure and dP/dt until peak dP/dt is detected. At this time, the ratio of maximum dP/dt to the time integral of isovolumic pressure (the area under the left ventricular pressure curve) is computed and returned to the chart recorder as an analogue signal. Evaluation of this index ($dP/dt \div$ integrated isometric tension, units = s^{-2}) as a measure of myocardial contractility has shown that, within a reasonable range, it is not influenced by changes in heart rate, preload or afterload (Goodman *et al.*, 1972).

All cardiovascular parameters were continuously recorded (Grass Model 7 Polygraph). Following instrumentation, the animals were allowed to stabilize for 30 min before recording of baseline cardiovascular variables.

Dose-response curves for dopexamine were obtained by infusion of increasing doses (IMED volumetric infusion pump 922). Infusion at each dose rate was continued until cardiovascular variables were maintained at a steady level and then the infusion rate was increased to administer the next dose. The cardiovascular variables were allowed to return to baseline.

Group 1 ($n = 5$) *Reproducibility of responses to dopexamine* The effect of dopexamine (5–20 μg kg^{-1} min^{-1}) on the cardiovascular system was examined. The dose-response curve was repeated three times in each animal to determine whether reproducible responses could be obtained. Cardiovascular variables were allowed to return to baseline between trials.

Group 2 ($n = 6$) *Effect of amitriptyline on dopexamine dose-response curves* A control dose-response curve (5–20 μg kg^{-1} min^{-1}) was obtained in each animal. The cardiovascular variables were allowed to return to baseline and then amitriptyline (2 mg kg^{-1}, i.v.) was administered. The effectiveness of Uptake$_1$ blockade by this dose of amitriptyline was tested by the administration of tyramine (25 μg kg^{-1}) before and after amitriptyline. The dose-response curve for dopexamine was then repeated. The test dose of tyramine was injected at the end of the experiment to test whether Uptake$_1$ blockade was still effective.

Group 3 ($n = 6$) *Effect of autonomic denervation and β_2-adrenoceptor blockade on cardiovascular responses to dopexamine* Control dose-response curves for dopexamine (5–50 μg kg^{-1} min^{-1}) were obtained as above. The vagi were then cut and sympathetic denervation of the heart was achieved by bilateral crushing of the *ansae subclavia*. The completeness of the denervation was assessed by comparing the cardiac response to 15 s bilateral carotid occlusion before and after denervation. The infusion of increasing doses of dopexamine was then repeated. When the cardiovascular variables had returned to baseline, the selective β_2-adrenoceptor antagonist, ICI 118551 (0.2 mg kg^{-1} bolus followed by 0.2 mg kg^{-1} h^{-1}) was administered and the dose-response curve to dopexamine was repeated.

Group 4 ($n = 11$) *Effect of raclopride on dopexamine dose-response curves* Dose-response curves for dopexamine were obtained in six anaesthetized animals. In five other animals, the selective D_2 receptor antagonist, raclopride (Ross & Jackson, 1989) was administered (0.2 μmol kg^{-1}, p.o.) 30 min before anaesthesia. A dose-response curve for dopexamine was obtained in these animals following instrumentation.

Experiments in conscious dogs

Konigsberg micromanometers were implanted in the left ventricles of mongrel dogs ($n = 5$) under halothane anaesthesia. The animals were allowed at least 6 weeks to recover before any experimental procedures were conducted. During the experiments, the dogs lay unrestrained on a mattress. Lead II ECG was recorded using surface leads attached to the paws. Cardiac contractility was measured, as above, using the signal from the implanted micromanometer. Systemic blood pressure was measured with a Critikon Dynamap Vital Signs Monitor. Dose-response curves to dopexamine were obtained by the intravenous infusion of increasing doses of dopexamine (1–50 μg kg^{-1} min^{-1}), as described above. In order to prevent the emetic effect of higher doses of dopexamine in conscious animals, the dogs were dosed with raclopride (0.2 μmol kg^{-1}, p.o.) approximately 2 h before dopexamine infusions.

Analysis of results

Dose-response curves to dopexamine were compared by repeated measures analysis of variance. Paired t tests were used to test for the significance of the effects of Uptake$_1$ blockade, denervation and β_2-adrenoceptor blockade on resting levels of cardiovascular variables in anaesthetized animals. Unpaired t tests were used to compare resting cardiovascular variables in anaesthetized and conscious animals which had been pretreated with raclopride. Results were considered to be significant when $P < 0.05$.

Results

Acute experiments

Group 1: Reproducibility of dopexamine dose-response curves Infusion of increasing doses of dopexamine increased cardiac contractility and heart rate and decreased blood pressure (Figure 1). The drug had a rapid onset of action, taking 2–10 min to reach steady state, depending on the rate of infusion. The effects wore off within 20 min of the termination of infusion. Analysis of variance indicated that there were no significant ($P > 0.05$) differences in any of the cardiovascular responses over time.

Group 2: Effect of amitriptyline on the dose-response curves to dopexamine Amitriptyline abolished the response to tyramine and remained effective after the dopexamine infusion.

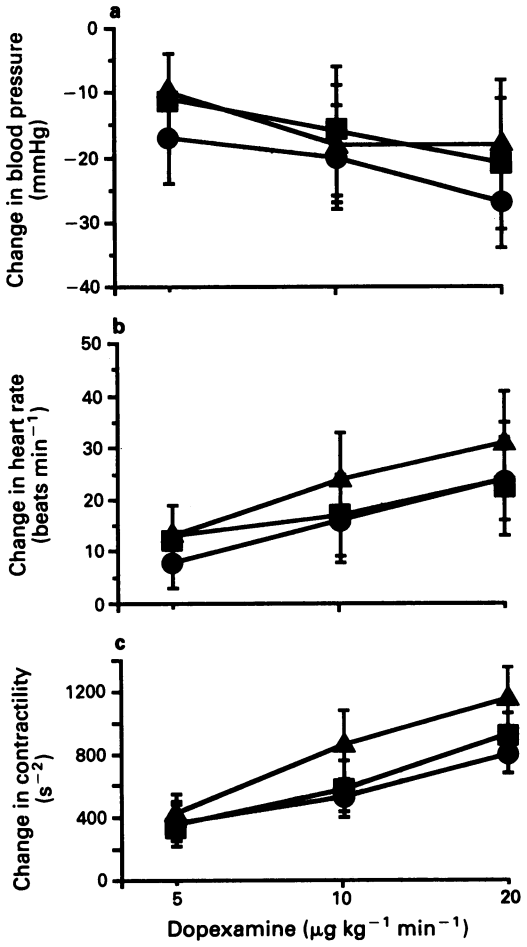

Figure 1 Effect of dopexamine on (a) blood pressure, (b) heart rate and (c) myocardial contractility (●) and on the same parameters when infused a second (▲) and third (■) time. Points shown represent the mean (± s.e.mean) of responses in six dogs.

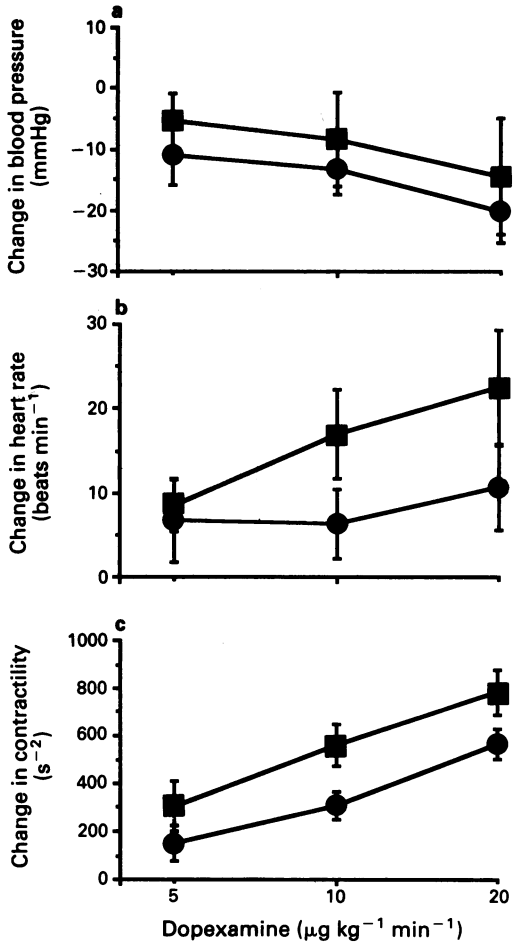

Figure 2 Effect of dopexamine on (a) blood pressure, (b) heart rate and (c) myocardial contractility before (■) and after (●) administration of amitriptyline (2 mg kg^{-1}). Points shown represent the mean (± s.e.mean) of responses in six dogs.

Table 1 Resting levels of blood pressure, heart rate and myocardial contractility before and after administration of amitriptyline

	Control	*Amitriptyline*
Blood pressure (mmHg)	121 ± 10	127 ± 7
Heart rate (min^{-1})	158 ± 4	155 ± 11
Myocardial contractility (s^{-2})	1580 ± 100	1600 ± 100

Values shown are mean (± s.e.mean).

The resting levels of blood pressure, heart rate and contractility before and after the administration of amitriptyline are shown in Table 1 where it can be seen that amitriptyline had no significant ($P>0.05$) effect on the resting cardiovascular parameters. The mean of the responses to increasing doses of dopexamine before and after amitriptyline are shown in Figure 2. Analysis of variance indicated that although there was a trend to reduced chronotropic responses and enhanced depressor responses to dopexamine after amitriptyline, the shifts in the dose-response curves were not significant ($P>0.05$). The reduction in inotropic responses to dopexamine after amitriptyline just failed to reach significance ($P<0.06$).

Group 3: Effect of autonomic denervation and β_2-adrenoceptor blockade on cardiovascular responses to dopexamine Resting levels of blood pressure, heart rate and contractility in this group of dogs were similar to those in Group 2 (Tables 1 and 2). Cardiac denervation abolished the chronotropic response to bilateral carotid occlusion. After denervation there was a significant decrease in heart rate ($P<0.005$; t test, 10 d.f.) and contractility ($P<0.001$; t test, 10 d.f.) but there was no significant change in blood pressure ($P>0.05$) (Table 2). Dose-response curves for dopexamine before and after cardiac denervation are shown in Figure 3. Analysis of variance indicated that only the chronotropic response was significantly reduced by denervation ($P<0.05$).

Administration of the β_2-adrenoceptor antagonist, ICI 118551 caused a further small, but significant ($P<0.05$; t test, 10 d.f.), reduction in heart rate and an insignificant ($P>0.05$) increase in contractility (Table 2). After receptor blockade, the inotropic dose-response curve to dopexamine was significantly reduced when compared to the results in the denervated animals ($P<0.01$; ANOVA). Although the depressor and chronotropic responses appeared to be reduced, these effects were not statistically significant ($P>0.05$). The difference between the chronotropic dose-response curve in the control period and that after ICI 11851 was significant ($P<0.01$, ANOVA).

Group 4: Effect of raclopride on cardiovascular responses to dopexamine The resting levels of blood pressure, contractility and heart rate in the groups of dogs which received

dopexamine alone and dopexamine after pretreatment with raclopride are shown in Table 3. Raclopride had no significant effect on any of these parameters. The mean of the responses to increasing doses of dopexamine in the animals pretreated with raclopride and those with no pretreatment are shown in Figure 4. Analysis of variance indicated that raclopride had no significant effect on any of the responses in the anaesthetized animals.

Table 2 Resting levels of blood pressure, heart rate and myocardial contractility before and after denervation and administration of ICI 118551

	Control	*Denervation*	*ICI 118551*
Blood pressure (mmHg)	128 ± 10	118 ± 3	130 ± 5††
Heart rate (min^{-1})	158 ± 6	127 ± 4*	119 ± 5†
Myocardial contractility (s^{-2})	1820 ± 80	1360 ± 170**	1500 ± 180

Values shown are mean (± s.e.mean).
*Significantly different from control ($P<0.05$);
**Significantly different from control ($P<0.01$);
†Significantly different from denervation ($P<0.05$);
††Significantly different from denervation ($P<0.005$).

Experiments in conscious dogs

Resting levels of contractility in the conscious dogs (1570 ± 180 s^{-2}) were similar to those in the anaesthetized animals which had been pretreated with raclopride, although heart rate (78 ± 3 min^{-1}) and mean arterial pressure (80 ± 2

Table 3 Resting levels of blood pressure, heart rate and myocardial contractility in anaesthetized dogs before and after administration of raclopride

	Control ($n=6$)	*Raclopride* ($n=5$)
Blood pressure (mmHg)	102 ± 7	120 ± 3
Heart rate (min^{-1})	149 ± 8	148 ± 8
Myocardial contractility (s^{-2})	1610 ± 150	1630 ± 130

Values shown are mean (± s.e.mean).

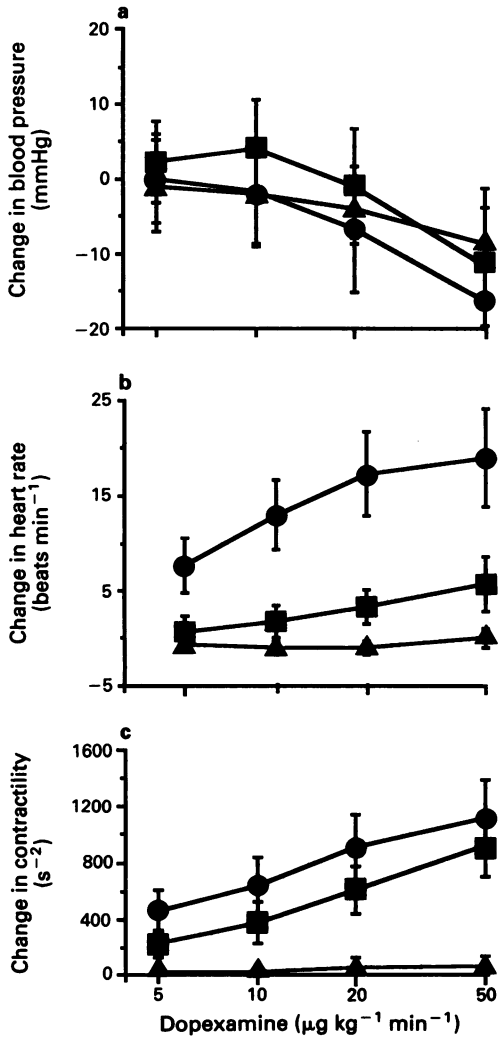

Figure 3 Effect of dopexamine on (a) blood pressure, (b) heart rate and (c) myocardial contractility before (●) and after (■) cardiac denervation and after (▲) administration of ICI 118551 (0.2 mg kg^{-1}, followed by 0.2 mg kg^{-1} h^{-1}). Points shown represent the mean (± s.e.mean) of responses in six dogs.

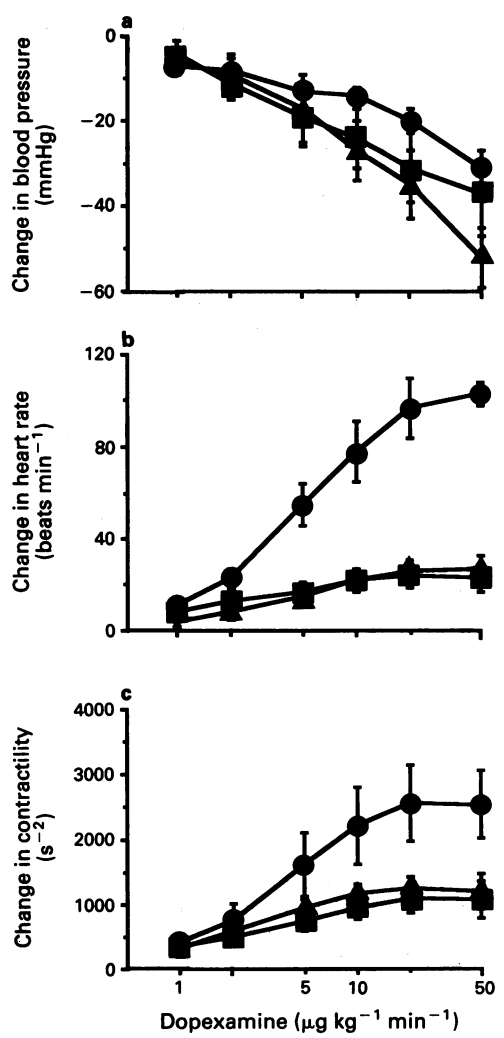

Figure 4 Effect of dopexamine on (a) blood pressure, (b) heart rate and (c) myocardial contractility in control anaesthetized dogs (■) ($n=6$), anaesthetized dogs pretreated with raclopride ($n=5$) (▲) and conscious dogs pretreated with raclopride ($n=5$) (●). Points shown represent the mean (± s.e.mean) of responses in each group.

mmHg) were significantly lower (heart rate: $P<0.001$; t test, 8 d.f.; blood pressure: $P<0.001$, t test, 8 d.f.). Dopexamine produced dose-related increases in both heart rate and contractility and reductions in blood pressure; the dose-response curves obtained in the conscious animals are shown in Figure 4. From this figure, it can be seen that the inotropic and chronotropic responses to dopexamine in the conscious animals were more marked than those in anaesthetized animals, while the depressor effect was similar. The difference in chronotropic responses were shown to be statistically significant when the data from anaesthetized animals which had been pretreated with raclopride were compared (ANOVA) to those from conscious animals. The differences between inotropic responses in anaesthetized and conscious animals failed to reach statistical significance. This was probably due to the greater variability in dose-response curves in the conscious animals.

Discussion

The effects of dopexamine on the cardiovascular system of anaesthetized dogs in the present experiments were similar to those reported by Smith *et al.* (1987) and Bass *et al.* (1987). As in these reports, the changes in blood pressure and heart rate in response to dopexamine in the present experiments were not as marked as the increase in cardiac contractility. In conscious animals, dopexamine also had positive inotropic and chronotropic actions and lowered blood pressure; responses which were qualitatively similar to those reported by Brown *et al.* (1985). In the present study, the animals were pretreated with raclopride and thus it was possible to administer higher doses of dopexamine without inducing panting, licking and emesis, which could all have influenced the cardiovascular system. It was assumed that raclopride did not alter the responses to dopexamine observed in conscious animal since the responses in anaesthetised dogs were shown to be unaffected by pretreatment with this selective D_2-antagonist.

It has been shown that dopexamine inhibits noradrenaline Uptake$_1$ (Mitchell *et al.*, 1987; Smith & O'Connor, 1988), and it has been suggested that this action contributes to the cardiac response to dopexamine (Bass *et al.*, 1987). The doses of dopexamine used in this study are effective in blocking Uptake$_1$ in dogs (Smith & Naya, 1987) and the resting heart rate of the anaesthetized animals indicates a high background level of sympathetic tone. Thus, the cardiac stimulant action of dopexamine in these animals could involve a potentiation of endogenous noradrenaline. If Uptake$_1$ blockade were a significant factor, the effects of dopexamine should be reduced in animals treated with an Uptake$_1$ blocker, such as amitriptyline. However, in the present experiments, although the abolition of the response to tyramine indicated effective Uptake$_1$ blockade, treatment with amitriptyline did not significantly alter the resting levels of the cardiovascular variables. Furthermore, despite a trend towards reduced responses in the amitriptyline-treated animals, the inotropic and chronotropic dose-response curves after blockade were not significantly different from those in the control period. It is unlikely, therefore, that the inhibition of Uptake$_1$ by dopexamine is of major importance in its cardiac stimulant action.

Dopexamine reduces blood pressure by a vasodilator action at β_2-adrenoceptors and dopamine receptors and it has been suggested that the baroreceptor response to the fall in pressure is 'a major contributor' to the increase in heart rate and cardiac contractile force produced by dopexamine (Bass *et al.*, 1987). This claim was based on experiments in anaesthetized dogs in which the cardiac response to bolus doses of dopexamine were almost abolished after ganglion blockade. Others, however, found that although the cardiac effects of infusion of low doses of dopexamine were reduced after ganglion blockade, the responses to higher doses were not affected or were even potentiated (Smith *et al.*, 1987). One explanation for this apparent discrepancy is the difference in the method of drug administration. The baroreceptor response is most pronounced during changes in blood pressure and would be expected to be of greater significance in the experiments where bolus doses were given. In those experiments where infusions were used, the baroreceptors 'adjust' to the new, lower pressure and their influence declines. Thus, in the steady state, the importance of the baroreceptor input would be reduced. Infusion of dopexamine was used as the method of administration in the present experiments since it is the route used clinically and thus is of most relevance.

In order to evaluate the contribution of the baroreceptors to the dopexamine response in anaesthetized dogs, the drug was administered to dogs after surgical removal of autonomic innervation to the heart. This technique has the advantage of maintaining the autonomic tone in the circulation, thus minimizing changes which may occur secondary to the very low blood pressure seen after pharmacological ganglion blockade. The resting heart rate and myocardial contractility were reduced after denervation since animals under pentobarbitone anaesthesia tend to have increased sympathetic tone.

Denervation significantly reduced the chronotropic response to dopexamine and the shift of the dose-response curve reflects the importance of the baroreceptor component in this response. It should be noted that after denervation dopexamine still produced a significant dose-related increase in heart rate. Thus, it has some direct chronotropic activity. The results from this group of dogs also suggest that the baroreceptors are not of major significance in the inotropic response in anaesthetized animals, since denervation produced only a slight and non-significant reduction in the increase in contractility due to dopexamine.

The contribution of the baroreceptor reflex to the cardiac response to dopexamine was likely to have been far greater in the conscious animals, where no anaesthetic was present to depress reflex activity. Indeed, it is quite likely that this was the main reason for the marked difference in the chronotropic responses in anaesthetized and conscious animals. As in the denervation studies in anaesthetized animals, the contribution of the baroreceptor reflex to the inotropic response appeared to be less marked.

The effect of dopexamine at neuronal D_2 receptors and β_2-adrenoceptors also requires consideration. The positive inotropic or chronotropic response may be attenuated by a reduction in noradrenaline release mediated *via* presynaptic D_2 receptors (Kohli *et al.*, 1983; Brown *et al.*, 1985b). If this were the case, blockade of D_2 receptors by raclopride would significantly affect the response to dopexamine and the response in denervated animals, in which there is no noradrenaline release, should be greater than that in the control animals. Since raclopride had no effect on the dose-response curves and the effect of denervation was to reduce the cardiac response to dopexamine, it is unlikely that presynaptic effects modulate the cardiac stimulant action of dopexamine. It also appears from the present results that D_2 receptor-induced reduction of neuronal release of noradrenaline does not contribute significantly to the depressor effect of dopexamine. It is also possible that in the presence of sufficient concentrations of dopexamine, stimulation of prejunctional β_2-adrenoceptors could facilitate release of noradrenaline. Appropriately designed studies of transmitter release are required to evaluate definitively the contribution of these prejunctional effects to the cardiovascular responses to dopexamine.

The cardiac effects of dopexamine in the denervated animals were abolished by ICI 118551. These results confirm the conclusions that the direct inotropic and chronotropic responses are mediated *via* β_2-adrenoceptors in the heart (Smith *et al.*, 1987). The depressor response appeared to be reduced by ICI 118551, although the difference was not statistically significant. The reduction in blood pressure which occurred after β_2-adrenoceptor blockade was presumably due to vaso-

dilatation mediated *via* dopamine D_1 receptors in the renal vascular bed.

The results of the present study confirm the positive inotropic and chronotropic effects of dopexamine and its vasodepressor activity. The fall in blood pressure has been shown to be largely due to β_2-adrenoceptor stimulation. The positive inotropic effect is predominantly due to direct stimulation of cardiac β_2-adrenoceptors. Both a direct, β_2-adrenoceptor-mediated response and the baroreceptor reflex contribute to the tachycardia.

The authors are grateful to Fisons plc, U.K. for their generous support and to Mr Kevin Woodman for expert technical assistance.

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(Received May 13, 1993
Revised August 18, 1993
Accepted September 29, 1993)

Br. J. Pharmacol. (1994), **111**, 205–212

L-NAME, nitric oxide and jejunal motility, blood flow and oxygen uptake in dogs

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1 The effects of the inhibitor of nitric oxide (NO) synthesis, N^G-nitro-L-arginine methyl ester (L-NAME), on systemic arterial blood pressure and jejunal motility, blood flow, and oxygen uptake have been investigated in anaesthetized dogs.

2 L-NAME (cumulative doses of 0.1–20 mg kg^{-1}, i.v.) dose-dependently increased blood pressure and jejunal motility and decreased heart rate. The maximal response of these three variables occurred at doses, 3, 10 and 10 mg kg^{-1}, respectively. L-NAME (cumulative doses of 0.5–5 mg kg^{-1}) also dose-dependently induced jejunal vasoconstriction. The jejunal vascular resistance returned to control values as the cumulative doses reached 10 and 20 mg kg^{-1}, which corresponded to the maximal increase in jejunal motility.

3 A single intravenous injection of L-NAME (10 mg kg^{-1}) produced a prompt increase in blood pressure, which lasted for at least 50 min.

4 L-NAME (10 mg kg^{-1}) produced a progressive rise in jejunal motility reaching its maximum (47 ± 6 mmHg) 15 min after the administration, and lasting for 40–50 min. Both the basal lumen pressure and the amplitude of rhythmic contractions increased during this period.

5 L-NAME (10 mg kg^{-1}) produced a triphasic change in jejunal vascular resistance and blood flow measured by timed collection of venous outflow. The blood flow decreased initially (– 43% at 5 min), increased (+ 35%) and returned to control value between 15 and 35 min, then decreased (– 35%) 40–50 min post-infusion. Jejunal vascular resistance reflected the blood flow response (+ 88% at both 5 and 50 min). The time during which the reversal of the vasoconstriction occurred (15–35 min) corresponded to the time of marked increase in motility, and was accompanied by a significant increase in jejunal oxygen uptake (+ 18%).

6 The L-NAME-induced increase in motility was prevented by L-arginine (1 g kg^{-1}, i.v.) but not by D-arginine pretreatment. The interim (15–35 min) changes in jejunal blood flow, vascular resistance and oxygen uptake were also prevented by L-arginine pretreatment.

7 L-Arginine pretreatment attenuated L-NAME-induced hypertension for 5 min.

8 The L-NAME-induced increases in jejunal vascular resistance and motility were inhibited by either local intra-arterial infusion of L-arginine (32 mM local arterial blood concentration) or topical application of 2 μM nitroglycerin. Infusion of D-arginine (32 mM local arterial blood concentration) had no such effect.

9 The L-NAME-induced increase in blood pressure was not the mechanism by which jejunal motility was increased, because similar increases in blood pressure by mefenamate (10 mg kg^{-1}, i.v.) had no such effect.

10 Thus, inhibition of nitric oxide synthesis by L-NAME increased jejunal motility and vascular resistance and the marked increase in motility can abolish or reverse the vasoconstriction. Endogenous nitric oxide may play a role in regulating motility and blood flow in the resting canine jejunum.

Keywords: Intestinal motility; intestinal blood flow; intestinal oxygen consumption; nitric oxide; L-NAME; L-arginine; D-arginine; nitroglycerin; enteric nervous system

Introduction

Nitric oxide (NO), which is synthesized endogenously from the guanidino nitrogen atoms of L-arginine (Palmer *et al.*, 1987; Moncada *et al.*, 1988; Ignarro, 1989), is a smooth muscle relaxant. Furthermore, administration of nitroglycerin or other nitrate increases blood flow to the small intestine and other organs via generation of NO (Frohlich *et al.*, 1965; Ignarro, 1989). Therefore it has been suggested that basal release of NO plays a physiological role in regulation of the skeletal muscle, renal, splanchnic, coronary and internal carotid blood flows, and systemic arterial blood pressure (Whittle *et al.*, 1989; Gardiner *et al.*, 1990a,b; Rees *et al.*, 1990; Iwata *et al.*, 1992; Jones & Brody, 1992; Pique *et al.*, 1992). In the gastrointestinal tract, NO has also been suggested to play a role in regulation of gastrointestinal motility, particularly in the neurally mediated relaxations of the oesophagus, pylorus, jejunum, colon and internal anal sphincter (Dalziel *et al.*, 1991; Allesher *et al.*, 1992; Calignano *et al.*, 1992; Knudsen *et al.*, 1992; Ward *et al.*, 1992; Yamato *et al.*, 1992). These studies suggest NO as a non-adrenergic non-cholinergic inhibitory transmitter in the gastrointestinal tract.

One technique commonly used to investigate the role of NO *in vivo* is systemic or local administration of NO synthesis inhibitors such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NNA), and N-iminoethyl-L-ornithine (L-NIO). Inasmuch as the actions of these inhibitors may change, depending on the time following their administration, it is important to determine the time course of their actions. The utilization of these analogues also has a potential problem in the elucidation of the role of NO in the gastrointestinal tract. Previous studies, which utilized NO synthesis inhibitors showed that these inhibitors not only decrease gastrointestinal blood flow

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(Gardiner et al., 1990a,b; Iwata et al., 1992; Pique et al., 1992), but also increase gut motility (Dalziel et al., 1991; Allesher et al., 1992; Calignano et al., 1992; Knudsen et al., 1992; Ward et al., 1992; Yamato et al., 1992). None of these studies, however, reported the influence of the increased motility on blood flow. It is known that spontaneous, as well as chemically-induced intestinal motility, can influence intestinal blood flow (Kvietys et al., 1986; Chou, 1989). The increase in motility induced by an NO synthesis inhibitor, therefore, might influence and complicate the induced decrease in blood flow.

The first objective of the present study was to determine the dose-response curves of the action of L-NAME on systemic arterial blood pressure, jejunal motor activity, blood flow, vascular resistance, and oxygen uptake in anaesthetized dogs. The second objective was to determine the time course of L-NAME actions on systemic arterial blood pressure and jejunal motor activity, blood flow, vascular resistance and oxygen uptake for 50 min following a single administration, and observe the interaction between the induced changes in blood flow, oxygen uptake and motility. The third objective was to determine (by use of L-arginine, D-arginine, and nitroglycerin) whether the actions of intravenous L-NAME were due to inhibition of endogenous nitric oxide synthesis.

Methods

Experiments were conducted on 56 mongrel dogs of either sex (15–25 kg), fasted for 24 h and anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.). All animals were ventilated with a positive pressure respirator (Harvard Apparatus, Millis, MA) that was adjusted to achieve normal blood pH, O_2 tension, and CO_2 tension before each experiment. Systemic arterial pressure was continuously monitored through a cannula in the femoral artery.

Seven series of experiments were performed. In all series, a midline abdominal incision was made and a segment of the jejunum (20–40 g) about 30 cm aboral to the ligament of Treitz was exteriorized (Chou et al., 1989). Heparin sodium (500 u kg^{-1}) was administered intravenously before cannulation of blood vessels. The single vein draining the jejunal segment was cannulated for measurement of venous outflow by timed collection with stopwatch and graduated cylinder. Blood flow was also continuously monitored by an extracorporeal flow transducer (BL 2048-EO4, Biotronex Lab, Silver Spring, MD, U.S.A.), placed in the venous outflow line and connected to an electromagnetic flowmeter (BL 610, Biotronex Lab, Silver Spring, MD, U.S.A.). Jejunal arteriovenous oxygen content [(a-v)O_2] difference was determined continuously by perfusing femoral arterial blood and a portion of the venous outflow at 6 ml min^{-1} through separate cuvettes of an (a–v)O_2 difference analyser (A-Vox Systems, San Antonio, TX, U.S.A) (Shepherd & Burgar, 1977) with a Gilson pump (Minipuls 2, Gilson Medical Electronics, Middleton, WI, U.S.A.). The venous outflow and outflows from the cuvettes were directed to a reservoir. The blood in the reservoir was pumped back to the animal via a femoral vein at a rate equal to the total outflows. Oxygen uptake was calculated as the product of blood flow and (a–v)O_2 difference. The time required for venous outflow to reach the (a–v)O_2 difference analyser was 30 s and was compensated for calculation of oxygen uptake. After a rubber balloon containing 10 ml of saline was placed into the lumen of the segment for continuous recording of lumen pressure by a pressure transducer (Statham P23Gb), both ends of the segment were tied and cut away from the adjacent jejunum to exclude collateral flow (Chou et al., 1989). The segment was covered with a plastic sheet and kept at 37°C with a heat lamp and a thermoregulator (Yellow Springs Instruments, Model 63RC, Yellow Springs, OH, U.S.A.).

Series I ($n = 8$). This series of experiments determined the effects of intravenous injections of L-NAME on systemic arterial blood pressure, heart rate, and jejunal blood flow, lumen pressure and oxygen uptake over the dosages 0.1–20 mg kg^{-1}. When all measured variables reached a steady state L-NAME (0.1 mg kg^{-1}) was infused i.v. Then, the sequentially increasing doses were given to achieve cumulative doses of 0.5, 1.0, 3.0, 5.0, 10 and 20 mg kg^{-1}. The responses to each infusion were observed for 10 min.

Series II ($n = 6$). The objective was to observe the time course of the changes in systemic arterial blood pressure, jejunal blood flow, lumen pressure and oxygen uptake for a period of 50 min following an i.v. administration of L-NAME (10 mg kg^{-1}). Based on series I studies, 10 mg kg^{-1} was chosen as the minimum dose of L-NAME that produced maximum actions on jejunal motility. In this as well as the following series, L-NAME was infused i.v. slowly over a 1–2 min period.

Series III ($n = 11$). This series of experiments determined whether or not the effects of L-NAME were due to competitive inhibition of endogenous nitric oxide synthesis. L-Arginine, a substrate for endogenous nitric oxide synthesis, and D-arginine, an enantiomer of L-arginine, were used for this purpose. It was found in preliminary studies ($n = 3$) that L-arginine (100 mg kg^{-1}, i.v.) did not reverse any of the effects of L-NAME. Therefore, a dose of 1 g kg^{-1} was used and infused slowly i.v. over a period of 15 min ($n = 6$). In five additional dogs, D-arginine (1 g kg^{-1}, i.v.) was infused as a control for L-arginine. Both arginines were infused slowly over 15 min in order to avoid a decrease in arterial pressure. When all measured variables reached a steady state after the infusion, L-NAME (10 mg kg^{-1}) was infused i.v. and the responses observed for 50 min.

Series IV and V. In series V, L-arginine ($n = 6$) or D-arginine ($n = 6$) was infused i.a. about 20 min after administration of L-NAME (10 mg kg^{-1}, i.v.) in order to determine if L- or D-arginine would alter the L-NAME-induced changes in jejunal blood flow, oxygen uptake, and motility. Either arginine was infused into the artery perfusing the jejunal segment via a catheter inserted into a branch of the artery with a Harvard infusion pump (Harvard Apparatus Co., Millis, MA, U.S.A.) at a rate of 100 mg min^{-1} to achieve arterial blood concentration of 32 mM. This blood concentration was selected from the Series IV experiments ($n = 4$) in which the effects of local arterial blood concentrations of 4–95 mM L- or D-arginine on jejunal blood flow and oxygen uptake were determined. It was found that 32 mM was the maximum blood concentration at which L- or D-arginine did not significantly alter all the measured variables under resting conditions.

Series VI ($n = 5$). The objective of this series of experiments was to determine the effects of a nitric oxide donor, nitroglycerin, on the L-NAME-induced changes. Nitroglycerin (2 μM) was applied topically to the serosal surface of the jejunal segment when L-NAME (10 mg kg^{-1}, i.v.) increased both jejunal vascular resistance and motility.

Series VII ($n = 7$). Following the observations that L-NAME produces a sustained increase in systemic arterial blood pressure and motility, we determined whether or not the jejunal motility is a consequence of the increase in systemic arterial blood pressure. Previously we have shown that a single intravenous injection of mefenamate (10 mg kg^{-1}) produces a sustained increase in blood pressure (Mangino & Chou, 1986; Chou et al., 1989). Therefore, utilizing the same jejunal preparation and the same protocol as in series II, jejunal lumen pressure and blood flow and systemic arterial pressure were recorded before and after a single intravenous injection of mefenamate (10 mg kg^{-1}) for 1 h.

In all series, the jejunal motor activity was quantitated from the lumen pressure tracings. The basal lumen pressure was used as an index of intestinal tonic contractions. A motility index was calculated by dividing all the pressure wave peaks by the number of pressure waves during a 1 min period, and reflects the average strength of intestinal rhythmic contractions (Chou, 1989). The basal lumen pressure and the motility index were expressed in mmHg. The jejunal vascular resistance was calculated by dividing the aortic pressure by jejunal blood flow.

Chemicals

N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), L-arginine hydrochloride, D-arginine hydrochloride and mefenamic acid were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Nitroglycerin (Parke-Davis, Mount Plains, NJ, U.S.A.) was obtained from a local apothecary. The vehicle for all compounds used was normal saline. All drugs were prepared the day of the experiment.

Statistical analysis

The data were analysed using Student's t test modified for comparison of two-paired sample means, analysis of variance and Duncan's range test. Statistical significance was set at $P<0.05$. All values were expressed as mean ± s.e.mean.

Results

Dose response

The dose-response curves of the actions of L-NAME (0.1 to 20 mg kg^{-1}, i.v.) on systemic mean arterial blood pressure, heart rate, and jejunal basal lumen pressure and motility index are shown in Figure 1. L-NAME significantly increased the arterial pressure (Figure 1a) and decreased heart rate (Figure 1b) at the cumulative doses above 1 mg kg^{-1}. The doses required to produce a maximal increase in blood pressure and a maximal decrease in heart rate were 3 and 10 mg kg^{-1}, respectively. L-NAME also increased jejunal basal lumen pressure (Figure 1c) and motility index (Figure 1d) in a dose-dependent fashion. The dose of L-NAME required to cause a maximal increase in lumen pressure and motility index was 10 mg kg^{-1}.

Figure 2 shows the dose-response curves of the actions of L-NAME on jejunal blood flow, vascular resistance, $(a-v)O_2$ difference, and oxygen uptake. L-NAME dose-dependently decreased jejunal blood flow (Figure 2a) and increased vascular resistance (Figure 2b) between doses of 0.5 and 5 mg kg^{-1}. With L-NAME 5 mg kg^{-1}, blood flow was decreased maximally from 56 ± 6 to 38 ± 2 ml min^{-1} 100 g^{-1} and vascular resistance increased maximally from 2.43 ± 0.37 to 3.79 ± 0.39 mmHg ml^{-1} min^{-1} 100 g^{-1}. However, as the cumulative doses of L-NAME reached 10 and 20 mg kg^{-1}, the decreased blood flow and increased vascular resistance were reversed to near control levels. Jejunal $(a-v)O_2$ difference (Figure 2c) significantly increased at all doses, whereas oxygen uptake (Figure 2d) remained unchanged until the dosages of L-NAME reached 10 and 20 mg kg^{-1}, when it was significantly increased. This increase in oxygen uptake occurred concurrently with the maximal increase in motility index (Figure 1d).

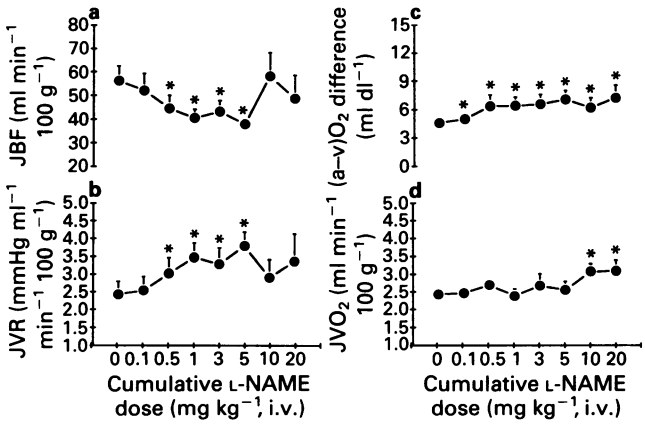

Figure 2 Effects of intravenous administration of cumulative doses (0.1–20 mg kg^{-1}) of N^G-nitro-L-arginine methyl ester (L-NAME) on jejunal blood flow (JBF) (a), vascular resistance (JVR) (b), $(a-v)O_2$ difference (c) and oxygen uptake (JVO_2)(d) ($n = 8$). Data are shown as mean ± s.e.mean. Significant difference from control (0) value is shown as $*P<0.05$.

Time course of action of L-NAME

Figures 3a,b and 4a,b,c show the time course of the actions of L-NAME (10 mg kg^{-1}, i.v.) on the variables measured.

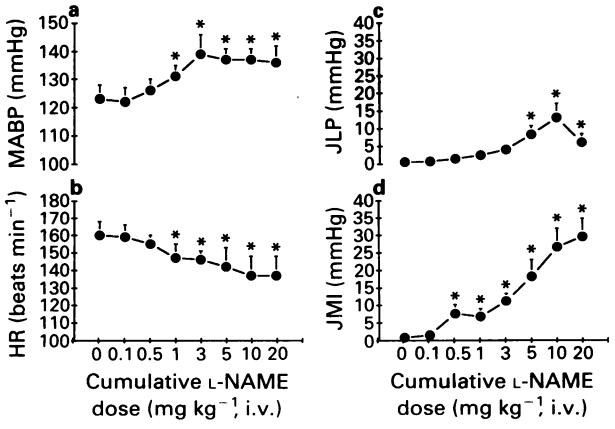

Figure 1 Effects of intravenous administration of cumulative doses (0.1–20 mg kg^{-1}) of N^G-nitro-L-arginine methyl ester (L-NAME) on mean arterial blood pressure (MABP) (a), heart rate (HR) (b), jejunal lumen pressure (JLP) (c) and motility index (JMI) (d) ($n = 8$). Data are shown as mean ± s.e.mean. Significant difference from control (0) value is shown as $*P<0.05$.

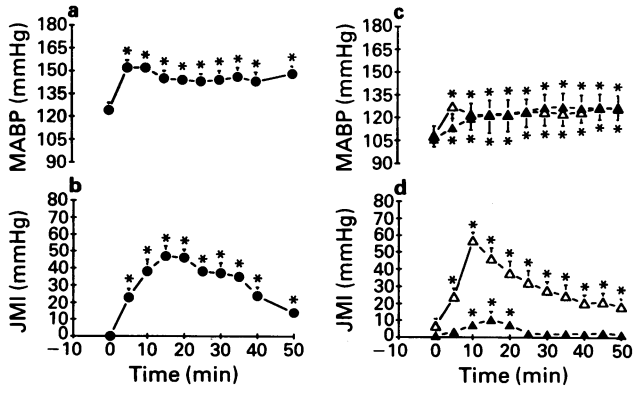

Figure 3 The time course of changes in mean arterial blood pressure (MABP) (a and c) and jejunal motility index (JMI) (b and d) after intravenous infusion of N^G-nitro-L-arginine methyl ester (L-NAME 10mg kg^{-1}) in dogs untreated ($n = 6$) (a and b) and pretreated (c and d) with either D-arginine ($n = 5$) (Δ) (1 g kg^{-1}, i.v.) or L-arginine ($n = 6$) (▲) (1 g kg^{-1}, i.v.). Data are shown as mean ± s.e.mean. Significant difference from control (0) value is shown as $*P<0.05$.

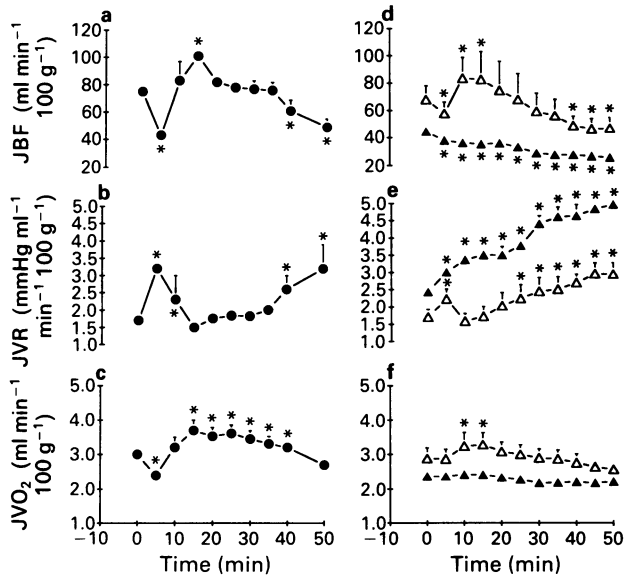

Figure 4 The time course of changes in jejunal blood flow (JBF) (a and d), vascular resistance (JVR) (b and e), and oxygen uptake (JVO_2) (c and f) after intravenous infusion of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) in dogs untreated ($n = 6$) (a,b and c) and pretreated (d,e and f) with either D-arginine ($n = 5$) (Δ) (1 g kg^{-1}, i.v.) or L-arginine ($n = 6$) (▲) (1 g kg^{-1}, i.v.). Data are shown as mean ± s.e.mean. Significant difference from control (0) value is shown as $^*P < 0.05$.

One objective was to determine if the actions of L-NAME would remain constant, and if so, for how long. Within 5 min following its infusion, L-NAME increased arterial blood pressure (Figure 3a) and jejunal motility and vascular resistance (Figures 3b and 4b), and decreased jejunal blood flow and oxygen uptake (Figures 4a and 4c). The increased blood pressure reached its maximum 5 min after the infusion, and this increase was maintained for the entire 50 min observation period. Jejunal motility increased progressively, reaching its maximum 10–20 min following the injection. Both the basal lumen pressure and the amplitude of rhythmic contractions increased during this period (Figure 5a). The motility index remained elevated until 35 min post-infusion, when it started to decrease (Figure 3b). At 50 min the motility index was 14 ± 4 mmHg, which was still significantly ($P < 0.05$) greater than pre-infusion levels.

Jejunal blood flow, vascular resistance, and oxygen uptake (Figure 4a,b and c) showed a triphasic change. The blood flow significantly ($P < 0.05$) decreased at 5 min, increased at 15 min, returning to control values between 20 and 35 min, then started to decrease thereafter. The flows at 40 and 50 min were significantly lower than the control. Jejunal vascular resistance reflected the blood flow responses. It significantly ($P < 0.05$) increased at 5 min, returning to control values between 15 and 35 min, then significantly increased at 40 and 50 min ($P < 0.05$). Jejunal oxygen uptake (Figure 4c) significantly ($P < 0.05$) decreased at 5 min, increased between 15 and 40 min and returned to the control value at 50 min. The time during which the increased oxygen uptake, as well as the reversal of blood flow and vascular resistance occurred (15–35 min), corresponded to the time of

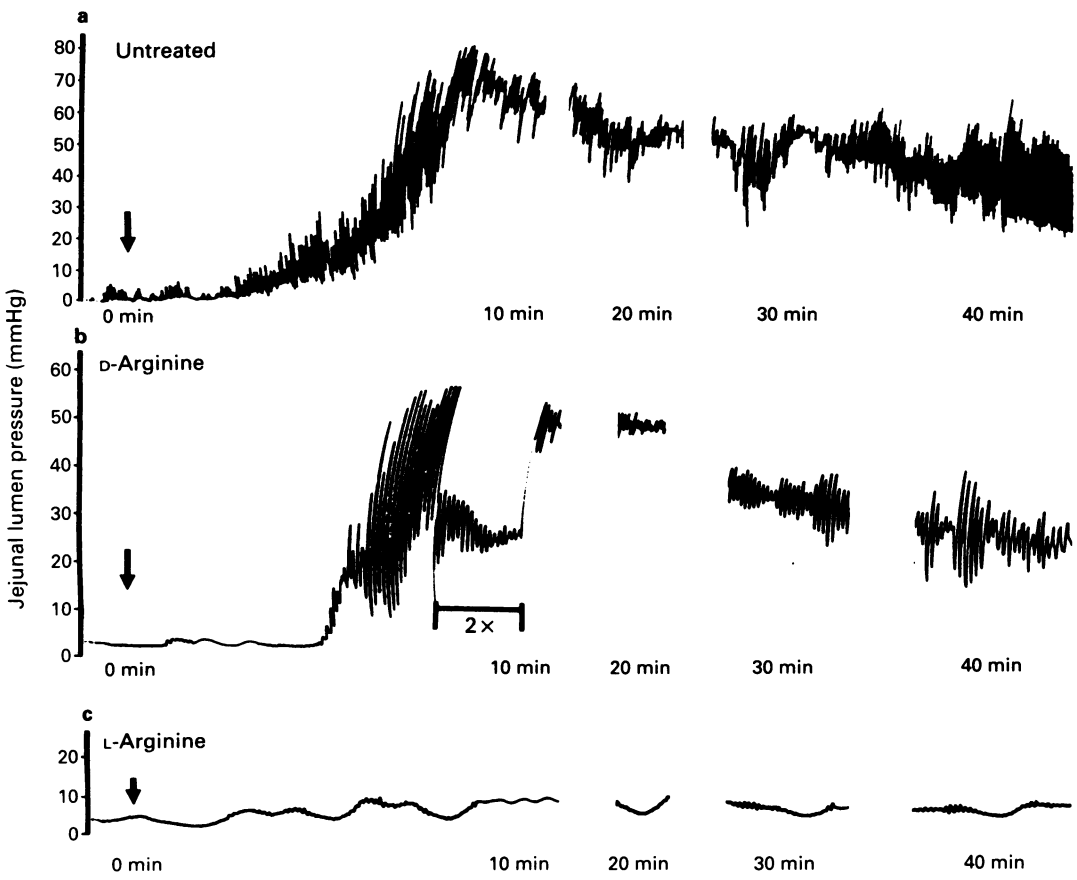

Figure 5 Typical tracings of jejunal lumen pressure following infusion of N^G-nitro-L-arginine methyl ester (L-NAME 10 mg kg^{-1}, i.v.) in dogs untreated (a) and pretreated with either D-arginine (1 g kg^{-1}, i.v.) (b) or L-arginine (1 g kg^{-1}, i.v.) (c). 2X in the middle tracing (b) indicates doubling of the attenuation of the pressure recording. Here, 30 mmHg = 60mmHg. Arrows indicate the end of L-NAME infusion, i.e., 0 min.

the markedly enhanced motility (Figure 3b). The subsequent decrease in blood flow and increase in vascular resistance (40–50 min), on the other hand, corresponded to the declining phase of jejunal motility.

Effects of L- or D-arginine on action of L-NAME

The objectives of the next three series of experiments were to determine whether or not the actions of L-NAME described above were due to inhibition of endogenous nitric oxide synthesis. In series III, dogs were pretreated with either L-arginine (1 g kg^{-1}, i.v.), a substrate for nitric oxide synthesis, or D-arginine (1 g kg^{-1}, i.v.), a control for L-arginine, before administration of L-NAME (10 mg kg^{-1}, i.v.). Figures 5b and 5c show typical tracings of the effect of L-NAME on jejunal lumen pressure in dogs pretreated with either D-arginine or L-arginine. As in untreated dogs (Figure 5a), L-NAME produced marked increases in rhythmic and tonic contractions 5–15 min following its administration in dogs pretreated with D-arginine. The enhanced motility lasted for 40–50 min. L-Arginine pretreatment (Figure 5c), on the other hand, completely prevented the L-NAME-induced increase in jejunal motility.

Figure 3c,d and Figure 4d,e,f summarize the data obtained in 11 dogs in series III. L-NAME increased arterial blood pressure in both L-arginine and D-arginine groups. However, in contrast to untreated (Figure 3a) and D-arginine-treated dogs (Figure 3c, open triangles), in which L-NAME promptly increased blood pressure by 23 ± 4% and 18 ± 4%, respectively, within 5 min of its administration, the increased blood pressure in L-arginine-treated dogs (Figure 3c, closed triangles) at the corresponding time was only 6 ± 2%. This value was significantly lower than the above two values. Thus, L-arginine, but not D-arginine, significantly attenuated the L-NAME-induced initial prompt increase in blood pressure. While the hypertension reached its maximum in 5 min in both untreated and D-arginine-treated dogs, it took 35 min in the L-arginine group to reach the maximum hypertension. The ranges of increased blood pressure during the 10–50 min period in untreated, L-arginine, and D-arginine groups were 16–21%, 13–20%, and 13–18%, respectively. Thus, neither L- nor D-arginine pretreatment significantly altered the level of L-NAME-induced increase in blood pressure during this period 10–50 min post-infusion. However, one of the six L-arginine pretreated dogs responded to L-NAME with no significant change in blood pressure.

The response of the motility to L-NAME in the L-arginine group was clearly different from that in either the untreated or D-arginine group. L-Arginine pretreatment significantly ($P<0.05$) attenuated or abolished the L-NAME-induced increase in jejunal motility index, whereas the response in the D-arginine group was similar to that in untreated dogs (Figure 3b and d). The maximum increases in motility index in D-arginine, untreated, and L-arginine groups were 60 ± 5, 47 ± 6, and 11 ± 5 mmHg, respectively. This indicates that L-, but not D-arginine, abolished or significantly attenuated the L-NAME-induced increase in motility (Figures 5 and 3d). L-NAME produced a triphasic change in jejunal blood flow and vascular resistance in the D-arginine group, whereas it only decreased jejunal blood flow and increased vascular resistance in the L-arginine group in a lineal fashion (Figures 4d and 4e). Thus, L-arginine abolished the triphasic vascular action of L-NAME.

Figure 4f shows the actions of D- or L-arginine on the L-NAME-induced changes in oxygen uptake. The L-NAME-induced increases in oxygen uptake as shown in Figure 4c were abolished in the L- but not in the D-arginine group.

In series IV, the actions of local i.a. infusions of L- or D-arginine were determined in order to select the dosages to be used in the next series of experiments. Neither L- nor D-arginine significantly altered blood pressure, heart rate, and jejunal motility. As shown in Table 1, neither L- nor D-arginine significantly affected jejunal blood flow, vascular resistance, and oxygen uptake until the arterial concentration reached 95 mM. At this dose both drugs significantly ($P<0.05$) increased jejunal blood flow and oxygen uptake, while decreasing the vascular resistance.

In the next series of experiments, L- or D-arginine was infused into the local jejunal artery 20 min after the administration of L-NAME. The objective was to determine if local infusion of either arginine at a rate calculated to achieve an arterial blood concentration of 32 mM would alter the L-NAME-induced changes in jejunal blood flow and motility. As shown in Figure 6, L-NAME produced a similar pattern and magnitude of increases in blood pressure and jejunal motility index, and a biphasic change in jejunal blood flow and vascular resistance before administration of either L- or D-arginine. These responses were similar to those in series II as shown in Figures 3a, 3b, 4a and 4b. When the increased motility reached a steady state, L- or D-arginine was infused i.a. (at time 20 min). Neither L- nor D-arginine affected the L-NAME-induced increase in blood pressure, because they were given by slow infusion into the local jejunal artery. However, the increased motility index was significantly inhibited by L-arginine, but not by D-arginine (Figure 6b). L-Arginine also significantly increased blood flow for 5 min (Figure 6c). The flow then decreased, and at 20 min after the start of L-arginine infusion, the blood flow was significantly lower than that before either L-NAME nor L-arginine infusion. The action of L-arginine on jejunal vascular resistance was exactly opposite to that on blood flow. D-Arginine, on the other hand, did not significantly alter blood flow and vascular resistance. This series of experiments appears to indicate that L-arginine, but not D-arginine, inhibited the L-NAME-induced increase in motility for the entire infusion period, and produced vasodilatation only during the first 5 min of the infusion.

Table 1 Effect of local intra-arterial infusion of L-arginine or D-arginine hydrochloride on jejunal blood flow (JBF, ml min^{-1} 100 g^{-1}), vascular resistance (JVR, mmHg ml^{-1} min^{-1} 100 g^{-1}), and oxygen uptake (JVO_2, ml min^{-1} 100 g^{-1})

	L-Arginine hydrochloride (mM)					
	0	4	8	18	32	95
JBF	54 ± 7	53 ± 5	52 ± 5	53 ± 5	64 ± 6	86 ± 7*
JVR	2.3 ± 0.3	2.1 ± 0.4	2.2 ± 0.4	2.1 ± 0.3	1.7 ± 0.3	1.2 ± 0.2*
JVO_2	1.9 ± 0.1	2.0 ± 0.2	1.9 ± 0.1	2.0 ± 0.1	2.6 ± 0.1	3.3 ± 0.2*
	D-Arginine hydrochloride (mM)					
	0	4	8	18	32	95
JBF	64 ± 12	62 ± 12	62 ± 12	73 ± 16	74 ± 16	90 ± 10*
JVR	2.0 ± 0.2	1.9 ± 0.4	1.9 ± 0.4	1.7 ± 0.4	1.6 ± 0.3	1.1 ± 0.2*
JVO_2	2.0 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	2.3 ± 0.3	2.5 ± 0.1	3.4 ± 0.4*

Values are mean ± s.e.mean ($n = 4$), where significant difference from control values (0 mM) is shown as $*P<0.05$.

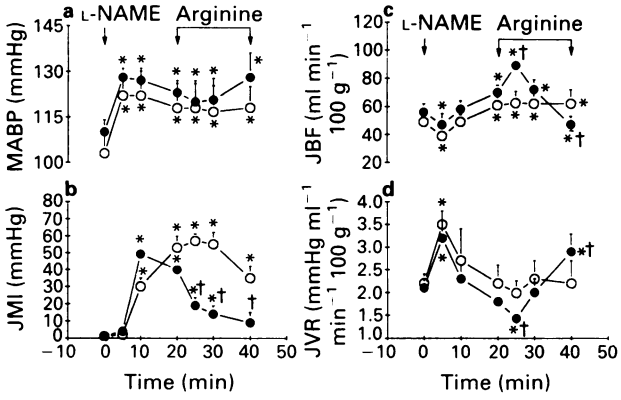

Figure 6 Effects of local intra-arterial infusion of L-arginine (●) ($n = 6$) or D-arginine (○) ($n = 6$) (32 mM local arterial blood concentration each) on N^G-nitro-L-arginine methyl ester (L-NAME)-induced changes in mean arterial blood pressure (MABP) and jejunal motility index (JMI), blood flow (JBF), and vascular resistance (JVR). L- or D-arginine was infused during 20–40 min after intravenous administration of L-NAME (10 mg kg^{-1}). Data are shown as mean ± s.e.mean. Significant difference from control (time 0) is shown as $*P < 0.05$, and from respective time 20 is shown as $\dagger P < 0.05$.

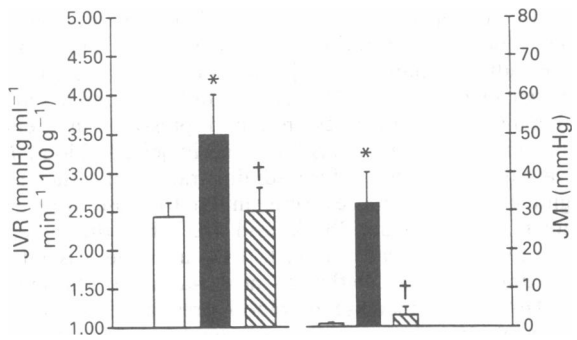

Figure 7 Effects of topical serosal application of nitroglycerin (GTN; 2 μM) on the N^G-nitro-L-arginine methyl ester (L-NAME)-induced increases in jejunal vascular resistance (JVR) and motility index (JMI). Resting values before L-NAME administration (open columns), values following L-NAME and prior to the application of GTN (solid columns), values observed following the application of GTN (hatched columns) ($n = 5$). Data are shown as mean ± s.e. mean. Significant difference from resting value is shown as $*P < 0.05$, and from L-NAME value, as $\dagger P < 0.05$.

Effects of nitroglycerin on action of L-NAME

In series VI ($n = 5$) we determined the effects of nitroglycerin on the L-NAME-induced increases in jejunal vascular resistance and motility index. Nitroglycerin (2 μM) was applied topically on the jejunal serosal surface when the L-NAME-induced increase in jejunal vascular resistance and motility reached a steady state. As shown in Figure 7, nitroglycerin promptly and completely inhibited the L-NAME-induced increases in jejunal vascular resistance and motility index. The effects of topical nitroglycerin lasted for only 1 min.

Effects of increased systemic arterial blood pressure on jejunal motility

In series VII ($n = 7$) experiments, we determined whether or not the L-NAME-induced jejunal motility is a consequence of the increase in systemic arterial blood pressure. A single intravenous injection of mefenamate (10 mg kg^{-1}) produced a sustained increase in blood pressure from resting values of 125 ± 4 to 147 ± 4 mmHg and a decrease in jejunal blood flow from resting values of 54 ± 7 to 32 ± 2 ml min^{-1} 100 g^{-1} ($P < 0.05$), but did not induce significant changes in jejunal motility. The lumen pressures before and following injection of mefenamate were 4 ± 1 and 6 ± 1 mmHg, respectively. The absolute and percentage increases in blood pressure produced by mefenamate (+ 22 mmHg and 18%), were not significantly ($P > 0.05$) different from those produced by L-NAME (+ 28 mmHg and 23%). Furthermore, jejunal blood flow remained below resting levels for more than 1 h.

Discussion

L-NAME has been used by many investigators to determine the role of endogenous nitric oxide in various physiological and pathophysiological conditions, using doses ranging between 1 and 100 mg kg^{-1} by intravenous bolus injections. The doses required to produce the maximal action, as assessed from the level of the increased blood pressure, ranged from 1 to 30 mg kg^{-1} (Gardiner *et al.*, 1990b; Rees *et al.*, 1990; Yamato *et al.*, 1992; Calignano *et al.*, 1992). In our study on anaesthetized dogs, intravenous infusion of L-NAME dose-dependently altered systemic arterial pressure, heart rate, and jejunal motility and blood flow. The doses required to increase blood pressure and jejunal motility maximally and to decrease heart rate maximally were 3, 10 and 10 mg kg^{-1}, respectively. L-NAME also dose-dependently decreases jejunal blood flow and increases vascular resistance, and the maximal response occurs at a dose of 5 mg kg^{-1}. Above this dose, both jejunal blood flow and vascular resistance return to control levels. As will be discussed below, the return of blood flow to its control levels is related to the increase in jejunal motility.

The objective of series II experiments was to determine the time course of the actions of L-NAME on systemic arterial blood pressure and jejunal motility, blood flow, vascular resistance and oxygen uptake, and to observe the interaction among the changes in jejunal motility, blood flow and oxygen uptake. L-NAME (10 mg kg^{-1}) was administered i.v., and its action was observed for 50 min. This dose of L-NAME is within the range used by others (Gardiner *et al.*, 1990b; Rees *et al.*, 1990; Allescher *et al.*, 1992; Calignano *et al.*, 1992; Yamato *et al.*, 1992) and in our preparation it induces the maximal increase in jejunal motility.

As shown in Figure 3, L-NAME produced a prompt increase in mean arterial blood pressure, which was maintained for the entire 50 min observation period. A similar degree of blood pressure increase has been reported after a single intravenous injection of L-NAME (Gardiner *et al.*, 1990b; Rees *et al.*, 1990; Yamato *et al.*, 1992; Calignano *et al.*, 1992), L-NMMA (Whittle *et al.*, 1989; Rees *et al.*, 1990; Gardiner *et al.*, 1990a; Pique *et al.*, 1992), or L-NOARG (Iwata *et al.*, 1992). Jejunal motility increased progressively, reaching its maximum value 10–20 min following the administration and remained elevated until 40 min, when it started to decrease. Both basal lumen pressure (tonic contraction) and the amplitude of phasic rhythmic contractions increased. The L-NAME-induced jejunal motility was unlikely to be the consequence of the increase in blood pressure, since mefenamate which also produced an increase in blood pressure similar to that induced by L-NAME, did not induce jejunal motility.

Jejunal blood flow showed a triphasic response to L-NAME: an initial decrease, followed by an increase above and around the resting levels for 25 min and an eventual significant decrease below resting levels at 40–50 min post-infusion. The response of jejunal vascular resistance was exactly opposite to the response of blood flow. The resistance increased at both 5 and 40–50 min post-infusion. Inasmuch as the increased blood pressure was maintained at a steady level for 50 min, the reversal of the vasoconstriction is unlikely to be a consequence of a reflex induced by the

hypertension. Furthermore, in series VII experiments, the mefenamate-induced sustained hypertension without changes in jejunal motilty was accompanied only by a sustained vasoconstriction. The reversal of the vasoconstriction in series II experiments between 15–35 min and in the dose-response experiments at cumulative doses of 10 and 20 mg kg^{-1} is most probably due to the enhanced jejunal motility that occurred during these periods. Several findings of the present study support this thesis. First, the reversal of the vasoconstriction was accompanied by an increase in jejunal oxygen uptake and the marked increase in motility. As the enhanced motility started to wane over 40–50 min, oxygen uptake returned towards control levels and the vasoconstriction reappeared. Furthermore, prevention of the L-NAME-induced marked increase in jejunal motility by L-arginine prevented the reversal of vasoconstriction, as well as the increase in oxygen uptake. L-NAME produced only a unidirectional increase in jejunal vascular resistance in L-, but not in D-arginine pretreated dogs. Unlike L-arginine, D-arginine did not alter the L-NAME-induced increase in gut motility.

It is known that spontaneous as well as chemically-induced intestinal motility can influence intestinal blood flow. The rhythmic contractions can be accompanied by a decrease, an increase, or no change in blood flow, depending on the strength and pattern of contractions (Kvietys *et al.*, 1986; Chou, 1989). The increase in blood flow during muscle contractions is usually accompanied by an increase in oxygen uptake. As discussed above, the reversal of L-NAME-induced decrease in jejunal blood flow was accompanied by an increase in oxygen uptake and motility. Thus, the L-NAME-induced marked increase in motility could influence the L-NAME-induced vasoconstriction and local oxygen uptake.

The objective of the next four series of experiments was to determine if the above actions of L-NAME were due to inhibition of endogenous nitric oxide. L- and D-arginine have been regularly used for this purpose. Pretreatment with L-arginine but not with D-arginine, abolished, or significantly attenuated the L-NAME-induced marked increase in motility. Furthermore, local i.a. infusion of L-arginine but not D-arginine, inhibited the L-NAME-induced increase in jejunal motility. Our findings are in agreement with those of Calignano *et al.* (1992) who have shown that L-arginine but not D-arginine, inhibited the L-NAME-induced increase in jejunal intraluminal pressure and phasic intestinal contractions in rats. Furthermore, others also have shown that L-arginine reverses the following actions of L-arginine analogues (L-NAME, L-NMMA, L-NNA): an increase in the velocity of oesophageal peristalsis (Yamato *et al.*, 1992) and inhibition of the gastric receptive relaxation and small intestinal and colonic inhibitory reflexes (Hata *et al.*, 1990; Desai *et al.*, 1991; Sanders & Ward, 1992). Thus, the L-NAME-induced increase in jejunal motility in our study is most probably due to an inhibition of the L-arginine-NO synthesis pathway. This thesis is supported by the finding that application of nitroglycerin, a source of nitric oxide, promptly abolished the L-NAME-induced increase in jejunal motility.

Our study also shows that L-NAME produced a sustained increase in systemic arterial blood pressure and a triphasic change in jejunal blood flow and vascular resistance. As discussed above, the interim increase in blood flow and decrease in vascular resistance were most likely due to the influence of the L-NAME-induced increase in jejunal motility. Thus, the direct action of L-NAME in the canine jejunum is most likely vasoconstriction.

Whether or not the L-NAME-induced sustained hypertension and jejunal vasoconstriction were due to inhibition of the L-arginine-NO synthesis pathway is not clear from the data of the present study. Unlike the sustained inhibitory action of L-arginine on L-NAME-induced increase in jejunal motility, L-arginine inhibited the L-NAME-induced hypertension and jejunal vasoconstriction only transiently. L-arginine, but not D-arginine, significantly attenuated the level of the L-NAME-induced hypertension for the initial 5–10 min. However, the later hypertension was not affected by either L- or D-arginine. L-NAME produced the initial and later increases in jejunal vascular resistance at 5 and 40–50 min. The initial jejunal vasoconstriction was significantly attenuated by either L- or D-arginine pretreatment but the later vasoconstriction was not affected. However, as shown in Figure 6, local i.a. infusion of L-arginine but not D-arginine, promptly increased jejunal blood flow and decreased vascular resistance for the initial 5 min in L-NAME treated dogs. Our findings are in agreement with those of Jones & Brody (1992), who have shown also that L-arginine reversed the L-NAME-induced hypertension and coronary vasoconstriction for 1 to 5 min. It thus appears that the effect of L-arginine on L-NAME-induced hypertension and jejunal and coronary vasoconstriction is different from its effect on the increased jejunal motility. The underlying mechanism for this difference is unclear. Gardiner *et al.* (1990a) have reported that the action of L-arginine on L-NMMA-induced vasoconstriction is complete in the kidney but the reversal of vasoconstriction in the superior mesenteric, internal carotid and hindlimb vascular beds is transient and incomplete. Furthermore, the NO synthase enzyme subtype (Rees *et al.*, 1990) in various tissues, e.g. endothelial cells responsible for cardiovascular changes (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987; 1988; Moncada *et al.*, 1988) and enteric neurones responsible for gut motility changes (Bredt *et al.*, 1990; Bult *et al.*, 1990; Sanders & Ward, 1992), might be different. This difference may account for the differential actions of L-arginine on the L-NAME-induced changes in cardiovascular system and gut motility. These possibilities should be investigated in the future. In our study the maximal increase in blood pressure and jejunal vascular resistance occurred at lower doses of L-NAME (3 and 5 mg kg^{-1}) and within 5 min of its administration. The maximal increase in jejunal motility, however, occurred at higher doses (10–20 mg kg^{-1}), and it took 15–20 min to reach the maximal increase in motility.

In conclusion, the present study demonstrates that inhibition of endogenous NO synthesis results in an increase in systemic arterial blood pressure and jejunal motility and vascular resistance. The marked increase in motility can abolish or reverse the vasoconstriction. Endogenous NO may play a role in regulating motility and blood flow in resting canine jejunum.

This work was supported by research grant HL-15231 from the National Institutes of Health. The authors are grateful for the excellent technical assistance of Dr S.Z. Li, a visiting scholar from P.R. of China.

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(Received May 19, 1993
Revised September 10, 1993
Accepted September 29, 1993)

Br. J. Pharmacol. (1994), **111**, 213–218

Evidence for an inhibitory 5-HT_4 receptor in urinary bladder of *Rhesus* and *Cynomolgus* monkeys

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1 The present study shows that 5-hydroxytryptamine (5-HT) inhibits electrically-evoked contractions of isolated urinary bladder strips from *Rhesus* and *Cynomolgus* monkeys via activation of 5-HT_4 receptors.

2 5-HT (0.1 nM–10 μM) produced concentration-dependent inhibition of the contractile response to electrical stimulation yielding a pEC_{50} of 7.8 (*Rhesus* monkey) and 7.6 (*Cynomolgus* monkey). This action of 5-HT was mimicked by 5-methoxytryptamine, renzapride and BIMU 8, each of which behaved as a full agonist relative to 5-HT. However, the potency estimate for BIMU 8 ($pEC_{50} = 6.5$) in *Cynomolgus* monkey was low, relative to 5-HT, indicating a possible heterogeneity of 5-HT_4 receptors.

3 The inhibitory action of 5-HT was resistant to antagonism by methysergide (1 μM) and ondansetron (5 μM), thereby eliminating a role for 5-HT_1, 5-HT_2 and 5-HT_3 receptors. The 5-HT_4 receptor antagonists, GR 113808 (10 nM), DAU 6285 (1–10 μM) and RS 23597-190 (1 μM), produced parallel, dextral displacements of the concentration-effect curves to 5-HT and other related agonists with affinity estimates in agreement with those defined previously in other 5-HT_4 receptor assay systems.

4 Experiments using direct electrical stimulation of bladder smooth muscle indicate that the 5-HT_4 receptors are located post-junctionally.

5 The inhibitory action of 5-HT in isolated urinary bladder of monkey differs from the excitatory effect of 5-HT in urinary bladder of man. Species variation and its implications for the development of therapeutic agents are discussed.

Keywords: 5-HT; 5-HT_4 receptor; urinary bladder of monkey; GR 113808; DAU 6285; BIMU 8; 5-methoxytryptamine; RS 23597-190; *Cynomolgus* monkey; *Rhesus* monkey

Introduction

Pharmacological studies on the urinary bladder have demonstrated species variation with regard to the nature of responses to 5-hydroxytryptamine (5-HT) and the subtype(s) of 5-HT receptor involved. For example, in dog, 5-HT contracts the isolated bladder via 5-HT_2 receptors (Cohen, 1990). In urinary bladder of the anaesthetized cat, biphasic excitatory concentration-effect curves to 5-HT have been reported and are mediated via 5-HT_3 and 5-HT_2 receptors (Saxena *et al.*, 1985). In isolated bladder of mouse, potentiation of electrically evoked contractions by 5-HT is mediated through 5-HT_{1B} and 5-HT_2 receptors (Cleal *et al.*, 1989). By contrast, 5-HT relaxes isolated bladder neck of pig, an effect blocked by methysergide (10 μM; an antagonist at 5-HT_1 and 5-HT_2 receptors), but not ketanserin (1 μM; a selective 5-HT_2 receptor antagonist) (Hills *et al.*, 1984). In bladder of bullfrog, 5-HT-induced inhibitions of electrically evoked contractions are insensitive to methysergide (Bowers & Kolton, 1987) as are 5-HT-induced contractions of the isolated bladder of guinea-pig (Callahan & Creed, 1981).

In view of therapeutic potential, there has been interest in the role of 5-HT in the physiology of micturition (Delaere *et al.*, 1987). However, pharmacological characterization of 5-HT receptors in urinary bladder of man has been hampered by a lack of specific ligands (Klarskov & Hørby-Petersen, 1986). Early suggestive evidence for a putative 5-HT_4 receptor can be found in the work of Hindmarsh *et al.* (1977) who reported potentiation of electrically induced contractions of human isolated bladder by low concentrations of 5-HT, an effect insensitive to blockade by methysergide and morphine. Recently, Corsi *et al.* (1991) described an 'atypical' 5-HT receptor in human bladder that mediates potentiation of contractile responses to electrical field stimulation. Elements of the agonist and antagonist profile for this receptor resemble that of the 5-HT_4 receptor, including potent agonism by 5-HT ($pEC_{50} = 8.0$) and 5-methoxytryptamine (5-MeOT), agonism by certain substituted benzamide derivatives, antagonism by micromolar concentrations of tropisetron (ICS 205-930), and resistance to inhibition by certain 5-HT_1, 5-HT_2 and 5-HT_3 receptor antagonists (see Bockaert *et al.*, 1992; Ford & Clarke, 1993). However, antagonism by tropisetron in human bladder deviates from competition and in high concentrations tropisetron behaves as an agonist (Corsi *et al.*, 1991). Furthermore, the potentiating action of 5-HT is antagonized, in part, by methysergide (1 μM).

To date, the nature of the 5-HT receptor in isolated urinary bladder of monkey has not been described. Our preliminary experiments revealed a potent inhibitory effect of 5-HT that was blocked by the 5-HT_4 receptor antagonist, DAU 6285 (Waikar *et al.*, 1992). The present study was undertaken therefore, to isolate pharmacologically and characterize further the putative 5-HT_4 receptor in the urinary bladder from *Rhesus* and *Cynomolgus* monkeys. Preliminary accounts of this work have been presented at the British Pharmacological Society Meeting, July, 1992 (Waikar *et al.*, 1992) and the 2nd International Symposium on Serotonin, September, 1992 (Ford *et al.*, 1992b).

Methods

Preparation of urinary bladder strips

Rectangular strips (2 cm × 0.5 cm) of urinary bladder from *Rhesus* and *Cynomolgus* monkeys of either sex (5–9 kg) were

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taken from the posterior medial smooth muscle layer and mounted vertically been two platinum electrodes in 10 ml tissue baths containing Tyrode solution (37°C; pH 7.4; gassed with O_2/CO_2, 95:5%). Cocaine (30 μM), corticosterone (30 μM), methysergide (1 μM), ondansetron (5 μM) and indomethacin (10 μM) were added to the Tyrode solution in order to achieve equilibrium conditions and isolate pharmacologically the putative 5-HT_4 receptor for study. Responses were recorded isometrically using a Hugo Sachs Elektronik (Biegestab K30) transducer coupled to Graphtec (linearcorder WR3310) four channel chart recorders.

Response to electrical stimulation

Electrical stimulation parameters were modified from Corsi *et al.* (1991). Tissues were placed under an initial tension of 10 mN and subjected to electrical field-stimulation (Grass S88 stimulator; Buxco Electronics Stimulus Distributor). Trains of electrical pulses (5 s duration, supramaximal voltage, 1 ms pulse width, 20 Hz) were applied at 1 train per min, yielding reproducible contractile responses. The effects of tetrodotoxin (TTX, 3 μM) and atropine (1 μM) on the responses to field stimulation were measured.

Effect of 5-HT_4 receptor agonists and antagonists on response to field stimulation

Following stabilization of electrically evoked contractions (≈30 min, washing every 10 min), cumulative concentration-inhibition (E/[A]) curves to agonist were constructed at 0.5 or 1 $\log_{10}$ unit intervals. Upon washout of agonist and subsequent recovery of the response to field stimulation (30–45 min), tissues were equilibrated with antagonist for 30–60 min and second E/[A] curves were constructed. Strips from some bladders failed to recover completely after the first E/[A] curve and were not used for antagonist studies.

Inhibition of carbachol-induced contracture

Segments of urinary bladder, not subjected to electrical stimulation, were contracted with carbachol (1 μM). Following the development of a sustained contracture (≈30 min), attempts were made to relax the tissue with 5-HT (10 nM–10 μM), isoprenaline (10 μM), forskolin (30 μM) and TTX (3 μM).

Direct stimulation of muscle

Direct electrical stimulation of the smooth muscle of bladder was conducted as described by Corsi *et al.* (1991). After a 15–30 min period of electrical stimulation (using the parameters given above), TTX (3 μM) and atropine (1 μM) were administered so as to inhibit completely the neurogenic contractile response. Subsequently, the pulse width of the electrical stimulation was increased 10 fold to 10 ms in order to evoke reproducible contractile responses of muscle similar in magnitude to those generated neurogenically. For the generation of E/[A] curves 5-HT was added cumulatively. BIMU 8 was used in a single concentration (10 μM).

Study of different anatomical regions of bladder and drug-free Tyrode solution

In order to investigate differences between results with monkey bladder (present study) and those reported in human bladder (Corsi *et al.*, 1991), strips of monkey bladder were taken from the dome region and suspended in Tyrode solution free of cocaine, corticosterone, ondansetron, methysergide and indomethacin. These conditions mimicked the experimental conditions employed by Corsi *et al.* (1991) on human bladder.

Data analysis

E/[A] curves are expressed as percentage inhibition of field stimulated contractions measured just prior to the addition of agonist. Estimates of maximal inhibition (E_{max}) and the concentration of agonist ([A]) giving half maximal inhibition (EC_{50}), were generated with a non-linear, iterative curve-fitting programme (Kaleidagraph, Synergy Software, PCS Inc., Reading, PA, U.S.A.) using the following form of the logistic function: $E/E_{max} = [A]^n/([A]^n + EC_{50}^n)$ where n is a parameter defining slope. Antagonist pA_2 estimates were made by comparing agonist EC_{50} values in the absence and presence (EC_{50}') of a single concentration ([B]) of antagonist ($CR = EC_{50}/EC_{50}'$), such that $pA_2 = -\log([B]/(CR-1))$.

Ninety five percent confidence limits (CL) and statistical significance of differences between samples (single comparisons; Student's unpaired *t* test, two-tailed) were determined using Statview IV (Brain Power Inc., Calabassas, CA, U.S.A.). Although several strips were obtained from each bladder, *n* refers to the number of bladders used.

Chemicals

The following were obtained as stipulated: BIMU 8 (endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl) ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) and DAU 6285 (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct 3 yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride) (Dr C.A. Rizzi, Boehringer Ingelheim Italia, Institute De Angeli, Milan, Italy); 5-hydroxytryptamine creatinine sulphate, 5-methoxytryptamine hydrochloride, corticosterone, carbamylcholine chloride, indomethacin, lignocaine hydrochloride, cocaine hydrochloride, atropine sulphate, tetrodotoxin, forskolin (Sigma Chemical Co., St. Louis, MO, U.S.A.); renzapride, ondansetron, RS 23597-190 (3(piperidine-1-yl)-propyl-4-amino-5-chloro-2-methoxy benzoate HCl) and GR 113808 ([1-[2-methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate) were synthesized on site (Syntex Research, Palo Alto, CA, U.S.A.); methysergide maleate (Sandoz, Basel, Switzerland); ω-conotoxin GVIA (Bachem California Inc., U.S.A.); 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; RBI, Natick, MA, U.S.A.).

All drugs were dissolved in deionised water with the following exceptions: corticosterone and methysergide (dimethylsulphoxide; DMSO), indomethacin (0.5% Na_2CO_3 in water), ω-conotoxin GVIA (10% bovine serum albumin in Tyrode solution) and forskolin (70% DMSO in water).

Results

Response to electrical stimulation

Transmural electrical field stimulation (supramaximal voltage, 30–90 V; pulse width, 1 ms; frequency 20 Hz; pulse train, 5 s; train interval, 55 s) evoked reproducible contractile responses in strips of monkey urinary bladder (Figure 1). These responses were blocked by TTX (3 μM) and atropine (1 μM).

Effect of 5-HT_4 receptor agonists and antagonists

Figure 1 shows that cumulative addition of 5-HT (0.3 nM–1 μM) inhibited field stimulated contractile responses in a concentration-dependent manner in bladder strips from both *Rhesus* ($pEC_{50} = 7.8$; 95% CL 7.6–8.0; $n = 13$) and *Cynomolgus* monkey ($pEC_{50} = 7.6$; 95% CL 7.4–7.8; $n = 10$). Iteratively fitted mean E/[A] curves for 5-HT and other 5-HT_4 receptor agonists are shown in Figure 1b. 5-Methoxytryptamine (5-MeOT), and, possibly, renzapride ($n = 1$) acted as full agonists, mimicking the inhibitory action of 5-HT (see Table 1 for data regarding agonists potency).

BIMU 8, a selective 5-HT$_4$ receptor agonist, also behaved as a full agonist relative to 5-HT but its estimated potency (6.5, 95% CL 6.1–6.8; *Cynomolgus* monkey, $n = 4$; Figure 1b) is lower than expected for a 5-HT$_4$ receptor. The selective 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT was without effect (*Cynomolgus* monkey; data not shown).

Table 1 summarizes data with regard to antagonist affinity. 5-HT$_4$ receptor antagonists, GR 113808 (10 nM; Figure 2a) and DAU 6285 (1 μM; Figure 2b), produced parallel dextral displacements of E/[A] curves to 5-HT yielding antagonist pA$_2$ estimates of 9.5 (95% CL 9.1–9.8; $n = 5$; *Cynomolgus* monkey) and 7.0 (95% CL 6.8–7.3; $n = 4$; *Rhesus* monkey) respectively. pA$_2$ estimates ranging from 6.8–7.4 were also generated for DAU 6285 against 5-MeOT, BIMU 8 and renzapride (Table 1). Another 5-HT$_4$ receptor antagonist, RS 23597-190, yielded a pA$_2$ estimate of 7.3 (95% CL 6.8–7.9; $n = 5$) against 5-HT in strips of bladder from *Rhesus* monkey (Table 1).

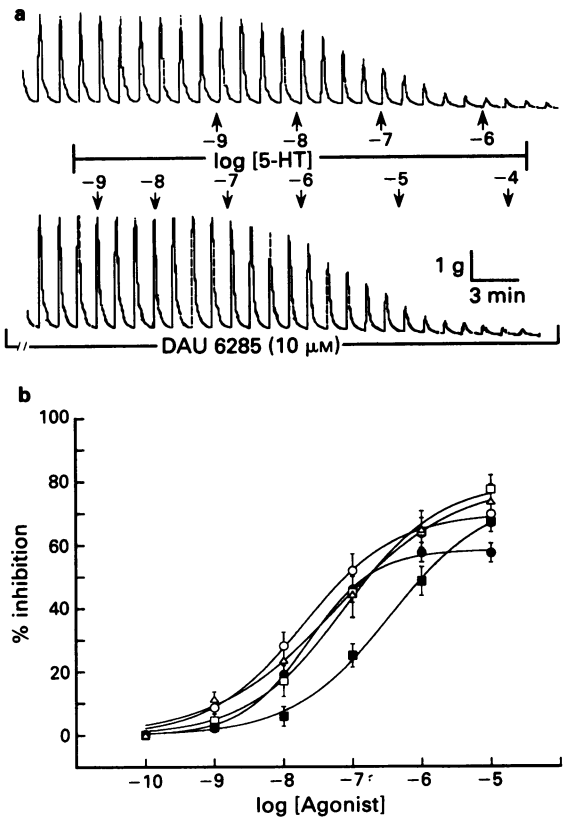

Figure 1 (a) Cumulative additions of 5-HT caused inhibition of electrically stimulated contractions in strips of *Rhesus* monkey urinary bladder (upper trace), an effect that was blocked in the presence of the selective 5-HT$_4$ receptor antagonist DAU 6285 (10 μM; lower trace). (b) Agonist concentration-inhibition curves to 5-HT (○, *Rhesus* monkey; ●, *Cynomolgus* monkey), BIMU 8 (□, *Rhesus*; ■, *Cynomolgus*), and 5-methoxytryptamine (△, *Rhesus*). Each point represents the arithmetic mean ± s.e.mean for 2–13 experiments (see Table 1 for potency estimates and *n* values).

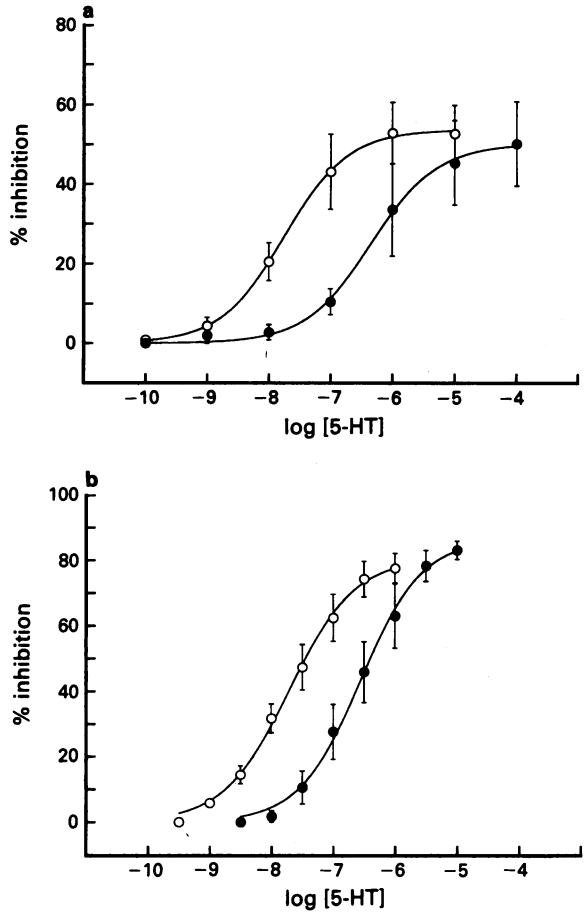

Figure 2 (a) Cumulative concentration-inhibition curves to 5-HT in the absence (○) and presence (●) of the novel, selective 5-HT$_4$ receptor antagonist, GR 113808 (10 nM) in electrically field-stimulated strips of *Cynomolgus* monkey urinary bladder. Each point represents the arithmetic mean ± s.e.mean for 5 experiments. (b) Cumulative concentration-inhibition curves to 5-HT in the absence (○) and presence (●) of DAU 6285 (1 μM) in electrically field stimulated strips of *Rhesus* monkey urinary bladder. Each point represents the arithmetic mean ± s.e.mean for 4 experiments.

Table I Agonist potencies and antagonist affinity estimates at the 5-HT$_4$ receptor in urinary bladder of *Rhesus* and *Cynomolgus* monkey

Agonist	*pEC$_{50}$ (95% CL)*	n	*Antagonist*		*[a]pA$_2$ (95% CL)*	n
Rhesus						
5-HT	7.8 (7.6–8.0)	13	DAU 6285	(1 μM)	7.0 (6.8–7.3)	4
			DAU 6285	(10 μM)	7.5 (7.5–7.6)	4
			RS 23597-190	(1 μM)	7.3 (6.8–7.9)	5
5-MeOT	7.1 (6.4–7.8)	2	DAU 6285	(1 μM)	7.0	2
Renzapride	7.2	1	DAU 6285	(3 μM)	6.9	1
BIMU 8	7.1 (6.7–7.5)	2	DAU 6285	(1 μM)	7.4	2
Cynomolgus						
5-HT	7.6 (7.4–7.8)	10	DAU 6285	(1 μM)	7.1 (7.0–7.3)	4
			GR 113808	(10 nM)	9.5 (9.1–9.8)	5
BIMU 8	6.5 (6.1–6.8)	4	DAU 6285	(1 μM)	6.8	1

[a]Single point analyses.

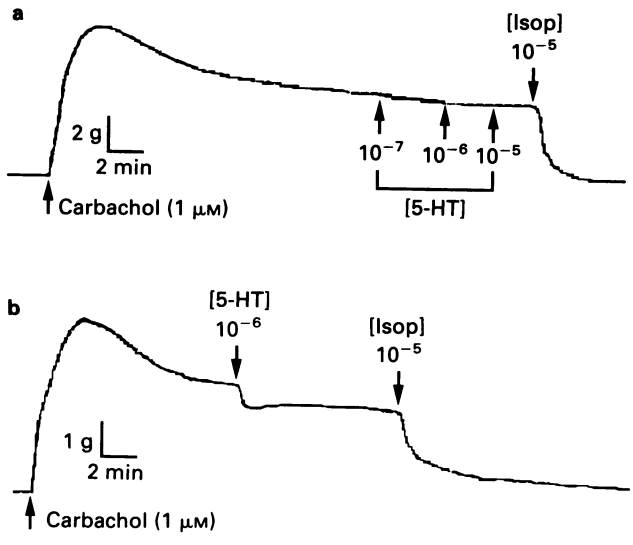

Figure 3 Two strips from the same *Rhesus* monkey urinary bladder responded differently to 5-HT following contraction with carbachol (1 μM). (a) One strip failed to relax upon addition of 5-HT whereas a corresponding strip from the same bladder (b) relaxed upon addition of 5-HT (1 μM). Further relaxation in the responsive strip (b) was achieved with isoprenaline (Isop, 10 μM). These two strips are representative of the inconsistency that hampered studies with carbachol-induced contraction of urinary bladder.

Inhibition of carbachol-induced contracture

Contractures induced by the addition of carbachol (1 μM) were strong (40–100 mN) and slow to develop (Figure 3). However, subsequent addition of 5-HT (up to 10 μM) caused relaxation in only some strips of bladder (Figure 3). Relaxation to 5-HT, when present, was unaffected by TTX (3 μM), suggesting a non-neuronally-mediated effect. Furthermore, relaxation by 5-HT versus carbachol was proportionately less than that induced by 5-HT against contractions to field stimulation (see below). Isoprenaline (10 μM) and forskolin (30 μM; data not shown), on the other hand, caused significant and consistent relaxation of the carbachol-induced contracture (Figure 3) independently of the effectiveness of 5-HT.

Localizing the 5-HT$_4$ receptor using direct muscle excitation

Figure 4 shows that electrically-evoked contractile responses were abolished by TTX (3 μM) and greatly inhibited ($\geqslant$ 90%) by atropine (1.0 μM), suggesting neuronal release of acetylcholine (ACh) as a mechanistic base for the contractile response. Lignocaine (100 μM) and ω-conotoxin GVIA (0.1 μM) also inhibited the responses (Figure 4).

In the presence of TTX (3 μM) and atropine (1 μM), electrical stimulation (using an increased pulse width of 10 ms; all other parameters unchanged) evoked direct contractions of smooth muscle that were similar in magnitude to those evoked neurogenically (Figure 4). Figure 4 shows that these atropine and TTX-insensitive contractions were also resistant to lignocaine (100 μM) and ω-conotoxin GVIA (0.1 μM), militating against the possibility of TTX and atropine-resistant neuronal transmission. However, the responses were inhibited significantly by single doses of 5-HT (1 μM; Figure 4) and BIMU 8 (10 μM; data not shown). Full E/[A] curves to 5-HT yielded a pEC_{50} estimate of 7.9 ($n = 2$; Figure 5b). Inhibition by 5-HT was reversed rapidly by addition of a selective 5-HT$_4$ receptor antagonist, GR 113808 (Figure 5a), at a concentration (3 μM) approximately $10^4 \times K_D$ (Grossman *et al.*, 1992). These findings suggest a post-junctional location

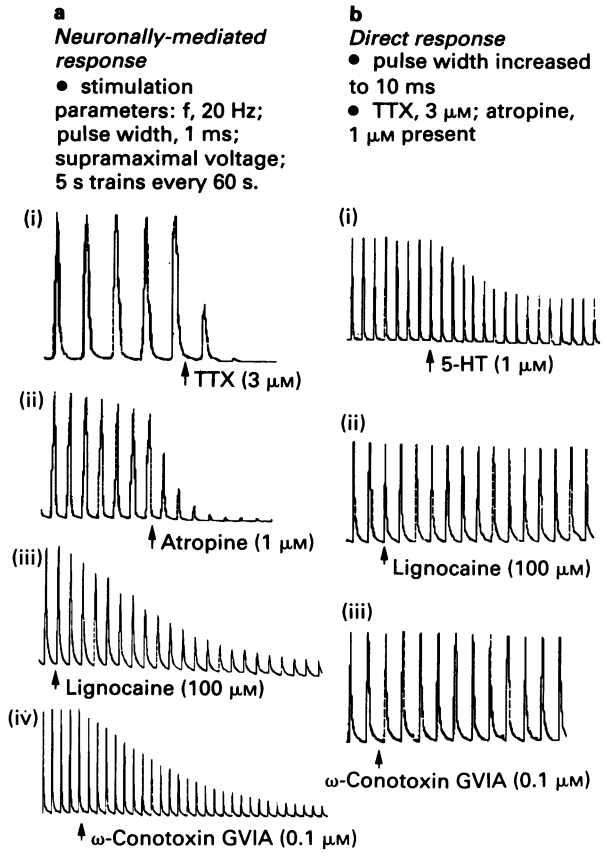

Figure 4 Location of 5-HT$_4$ receptors in strips of urinary bladder of *Rhesus* and *Cynomolgus* monkey by comparison of contractions evoked neurogenically and by direct stimulation of muscle. (a) Neurogenically-evoked contractions were abolished by tetrodotoxin TTX (3 μM; (i)) and greatly inhibited by atropine (1 μM; (ii)) indicating a neuronal release of ACh as the basis for the contractile response. Other inhibitors of neuronal function, lignocaine (100 μM; (iii)) and ω-conotoxin GVIA (0.1 μM; (iv)) also inhibited responses. (b) Contractions of similar magnitude were evoked in the presence of TTX (3 μM) and atropine (1 μM) by increasing the pulse width from 1 ms to 10 ms. These responses were resistant to inhibition by lignocaine (100 μM; b (ii)) and ω-conotoxin GVIA (0.1 μM; (b) (iii)) indicating that they were not the result of TTX-resistant neuronal transmission. Contractions elicited via direct stimulation of muscle (b(i)) were inhibited consistently by 5-HT (1 μM) or BIMU 8 (10 μM; data not shown), demonstrating the presence of post-junctional 5-HT$_4$ receptors in urinary bladder of monkey.

of the 5-HT$_4$ receptor. Due to limited tissue availability, concentration-inhibition curves to other 5-HT$_4$ receptor agonists were not constructed.

Use of different anatomical regions of the bladder and drug free Tyrode

Electrical stimulation of strips taken from other regions of the bladder (dome and bladder neck) evoked contractile responses of consistent magnitude which were inhibited by 5-HT ($n = 1$, data not shown), suggesting that the response to 5-HT (inhibitory versus excitatory) in monkey bladder is not a function of anatomical region. Furthermore, the use of Tyrode solution free from drugs to facilitate agonist equilibrium and 5-HT$_4$ receptor isolation did not affect the potency of 5-HT ($pEC_{50} = 7.7$; $n = 1$; data not shown) and BIMU 8 ($pEC_{50} = 6.4$; $n = 1$; data not shown) nor the affinity estimate for DAU 6285 ($pA_2 = 6.8$ vs. BIMU 8; $n = 1$; data not shown). Nevertheless, all other experiments conducted in the present study used Tyrode solution containing 5-HT receptor antagonists and drugs to promote equilibrium conditions.

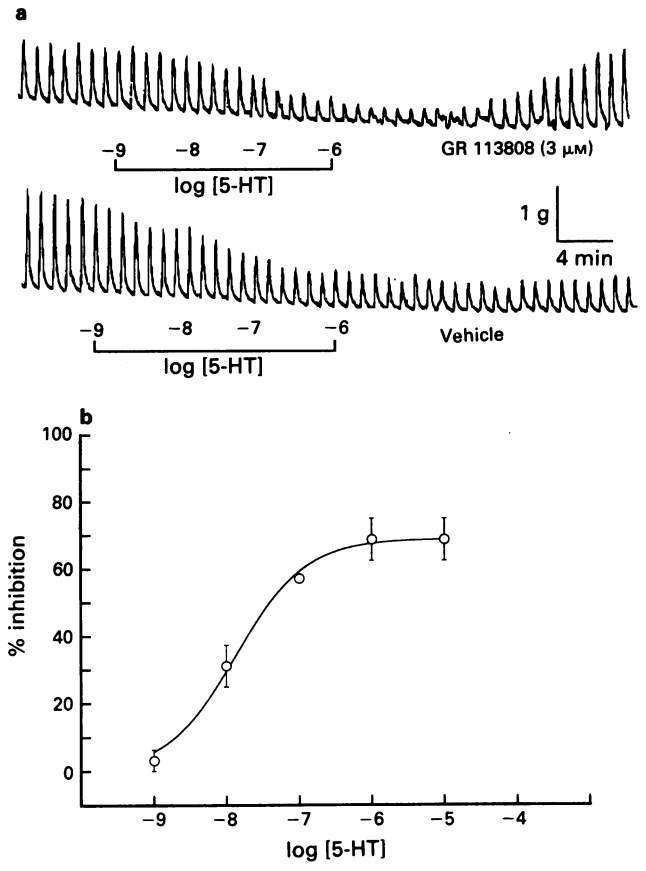

Figure 5 Determining the location of the 5-HT$_4$ receptor in monkey urinary bladder. (a) Increasing pulse width to 10 ms in the presence of tetrodotoxin (TTX, 3 μM) and atropine (1 μM) evoked contractions in strips of *Cynomolgus* monkey urinary bladder via direct stimulation of the muscle which were sensitive to inhibition by 5-HT (1 nM–1 μM). This inhibitory effect of 5-HT was readily reversed by the novel, selective 5-HT$_4$ receptor antagonist GR 113808 (3 μM; upper trace) whereas the addition of vehicle to a corresponding strip (lower trace) did not affect the inhibition caused by 5-HT. (b) Concentration-inhibition curve to 5-HT under conditions of direct stimulation of muscle yields a pEC$_{50}$ value of 7.9 in *Cynomolgus* monkey urinary bladder. Each point represents the arithmetic mean ± range for 2 experiments.

Discussion

The present study demonstrates that 5-HT inhibits electrically-evoked contractions of isolated urinary bladder from *Rhesus* and *Cynomolgus* monkeys through activation of a 5-HT$_4$ receptor. This inhibitory action of 5-HT contrasts with its excitatory action in strips of isolated bladder of man (Corsi *et al.*, 1991).

The presence of a 5-HT$_4$ receptor in the bladder of monkey is indicated strongly by the fact that 5-HT$_4$ receptor agonists from three distinct structural classes (indoleamine derivatives, 5-HT, 5-methoxytryptamine; substituted benzamides, renzapride; benzimidazolones, BIMU 8) evoked inhibition. Furthermore, three antagonists at the 5-HT$_4$ receptor caused parallel displacements of E/[A] curves to 5-HT (and other agonists) yielding single point pA$_2$ estimates similar to those generated in standard 5-HT$_4$ assay systems (GR 113808, Grossman *et al.*, 1992; DAU 6285, Bockaert *et al.*, 1992; RS 23597-190, Eglen *et al.*, 1992). Due to a limited supply of tissue, Schild regression analyses for unequivocal estimates of antagonist affinity were not possible.

One important point regarding the currently described pharmacological profile is the relatively low agonist potency of BIMU 8 (8–10 fold lower than 5-HT). This is not in accord with 5-HT$_4$ receptors defined previously where BIMU 8 is usually equipotent with 5-HT (Bockaert *et al.*, 1992). Indeed, the potency estimate for BIMU 8 in the *Cynomolgus* monkey (pEC$_{50}$ = 6.6) is lower than its estimated binding affinity at 5-HT$_4$ receptors in guinea-pig striatum (p*Ki* = 7.9; Grossman *et al.*, 1992). This result is not in agreement with receptor theory and, if reproducible, may point to the existence of heterogeneity among 5-HT$_4$ receptors as has been discussed elsewhere (Ford & Clarke, 1993).

Attempts were made to determine a pre- or postsynaptic location for the 5-HT$_4$ receptor in urinary bladder of monkey. Near-complete inhibition of electrically-stimulated contractions by TTX and atropine indicate that contractions are mediated neurogenically, largely through the release of ACh. In the presence of TTX, 5-HT inhibited carbachol-induced contracture, implying that 5-HT is not acting through neuronally based receptors. However, the interaction of 5-HT with carbachol yielded ambiguous results as the response to 5-HT was inconsistent and less effective (proportionately) than versus neuronally-mediated responses. Compared with neuronally-mediated contractile responses (evoked electrically), contractions to carbachol were sustained, greater in magnitude and slower to develop, indicating a certain lack of congruity between the two methods of evoking contractions.

In order to investigate better the location of the 5-HT$_4$ receptor, direct stimulation of the smooth muscle was employed. Under these conditions (increased pulse width, in the presence of TTX and atropine), responses were obtained which were equivalent to neuronally-mediated contractions with regard to time-course and tension development. Furthermore, ω-conotoxin GVIA (0.1 μM) and lignocaine (100 μM) failed to inhibit the responses but 5-HT and BIMU 8 caused inhibition of induced contractions. E/[A] curves to 5-HT yielded potency values similar to those generated against neurogenically-mediated contractions. Furthermore, 5-HT-induced inhibition of contractions was reversed completely by addition of the selective 5-HT$_4$ receptor antagonist GR 113808 (Grossman *et al.*, 1992), indicating that inhibition is mediated by post-junctional 5-HT$_4$ receptors. The possibility of some neuronal 5-HT$_4$ receptors, in addition to post-junctional 5-HT$_4$ receptors cannot, however, be eliminated entirely.

Previous reports have indicated that 5-HT$_4$ receptors in many tissues, including smooth muscle, couple preferentially to the stimulation of adenylyl cyclase and elevate intracellular cyclic AMP levels (see Bockaert *et al.*, 1992; Ford *et al.*, 1992a). Theoretically, if such a coupling mechanism were operational for 5-HT$_4$ receptors in the bladder of monkey, a locus of action on smooth muscle cells (where elevating cyclic AMP is characteristically relaxant) would be a more attractive explanation of the data than one on neurones (where elevating cyclic AMP characteristically enhances neurotransmitter release).

It may be postulated that the differential responses to 5-HT on the isolated urinary bladder of man (potentiation of contractions, Corsi *et al.*, 1991) versus monkey (inhibition of contractions, present study) result from the use of anatomically distinct regions (anterior dome, man; dorsal midline, monkey). Indeed, Klarshov & Hørby-Petersen (1986) found in pig that 5-HT contracts the detrusor muscle yet relaxes the trigone, bladder neck and urethral smooth muscle. Initial studies using samples from dome and neck of monkey bladder suggest that the 5-HT$_4$ receptor-mediated relaxations described above may be widespread through this hollow organ; further investigation will be required for confirmation. However, no such studies in the bladder of man were reported by Corsi *et al.* (1991). Based on our preliminary experiments, the response to 5-HT in human bladder also appears to be independent of anatomical region (Waikar, Ford & Clarke, unpublished observations). Similarly, the difference between the present study in monkey and that of Corsi *et al.* (1991) in man is not related to the use of drugs to

isolate pharmacologically the 5-HT_4 receptor (present study). Based upon previous reports (see Introduction), the most likely explanation is species variation. Such species variability in the response to 5-HT_4 receptor stimulation does not appear to be restricted to the urinary bladder. The effect of 5-HT_4 receptor activation on ileal smooth muscle, for example, is inhibitory in rat (Tuladhar *et al.*, 1991) yet excitatory in guinea-pig (contraction of ileum and potentiation of electrically-stimulated ileal contractions; Craig *et al.*, 1990). Furthermore, certain reflexes that involve both contraction and relaxation are facilitated by 5-HT_4 receptor agonism in guinea-pig (peristaltic reflex; Buchheit & Buhl, 1991; Craig & Clarke, 1991) and ferrets (emesis; Bhandari & Andrews, 1991).

Given the long-standing role of 5-HT in neuromodulation (Hen, 1993), it is conceivable that the 5-HT_4 receptor plays a key physiological role in modulating neuromuscular activity, particularly with regard to the regulation of movement and co-ordinated rhythmic activity in mammalian hollow organs (alimentary tract, urinary bladder, atria of heart). Although the source of 5-HT for such a role is not readily apparent (at least in heart and urinary bladder) it must be recalled that 5-HT has been detected in peripheral tissues, in addition to the alimentary tract, and may be released from sympathetic postganglionic neurones or chromaffin cells or both (Verbeuren, 1989; 1992). It is postulated, therefore, that the 5-HT_4 receptor is integrated into neuromuscular circuits so as to facilitate co-ordinated rhythmic activity of a functional nature (e.g. peristaltic reflex; contraction of the urinary bladder). This control may be manifested as inhibition in some species or excitation in others. Nevertheless, it provides an opportunity for the development of therapeutic agents for rhythmic disorders such as supraventricular arrhythmias, gastro-oesophageal reflux disorders, gastro-paresis, irritable bowel syndrome and urinary incontinence.

In conclusion, the present study demonstrates, for the first time, that 5-HT_4 receptors function to relax smooth muscle in the urinary bladder of two monkeys of the macaque family. A role for 5-HT_4 receptors in the physiology and pathology of urinary bladder function is postulated.

The authors wish to thank Susan Winslow, Syntex Research, for provision of the tissues used in these studies and Dr C.A. Rizzi, Institute De Angeli, Milan, Italy, for his gifts of DAU 6285 and BIMU 8.

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(Received May 4, 1993
Revised August 14, 1993
Accepted October 4, 1993)

Br. J. Pharmacol. (1994), **111**, 219–226

A new method for estimating dissociation constants of competitive and non-competitive antagonists with no prior knowledge of agonist concentrations

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1 A method is presented which enables the dissociation constant (K_I) of a competitive, pseudo-irreversible or non-competitive antagonist-receptor complex to be estimated without knowledge of agonist concentrations.

2 The technique has been tested using sets of concentration-response data which simulated these various types of antagonism.

3 The points for each set of simulated data could be plotted both as agonist concentration-response curves at fixed antagonist concentrations and *vice versa*, producing paired data sets.

4 pK_I-values were estimated from such paired data sets using appropriate graphical and computer curve-fitting methods.

5 For competitive antagonism, for each paired data set the computer curve-fitting techniques gave the same value for pK_I, assuming drug-receptor interaction to be 1:1 and agonist concentrations to be known.

6 When agonist concentrations were assumed unknown, pK_Is could not be estimated by the conventional method (using agonist dose-ratios) but could still be obtained (for competitive, pseudo-irreversible and non-competitive antagonism) by the new method.

7 This new method should be especially useful for measuring dissociation constants of antagonists against neuronally- or ionophoretically-released agonists. It may also be useful when agonist is applied exogenously, especially if suitable drugs are not available to block agonist uptake and/or metabolism.

Keywords: Antagonists; dissociation constants; affinity constants; pK_I values; pA_2 values; neurotransmitters; ionophoresis

Introduction

The classical method for estimating the dissociation constant of a competitive antagonist-receptor complex requires a comparison of agonist concentration-response curves measured on isolated cells or tissues in the absence and in the presence of a range of fixed concentrations of the antagonist (Arunlakshana & Schild, 1959). Such results allow estimation of the dissociation constant, usually expressed as a pA_2 or pK_I-value (i.e. $-\log_{10}$ (dissociation constant)), and provide a test for deviation from competitive antagonism. The classical method of analysis is based on the assumption that the concentrations of agonist and of antagonist at the receptors are the same as those in the surrounding medium. If these latter assumptions are not valid then incorrect dissociation constants may be obtained (see e.g. Kenakin, 1984). For these reasons it is sometimes necessary to add other substances to the medium to block uptake and/or metabolism of the agonist or antagonist. Since the classical method requires a knowledge of agonist dose-ratios it can be applied only to curves obtained with exogenously applied agonist.

More recently an alternative method for estimating pA_2-values has been suggested by Poch *et al.* (1990, 1992). Their method is based on the use of antagonist concentration-response curves derived from either complete or incomplete agonist concentration-response curves, but still depends on a knowledge of agonist dose-ratios. The aim of the present paper is to set out in detail the basis of a new method, previously presented in outline (Mackay, 1992), for estimating dissociation constants of competitive and non-competitive antagonists from antagonist concentration-response curves without knowledge of agonist concentrations. The advantages and limitations of the new method will be compared with those of the conventional method.

Theory

If several log concentration-response curves are measured for an agonist, each in the presence of a different fixed concentration of a competitive antagonist, then the curves obtained would be expected to be parallel and appropriately displaced (see Figure 1a). Poch *et al.* (1990) pointed out that antagonist log concentration-response curves, can be derived from such agonist log concentration-response curves (see Figure 1b). Intuitively this suggests that both sets of curves may contain the same information.

One approach for obtaining information from antagonist curves is to derive a suitable null equation for antagonist concentrations which produce equal responses. Each antagonist concentration-response curve is measured at a fixed agonist concentration. For two curves (such as those fitted with the solid lines in Figure 1b) at any chosen response level molar concentrations $[A]'$ of agonist and $[I]'$ of antagonist, measured for one curve, produce the same response as concentrations $[A]''$ and $[I]''$ measured from the other curve. If the antagonism is assumed to be competitive then the proportion of receptors occupied by agonist at $[A]'$ is

$$p'_A = \frac{1}{1 + \dfrac{1 + [I]'/K_I}{[A]'/K_A}} \qquad (1)$$

where K_I is the dissociation constant of the antagonist-receptor complex and K_A is an apparent dissociation constant of the agonist-receptor complex. A similar equation would apply for p_A'', the proportion of receptors occupied when

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concentration $[A]''$ of agonist produces a steady-state occupancy of receptors in the presence of concentration $[I]''$ of the antagonist. Then for equal responses, from equal receptor occupancies by agonist,

$$1 + \frac{1 + [I]'/K_I}{[A]'/K_A} = 1 + \frac{1 + [I]''/K_I}{[A]''/K_A} \quad (2)$$

which can be rearranged to give

$$\frac{1 + [I]''/K_I}{1 + [I]'/K_I} = \frac{[A]''}{[A]'} \quad (3)$$

Equation 3 is a generalized form of the Schild equation (using dissociation constants in place of affinity constants and with $[I]'$ not equal to zero) and should apply whether the curves compared are obtained by varying values of $[A]$ with fixed values of $[I]$, or *vice versa*. If however the values of $[A]''$ and $[A]'$ are kept constant while the antagonist concentrations are varied then the ratio $[A]''/[A]'$ is constant at all response levels for the two curves being compared. For these conditions equation 3 can be rearranged to give

$$[I]'' = (m - 1)K_I + m[I]' \quad (4a)$$

where

$$m = [A]''/[A]' \quad (4b)$$

A plot of $[I]''$ versus $[I]'$ for equal responses should therefore give a straight line of slope m and intercept $(m - 1)K_I$, so that

$$K_I = \text{intercept}/(\text{slope} - 1) \quad (5)$$

If the binding of the antagonist is reversible but it is not displaced to any significant extent by agonist during the chosen agonist contact time then it behaves pseudo-irreversibly. Under such conditions

$$p'_A\,(1 - p'_I) = p''_A\,(1 - p''_I) \quad (6)$$

where p_I represents the proportion of receptors occupied by the antagonist. This equation again leads to equations 4a and 5, but with the constant

$$m = \left\{1 + \frac{K_A}{[A]'}\right\} \Big/ \left\{1 + \frac{K_A}{[A]''}\right\} \quad (7)$$

Equations 4a and 5 can also be derived for an antagonist which blocks the action of an agonist by binding to another site on the same receptor. Equation 4a should therefore be capable of providing estimates of the dissociation constant of an antagonist-receptor complex whether the antagonist is competitive or non-competitive acting under equilibrium conditions, or is competitive but acting pseudo-irreversibly, provided that the agonist and antagonist interact with the receptor on a 1:1 molecular basis.

Generation of simulated data

In order to test the ideas set out in the previous section an ideal agonist concentration-response curve was generated using the general logistic equation

$$r = \frac{(a - d)}{1 + \left\{\frac{[A]}{c}\right\}^b} + d \quad (8)$$

where r is the response, $[A]$ is the chosen concentration of agonist and a, b, c and d are parameters with chosen numerical values. Since each agonist concentration-response curve produced in the presence of a chosen concentration of a competitive antagonist under equilibrium conditions is related to the first agonist curve through the modified Schild equation

$$[A]'/[A] = (1 + [I]'/K_I) \quad (9)$$

it follows that each curve in the family of curves should fit the equation

$$r = \frac{(a - d)}{1 + \left\{\frac{[A]}{c\,(1 + [I]/K_I}\right\}^b} + d \quad (10)$$

where $[A]$ is now the agonist concentration applied in the presence of any antagonist concentration $[I]$. When $[I]$ is zero equation 10 simplifies to equation 8.

In order to simulate the results of experiments carried out with a competitive antagonist under equilibrium conditions ideal responses were calculated using the above equation for fixed values of a, b, c, d and K_I, and for a range of chosen values of $[A]$ and $[I]$. Random errors were superimposed on these ideal responses to generate thirty sets of non-ideal simulated data. The random errors were obtained from a normal population with a standard deviation of 2.4 response units (i.e. 3% of the maximal response) regardless of the response level at which they were applied.

Ten sets of simulated data were also produced essentially as described above assuming the antagonist to behave pseudo-irreversibly. However in this case the equation, which was derived from equation 6 and used to calculated the ideal responses, was

$$r = \frac{(a - d)}{1 + \left\{\frac{[A]}{c\,([I][A]/K_IK_A + (1 + [I]/K_I))}\right\}^b} + d \quad (11)$$

Equation 11 can also be derived for a non-competitive antagonist which blocks the action of an agonist by interacting with another site on the same receptor. Use of equation 11 required additionally the choice of a numerical value for K_A.

A typical example of one of the sets of simulated data for a competitive antagonist under equilibrium conditions has been plotted in Figure 1a as a series of agonist log concentration-response curves at fixed antagonist concentrations. The same data points have been plotted as antagonist concentration-response curves in Figure 1b. Analogous plots of a set of simulated data for pseudo-irreversible conditions are shown in Figures 2a and 2b.

Graphical analysis of simulated data

The set of simulated agonist log concentration-response curves shown in Figure 1a has been analysed by the classical graphical method. For purely illustrative purposes continuous curves have been drawn through ideal results calculated with no random error. Dose-ratios measured at the 50% response level were used to obtain an Arunlakshana-Schild (AS) plot (Figure 3a).

The same simulated experimental data points have been plotted, at fixed agonist concentrations, in Figure 1b. Here too the smoothed curves have been drawn through ideal results calculated with no random error. Two of the curves, drawn with solid lines cover a wide range of responses. To illustrate application of the new method for estimating pK_I, these two curves were compared at various response levels (each 5 units apart) in the response range 50 to 20 units. The antagonist concentrations $[I]''$ and $[I]'$ which produced those chosen responses were read from the curves and plotted one against the other as shown in Figure 3b.

Use of simultaneous non-linear regression to analyse the simulated data

Since the simulated data points at fixed concentrations of competitive antagonist were generated by adding random

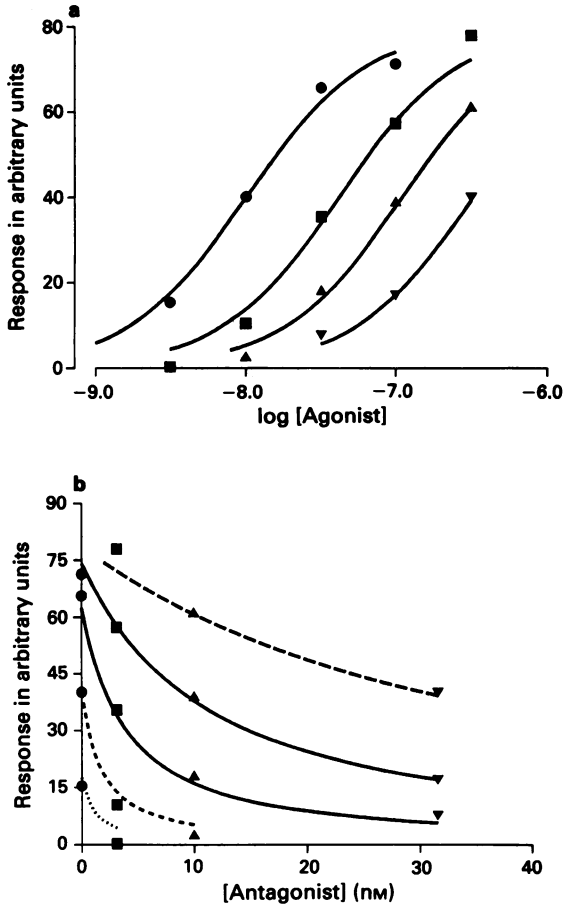

Figure 1 (a) A typical set of data simulating results expected using a competitive antagonist. Data are plotted as agonist log concentration-response curves as fixed concentrations of antagonist. The smoothed curves have been drawn through calculated ideal results. Data points were obtained from the ideal results by superimposing errors chosen randomly from a normal distribution with a standard deviation of 2.4 response units regardless of the response level. The antagonist concentrations 0, 3.16, 10 and 31.6 nM are indicated by the symbol (●), (■), (▲) and (▼), respectively. (b) A typical set of data simulating results expected using a competitive antagonist. Data are plotted as antagonist concentration-response curves at fixed agonist concentrations of 316, 100, 31.6, 10 and 3.16 nM. The smoothed curves have been drawn through calculated ideal results. Data points were obtained from ideal results by superimposing errors chosen randomly from a normal distribution with a standard deviation of 2.4 response units regardless of the response level. The responses at antagonist concentrations 0, 3.16, 10 and 31.6 nM are indicated by the symbols (●), (■), (▲) and (▼), respectively. Data points are the same as those plotted as agonist log concentration-response curves in (a).

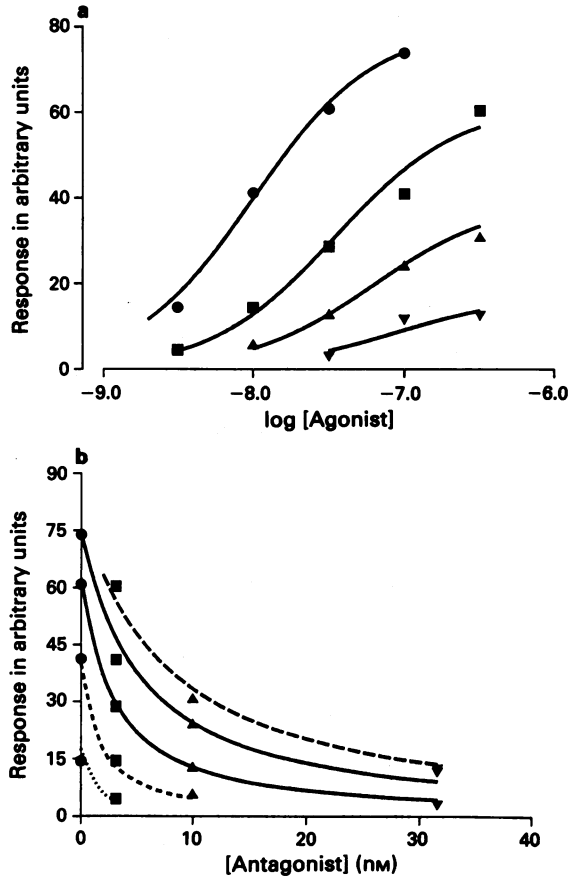

Figure 2 (a) A typical set of data simulating results expected using a competitive antagonist acting under pseudo-irreversible conditions, or alternatively a non-competitive antagonist. Data are plotted as agonist log concentration-response curves at fixed concentrations of antagonist. The smoothed curves have been drawn through calculated ideal results. Data points were obtained from the ideal results by superimposing errors chosen randomly from a normal distribution with a standard deviation of 2.4 response units regardless of the response level. The antagonist concentrations 0, 3.16, 10 and 31.6 nM are indicated by the symbols (●), (■), (▲) and (▼), respectively. (b) A typical set of data simulating results expected using a competitive antagonist acting under pseudo-irreversible conditions, or alternatively a non-competitive antagonist. Data are plotted as antagonist concentration-response curves at fixed agonist concentrations of 316, 100, 31.6, 10 and 3.16 nM. The smoothed curves have been drawn through calculated ideal results. Data points were obtained from ideal results by superimposing errors chosen randomly from a normal distribution with a standard deviation of 2.4 response units regardless of the response level. The responses at antagonist concentrations 0, 3.16, 10 and 31.6 nM are indicated by the symbols (●), (■), (▲) and (▼), respectively. The data points are the same as those plotted as agonist log concentration-response curves in (a).

errors to ideal data which fitted equation 10, it would have been possible to use that equation to obtain estimates of log K_I from the mock data, assuming antagonism to be competitive. However, the classical test for competitive antagonism is to test if the slope of an AS plot deviates significantly from unity, though this test does not check directly for non-parallelism of a set of agonist concentration-response curves. A linear AS plot with a slope of N, where N differs from unity, could indicate that the antagonist produces parallel displacement of agonist concentration-response curves but the dose-ratios do not fit equation 9. Such results could be obtained if the antagonist concentration in equation 9 is raised to the power N. Although this classical test for deviation from competitive antagonism is not entirely satisfactory, equation 10 has been modified by raising $[I]$ to the power N, producing equation 12 for fitting agonist concentration-response curves at fixed antagonist concentrations

$$r = \frac{(a-d)}{1+\left\{\dfrac{[A]}{c\,(1+[I]^N/K_I)}\right\}^b} + d \qquad (12)$$

A different equation was used to fit antagonist concentration-response curves at fixed agonist concentrations. If the curve at the highest agonist concentration (Figure 1b) is taken as a reference curve and if the data points can be fitted satisfactorily to an appropriate equation (e.g. a logistic eqn.) then it should be possible to combine that with equation 4a to obtain a more general equation which will fit each member

of the family of curves shown in Figure 1b. On the basis of these assumptions an appropriate general equation would be

$$r = \frac{\alpha - \delta}{1 + \left\{ \frac{(m-1)K_I + m[I]}{\gamma} \right\}^{\beta}} + \delta \qquad (13)$$

where r, m, K_I and $[I]$ have the same meanings as before and α, β, γ and δ are the adjustable parameters for fitting the logistic equation to the reference curve with m set equal to unity for that particular case. Equation 13 should also be able to fit antagonist concentration-response curves for a competitive antagonist behaving pseudo-irreversibly or for a non-competitive antagonist (see theoretical section). If however, antagonist concentrations are raised to the power N for reasons discussed above, then the appropriate equation for fitting antagonist concentration-response curves at fixed agonist concentrations becomes

$$r = \frac{\alpha - \delta}{1 + \left\{ \frac{\{(m-1)K_I + m[I]^N\}^{(1/N)}}{\gamma} \right\}^{\beta}} + \delta \qquad (14)$$

The computer programme ALLFIT (De Lean *et al.*, 1978) was modified, as described in Appendices A and B, to enable equations 12 and 14 to be fitted to each of the 30 paired sets of mock data, of the kind shown in Figures 1a and 1b respectively. Equation 14 has also been used to fit the 10 sets of simulated antagonist concentration-response curves, of the kind shown in Figure 2b, which can mimic either pseudo-irreversible or non-competitive antagonism. When fitting these equations to any data set all of the data points, from complete and from incomplete curves, were used.

When fitting equation 12 to agonist concentration-response curves, the values of a, b, c, d and K_I were forced to be the same, though adjustable, for each curve in any one data set. These assumptions imply that the tissue sensitivity does not change during the experiment and that K_I is a true constant.

The question of whether N deviated significantly from unity could be tested either for each individual data set or over all datasets. In the former case for each data set competitive antagonism could either be assumed by setting the value of N to unity (Fit 1) or be tested for by allowing N to deviate from unity (Fit 2). The 'goodness' of these fits, expressed in terms of the sums of squares about regression, could be compared to assess the statistical significance of any deviation of N from unity. The test was based on variance ratio

$$F = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{SS_2/DF_2} \qquad (15)$$

where SS_1 is the greater sum of squared deviations, corresponding to the smaller number of adjustable parameters and therefore to the greater number of degrees of freedom DF_1. In this instance SS_1 and DF_1 refer to the first fit with N forced equal to 1.00 and SS_2 and DF_2 refer to the second fit with N allowed to optimise. A statistically significant value for F would suggest that N differs significantly from unity for that data set. Deviation of N from unity could also be tested by using a t test to compare the mean value of N, for a group of data sets, with the theoretical value of unity.

When fitting equation 14 to antagonist curves for each simulated data set the values of α, β, γ, δ and K_I were assumed to be the same for each curve in that data set. For the 30 data sets simulating equilibrium competitive antagonism, if agonist concentrations were assumed to be known then each parameter m could be given a fixed, accurate numerical value. Under these conditions the value of N could either be set equal to 1.00 or allowed to optimise, as described above. Alternatively, with the value of N fixed at 1.00, values of m could be allowed to optimise and these values could be compared with the true values known from the initially chosen agonist concentrations. In many cases it was also possible to allow the values of pK_I, m and N to optimise simultaneously.

The basic principles outlined above were also applied when equation 14 was used to analyse the 10 sets of data capable of simulating either pseudo-irreversible or non-competitive antagonsim.

Results

Graphical analysis of the smoothed curves shown in Figure 1a produced an AS plot (Figure 3a) which gave a straight line of slope 0.999 and a pA_2 of 8.99. Comparison of the smoothed curves shown as thick lines in Figure 1b produced a plot of $[I]''$ vs $[I]'$ (Figure 3b) which gave a straight line with a slope of 3.096 and an intercept of 2.016 nM. From equation 5 the value of pK_I was therefore estimated to be 9.02. The true value of pK_I was 9.00. The actual data points shown in Figure 1b gave pK_I as 8.971 when analysed by the curve-fitting programme for agonist concentration-response curves and the very same value when analysed by the programme for antagonist concentration-response curves. In both cases N was set equal to 1.00 and the agonist concentrations were assumed to be known.

The mean results obtained using the computer curve-fitting programme to fit the 30 paired sets of simulated data, with

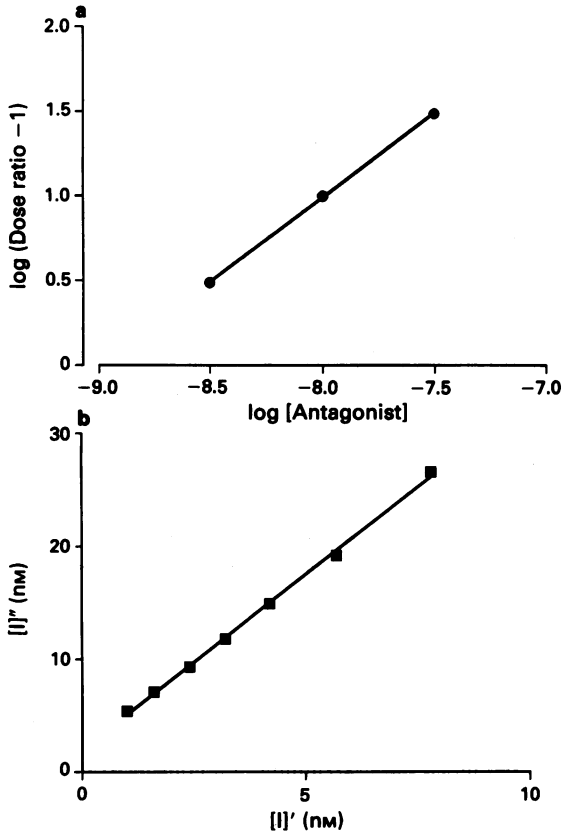

Figure 3 (a) A Schild plot produced from the simulated agonist log concentration-response curves determined at fixed concentrations of competitive antagonist, shown in Figure 1a. The best straight line has a slope of 0.999 and gives a pA_2 value of 8.99. (b) A plot of [I]'' vs [I]' read from two antagonist concentration-response curves drawn with solid lines in Figure 1b. Each agonist curve was determined at a fixed agonist concentration. [I]'' and [I]' are the concentrations of antagonist which, for the pair of curves, produce equal responses at chosen response levels. In this plot the agonist concentrations are assumed to be unknown.

Table 1 Estimates of various parameters, with standard errors, from 30 sets of data simulating results of competitive antagonism, considered as agonist concentration-response curves at fixed antagonist concentrations and *vice versa*

Curve analysis	N†	pK_I††	m_2*	m_3*	m_4*	m_5*
(a) Agonist	1.00	9.004	–	–	–	–
curves	fixed	±0.009	–	–	–	
(b) Agonist	1.020	9.165	–	–	–	–
curves	±0.009	±0.074	–	–	–	–
(c) Antagonist	1.00	9.004	3.16	10.00	31.6	100.0
curves	fixed	±0.009	fixed	fixed	fixed	fixed
(d) Antagonist	1.021	9.194	3.16	10.00	31.6	100.0
curves	±0.010	±0.072	fixed	fixed	fixed	fixed
(e) Antagonist	1.00	8.992	3.066**	9.539**	32.74**	98.48**
curves	fixed	±0.022	±0.061	±0.273	±1.85	±8.30

Notes: †N is the power to which the antagonist concentration is raised, and should be 1.00 for competitive antagonistm
††The true value of pK_I was 9.000.
*m_x represents the agonist concentration-ratio of the control curve relative to curve x, either fixed when agonist concentrations are assumed known, or estimated from antagonist concentration-response curves when agonist concentrations are not known.
**The true values of these agonist concentration-ratios were 3.16, 10.00, 31.6 and 100.

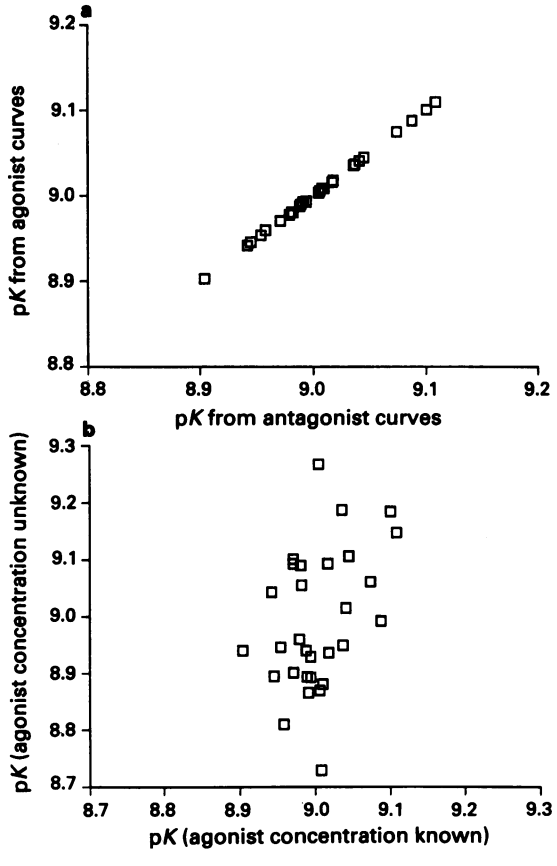

Figure 4 (a) A comparison of pK_I-values estimated from agonist concentration-response curves at fixed antagonist concentrations with those estimated from antagonist curves at fixed agonist concentrations. In these analyses drug:receptor interaction was assumed to be 1:1 and agonist concentrations were assumed known. The results are based on 30 paired sets of simulated data. The slope of the linear correlation is 1.002 and the correlation coefficient is 0.999 ($P<0.001$). (b) A comparison of pK_I-values estimated from antagonist concentration-response curves at fixed agonist concentrations assuming no knowledge of agonist concentrations, with those values obtained from the same data assuming the agonist concentrations to be known. In these analyses drug:receptor interaction was assumed to be 1:1. The results are based on 30 sets of simulated data. The slope of the linear correlation is 1.007 and the correlation coefficient is 0.3886 ($P<0.05$).

various assumptions, are summarized in Table 1 and in Figures 4a and 4b.

Analyses (a) and (b) (Table 1) show respectively estimates of pK_I from the sets of agonist concentration-response curves (a) assuming N to be unity and (b) allowing N to deviate from unity. Analyses (c) and (d) (Table 1) show the results of analysing the corresponding sets of antagonist concentration-response curves (c) assuming N to be unity and (d) allowing N to deviate from unity. For each paired dataset the numerical value of pK_I obtained from analysis of the agonist concentration-response curves was extremely close to that obtained from analysis of the antagonist concentration-response curves, assuming N to be unity in both cases. The correlation between these estimates of pK_I, from analyses (a) and (c), is shown in Figure 4a.

Analyses were also carried out on each set of antagonist concentration-response curves (assuming N to be unity but agonist concentrations to be unknown) to obtain estimates of pK_I and of the agonist concentration-ratio for each curve relative to the control curve (analysis (e), Table 1). The correlation between values of pK_I obtained in analysis (e) and those obtained in analysis (c) is shown in Figure 4b. When pK_I, m and N were all allowed to vary simultaneously, successful optimisation was achieved for only 21 out of the 30 datasets. Allowing N to deviate from unity did not produce a statistically significantly improvement in fit in any of these 21 cases (see equation 15).

When equation 14 was used to analyse the 10 sets of data which simulated the results expected for a competitive antagonist acting under pseudo-irreversible conditions (and could equally well represent results obtained with a non-competitive antagonist) the mean value of pK_I was 9.11 ± 0.06. In these analyses the value of N was assumed to be unity and the agonist concentrations assumed to be unknown. The mean best fit estimates of m were 1.58 ± 0.04, 3.53 ± 0.18, 10.74 ± 1.04 and 33.09 ± 4.77 which should be compared with the expected values 1.52, 3.16, 8.36 and 24.8 (calculated from equation 7). By contrast the actual applied concentration ratios were 3.16, 10.00, 31.6 and 100. When pK_I, m and N were all allowed to vary simultaneously, successful optimisation was achieved for 9 out of the 10 datasets. However allowing N to deviate from unity did not produce a statistically significant improvement in fit in any of these 9 cases.

Discussion

Analysis of a typical single set of simulated data shown as alternative plots in Figures 1a and 1b, gave very similar values for pK_I, when analysed by the classical AS plot and by a plot of $[I]''$ vs. $[I]'$, the latter being the graphical plot for the new method. However from Figures 1a and b it can be seen that it is easier to draw by eye a series of parallel agonist log concentration-response curves through a limited number of data points than it is to draw a series of non-

parallel antagonist concentration-response curves through the same data points. Indeed graphical methods which require drawing of curves by eye may be liable to appreciable bias and generally do not make efficient use of all data points. Additional errors can be introduced from reading equieffective drug concentrations from the curves, whether concentrations are plotted linearly or logarithmically. Another limitation of the classical AS plot is that it depends on the availability of the agonist curve obtained at zero concentration of antagonist. This limitation does not apply to the computer curve-fitting methods based on equation 3. The latter can be applied over any range of antagonist concentrations and are much less laborious.

Analysis of the simulated data sets, using the appropriate computer programme to fit all the data points on complete and incomplete agonist concentration-response curves in each dataset, gave a mean value of pK_I which was practically the same as the true chosen value when antagonism was assumed to be competitive (i.e. analysis (a), $N = 1.00$, Table 1). When N was allowed to optimise (analysis (b), Table 1) the deviation of N from unity was very small but statistically significant ($P = 0.04$). The corresponding value for pK_I was 9.165 ± 0.074. The deviation from the true value of 9.00 was again small but statistically significant ($P = 0.04$). The distribution of the individual estimates of N and of pK_I were as expected for a normal population even though the group means were very slightly biased from the true values. It should also be pointed out that the magnitude of random errors introduced into the simulated data was greater than that likely to be encountered with good experimental data. Analysis of the 30 sets of simulated data using the appropriate computer programmes to fit the antagonist concentration-response curves and the corresponding agonist concentration response curves in each data set, gave mean values for pK_I which were identical when N was fixed at 1.00 and the agonist concentrations were taken as known (compare analyses (a) and (c), Table 1). For each paired data set the numerical value of pK_I obtained in analysis (a) was practically identical to that obtained in analysis (c), as will be seen from the very good correlation ($P < 0.001$) shown in Figure 4a. When N was allowed to optimise for antagonist curves (analysis (d) Table 1), the value of N obtained and the corresponding value for pK_I were practically the same as values obtained from the corresponding analysis (b) of the agonist curves. For antagonist concentration-response curves with N fixed at 1.00, values could be estimated for pK_I and for the agonist concentration-ratio for each curve relative to the control curve (analysis (e) Table 1). In this case the estimated mean values of pK_I and of the agonist concentration-ratios were not significantly different from the original chosen values. However the paired values of pK_I obtained in analyses (e) and (c) did not correlate nearly as well as did those from analyses (a) and (c) (cf. Figure 3b and 3a)) though the correlation was still statistically significant ($P < 0.05$).

Attempts to fit each set of antagonist concentration-response curves while allowing N, pK_I and the concentration-ratios to optimise simultaneously were not always successful, and did not produce statistically significant improvements in fit of the theoretical equations to the data points. This applies to the simulated data for pseudo-irreversible antagonism and non-competitive antagonism as well as to that for equilibrium competitive antagonism. It is clear from analysis of simulated data, and from equations 4a, 4b, 5 and 7, that antagonist concentration-response curves do not permit any distinction between competitive antagonism under equilibrium and pseudo-irreversible conditions, or non-competitive antagonism, unless agonist concentrations are known.

These analyses of simulated data show that the null equations and computer programmes described here can be used to estimate parameters from agonist concentration-response curves at fixed antagonist concentrations and from antagonist concentration-response curves at fixed agonist concentrations. The programmes are based on a general technique for estimating parameters of null equations from concentration-response curves (Mackay, 1988), which in turn was an extension of methods described by Waud & Parker (1971). The methods used here depend on use of modified versions of the programme ALLFIT which allows simultaneous non-linear curve-fitting to a series of data sets. An example of the use of a commerically available programme, PCNONLIN, to produce simultaneous fits of concentration-response curves is given by James *et al.* (1989).

The analyses discussed above show that agonist concentration-response curves at fixed antagonist concentrations and antagonist concentration-response curves at fixed agonist concentrations can give the same information if agonist and antagonist concentrations at the receptors are known. They also show that the dissociation constant of a competitive antagonist can be estimated from antagonist concentration-response curves measured at fixed agonist concentrations without knowledge of the actual agonist concentrations. However for this to be feasible the antagonist must be allowed adequate time to equilibrate with the receptors. It must not be taken up or metabolised to any appreciable extent by the tissue. The antagonist may act competitively, pseudo-irreversibly or non-competitively provided that agonist and antagonist each interact with the receptor on a 1:1 molecular basis. Under these conditions the value of N in equation 14 can be set equal to unity. However the limitations concerning uptake and metabolism need not apply to the agonist.

Agonist concentrations at the receptors can be assumed known if the agonist is applied exogenously to a tissue in an organ bath, adequate time is allowed for equilibration of agonist with the receptors, and uptake and metabolism of the agonist by the tissue are blocked. If only graphical methods of analysis can be applied to such results then the classical AS plot is best, though a test of parallelism (such as that provided by the standard ALLFIT programme) might be a useful extra. On the other hand if general curve-fitting programmes are available, such as those used here, then analysis of agonist or of antagonist-concentration-response curves should be equally easy and equally satisfactory.

However the new method should have advantages over the classical method if the concentration of agonist at the receptors can be considered to be constant, though unknown, over a suitable period of time while the effect of the antagonist is being measured. This situation could arise if agonist is applied exogenously but blockers of agonist uptake and metabolism are not available. It could also arise if agonist is released from electrically stimulated nerves within the tissue or applied to cells by ionophoresis. In these latter cases the antagonist is likely to be known to act either competitively or pseudo-irreversibly, with N equal to unity, and to have been chosen as a pharmacological tool to characterize receptors.

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(Received August 9, 1993
Accepted October 5, 1993)

Appendix A

Modifications to the standard ALLFIT programme (written in Basic), required to fit equation 12 to a maximum of 10 curves, are listed below:

```
755  Dim I0(10)

1440 N = 6
     N$(1) = "A"
     N$(2) = "B"
     N$(3) = "C"
     N$(4) = "D"
     N$(5) = "E"
     N$(6) = "F"
     P(3) = 1

1510 PRINT"A IS THE RESPONSE AT ZERO
     AGONIST CONCENTRATION"
     PRINT"B IS THE SLOPE-FACTOR FOR THE
     CURVE"
     PRINT"C IS THE EC50 FOR THE AGONIST-
     ONLY CURVE"
     PRINT"D IS THE RESPONSE AT VERY HIGH
     AGONIST CONCENTRATIONS"
     PRINT"E IS -LOG(DISSOCIATION CONSTANT)
     OF THE ANTAGONIST-RECEPTOR COMPLEX"
     PRINT"F IS A 'HILL' FACTOR FOR THE
     ANTAGONIST CONCENTRATION"

1520 DEF FNF (X9,L9,I0)
     IF X9<=0 THEN X9 = 1E-20
     IF B(1 + N*(L9-1)) = 0 THEN B(1 + N*(L9-1)) =
     1E-3
     IF B(4 + N*(L9-1)) = 0 THEN B(4 + N*(L9-1)) =
     1E-3
     IF B(5 + N*(L9-1))<=2 THEN B(5 + N*(L9-1)) = 2
     Z1 = 1 + (10^B(5 + N*(L9-1)))*(I0^B(6 + N*(L9-1)))
     Z1 = (X9/(Z1*EXP(B(3 + N*(L9-1)))))^B(2 + N*(L9-1))
     A = ((B(1 + N*(L9-1))-B(4 + N*(L9-1)))/(1 + Z1))
                                  + B (4 + N*(L9-1))
     FNF = A
     END DEF

1660 DEF FND(X9,L9,I9,I0)
     D1 = FNF(X9,L9,I0)
     B(I9 + N*(L9-1)) = 1.001*B(I9 + N*(L9-1))
     D2 = FNF(X9,L9,I0)
     B(I9 + N*(L9-1)) = B(I9 + N*(L9-1))/1.001
     FND = (D2-D1)/(0.001*B(I9 + N*(L9-1)))
     END DEF
```

The remainder of the standard programme may be left unchanged except that

(a) all references to FNF and FND within the programme should be changed to include the term I0 and all reference to automatic initial parameter estimation deleted,

(b) the programme should be modified to read from the data, the concentration of antagonist (given the generalised symbol I0) for each agonist concentration-response curve, and

(c) a final printout of observed and calculated responses is useful.

Appendix B

Modifications to the standard ALLFIT programme (written in Basic), required in order to fit equation 14 are listed below:

```
1440 N = 7
     N$(1) = "A"
     N$(2) = "B"
     N$(3) = "C"
     N$(4) = "D"
     N$(5) = "E"
     N$(6) = "F"
     N$(7) = "G"
     P(3) = 1

1510 PRINT" TAKE THE CURVE WITH THE
     HIGHER INITIAL RESPONSE AS THE CON-
     TROL CURVE"
     PRINT"A IS THE LIMITING RESPONSE AT
     VERY HIGH CONCENTRATIONS OF
     ANTAGONIST FOR THE CONTROL CURVE"
     PRINT"B IS THE SLOPE-FACTOR FOR THE
     CONTROL CURVE, AND MAY BE SET
     EQUAL TO -1.0 INITIALLY"
     PRINT"C IS THE EC50 FOR THE CONTROL
     CURVE"
     PRINT"D IS THE LIMITING RESPONSE AT
     VERY LOW CONCENTRATIONS OF
     ANTAGONIST, FOR THE CONTROL CURVE"
     PRINT"E IS -LOG(DISSOCIATION CONSTANT)
     OF THE ANTAGONIST-RECEPTOR COMPLEX"
     PRINT"F IS THE APPARENT AGONIST CON-
     CENTRATION RATIO; FIXED AT 1 FOR THE
     CONTROL CURVE AND VALUES >1 FOR
     THE OTHER CURVES"
     PRINT"G IS THE POWER TO WHICH [I] IS
     RAISED"

1520 DEF FNF(X9,L9)
     IF X9<=0 THEN X9 = 1E-20
     IF B(1 + N*(L9-1)) = 0 THEN B(1 + N*(L9-1)) =
     1E-3
     IF B(4 + N*(L9-1)) = 0 THEN B(4 + N*(L9-1)) =
     1E-3
     IF B(5 + N*(L9-1))<=2 THEN B(5 + N*(L9-1)) = 2
     Z1 = (B(6 + N*(L9-1))-1)/(10^B(5 + N*(L9-1)))
     Z1 = Z1 + B(6 + N*(L9-1))*(X9^B(7 + N*(L9-1)))
     Z1 = Z1^(1/B(7 + N*(L9-1)))
     IF Z1<=0 THEN Z1 = 1E-20
     Z1 = (Z1/EXP(B(3 + N*(L9-1))))^B(2α N*(L9-1))
     A = ((B(1 + N*(L9-1))-B(4 + N*(L9-1)))/(1 + Z1))
                                  + B (4 + N*(L9-1))
     FNF = A
     END DEF

1660 DEF FND(X9,L9,I9)
     D1 = FNF(X9,L9)
     B(I9 + N*(L9-1)) = 1.001*B(I9 + N*(L9-1))
```

```
D2 = FNF(X9,L9)
B(I9 + N*(L9-1)) = B(I9 + N*(L9-1))/1.001
FND = (D2-D1)/(0.001*B(I9 + N*(L9-1)))
END DEF
```

The remainder of the programme can be left unchanged, except that all reference to automatic initial parameter estimation should be deleted, and a printout of observed and calculated responses is useful.

Br. J. Pharmacol. (1994), **111**, 227–232

pK_I values of prazosin and idazoxan for receptors stimulated by neuronally released transmitter in the epididymal portion of rat isolated vas deferens

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1 A new method has been used to measure pK_I values of prazosin and idazoxan against neuronally-released transmitter in the epididymal portion of the rat isolated vas deferens. The most reproducible results were obtained with a prolonged antagonist equilibration time (1 h).

2 Under these conditions the pK_I of prazosin was practically unaffected by addition of α, β-methylene-adenosine-5′-triphosphate (10 μM) to desensitize purinoceptors. Addition of desmethylimipramine (DMI) (0.3 μM) produced a small, but statistically non-significant, reduction.

3 The same method has been used to measure the pK_I of prazosin against exogenous noradrenaline. In the latter case addition of DMI (0.3 μM) and corticosterone (30 μM) together produced a statistically significant reduction in the apparent pK_I of prazosin.

4 The new method for estimating pK_I values shows that DMI itself acts either pseudo-irreversibly or non-competitively and may be reducing the apparent pK_I of prazosin.

5 The pK_I values obtained for prazosin and idazoxan against neuronally-released transmitter are in good agreement with those obtained by other workers for the actions of these drugs on α-adrenoceptors.

Keywords: Competitive antagonists; dissociation constants; affinity constants; pA_2 values; pK_I values; rat vas deferens; neurotransmitters; prazosin; idazoxan

Introduction

Dissociation constants, K_I, of antagonist-receptor complexes have been widely used to characterize receptors in isolated tissues. The classical method for estimating the pA_2 (or pK_I) of a competitive antagonist for its receptor is to measure, on a cell or isolated tissue, several agonist concentration-response curves, each curve at a fixed concentration of antagonist. Such results provide agonist dose-ratios (DR) for each concentration of antagonist [I]. The classical plot of log (DR − 1) against log [I] then provides a test for competitive antagonism and an estimate of the pA_2 (Arunlakshana & Schild, 1959). The classical method can only be used dependably if agonist and antagonist concentrations at the receptors are known. This usually limits the technique to exogenously applied agonist and often requires the presence of other drugs to block uptake or metabolism of agonist (or antagonist) by the tissue.

In the previous paper (Mackay, 1994) it was shown that if two or more antagonist concentration-response curves are measured, each at a fixed agonist concentration, then these curves can be analysed to give estimates of pK_I and of the agonist concentration-ratio(s). This method should be applicable if antagonism is competitive under equilibrium or pseudo-irreversible conditions, or non-competitive, provided that the agonist and antagonists all interact with the receptor on a 1:1 molecular basis. The present paper shows how this technique has been used to measure the pK_I values of prazosin and of idazoxan for receptors acted on by neurotransmitter released by field stimulation of the rat isolated vas deferens.

Theory

The theoretical basis of the technique, for measuring the dissociation constant of a competitive antagonist without knowledge of agonist concentrations, has been set out and analysed in the preceding paper. Initially, the method was based on the general null equation for competitive antagonism under equilibrium conditions, namely

$$\frac{1+[I]''/K_I}{1+[I]'/K_I} = \frac{[A]''}{[A]'} \quad (1)$$

where concentration $[A]'$ of agonist with $[I]'$ of antagonist produce the same response, from an isolated cell or tissue, as that produced by $[A]''$ of agonist with $[I]''$ of antagonist. If each antagonist concentration-response curve is measured at a fixed agonist concentration then equation 1 can be rearranged to give

$$[I]'' = (m-1)K_I + m\,[I]' \quad (2)$$

where the fixed agonist concentration-ratio is

$$m = [A]''/[A]' \quad (3)$$

As outlined in the previous paper, equation 2 can also be derived if the antagonist is competitive but acts under pseudo-irreversible conditions or if the antagonist acts non-competitively on the receptor. However, in these cases m is not the agonist concentration-ratio but is given by a more complex expression such as

$$m = \left\{1+\frac{K_A}{[A]'}\right\} \Big/ \left\{1+\frac{K_A}{[A]''}\right\} \quad (4)$$

where K_A is an apparent dissociation constant of the agonist-receptor complex.

If the responses measured at the higher agonist concentration, $[A]''$, can be fitted by the general logistic equation

$$r = \frac{\alpha-\delta}{1+\left\{\frac{[I]}{\gamma}\right\}^{\beta}} + \delta \quad (5)$$

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(where r is the response, $[I]$ is the molar concentration of antagonist and α, β, γ and δ are adjustable parameters) then equations 2 and 5 can be combined to produce a general equation which can fit each member of a family of curves with common values of α, β, γ, δ and K_I but with different values of m. The appropriate equation is

$$r = \frac{\alpha - \delta}{1 + \left\{ \frac{(m-1)\ K_I + m\ [I]}{\gamma} \right\}^{\beta}} + \delta \qquad (6)$$

which reduces to equation 5 for the control curve when $m = 1$. Equation 6 can be used to obtain estimates of pK_I and m from two or more antagonist concentration-response curves whether the antagonism is competitive, pseudo-irreversible or non-competitive, provided that the agonist and antagonists all interact with the receptor on a 1:1 molecular basis.

The usefulness of equation 6 can be extended by deriving a null equation for two competitive antagonists, I and J, acting simultaneously against agonist A which acts on one type of receptor on the cell or tissue. When the antagonists are acting under equilibrium conditions the proportion of receptors occupied by the agonist would be

$$P_A = \frac{[A]/K_A}{\{\ 1 + [A]/K_A + [I]/K_I + [J]/K_J\ \}} \qquad (7)$$

From equation 7 a null equation can be derived relating those concentrations $[I]''$ and $[I]'$ of the first competitive antagonist which produce equal reponses in the presence of fixed concentrations $[A]''$ and $[A]'$ of the agonist in the presence of a fixed concentration $[J]$ of the second competitive antagonist. The resulting equation is of the same form as equation 2 and therefore leads to equation 6 but with K_I replaced by an apparent dissociation constant

$$K_{Iapp} = K_I\ \{1 + [J]/K_J\} \qquad (8)$$

If K_I is already known then K_J can be calculated from K_{Iapp}.

Methods

Experimental techniques

Male Wistar rats (250–350 g) were killed by cervical dislocation and bled out. The vasa deferentia were removed and the epididymal portions placed in Krebs-Henseleit solution of the following composition (mM): Na^+ 143.3, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128.3, HCO_3^- 24.9, SO_4^- 1.2, $H_2PO_4^-$ 2.2, ascorbic acid 0.057 and glucose 10.0. The physiological saline was gassed with 95% O_2/5% CO_2.

Two different protocols were used in the experiments.

Protocol A

Each epididymal portion of vas deferens was cleared of excess fat and tissue, and mounted in an organ bath at 37°C. Contractions produced either by field stimulation via platinum electrodes positioned parallel to the tissue, or by addition of noradrenaline to the organ bath, were recorded on a Lectromed MX216 recorder via a Dynamometer UF1 isometric transducer. The resting tension on the tissue was 0.5 g.

After being set up in the organ bath, tissues were initially washed twice at 15 min intervals and then exposed to noradrenaline (1.25 μM and 12.5 μM) for 30 s periods, or stimulated with 30 s trains (alternatively at 12.5 and 25 Hz, pulse width 0.5 ms, supramaximal current (400 mA)) every 10 min for 30 min. After this period tissues were exposed to noradrenaline (1 μM and 2 μM) or stimulated (12.5 Hz and 25 Hz) every 5 min for about 30 min by which time reproducible responses were obtained. During this time and thereafter, desmethylimipramine (0.3 μM) and propranolol (0.3 μM) were present in the bathing medium to block neuronal uptake of noradrenaline and effects on β-adrenoceptors. The effects of increasing concentrations of antagonist were then tested, each concentration being allowed to act for 30 min. When the responses were elicited by exogenous noradrenaline the antagonist was replaced after washout, either with the same or with a higher concentration as appropriate. When the responses were produced by field stimulation the organ bath was washed out and a higher antagonist concentration applied every 30 min. In some experiments corticosterone (30 μM) was also present to block extraneuronal uptake of noradrenaline.

Protocol B

Each epididymal portion of vas deferens was cleared of fat, desheathed, and mounted in an organ bath at 37°C. Contractions of the vas deferens were produced either by field stimulation via platinum electrodes positioned parallel to the tissue, or by addition of noradrenaline to the organ bath. In this protocol the contractile responses were recorded on a Lectromed MX216 recorder via a Gould-Statham isometric transducer, and a damped output ($t_{1/2} = 1.5$ s) was fed from the MX216 to a Chessel flat-bed recorder to produce more easily measurable traces. The resting tension on the tissue was 0.5 g.

After being set up in the organ bath the tissues were stimulated with 30 s trains (alternately at 5 and 10 Hz, pulse width 0.5 ms, supramaximal current (400 mA)) every 5 min for 30 min then every 10 min for 60 min. During this time the tissue was washed by overflow every 15 min. Any additional drugs required to be present during the experiment were added at this stage and replaced after completion of each subsequent wash. When noradrenaline concentration-response curves were being measured the responses were produced by bolus injections of the agonist. The contact time of the agonist was that required to obtain plateau or peak responses (at least 25 s). After draining and refilling the organ bath 3 times, a rest period of 7 min was allowed before adding the next bolus injection of noradrenaline. After measurement of the first agonist concentration-response curve a rest period of 1 h was allowed before the above procedure was repeated. After the second curve, which was usually very similar to the first, a chosen concentration of antagonist was added to the organ bath and allowed to act for 1 h before the next agonist concentration-response curve was obtained. When antagonist was also present in the organ bath it was replaced each time the agonist was washed out. The above procedure was repeated until the effect of a suitable number of concentrations of antagonist had been tested. When the effect of the antagonist was being measured on responses to various frequencies of nerve stimulation (range 10 Hz to 1 Hz) the procedure was essentially the same as that described above except that the organ bath was washed out and antagonist or other drugs added only after the completion of each frequency-response curve.

Materials and solutions

Prazosin hydrochloride, desmethylipramine hydrochloride (DMI), corticosterone, α,β-methylene-adenosine 5'-triphosphate (α,β-Me ATP) and (−)-noradrenaline bitartrate, were all supplied by Sigma Chemical Co. (±)-Propranolol hydrochloride was a gift from I.C.I. All inorganic salts were of A.R. grade.

Solutions of noradrenaline were prepared daily in ascorbic acid solution (100 μM) and stored on ice. Prazosin hydrochloride was dissolved in either methanol or ethanol, dilutions being made with ethanol. Solutions of corticosterone were

also prepared in ethanol. The volumes of alcoholic solutions added to the organ bath were kept low, so that the alcohols themselves had no detectable effect on tissue responses.

Analysis of results

The general curve-fitting programme ALLFIT was modified, as described in the appendix of the preceding paper, to enable equation 6 to be fitted to sets of data, each set consisting of two or more antagonist concentration-response curves measured at constant agonist concentrations on a single sample of tissue. In some experiments these constant agonist concentrations were produced by applying exogenous agonist to the tissue. In others, field stimulation was applied at different frequencies, for sufficiently long periods of time to produce either steady or peak responses. These responses were assumed to correspond to steady, though unknown, interstitial concentrations of neurotransmitter.

Each experiment gave an estimate of pK_I and of the agonist concentration-ratio(s). Unless otherwise stated values are given as mean ± s.e.mean based on n results. Depending on the experimental design, tests of significance between groups were based either on the Kruskal-Wallis non-parametric test followed by distribution-free multiple comparisons, or on Student's paired t tests (Dunn, 1964; Snedecor & Cochran, 1967).

Results

Protocol A

Preliminary experiments showed that when this protocol was followed the tissue was capable of giving reproducible responses for about 4 h. Typical results obtained with exogenous noradrenaline and field stimulation are shown in Figure 1a and b respectively. Using prazosin as an antagonist of exogenous noradrenaline, values of pK_I and of the agonist concentration-ratio, m, were measured in the presence of propranolol and DMI, with and without corticosterone. Analogous experiments were carried out using field stimulation instead of exogenous noradrenaline. In the latter case the effects of α,β-Me-ATP on pK_I and m were also tested. These results are summarised in Table 1.

Application of the Kruskall-Wallis test suggested the presence of significant differences between the groups of results shown in Table 1 ($P<0.05$). Distribution-free multiple comparisons indicated no significant difference between individual groups but a significant difference when the results obtained with exogenous noradrenaline were compared with those obtained using electrical stimulation ($P<0.05$).

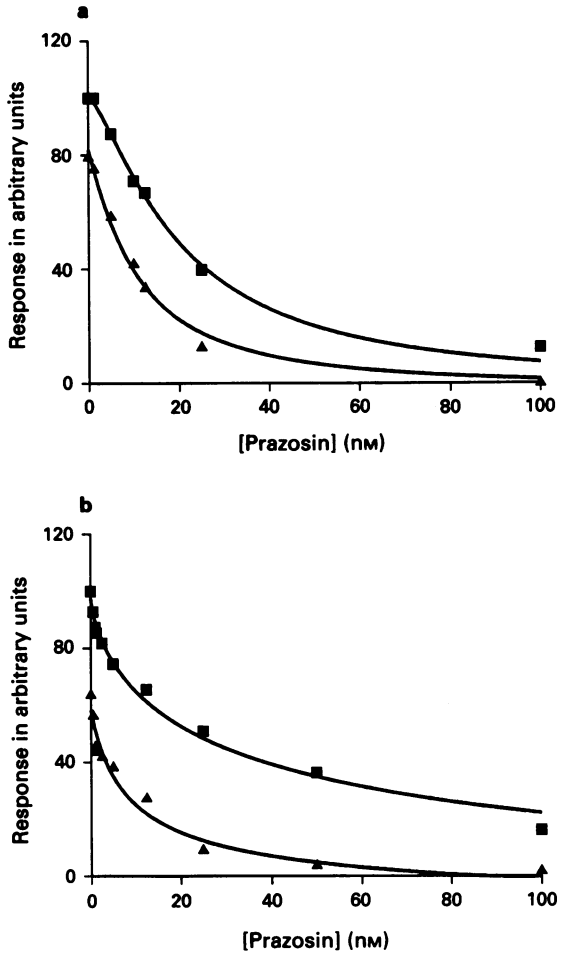

Figure 1 (a) Antagonist concentration-response curves for prazosin acting against 2 μM (■) and 1 μM (▲) noradrenaline on the epididymal portion of the rat isolated vas deferens at 37°C. Each concentration of antagonist was allowed to act on the tissue for 30 min. Other drugs present were propranolol (0.3 μM), desmethylimipramine (0.3 μM) and corticosterone (30 μM). The pK_I of prazosin estimated from these curves was 8.14. (b) Antagonist concentration-response curves for prazosin acting against neurotransmitter released by field stimulation of the epididymal portion of the rat isolated vas deferens at 37°C. The stimulation frequencies were 25 Hz (■) and 12.5 Hz (▲). Each concentration of antagonist was allowed to act on the tissue for 30 min. Other drugs present were propranolol (0.3 μM), desmethylimipramine (0.3 μM) and corticosterone (30 μM). The pK_I estimated from these curves was 8.61.

Table 1 Estimates of pK_I from antagonist concentration-response curves for prazosin at 37°C, with a 30 min contact time*

	Stimulus	pK_I	m_2	*No. of results*
(a)	Exogenous noradrenaline	8.02 ± 0.02	2.05 ± 0.11**	5
(b)	Exogenous† noradrenaline	8.17 ± 0.09	1.83 ± 0.08**	5
(c)	Field stimulation	8.51 ± 0.07	4.41 ± 0.50	6
(d)	Field† stimulation	8.52 ± 0.32	4.91 ± 1.83	4
(e)	Field†† stimulation	8.24 ± 0.12	2.77 ± 0.50	4

*The experiments were on the epididymal segment of rat isolated vas deferens. Desmethylimipramine (0.3 μM) and propranolol (0.3 μM) were always present. Values are given as mean ± s.e.mean. **With exogenous noradrenaline the applied agonist concentration-ratio was 2.0. †Corticosterone (30 μM) was also present during these experiments. ††α,β methylene-adenosine 5'-triphosphate (10 μM) was present during these experiments.

Protocol B

Preliminary experiments showed that when this protocol was followed the tissue was capable of giving reproducible responses for about 6 h. Typical results obtained with exogenous noradrenaline and field stimulation are presented as antagonist concentration-response curves in Figures 2a and b respectively. Using prazosin as an antagonist of exogenous noradrenaline, values of pK_I and of the agonist concentration-ratios, m, were measured in the presence of propranolol, with and without DMI plus corticosterone. Analogous experiments were carried out to test the effect of various combinations of propranolol, DMI, α,β-Me-ATP and idazoxan on estimates of pK_I using field stimulation instead of exogenous noradrenaline. These results are summarised in

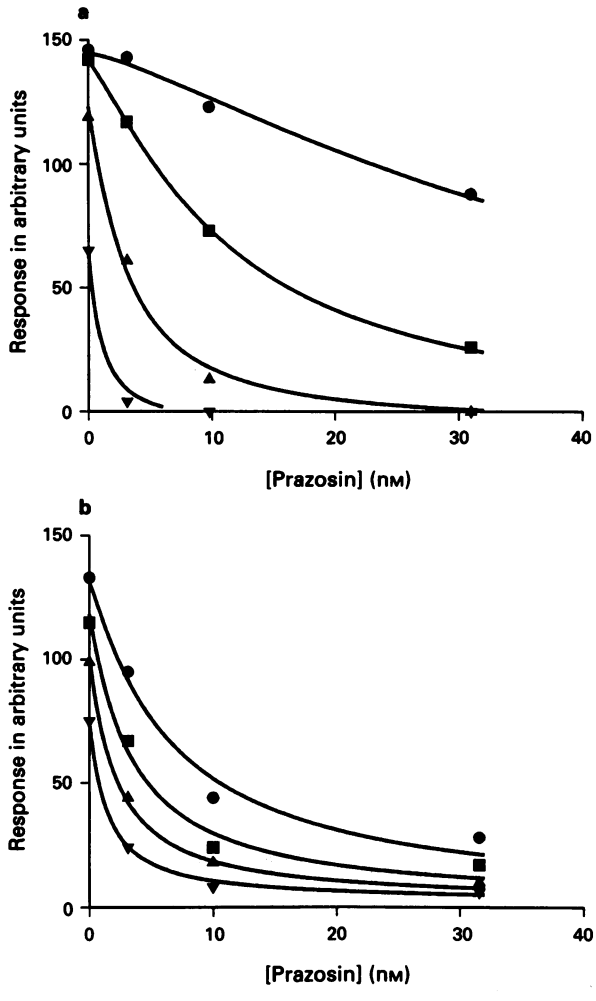

Figure 2 (a) Antagonist concentration-response curves for prazosin acting against 9.8 (●), 3.1 (■), 0.98 (▲) and 0.31 μM (▼) noradrenaline on the epididymal portion of the rat isolated vas deferens at 37°C. Each concentration of antagonist was allowed to act on the tissue for 1 h. Propranolol (0.3 μM) was also present. The pK_I of prazosin estimated from these curves was 9.08. (b) Antagonist concentration-response curves for prazosin acting against neurotransmitter released by field stimulation of the epididymal portion of the rat isolated vas deferens at 37°C. The stimulation frequencies were 10(●), 6.67 (■), 3.33 (▲) and 2.50 Hz (▼). Each concentration of antagonist was allowed to act on the tissue for 1 h. Propranolol (0.3 μM) was also present. The pK_I estimated from these curves was 9.04.

Table 2. Application of paired t tests gave $P<0.01$, $P>0.5$, $P>0.1$ and $P<0.01$ respectively for the pairs in groups (a), (b), (c) and (d) in Table 2.

Table 3 summarises estimates of pK_I and m for prazosin against exogenous noradrenaline, in the presence of various combinations of propranolol, DMI and corticosterone using protocols A and B.

Two experiments were also carried out to test the action of increasing concentrations of DMI against exogenous noradrenaline in the presence of propranolol (0.3 μM). For DMI these experiments gave $pK_I = 6.12 \pm 0.29$, $m_2 = 1.70 \pm 0.19$, $m_3 = 3.33 \pm 0.73$ and $m_4 = 7.07 \pm 2.27$. The applied agonist concentration-ratios, to be compared with the above estimates of m, were 3.16, 10.0 and 31.6 respectively.

Discussion

The theoretical basis of the new method for measuring pK_I values of antagonists was set out and tested in the previous paper. How well the modified version of ALLFIT can fit equation 2 to real antagonist concentration-response curves, obtained with either exogenous noradrenaline or nerve stimulation, can be judged by examining Figures 1a and b, 2a and b. An important assumption made when applying this method of analysis is that the agonist and antagonist are acting on only one type of receptor. For this reason propranolol was always present in these experiments to block any actions of noradrenaline on β-adrenoceptors. Electrical stimulation of the rat vas deferens is known to cause concurrent release of noradrenaline and adenosine-5'-triphosphate (McGrath, 1978; Brown *et al.*, 1983; Sneddon & Burnstock, 1984) which act independently on their postsynaptic receptors to produce contraction of the smooth muscle (Ventura & Pennefather, 1991). The P_2-purinoceptor agonist α,β-MeATP blocks the effects of ATP by desensitizing these receptors (Sneddon & Burnstock, 1983; Stjarne & Astrand, 1985)). In the experiments described here, α,β-MeATP produced a transient contraction after which addition of more α,β-MeATP failed to produce any response. The presence of α,β-MeATP caused the response to field stimulation to be reduced by up to 50%. Nevertheless, with protocol B, the presence of α, presence of α, β-MeATP caused no significant change in the pK_I obtained for prazosin, as shown in Table 2. A similar result was obtained with protocol A (Table 1). The reason for this finding is not entirely clear though it may be due to some cancellation of error. In particular it should be emphasised that the new method for estimating pK_I depends on relative positions of antagonist concentration-response curves, and is not sensitive to some kinds of curve distortion.

Table 2 Estimates of pK_I from antagonist concentration-response curves for prazosin at 37°C, with a 1 h contact time*

		Paired vasa deferentia			
		First vas deferens		*Second vas deferens*	
	Stimulus	pK_I	*Other drugs present*	pK_I	*Other drugs present*
(a)	Exogenous noradrenaline	9.12 ± 0.05 ($n = 4$)	Propranolol	8.69 ± 0.07 ($n = 4$)	propranolol DMI, corticosterone
(b)	Field stimulation	8.86 ± 0.06 ($n = 4$)	Propranolol DMI	8.81 ± 0.16 ($n = 4$)	propranolol DMI, α,β-Me-ATP
(c)	Field stimulation	8.81 ± 0.12 ($n = 9$)	Propranolol	8.60 ± 0.09 ($n = 9$)	propranolol, DMI
(d)	Field stimulation	8.94 ± 0.11 ($n = 3$)	Propranolol α,β-Me-ATP	7.70 ± 0.20 ($n = 3$)	propranolol, idazoxan, α,β-Me-ATP

*The experiments were on the epididymal segment of rat isolated vas deferens. Drug concentrations were propranolol 0.3 μM, DMI (desmethylimipramine) 0.3 μM, corticosterone 30 μM, α,β-Me-ATP (α,β methylene-adenosine 5'-triphosphate) 10 μM and idazoxan 10 μM. Values are given as mean ± s.e.mean based on n results.

Table 3 Estimates of pK_I for prazosin at 37°C, and noradrenaline concentration-ratios, *m*, from antagonist concentration-response curves*

*Protocol**	*Other drugs† present*	$log_{10}K_I$	m_2	m_3	m_4	*No. of results*
A	Propranolol,	8.03 ± 0.02	2.07 ± 0.14**	–	–	5
	DMI	8.01 ± 0.08	2.00 fixed**	–	–	
A	Propranolol, DMI,	7.99 ± 0.08	1.88 ± 0.10**	–	–	5
	corticosterone	8.06 ± 0.10	2.00 fixed**	–	–	
B	Propranolol	9.12 ± 0.05	3.25 ± 0.25††	11.3 ± 1.6††	40.7 ± 10.0††	4
		9.07 ± 0.08	3.16 fixed††	10.0 fixed††	31.6 fixed††	
B	Propranolol, DMI,	8.69 ± 0.07	2.69 ± 0.05††	9.2 ± 0.7††	30.6 ± 8.0††	4
	corticosterone	8.69 ± 0.05	3.16 fixed††	10.0 fixed††	31.6 fixed††	

*The experiments were on the epididymal segment of rat isolated vas deferens. The results were obtained using two protocols with different contact times for antagonist, 30 min for protocol A and 1 h for protocol B. Values are given as mean ± s.e.mean. †Drug concentrations were propranolol 0.3 μM, DMI (desmethylimipramine) 0.3 μM and corticosterone 30 μM. **The applied noradrenaline concentration-ratio was 2.0. ††The applied noradrenaline concentration ratios were 3.16, 10.0 and 31.6.

This is clear from the fact that this method can be used for competitive, pseudo-irreversible or non-competitive antagonism.

It was suggested in the preceding paper that the new method should permit measurement of pK_I values even when uptake or metabolism of the agonist is not blocked, provided that these processes remain constant during the experiment. In principle these comments should apply whether the agonist is applied exogenously or released endogenously. Statistical analysis of the pK_I estimates obtained with protocol A (see Table 1) detected no significant differences between individual groups. This suggests that neither corticosterone nor α, β-MeATP significantly affected the estimation of pK_I by the new method. However, further statistical analysis showed that the values of pK_I obtained with exogenous noradrenaline were significantly lower than those obtained using electrical stimulation. Since the latter effect was not found with protocol B, the lower pK_I values obtained with exogenous noradrenaline using protocol A are presumably due to some poorly controlled factor in these particular experiments. The main conclusion from examination of Table 1 is that use of protocol A, with a 30 min contact time, produced estimates of the pK_I for prazosin against exogenous noradrenaline which were lower than those measured against neuronally released transmitter, and both sets of values obtained with protocol A were lower than those obtained with protocol B (see Table 2).

Use of paired instead of unpaired experiments might be expected to enable detection of smaller treatment effects. Statistical analysis of the paired sets of results shown in Table 2, which were obtained using protocol B with a 1 h contact time for the antagonist, suggests that the pK_I-value for prazosin against exogenous noradrenaline was significantly reduced by the combined presence of DMI and corticosterone (group (a), $P<0.01$). Using protocol B, DMI without corticosterone seemed to produce a small reduction in the pK_I of prazosin which however is not statistically significant (group (c), $0.2>P>0.1$). As will be seen from the results section, DMI itself appeared to act as a non-competitive or pseudo-irreversible antagonist against exogenous noradrenaline since the values of *m* differed greatly from the applied agonist concentration ratios. The estimated pK_I of 6.12 for DMI can be compared with values of 6.3 to 6.7 reported by McCulloch & Story (1972). A pK_I of 6.8 was obtained by U'Pritchard *et al.* (1978) for DMI as a competitor at [^{3}H]-WB-4101 ('noradrenergic') binding sites in rat brain. If the effect of DMI on the rat isolated vas deferens can be considered 'competitive' at low concentrations then 0.3 μM DMI might be expected to reduce the apparent pK_I of prazosin by 0.15 to 0.40 log units.

The presence of idazoxan (10 μM) produced a highly significant reduction in the apparent pK_I of prazosin against released transmitter (Table 2, group (d), $P<0.01$). Assuming the true pK_I value of prazosin to be 8.81 (group (c), Table 2) the pK_I of idazoxan against released transmitter can be calculated from equation 8 to be 6.07. This is close to the value of 6.10 obtained for idazoxan acting as an antagonist of α_1-adrenoceptors on rat anococcygeus muscle (Doxey *et al.*, 1983).

It is worth emphasising that for those experiments in which agonist was applied exogenously, the values of the agonist concentration-ratios estimated by this new method of analysis are generally in good agreement with the ratios actually applied to the tissue (see Table 3). This agreement, which was found whether the contact time for the antagonist was 30 min or 1 h, suggests that in these experiments either prazosin was acting competitively or there was appreciable spare receptor capacity for the agonist. Since prazosin seems to equilibrate slowly with the receptors the latter explanation may be the correct one.

The values obtained with protocol B are in good agreement with the value of 8.97 reported by Minneman *et al.* (1982) and 9.26 reported by Aboud *et al.* (1993), who used 45 min and 60 min contact times respectively. Lower pK_I values were obtained with protocol A, probably because that involved less drastic removal of connective tissue and a shorter equilibration time for the antagonist.

The results discussed above provide general support for the new method for measuring pK_I values of antagonists from antagonist concentration-response curves. They also confirm the potential of the new method for use in situations where the concentration of agonist is controllable but unknown. In the present series of experiments with a 1 h equilibration time for antagonist, most estimates of the pK_I of prazosin against released transmitter were in the range 8.7 to 9.1, while that of idazoxan was close to 6.1. These estimates are consistent with values obtained by other workers for interaction of these antagonists with α_1-adrenoceptors.

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(Received August 9, 1993
Accepted October 5, 1993)

Br. J. Pharmacol. (1994), **111**, 233–237

Effects of cyclopiazonic acid on phenylephrine-induced contractions in the rabbit ear artery

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1 Upon stimulation with phenylephrine, the rabbit ear artery displays endothelium-regulated rhythmic contractions, which may be attributed to the periodical activation of the dihydropyridine-sensitive Ca^{2+} channel, presumably regulated by the Ca^{2+}-activated K^+ channel. The effect of cyclopiazonic acid (CPA), an inhibitor of the Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR), on phenylephrine-induced contractions was examined in endothelium-denuded rabbit ear arteries suspended in an organ chamber for isometric tension recordings.

2 Phenylephrine-induced tonic contractions were converted to rhythmic ones by the addition of CPA.

3 The CPA-induced rhythmic contractions were abolished by the blockade of the dihydropyridine-sensitive Ca^{2+} channel and the Ca^{2+}-activated K^+ channel by nifedipine and charybdotoxin, respectively. In contrast, glibenclamide, an ATP-sensitive K^+ channel antagonist, had no effect on the CPA-induced rhythmic responses.

4 CPA attenuated both Ca^{2+} repletion by the SR and Ca^{2+} influx across the plasmalemma without having a significant effect on Ca^{2+} release from the SR, as evaluated by phenylephrine-induced contractions. In contrast, these three parameters were not altered by the presence of the endothelium.

5 These findings indicate that the CPA-induced rhythmic contractions in the endothelium-denuded rabbit ear artery may be induced by the same ionic mechanism as endothelium-regulated rhythmic responses, by which the K^+ channel could regulate the probability of the Ca^{2+} channel being opened. The CPA-induced rhythmic contractions may correlate with the inhibitory effects of CPA on the SR function, although this is not true for the endothelium-regulated rhythmic contractions.

Keywords: Rhythmic contractions; cyclopiazonic acid; sarcoplasmic reticulum; nifedipine; charybdotoxin; rabbit ear artery

Introduction

The sarcoplasmic reticulum (SR), which is considered to be the cytosolic store of Ca^{2+} in vascular smooth muscle, releases Ca^{2+} with receptor activation by various agonists (Van Breemen & Saida, 1989). The SR also sequesters the Ca^{2+} entering from the extracellular space before it activates the contractile filaments, and this constitutes the buffer barrier to Ca^{2+} influx (Van Breemen *et al.*, 1986; Van Breemen & Saida, 1989). Recently, very useful pharmacological tools have been available to investigate the SR Ca^{2+} function in a variety of cells. One of them is cyclopiazonic acid (CPA), which is an indole tetramic acid metabolite of *Aspergillus* and *Penicillium*. CPA has been shown to be a highly selective inhibitor of the Ca^{2+}-ATPase on the SR in skeletal muscle (Seidler *et al.*, 1989). An inhibitory effect of CPA on the SR Ca^{2+}-ATPase also has been suggested in vascular smooth muscle of the rat aorta (Den & Kwan, 1991) and the dog mesenteric artery (Low *et al.*, 1992).

In our previous study, the rabbit ear artery displayed rhythmic contractions when stimulated with phenylephrine (Omote & Mizusawa, 1993). These rhythmic contractions may be attributed to the periodic Ca^{2+} influx via the dihydropyridine-sensitive Ca^{2+} channel, regulated by the Ca^{2+}-activated K^+ channel. Interestingly, these phenylephrine-induced rhythmic contractions were greatly attenuated by endothelium removal, although synthetic inhibitors of endothelium-derived nitric oxide or prostanoids had no effect on the rhythmic responses. Upon further investigation, we found that CPA induced rhythmic contractions in the endothelium-denuded artery contracted with phenylephrine. In the present study, the ionic mechanism of the CPA-induced rhythmic contractions in endothelium-denuded arteries was investigated and compared with previously reported mechanisms of endothelium-regulated rhythmic responses. The effects of CPA and the endothelium on the SR Ca^{2+} handling were also studied. The results demonstrated that CPA-induced rhythmic contractions in the endothelium-denuded arteries were elicited by the same ionic mechanism as the endothelium-regulated rhythmic contractions, although unlike CPA, the endothelium had little effect on SR function in the rabbit ear arteries.

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Methods

Tension recordings

Male albino rabbits (2.8–3.4 kg) were anaesthetized with sodium pentobarbitone (20 mg kg^{-1}, i.v.) and exsanguinated. The ear arteries were immediately removed from each rabbit, cleaned of adherent connective tissue, and cut into rings approximately 1.5 mm in width. The endothelium was removed by rubbing the intimal surface of the artery with a roughened needle. Each arterial ring was suspended in a 5 ml organ bath containing Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10) at 37°C and oxygenated (95% O_2:5% CO_2) for isometric tension recordings. The arterial rings were equilibrated for more than 90 min at a resting tension of 0.6 g.

Protocol

After the equilibration period, the ear arteries were stimulated with phenylephrine (100 μM). The presence or the absence of endothelium was confirmed by the addition of substance P (0.1 μM) during phenylephrine-induced contractions. Substance P has been shown to be a more sensitive

indicator of damage to the endothelium than carbachol (Bolton & Clapp, 1986). The arteries were then washed and equilibrated for another 60 min before the subsequent experiment. The effects of CPA (3 μM) were examined in the endothelium-denuded arteries contracted with phenylephrine (1 μM). An antagonist was added after CPA induced reproducible rhythmic contractions.

The effects of CPA on the SR function, as well as on Ca^{2+} influx from the extracellular space, were examined in the endothelium-denuded artery, according to the method of Low *et al.* (1991). The role of the endothelium was also examined in the endothelium-intact artery. In brief, the phenylephrine (100 μM)-induced contraction in Ca^{2+}-containing solution was taken as 100% and all subsequent responses were expressed as percentages of this. The artery was washed and incubated for 50 min in Ca^{2+}-containing solution, followed by the introduction of Ca^{2+}-free solution. The artery was incubated for 10 min in the Ca^{2+}-free solution to remove extracellular Ca^{2+} and then contracted with phenylephrine; these contractions are referred to as 'release'. After removing the agonist, the artery was incubated for 50 min in Ca^{2+}-containing solution to replenish the SR with Ca^{2+}, followed by the subsequent phenylephrine-induced contraction in Ca^{2+}-free solution; the latter contraction is referred to as 'repletion'. Finally, the artery was contracted by restoring Ca^{2+} (2.5 mM) to the Ca^{2+}-free solution in the presence of the agonist; this is referred to as 'influx'. CPA (3 μM) was added to the bath 30 min before 'release' and was replaced each time throughout the study, after the artery was washed.

Drugs

Phenylephrine hydrochloride, CPA, nifedipine, substance P and ethylene glycol-*bis* (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), charybdotoxin and glibenclamide from Research Biochemicals Inc. (Natick, MA, U.S.A.). Ca^{2+}-free solution was prepared by omitting Ca^{2+} and adding EGTA to a final concentration of 50 μM (Guan *et al.*, 1988).

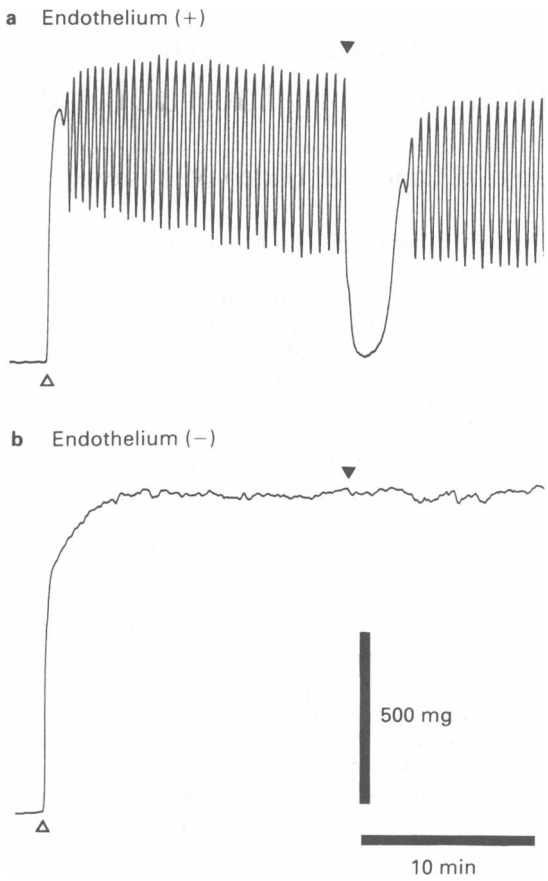

Figure 1 Typical recordings of changes in tension produced by phenylephrine in the rabbit ear artery (a) with endothelium and (b) without endothelium. The artery was stimulated with 1 μM phenylephrine (△), and then 0.1 μM substance P was added (▼). Successful removal of the endothelium was demonstrated by the lack of substance P-induced relaxation (b).

Results

Effects of CPA on phenylephrine-induced contractions

As we showed previously (Omoe & Mizusawa, 1993), phenylephrine (1 μM) induced rhythmic contractions in the rabbit ear artery with intact endothelium (Figure 1a). By comparison, the same concentration of phenylephrine induced tonic contractions in the endothelium-denuded artery (Figure 1b). When CPA (3 μM) was added during phenylephrine-induced tonic contractions in the endothelium-denuded artery, the contractions became rhythmic (Figure 2). These CPA-induced rhythmic contractions were observed in 47 of the 50 endothelium-denuded arteries isolated from 13 rabbits.

Effects of Ca^{2+} and K^+ channel antagonists

The contribution of Ca^{2+} and K^+ channels to the CPA (3 μM)-induced rhythmic contractions was investigated. CPA-induced rhythmic contractions in the endothelium-denuded artery contracted with phenylephrine (1 μM) were completely abolished by nifedipine (10 nM), the dihydropyridine-sensitive Ca^{2+} channel antagonist (Figure 3). These CPA-induced rhythmic responses were also eliminated by charybdotoxin (30 nM), an antagonist of the Ca^{2+}-activated K^+ channel (Farly & Rudy, 1988) (Figure 4a). In contrast, glibenclamide (10 μM), the ATP-sensitive K^+ channel antagonist (Nelson *et al.*, 1990), had no effect on the rhythmic responses (Figure 4b).

Effects of CPA and endothelium on phenylephrine-induced Ca^{2+} release, repletion and influx

Since the ionic mechanism of CPA-induced rhythmic contractions in the phenylephrine-contracted rabbit ear artery was found to be very similar to that of the endothelium-regulated rhythmic contractions, the effects of CPA and endothelium on SR Ca^{2+} release and repletion, as well as Ca^{2+} influx, were investigated by evaluating the phenylephrine (100 μM)-induced contractions, as shown in Figure 5.

In the endothelium-denuded arteries, the magnitudes of the phenylephrine-induced 'release', 'repletion', and 'influx' were 33, 18 and 97%, respectively of the maximum contraction (1,336.0 ± 103.7 mg, $n = 10$) obtained in the Ca^{2+}-containing solution. In the endothelium-denuded arteries treated with CPA (3 μM), both 'repletion' and 'influx' were decreased, while 'release' was not significantly affected. By comparison, the agonist-induced 'release', 'repletion' or 'influx' in the endothelium-intact artery were not different from those in the endothelium-denuded arteries.

Discussion

In the present study, CPA induced rhythmic contractions in the rabbit ear artery contracted with an α_1-adrenoceptor agonist, phenylephrine. Our findings that the CPA-induced rhythmic contractions were abolished by nifedipine and charybdotoxin may provide evidence that the activation of

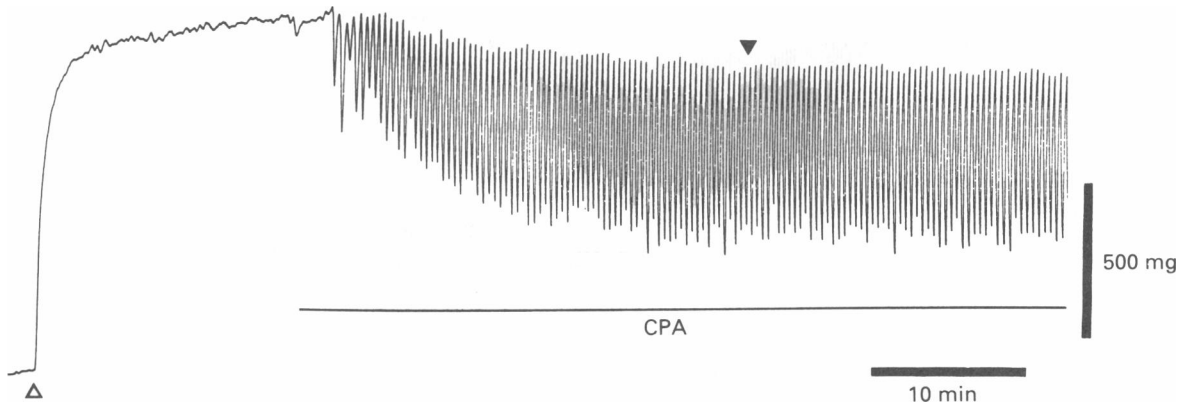

Figure 2 Typical recording of the changes in tension produced by phenylephrine and the subsequent addition of cyclopiazonic acid (CPA 3 μM) in the endothelium-denuded rabbit ear artery. The artery was stimulated with 1 μM phenylephrine (Δ). Successful removal of the endothelium was confirmed by the lack of effect of 0.1 μM substance P (▼).

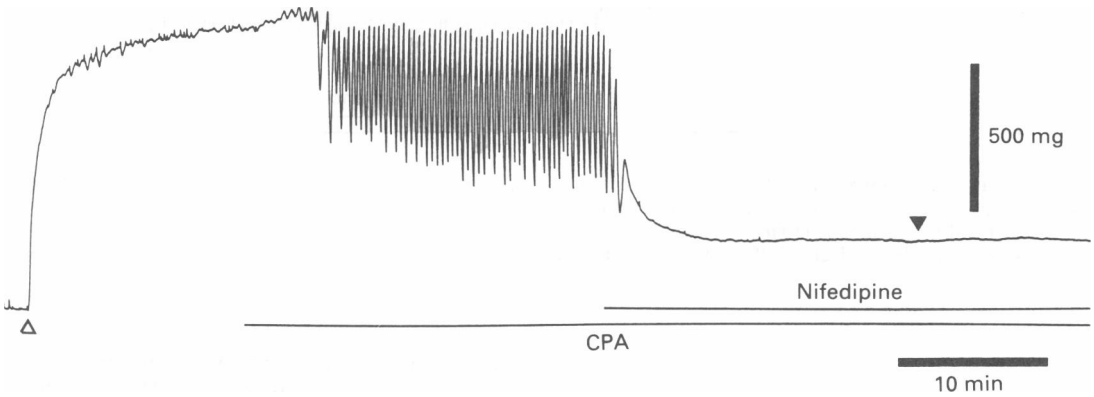

Figure 3 Effects of nifedipine on cyclopiazonic acid (CPA)-induced rhythmic contractions in an endothelium-denuded artery. The artery was contracted with 1 μM phenylephrine (Δ) and then 3 μM CPA was added. Nifedipine (10 nM) was added after CPA had induced reproducible rhythmic contractions. Successful removal of the endothelium was confirmed by the lack of effect of 0.1 μM of substance P (▼). Identical results were obtained in six other arteries.

the dihydropyridine-sensitive Ca^{2+} channel and the Ca^{2+}-activated K^+ channel contribute to the rhythmic responses. Since the inhibitory effects of these antagonists were obtained in the endothelium-denuded artery, the Ca^{2+} and the K^+ channels in the smooth muscle cells, but not in the endothelial cells, may play an obligatory role in the CPA-induced rhythmic contractions.

Rhythmic contractions observed in the vascular smooth muscles may correlate with the synchronized oscillations of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the cells, which have been shown to be elicited by either the membrane oscillator or the cytosolic oscillator (Berridge & Galione, 1988). The membrane oscillator depends on the activation of the Ca^{2+}-activated K^+ channel, which then regulates the probability of opening the voltage-sensitive Ca^{2+} channel. The cytosolic oscillator is due to the repetitive release of Ca^{2+} from the SR, which is charged with Ca^{2+} entering from the extracellular space. Therefore, CPA-induced rhythmic contractions may be associated with the $[Ca^{2+}]_i$ oscillation induced by the mechanism of the membrane oscillator. Since the membrane oscillator is initiated by the activation of the Ca^{2+}-activated K^+ channel (Berridge & Galione, 1988), CPA could have some stimulating effects on the K^+ channel. CPA, however, has been reported to have no direct effect on the probability of opening the Ca^{2+}-activated K^+ channel (Suzuki *et al.*, 1992b). We could not determine the precise effect of CPA on the K^+ channel in the present study. The inhibitory effects of CPA on the SR Ca^{2+}-ATPase may affect the K^+ channel indirectly. In fact, CPA inhibited SR Ca^{2+} sequestration at a concentration which induced rhythmic contractions. Thus, a possible explanation is as follows: inhibitory effects of CPA on the SR Ca^{2+}-ATPase can impair the SR buffer barrier to Ca^{2+} influx, which then allows an increase in $[Ca^{2+}]_i$ near the plasmalemma. The increased $[Ca^{2+}]_i$ would then activate the Ca^{2+}-activated K^+ channel. This hypothesis is inconsistent with other findings that CPA inhibits the Ca^{2+}-dependent K^+ current indirectly (Suzuki *et al.*, 1992b). However, these findings were obtained in the intestinal smooth muscle cell in which the activation of the K^+ channel depends on Ca^{2+} release from the SR (Benham & Bolton, 1986; Ohya *et al.*, 1987). It should be noted that in the rabbit ear artery the phenylephrine-induced contractions in the Ca^{2+} free solution as a result of Ca^{2+} release from the SR was 33% of the maximum contraction and the large part of the contraction was due to Ca^{2+} influx. Therefore, unlike the intestinal smooth muscle, the Ca^{2+} released from the SR may be insufficient for the activation of the Ca^{2+}-activated K^+ channel or the SR buffer barrier to Ca^{2+} influx may preserve the K^+ channel from the Ca^{2+}-dependent activation in the rabbit ear artery. CPA also reduced Ca^{2+} influx in the present study, although the agent has been shown to augment 5-hydroxytryptamine-induced contractions in the rat mesenteric artery (Shima & Blaustein, 1992). The reason for the inhibitory effects of CPA on Ca^{2+} influx in the rabbit ear artery is unknown. However, it could be due to a decrease in the probability of opening the dihydropyridine-sensitive Ca^{2+} channel as a result of the CPA-induced indirect activation of the Ca^{2+}-activated K^+ channel in the cell of this artery.

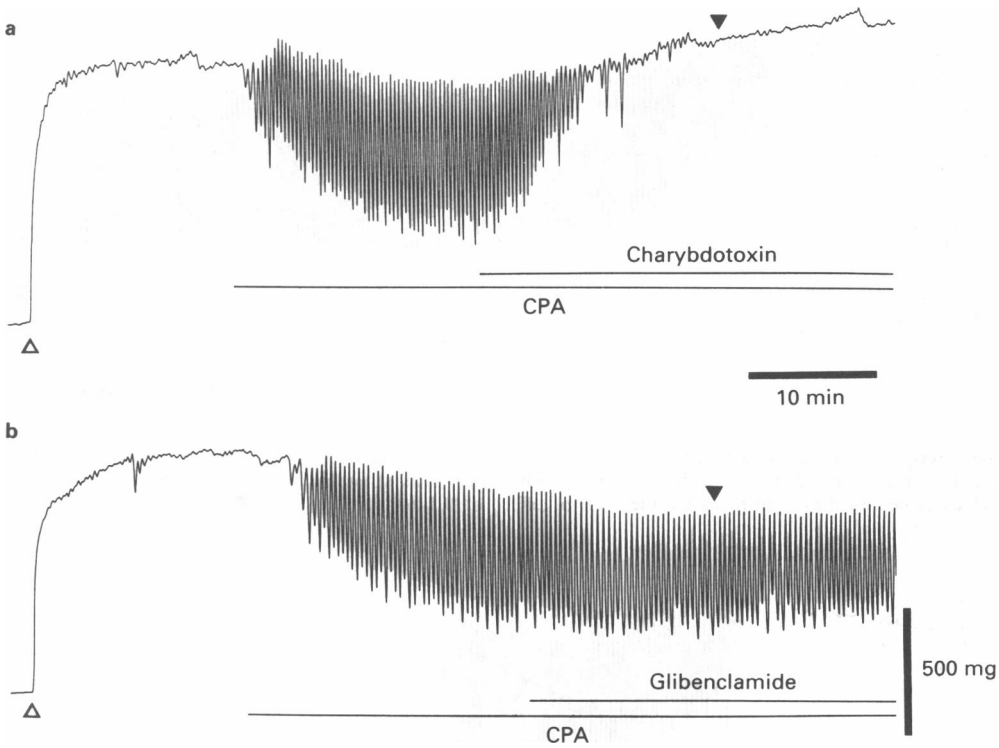

Figure 4 Effects of (a) charybdotoxin and (b) glibenclamide on cyclopiazonic acid (CPA)-induced rhythmic contractions in endothelium-denuded rabbit ear arteries. The arteries were contracted with 1 μM phenylephrine (△) and then 3 μM CPA was added. Charybdotoxin (30 nM) and glibenclamide (10 μM) were added after CPA had induced rhythmic contractions. Successful removal of the endothelium was confirmed by the lack of effect of 0.1 μM substance P (▼). Identical results were obtained in five other arteries.

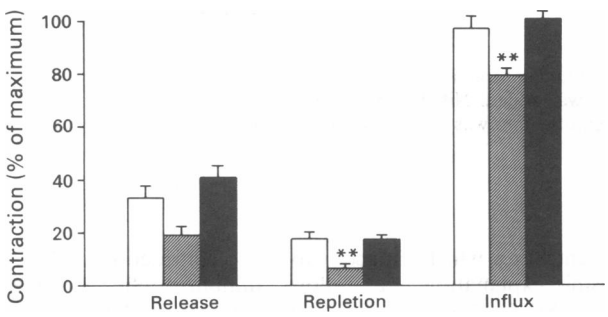

Figure 5 Effects of cyclopiazonic acid (CPA) and endothelium on Ca^{2+} release from the sarcoplasmic reticulum (SR), Ca^{2+} repletion by the SR and Ca^{2+} influx from extracellular space, evaluated by phenylephrine-induced contractions in the rabbit ear artery. Contractions induced by 100 μM phenylephrine in Ca^{2+}-containing solution were taken to be 100%. Phenylephrine-induced contractions in Ca^{2+}-free solution were performed twice, the second contraction being elicited after incubation of the artery with Ca^{2+}-containing solution, and this was followed by the contraction induced by restoring Ca^{2+} in the presence of the agonist. These contractions are referred to as 'release', 'repletion' and 'influx', respectively. Phenylephrine-induced contractions in the endothelium-denuded artery treated with 3 μM CPA (▨) and in the endothelium-intact artery (■) are compared with those in the endothelium-denuded artery without CPA (□). Each column represents the mean ± s.e.mean of 10 arteries. Asterisks (**) indicate statistically significant difference from the contractions in the endothelium-denuded artery at $P < 0.01$ by the Dunnett test.

Taken together, the rhythmic contractions induced by CPA in the rabbit ear artery may be the result of $[Ca^{2+}]_i$ oscillation with subsequent activation of the Ca^{2+}-activated K^+ channel. Impairment of the SR buffer barrier to Ca^{2+} influx by CPA could increase $[Ca^{2+}]_i$ near the plasmalemma, which would then activate the K^+ channel.

We previously showed that phenylephrine induces endothelium-regulated rhythmic contractions in the rabbit ear artery (Omote & Mizusawa, 1993). Since these rhythmic contractions have been found to be sensitive to both nifedipine and charybdotoxin, the ionic mechanism is expected to be identical to the CPA-induced rhythmic contractions, in which the activation of the Ca^{2+}-activated K^+ channel has an obligatory role. Therefore, we investigated whether the endothelium also has inhibitory effects on the SR function like CPA. Contrary to our expectation, endothelium had no effect on the SR buffer barrier function. Thus, although we could not determine the precise action of the endothelium on the K^+ channel in the present study, the endothelium would activate the K^+ channel by a mechanism distinct from that of CPA. One plausible candidate responsible for the endothelium-dependent activation of the K^+ channel could be endothelium-derived hyperpolarizing factor, which relaxes vascular smooth muscle by hyperpolarizing the membrane (Suzuki *et al.*, 1992a). The endothelium-dependent hyperpolarization of the rat aorta and the guinea-pig coronary artery has been reported to be due to the activation of the Ca^{2+}-activated K^+ channel (Chen *et al.*, 1991; Van de Voorde *et al.*, 1992). Further studies may be required to elucidate the mechanism for the action of the endothelium on the K^+ channel.

In conclusion, CPA was found to induce endothelium-independent rhythmic contractions in the rabbit ear artery and these responses may be regulated by the Ca^{2+}-activated K^+ channel. Impairment of the SR buffer barrier to Ca^{2+} influx could allow the increase in $[Ca^{2+}]_i$ sufficiently to activate the K^+ channel. Although this ionic mechanism of the CPA-induced rhythmic contractions was shown to be identical to that of previously observed endothelium-regulated rhythmic contractions, the impairment of the SR buffer barrier may not be involved in the endothelium-regulated activation of the K^+ channel.

The authors are grateful to Dr R.R. Fike, the director of Hirakata Center, for his support of this study.

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(Received July 26, 1993
Revised August 23, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 238–244

Potentiation by ACE inhibitors of the dilator response to bradykinin in the coronary microcirculation: interaction at the receptor level

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1 To examine the possibility that angiotensin-converting enzyme (ACE) inhibitors modulate the action of bradykinin at the receptor level, their effect on the dilator response to bradykinin was studied in the isolated saline-perfused heart of the rabbit.

2 Continuous infusion of bradykinin (10 nM) elicited a transient decrease in coronary perfusion pressure (CPP) and increased prostacyclin (PGI_2) release which returned to baseline values within 30 min.

3 Subsequent co-infusion of ramiprilat ($\geqslant$ 10 nM) or moexiprilat, but not of the less potent ACE inhibitor n-octyl-ramipril (RA-octyl), caused another fall in CPP and an increase in PGI_2 release, the magnitude and time course of which were almost identical to the first response to bradykinin. No change in CPP or PGI_2 release was observed when the ACE inhibitors were administered in the absence of exogenous bradykinin.

4 Infusion of D-Arg[Hyp^3]-bradykinin (10 nM), a specific B_2-receptor agonist which was significantly more resistant to degradation by ACE than bradykinin, produced virtually identical changes in CPP and PGI_2 release when compared to bradykinin. Subsequent co-infusion of ramiprilat was similarly effective in restoring the fall in CPP and increase in PGI_2 release elicited by D-Arg[Hyp^3]-bradykinin as in the presence of bradykinin.

5 In concentrations which should block the degradation of bradykinin by ACE in the coronary vascular bed, two ACE substrates, hippuryl-L-histidyl-L-leucine (0.2 mM) and angiotensin I (0.3 μM), were unable to elicit a significant change in CPP or PGI_2 release while ramiprilat and another ACE inhibitor, quinaprilat, were still active in the presence of these substrates.

6 To reveal the potential B_2-receptor action of ramiprilat, its effect on the constrictor response to bradykinin was studied in the rabbit isolated jugular vein. Ramiprilat (0.1 μM), but not RA-octyl (1 μM), potentiated the endothelium-independent, B_2-receptor-mediated constrictor response to bradykinin, but not that to the thromboxane-mimetic U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$). Moreover, ramiprilat but not RA-octyl caused a concentration-dependent, B_2-receptor antagonist-sensitive increase in tone when administered alone.

7 These findings suggest that an interaction of ACE inhibitors with the B_2-receptor or its signal transduction pathway rather than an accumulation of bradykinin within the vascular wall is responsible for the restoration of the endothelial response to bradykinin (dilatation, PGI_2 release) in the coronary vascular bed of the rabbit.

Keywords: Angiotensin-converting enzyme; bradykinin; endothelium; heart; rabbit

Introduction

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of essential hypertension and congestive heart failure due to their unique mechanism of action and favourable safety profile. Their acute blood pressure-lowering effect in patients with an activated renin-angiotensin system, but not in those with normal or low levels of renin, can be explained by an inhibition of systemic and local angiotensin II formation. Since ACE or kininase II (EC 3.4.15.1) also catalyzes the degradation of the potent vasodilator, bradykinin (Regoli & Barabé, 1980), it has long been suspected, but not yet proven, that an increase in the concentration of endogenous kinins also plays an important role in the antihypertensive effect of this class of compounds (Kiowski *et al.*, 1992). Studies on plasma kinin levels in hypertensive patients receiving ACE inhibitors do not support a major role for circulating kinins in the dilator response to these drugs (Iimura & Shimamato, 1989), so that an interference with the degradation of bradykinin within the vascular wall may be assumed. Indeed, vascular endothelial cells, like smooth muscle cells, contain considerable amounts of high-molecular weight kininogen (Schmaier *et al.*, 1988) which upon activation of kallikrein-like serine proteases can be cleaved to yield bradykinin (Bhoola *et al.*, 1992).

The potent vasodilator action of bradykinin is mainly brought about by the subsequent release of nitric oxide (NO) and prostacyclin (PGI_2) from the vascular endothelium through activation of B_2-receptors (Schini *et al.*, 1990). Although B_1 and B_2-receptors may coexist on the same cell, B_1 receptors are normally absent, but their expression in vascular cells may be enhanced after exposure to pro-inflammatory or noxious stimuli (Bhoola *et al.*, 1992). Both ACE and the B_2-receptor are located in the luminal plasma membrane of endothelial cells (Erdös, 1990). It is conceivable, therefore, that the proteolytic activity of this enzyme influences the local concentration of bradykinin at the receptor and hence the amounts of NO and PGI_2 being formed in response to the kinin. By promoting the accumulation of endogenous kinins, presumably bradykinin, we have recently shown that ACE inhibitors can enhance the basal

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release of both PGI_2 and NO from cultured endothelial cells, an effect which is mediated by a B_2-receptor-dependent increase in intracellular Ca^{2+} (Wiemer *et al.*, 1991; Busse & Lamontagne, 1991). In the absence of exogenous bradykinin, ACE inhibitors can also elicit endothelium-dependent, largely NO-mediated relaxations of bovine and porcine coronary arteries which are antagonized by the specific B_2-receptor antagonist, Hoe 140 (Hecker *et al.*, 1993a). Thus, in certain vascular beds there may be a continuous synthesis of vasoactive kinins from an endogenous source, the release of which can stimulate endothelial autacoid formation in an auto- or paracrine manner when their local concentration exceeds a critical threshold level.

However, besides promoting the accumulation of endothelium-derived kinins, ACE inhibitors may also amplify the action of bradykinin at the receptor level (Auch-Schwelk *et al.*, 1993; Hecker *et al.*, 1993a). To address this possibility further, we have (i) investigated the effects of an active and a very weakly active, structurally related ACE inhibitor on the vasodilator response to bradykinin in the isolated saline-perfused rabbit heart, (ii) compared the magnitude and time course of these responses with those elicited by the putatively ACE-resistant B_2-receptor agonist, D-Arg[Hyp^3]-bradykinin (Rhaleb *et al.*, 1990), and (iii) examined the effects of these ACE inhibitors on the constrictor action of bradykinin in ring segments of the rabbit jugular vein. Our findings demonstrate that ACE inhibitors enhance the efficacy of bradykinin by modulating its interaction with the B_2-receptor rather than by promoting its accumulation within the vascular wall.

Methods

Isolated rabbit heart (Langendorff preparation)

Hearts from anaesthetized mongrel rabbits (1.0–1.5 kg body weight) of either sex were excised and perfused with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution, pH 7.4 (composition in mM: Na^+ 142.0, K^+ 5.2, Cl^- 127.0, Ca^{2+} 2.5, Mg^{2+} 1.0, KPO_4^{2-} 1.2, HCO_3^- 24.0, D-glucose 5.0, pyruvate 2.0) as described previously (Lamontagne *et al.*, 1992). Coronary perfusion pressure (CPP) and isovolumetric left ventricular pressure (LVP) were measured by means of pressure transducers connected to a sidearm of the aortic perfusion cannula and to a fluid-filled latex balloon inserted into the left ventricle, respectively. Heart rate was derived from the LVP signal by a cardiotachometer, and the flow rate (20–30 ml min^{-1}) was adjusted to obtain a coronary perfusion pressure of approximately 60 mmHg.

Determination of PGI_2 release

The concentration of 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2, in the coronary effluent (collected for 20 s before and during the infusion of each substance at various intervals, double determination) was measured by a specific radioimmunoassay (Lamontagne *et al.*, 1992), and PGI_2 release was expressed as ng 6-keto-$PGF_{1\alpha}$ released per g heart weight and minute.

Bioassay

Rabbit jugular veins were cleaned of adventitial adipose and connective tissue and cut into rings of 5 mm width which were mounted between K30 force transducers and a rigid support for measurement of isometric force (*Schuler-Organbad*, kindly made available to us by Hugo Sachs Elektronik, March, Germany). Four rings were simultaneously incubated in 10 ml of warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution (pH 7.4), and basal (passive) tension was adjusted over a 30 min equilibration period to 1 g. In some experiments, the endothelium of the ring segments was removed mechanically and its absence confirmed by the lack of a relaxant response to the endothelium-dependent vasodilator acetylcholine (0.1–1 μM) after preconstriction with U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$, 10–30 nM) to 3 g of tension. Relaxations to the endothelium-independent vasodilator, glyceryl trinitrate (0.01–0.1 μM) were not different between endothelium-intact and -denuded ring segments, demonstrating that smooth muscle function was not affected by abrasion of the endothelium.

Preparation of rabbit lung microsomes

Rabbit lungs were rinsed with saline (0.9% NaCl, w/v), minced into small pieces with scissors and homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.8, ~10 ml per lung) containing 30 mM KCl, 5 mM $MgSO_4$ and 0.25 M sucrose (Das & Soffer, 1975) in a Potter-Elvehjem-type homogenizer fitted with a low-clearance Teflon pestle. Subcellular fractions were prepared by differential ultracentrifugation at 2,000 *g* (10 min), 10,000 *g* (20 min) and 100,000 *g* (60 min). The 100,000 *g*-sediment (microsomes) was resuspended in the same buffer containing 10% (v/v) glycerol. Protein concentrations were measured according to the method of Bradford (1976) by using the Bio-Rad protein assay and bovine serum albumin as a standard.

Determination of ACE activity

Aliquots of the microsomal fraction (33–100 μg of protein) were incubated with 1 mM [1-^{14}C]-hippuryl-L-histidyl-L-leucine (specific activity 0.33 Ci mol^{-1}) in 50 mM HEPES buffer, pH 8.0 (total volume of 100 μl) containing 100 mM NaCl and 600 mM Na_2SO_4 at 37°C. After 10, 30 and 60 min, samples were acidified to pH 3.0 with 80 μl 1 M HCl, and extracted with 200 μl ethyl acetate. A 100 μl-aliquot of the organic layer was mixed with 5 ml scintillation fluid and counted for radioactivity (d.p.m.) in a β-liquid scintillation counter. The calculation of ACE activity was based on the recovery of labelled material in the organic layer, the dilution factor (2) and an extraction coefficient of 0.693 (Ryan, 1984).

The ACE activity in rabbit isolated perfused (20 ml min^{-1}) hearts was determined by infusing [1-^{14}C]-hippuryl-L-histidyl-L-leucine (specific activity 0.66 Ci mol^{-1}) at a rate of 200 nmol min^{-1} over 25 min. A 1 ml-sample of the effluent was collected at 30 s intervals, acidified to pH 3.0 with 5 μl 5 M HCl, and stored at 0–4°C. Thereafter the samples were extracted with 1 ml ethyl acetate, and an 800 μl-aliquot of the organic layer was mixed with 10 ml scintillation fluid and counted for radioactivity as above.

Comparison of the degradation of bradykinin and D-Arg [Hyp^3]-bradykinin by ACE

The two kinins (final concentration 10 μM) were incubated with the microsomal ACE preparation (2.5 mu ACE activity) in 500 μl HEPES buffer (50 mM, pH 8.0) containing 100 mM NaCl and 600 mM Na_2SO_4 for 0–120 min at 37°C. In some experiments either hippuryl-L-histidyl-L-leucine (2 mM) or ramiprilat (1 μM) were incubated together with bradykinin for 0–90 min and 120 min respectively. At regular interals (10 min), an aliquot of the incubation mixture was removed (10 μl) and added to endothelium-intact ring segments of rabbit jugular vein suspended in 10 ml organ chambers. The resulting constrictions were compared graphimetrically to the control responses of the bioassay tissues in the presence of bradykinin or D-Arg[Hyp^3]-bradykinin (10 nM).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as mean ± s.e.mean. Statistical evaluation was performed by unpaired two-tailed Student's *t* test or one-way

analysis of variance (ANOVA), where appropriate, with a P-value <0.05 considered statistically significant.

Materials

Hoe 140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin), RA-octyl (n-octyl ester of ramipril, RA-octyl) and ramiprilat were kindly provided by Hoechst (Frankfurt/Main, Germany), moexiprilat by Schwarz Pharma (Monheim, Germany), and quinaprilat by Goedecke (Freiburg, Germany). D-Arg[Hyp^3]-bradykinin was generously supplied by Prof. D. Regoli (University of Sherbrooke, Canada); ICI-D8731 (2-ethyl-4-[(2'-(1H-1,2,3,4-tetrazol-5-yl)biphenyl-4-yl)-methoxy]quinoline hydrochloride) was from ICI Pharmaceuticals (Macclesfield, U.K.). [1-^{14}C]-hippuryl-L-histidyl-L-leucine and the [^{3}H]-6-keto-prostaglandin $F_{1\alpha}$ radioimmunoassay kit were from DuPont (Dreieich, Germany), and hippuryl-L-histidyl-L-leucine and angiotensin I from Bachem (Heidelberg, Germany). All other substances and chemicals were either from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany) or Boehringer Mannheim (Germany).

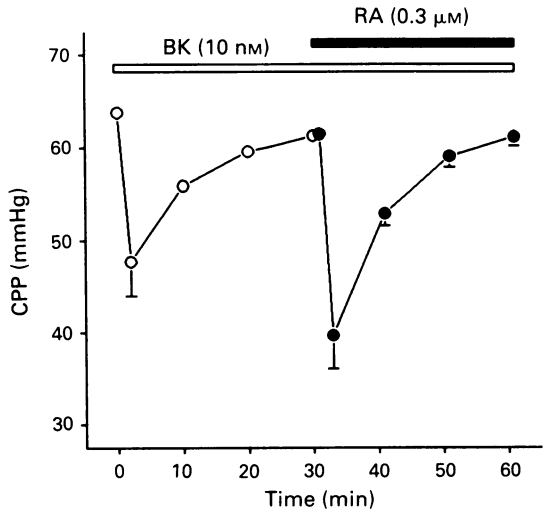

Figure 1 Change in coronary perfusion pressure (CPP) during the continuous infusion of bradykinin (BK) into the rabbit isolated heart and effect of the subsequent co-infusion of ramiprilat (RA; $n = 18$).

Results

The average weight, flow rate and coronary perfusion pressure (CPP) of the rabbit hearts were 5.1 ± 0.2 g, 26.7 ± 0.9 ml min^{-1} and 63.8 ± 0.5 mmHg ($n = 22$), respectively. Neither ramiprilat nor moexiprilat had any significant effect on CPP (101 ± 8 and 100 ± 1% of control respectively, $n = 8-14$) or basal PGI_2 release (99 ± 5 and 115 ± 16% of control respectively, $n = 7-14$) when administered alone at a concentration of 0.3 μM. In separate experiments bradykinin was found to elicit a half-maximal decrease in CPP when infused at a concentration of 10 nM (21 ± 2%, $n = 22$; maximum fall in CPP of 36 ± 3% at 1 μM bradykinin, $n = 5$). Despite the continuous infusion of the kinin, this dilator response was rapid and transient with CPP returning to baseline values within 30 min (Figure 1). Subsequent co-infusion of ramiprilat (0.3 μM), after a lag phase of 0.7 ± 0.1 min ($n = 15$), elicited another fall in CPP, the magnitude and time course of which was very similar to the initial response caused by bradykinin (Figure 1). Both bradykinin alone and the subsequent co-infusion of ramiprilat induced a substantial release of PGI_2 (Table 1). These effects of the ACE inhibitor were comparable at concentrations of 0.01 and 0.3 μM (25 ± 3 and 21 ± 2% decrease in CPP with bradykinin alone and 30 ± 2 and 28 ± 3% following the subsequent co-infusion of 0.01 and 0.3 μM ramiprilat respectively, $n = 4-18$; for PGI_2 release see Table 1). Moexiprilat produced very similar effects at a concentration of 0.3 μM (16 ± 3% decrease in CPP with bradykinin alone and 17 ± 3% following the subsequent co-infusion of the ACE inhibitor, $n = 3$; for PGI_2 release see Table 1). The B_2-receptor antagonist, Hoe 140 (0.1 μM) completely suppressed both the fall in CPP and the increase in PGI_2 release caused by both ACE inhibitors ($n = 3$, data not shown).

Increasing the concentration of bradykinin in the perfusate from 10 nM to 1 μM after 30 min also elicited another fall in CPP, the time course of which was virtually identical to the initial response elicited by 10 nM bradykinin. Its amplitude, however, was significantly greater (23 ± 3 vs. 40 ± 1% decrease in CPP, $n = 3$, $P<0.01$). The whole protocol with ramiprilat (0.3 μM) and bradykinin (10 nM) could be repeated at least once in the same heart after a washout period of 30 min ($n = 3$, data not shown). However, ramiprilat failed to elicit a fall in CPP when administered 10 min after stopping the infusion of bradykinin (21 ± 3 and 0 ± 0% decrease in CPP respectively, $n = 4$).

To verify that ramiprilat blocks the ACE activity present in the coronary vascular bed, we compared its effect on ACE

Table 1 Effects of different ACE inhibitors and substrates on the release of prostacyclin (PGI_2) from the rabbit isolated perfused heart (expressed as net increase in 6-keto-$PGF_{1\alpha}$ release (ng g^{-1} min^{-1}) over basal levels) elicited by infusions of bradykinin (BK) or D-Arg[Hyp^3]-bradykinin (D-Arg, 10 nM each)

ACE inhibitor (μM)	*BK alone*	Δ *6-keto-$PGF_{1\alpha}$ release* (ng g^{-1} min^{-1}) + *ACE inhibitor*		n
RA (0.01)	3.6 ± 1.4	3.0 ± 1.0		4
RA (0.3)	4.5 ± 1.6	9.5 ± 3.7		18
RA-octyl (0.01)	2.6 ± 0.9	0.0 ± 0.1*		3
RA-octyl (1.0)	2.6 ± 0.9	0.3 ± 0.1		3
Mxp (0.3)	7.8 ± 0.6	9.4 ± 2.9		6
	D-Arg alone	+ *ACE inhibitor*		
RA (0.3)	2.2 ± 0.3	0.6 ± 0.1**		5
	BK alone	+ *Hip-His-Leu*	+ *ACE inhibitor*	
RA (0.3)	1.8 ± 0.5	0.2 ± 0.2*	1.0 ± 0.3	4
		+ *AI/ICI-D8731*	+ *ACE inhibitor*	
Qlp (0.3)	8.9 ± 5.7	0.1 ± 0.1	17.3 ± 12.5	5

The asterisks (*$P<0.05$, **$P<0.01$) denote significant differences from the corresponding control group. Additional abbreviations and concentrations: RA, ramiprilat; RA-octyl, n-octyl ramipril; Mxp, moexiprilat; Qlp, quinaprilat; Hip-His-Leu, 0.2 mM; angiotensin I/ICI-D8731, 0.3/1.0 μM.

activity both *in vitro* (rabbit lung microsomes) and *in situ* (rabbit heart). Ramiprilat inhibited the microsomal ACE activity with an IC_{50} value of 10 ± 1 nM ($n = 4$). At this concentration, the ACE inhibitor almost completely inhibited the coronary ACE activity (Figure 2) which was approximately ten fold higher (50.4 ± 4.5 mu, $n = 3$) than in the microsomal preparation (5.2 ± 0.1 mu, $n = 4$). RA-octyl was less effective in inhibiting the microsomal ACE activity by two orders of magnitude (IC_{50}-value 1.22 ± 0.28 μM, $n = 3$, $P < 0.01$) as compared to ramiprilat. Moreover, RA-octyl was virtually inactive in the rabbit heart at a concentration of 10 nM, and affected ACE activity only when its concentration was raised to 1 μM (Figure 2). Unlike ramiprilat, RA-octyl did not restore the dilator response (25 ± 3 and 0 ± 0% decrease in CPP in the presence of bradykinin and bradykinin plus RA-octyl respectively, $n = 3$) or augment the release of PGI_2 (Table 1) during the continuous infusion of 10 nM bradykinin. Raising the concentration of RA-octyl in the perfusate from 10 nM to 1 μM after 10 min caused a fall in CPP (38 ± 11%, $n = 3$) which, however, was not associated with a concomitant increase in the release of PGI_2 (Table 1).

To examine the possibility that ACE inhibitors modulate the action of bradykinin at the receptor level, we studied the effects of ramiprilat on the responses to D-Arg[Hyp^3]-bradykinin, a more specific, and due to its N-terminal elongation, potentially ACE-resistant B_2-receptor agonist (Rhaleb *et al.*, 1990). To verify that D-Arg[Hyp^3]-bradykinin is indeed more resistant to inactivation by ACE than bradykinin, we compared the constrictor effect of both kinins after incubation with the rabbit lung ACE preparation on ring segments of endothelium-intact rabbit jugular veins. In control experiments, bradykinin and D-Arg[Hyp^3]-bradykinin elicited marked equipotent constrictions of the venous segments with ED_{50}-values of 6 ± 3 and 6 ± 2 nM respectively ($n = 3-5$). Hoe 140 (0.1 μM) completely prevented the constrictor responses to both kinins, while the B_1-receptor agonist des-Arg^9-bradykinin was completely inactive in concentrations ≤ 10 μM ($n = 3$, data not shown). Bradykinin and D-Arg [Hyp^3]-bradykinin (10 μM) were incubated with the microsomal ACE preparation for different periods of time and aliquots of the supernatant (equivalent to a final concentration of 10 nM in the organ bath) were assayed for their constrictor activity. To allow a more precise determination of the relative stability of the two kinins, the ACE activity in the incubation medium was reduced to 2.5 mu which, on the basis of the estimate that the molecular activity of purified rabbit lung ACE for hippuryl-L-histidyl-L-leucine is 30 times higher than that for bradykinin (Das & Soffer, 1975), should slowly degrade bradykinin over a period of 60–90 min. Under these conditions, bradykinin was significantly less stable ($t_{\frac{1}{2}} = 51 \pm 4$ min, $n = 5$) than D-Arg[Hyp^3]-bradykinin ($t_{\frac{1}{2}} = 123 \pm 15$ min, $n = 3$), while the addition of ramiprilat (1 μM) largely prevented the degradation of bradykinin (Figure 3).

In the rabbit isolated perfused heart, D-Arg[Hyp^3]-bradykinin (10 nM) was as potent as bradykinin in decreasing CPP (maximum fall in CPP of 16 ± 2 as compared to 14 ± 1 mmHg, $n = 5-18$) and also elicited a comparable increase in PGI_2 release (Table 1). However, during the infusion of the B_2-receptor agonist, co-infusion of ramiprilat was less effective in causing another fall in CPP as compared to bradykinin, while the time course of these dilator responses was virtually identical (lag phase in the presence of the ACE inhibitor: 0.7 ± 0.2 min, $n = 5$, Figure 4). There was a more marked difference between the two kinins in terms of the ramiprilat-induced PGI_2 release which was identical to the initial response in the presence of bradykinin, but reduced by approximately 75% in the presence of D-Arg[Hyp^3]-bradykinin (Table 1). It should be noted, however, that the release of PGI_2 elicited by the co-infusion of ramiprilat in the presence of D-Arg[Hyp^3]-bradykinin still represented a sizeable increase over basal levels (8.9 ± 1.9 fold, $n = 5$).

To exclude further an involvement of ACE in the restoration of the bradykinin-induced coronary dilatation by the ACE inhibitors, we tested the effects of two ACE substrates, hippuryl-L-histidyl-L-leucine and angiotensin I. At a concentration 20,000 times higher than that of bradykinin, the synthetic peptide was unable to elicit a significant increase in

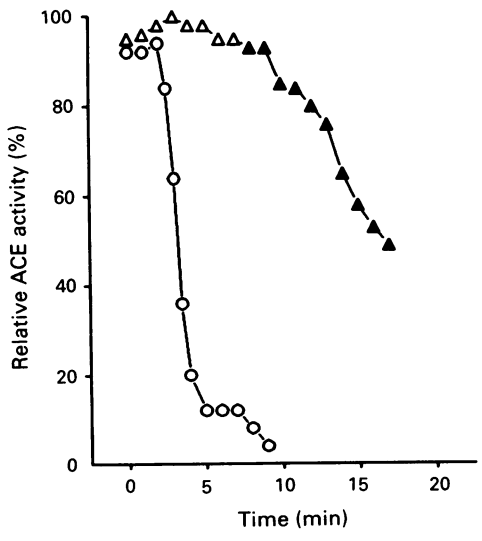

Figure 2 Inhibition of the ACE activity in the rabbit isolated heart by ramiprilat and RA-octyl. The figure shows two individual experiments representative of three experiments with each ACE inhibitor. Ramiprilat (○) or RA-octyl (△) were infused at a concentration of 10 nM or 1 μM (RA-octyl, ▲) respectively. Due to the difference in ACE activity between individual hearts, ACE activity was expressed as percentage of the mean activity measured prior to the infusion of the ACE inhibitors.

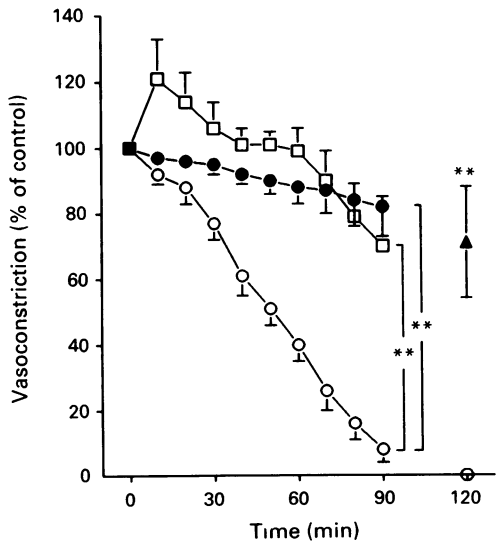

Figure 3 Comparison of the rate of inactivation by ACE of bradykinin (○) and D-Arg[Hyp^3]-bradykinin (□). The two kinins were incubated with the microsomal ACE preparation for 0–120 min. At regular intervals (10 min), aliquots of the incubation mixture (corresponding to a kinin concentration of 10 nM in the organ bath) were assayed for their constrictor effect on endothelium-intact rings of rabbit jugular vein. Constrictions were expressed relative to the control responses of the bioassay tissues to 10 nM bradykinin or D-Arg[Hyp^3]-bradykinin before and after the incubations. Note that the degradation of bradykinin was largely prevented by hippuryl-L-histidyl-L-leucine (2 mM, ●) and ramiprilat (1 μM, ▲) respectively. The asterisks denote significant differences ($P < 0.01$ by ANOVA) between the indicated curves, ($n = 3-5$).

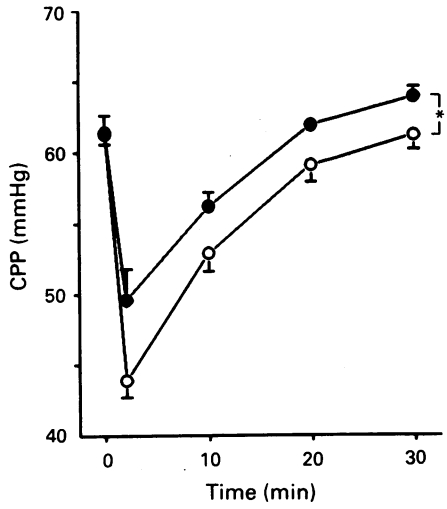

Figure 4 Comparison of the magnitude and duration of the dilator response (expressed as a percentage decrease in coronary perfusion pressure, CPP) to ramiprilat (0.3 μM) in the presence of bradykinin (○) or D-Arg[Hyp³]-bradykinin (●). The asterisk denotes a significant difference ($P<0.05$ by ANOVA) between the two curves ($n = 5-18$).

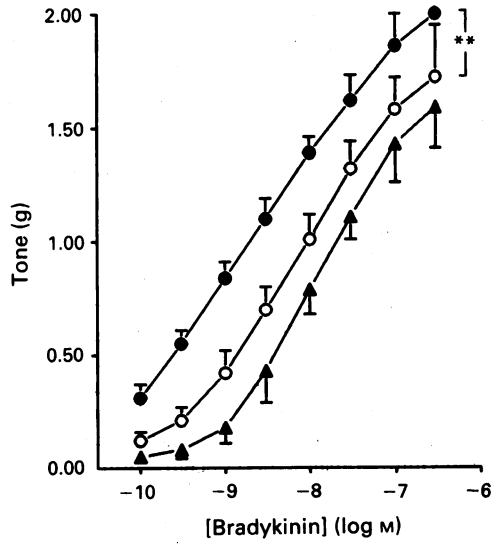

Figure 6 Comparison of the effects of ramiprilat (0.1 μM, ●) and RA-octyl (1 μM, ▲) on the constrictor responses to cumulative doses of bradykinin (○) in endothelium-denuded ring segments of rabbit jugular vein ($n = 3-12$). The asterisks denote a significant difference ($P<0.01$ by ANOVA) between the indicated curves.

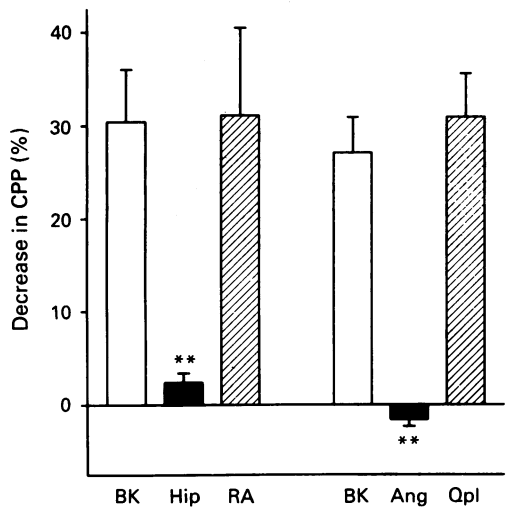

Figure 5 Co-infusions of ramiprilat (RA) and quinaprilat (Qpl, 0.3 μM each), but not hippuryl-L-histidyl-L-leucine (0.2 mM) or angiotensin I (Ang, 0.3 μM in the presence of 1 μM ICI-D8731), restore the decrease in coronary perfusion pressure (CPP, expressed as a percentage of basal pressure) elicited by bradykinin (BK, 10 nM) in the rabbit isolated perfused heart ($n = 4-5$; $^{**}P<0.01$ vs. bradykinin alone).

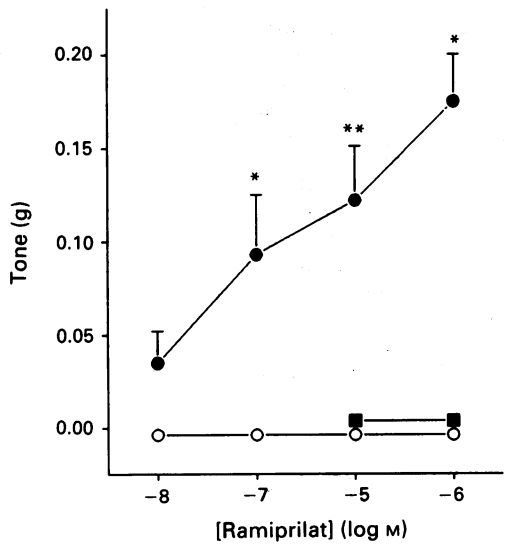

Figure 7 Effects of increasing concentrations of ramiprilat in the absence (●) or presence of Hoe 140 (0.1 μM, ○), on the tone (g) of endothelium-denuded ring segments of rabbit jugular vein ($n = 6-10$; $^{*}P<0.05$; $^{**}P<0.01$). Note that the small increase in tension caused by the ACE inhibitor (0.09 ± 0.03 g at 0.1 μM, $n = 10$) was subtracted when comparing the concentration-response curve of bradykinin in the absence and presence of ramiprilat. The figure also depicts the lack of effect of RA-octyl (■) at a concentration of 1 and 10 μM respectively.

CPP or PGI_2 release while ramiprilat was still active in the presence of the ACE substrate (Figure 5, Table 1). A 200 fold excess of hippuryl-L-histidyl-L-leucine, on the other hand, almost completely prevented the breakdown of bradykinin by the microsomal ACE preparation (Figure 3). To prevent the vasoconstrictor action of angiotensin I, its effect on the endothelial response to bradykinin was evaluated in the presence of the AT_1 receptor antagonist ICI-D8731. In control experiments, angiotensin I (0.3 μM) caused a 4 ± 1 mmHg increase in CPP in the presence of ICI-D8731 (1 μM, $n = 5$). Although the affinity of ACE for bradykinin and angiotensin I is virtually identical (Das & Soffer, 1975), a 30 fold higher concentration of angiotensin I failed to induce a significant change in CPP or PGI_2 release. However, like ramiprilat or moexiprilat, another ACE inhibitor, quinaprilat, completely restored the sensitivity of the rabbit isolated perfused heart to bradykinin despite the continued presence of angiotensin I (Figure 5, Table 1).

In a separate series of experiments, the effects of ramiprilat on the interaction of bradykinin with the B_2-receptor were studied in ring segments of the rabbit jugular vein. To minimize the possible contribution of endogenous ACE activity, the endothelium was mechanically removed from the veins, a treatment which did not enhance the potency of bradykinin (ED_{50}-value 11 ± 3 nM, $n = 9$). Co-incubation with RA-octyl (1 μM) slightly shifted the concentration-response curve of bradykinin to the right (ED_{50}-value

24 ± 15 nM, $n = 3$, Figure 6). This effect contrasted with the marked shift of the concentration-response curve to the left (ED_{50}-value 0.6 ± 0.1 nM, $n = 8$) in the presence of ramiprilat (0.1 μM, Figure 6). At this concentration, ramiprilat had no effect on the constrictor responses to U46619 (3–300 nM), another receptor-dependent agonist ($n = 7$, data not shown). Moreover ramiprilat, but not RA-octyl (in concentrations of up to 10 μM, Figure 7), elicited a distinct increase in tone even in the absence of exogenous bradykinin. This constrictor response was concentration-dependent and completely antagonized by Hoe 140 (0.1 μM, Figure 7).

Discussion

The present study shows that ACE inhibitors restore the endothelium-dependent dilator response and release of PGI_2 elicited by bradykinin in the coronary vascular bed of the rabbit. Moreover, these agents not only potentiate the contractile responses elicited by bradykinin in the rabbit isolated jugular vein, but also induce a concentration-dependent constriction on their own.

The rabbit isolated jugular vein is a suitable and sensitive bioassay tissue for studying the effects of B_2-receptor stimulation and/or blockade, even though it may contain some residual ACE activity (Regoli & Barabé, 1980). Judged by the complete lack of effect of des-Arg^9-bradykinin, the rabbit jugular vein is also devoid of any significant B_1-receptor activity (Regoli & Barabé, 1980). According to our data, however, there is very little, if any, ACE activity present in this vascular preparation, since removal of the endothelium, the primary source of ACE (Erdös, 1990), had no effect on the potency of bradykinin. Moreover, at a concentration which should block any remaining ACE activity in the smooth muscle, RA-octyl decreased rather than enhanced the constrictor response to bradykinin.

The amplification by ramiprilat of the constrictor response to bradykinin in this bioassay system, therefore, suggests that the ACE inhibitor potentiates the action of bradykinin independently of an inhibition of ACE. Since ramiprilat did not affect the constrictor response to another receptor-dependent agonist, U46619, the potentiation of the action of bradykinin is likely to be mediated by an effect of the ACE inhibitor at the level of the agonist-B_2-receptor complex. This notion is substantiated by the finding that ramiprilat, but not RA-octyl, also elicited a distinct Hoe 140-sensitive constrictor response in the absence of exogenous bradykinin. This rise in vascular tone was concentration-dependent and even increased at concentrations of the ACE inhibitor which should have completely suppressed any residual ACE activity in the endothelium-free bioassay tissue. Considering that an involvement of endogenous kinins is unlikely, these results point to a direct interaction of the ACE inhibitor with the B_2-receptor.

A direct effect of ACE inhibitors on the B_2-receptor also seems to be responsible for the instantaneous increase in the intracellular concentration of free Ca^{2+} elicited by moexiprilat or ramiprilat in cultured endothelial cells in the absence of exogenous bradykinin. This effect is completely antagonized by Hoe 140 (Hecker *et al.*, 1992). Moreover, ACE inhibitors can also relax bovine, canine, human and porcine coronary arteries in an endothelium-dependent manner when administered in the presence of subthreshold concentrations of bradykinin, and potentiate the dilator response to bradykinin (Mombouli *et al.*, 1992; Auch-Schwelk *et al.*, 1993; Hecker *et al.*, 1993a). Since the ACE activity present in these coronary artery preparations is apparently too low to influence the dilator response to bradykinin (Auch-Schwelk *et al.*, 1993), these findings support the concept that ACE inhibitors modulate the action of bradykinin at the receptor level or interfere with the subsequent signal transduction pathway.

It is important to note that the present investigation of the ACE inhibitor action in the rabbit isolated perfused heart differs from the aforementioned studies, because the restoration of the sensitivity to bradykinin and not the potentiation of its vascular action(s) was examined. Even though ACE inhibitors can promote an accumulation of endothelium-derived kinins in superfused bovine and porcine coronary arteries (Hecker *et al.*, 1993a), they do not have such an effect in the coronary vascular bed of the rabbit (Busse *et al.*, 1993; Hecker *et al.*, 1993b). Moreover, none of the ACE inhibitors tested in the present study had any effect on coronary perfusion pressure or PGI_2 release on their own. Thus, endogenously produced kinins do not seem to contribute to the restoration by these agents of the sensitivity to bradykinin in the rabbit coronary microcirculation. In addition, an accumulation of the exogenously administered kinin in or at the vascular wall is also unlikely to play a role, since the dilator response to D-Arg[Hyp^3]-bradykinin was affected by ramiprilat virtually in the same way as that to bradykinin, although the B_2-receptor agonist was clearly more resistant to inactivation by ACE than bradykinin. The rapid and identical fading of the dilator response and PGI_2 release elicited by both kinins, on the other hand, suggests that the duration of their action is governed by the desensitization of the B_2-receptor. On the contrary, if the coronary ACE activity would determine the duration of these responses, one would expect D-Arg[Hyp^3]-bradykinin to cause a more prolonged effect than bradykinin.

The finding that ramiprilat was somewhat less effective in restoring the sensitivity to D-Arg[Hyp^3]-bradykinin as compared to bradykinin may be explained by the fact that, due to its greater stability, the B_2-receptor agonist may have caused a greater degree of regulated receptor loss (down-regulation) than bradykinin (Bathon & Proud, 1991; Munoz & Leeb-Lundberg, 1992). As a consequence, the total number of available B_2-receptors would decrease, hence attenuating the subsequent response to the ACE inhibitor. Enhanced desensitization would also explain the more pronounced difference in PGI_2 release which requires a more pronounced increase in intracellular Ca^{2+}, i.e. a greater degree of cell activation than, e.g. NO release, to be initiated (Newby & Henderson, 1990).

Another argument against an accumulation of bradykinin in the presence of ramiprilat is the virtually identical time course after which the dilator response to bradykinin or the ACE inhibitor occurred. This brief lag phase contrasts with the significantly longer period of time that was necessary to attain a substantial blockade of the coronary ACE activity. Moreover, the dilator response to ramiprilat was comparable to that elicited by a 100 fold increase in the concentration of bradykinin in the perfusate. However, it is difficult to envision how bradykinin can accumulate in the vicinity of the B_2-receptor to such an extent and in such a short period of time.

It may be argued that the lack of effect of RA-octyl in the rabbit heart indicates that effective ACE blockade is a prerequisite for the dilator response to ramiprilat or moexiprilat. However, two ACE substrates, hippuryl-L-histidyl-L-leucine and angtiotensin I, in concentrations which should competitively block the metabolism of bradykinin by ACE or displace the kinin from the active site of the enzyme, were unable to restore the endothelial response to bradykinin in the coronary vascular bed (dilatation and PGI_2 release). In contrast, ramiprilat and quinaprilat were still active despite the presence of these peptides. Thus, it is more likely that, similar to its lack of effect in the jugular vein, RA-octyl is unable to interact with the B_2-receptor in the same way as the other three ACE inhibitors.

At present, we can only speculate about the possible mechanism involved in the restoration of the sensitivity to bradykinin by ACE inhibitors. Although a decrease in receptor affinity cannot be ruled out (Etscheid *et al.*, 1989), the majority of studies with cultured endothelial cells, fibroblasts and smooth muscle cells suggest that the desensitization of

the B_2-receptor is mainly due to an internalization of the ligand-receptor complex following bradykinin occupancy (Roscher *et al.*, 1984; Munoz & Leeb-Lundberg, 1992; Weintraub *et al.*, 1992). These sequestered receptors, however, can be recycled to the cell surface and due to the limited number of B_2-receptors on the surface, this process must be continuous to ensure an adequate responsiveness of the cells to bradykinin (Munoz & Leeb-Lundberg, 1992). It may be that ACE inhibitors such as ramiprilat, moexiprilat or quinaprilat accelerate the recycling of these receptors, e.g. by a flip-flop type of mechanism.

In summary, the pharmacological evidence obtained in this study supports the conclusion that ACE inhibitors restore the sensitivity of the B_2-receptor system to bradykinin in the rabbit isolated perfused heart independently of an inhibition of ACE. Presumably via a similar effect on the desensitization mechanism of the B_2-receptor, ramiprilat also potentiates the constrictor response to bradykinin in the rabbit jugular vein. Apart from blocking vascular angiotensin II formation and protecting endogenously produced kinins from inactivation by ACE, this additional mode of action of ACE inhibitors may play an important role in the vascular effects of this class of compounds.

This work was supported by the Deutsche Forchungsgemeinschaft (Bu 436/4-2, He 1587/2-1 and 3-1). I.P. is a research fellow of the Alexander-von-Humboldt Stiftung. We are indebted to Florens Nestler, Wolfgang Vorhoff and Xu Zheng for their help with the rabbit isolated heart preparation and to Roswitha Merazzi and Sigrid Weis for expert technical assistance.

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(Received July 26, 1993
Revised August 12, 1993
Accepted September 6, 1993)

Br. J. Pharmacol. (1994), **111**, 245–249

Suramin and reactive blue 2 are antagonists for a newly identified purinoceptor on rat megakaryocyte

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1 The effects of purinoceptor antagonists on ATP-induced oscillatory K^+-currents in rat isolated megakaryocytes were investigated.

2 Both reactive blue-2 (RB-2), a selective antagonist of the P_{2Y} purinoceptor, at concentrations of 0.3–10 μM and suramin, a non-selective P_2 purinoceptor antagonist, at 1–30 μM blocked the ATP-induced oscillation in a concentration-dependent manner.

3 RB-2 and suramin also blocked the ADP-induced K^+-current oscillation at the same concentration range as in the case of ATP. However, both suramin and RB-2 had no effect on thrombin- and inositol 1,4,5-trisphosphate (IP_3)-induced K^+ current oscillation, indicating that they act as specific purinoceptor antagonists.

4 Thus, the purinoceptors on megakaryocytes show the properties of the P_2 subtype according to their blockade by antagonists.

Keywords: Megakaryocytes; purinoceptors; suramin; reactive blue 2

Introduction

The potent extracellular actions of purine nucleosides and nucleotides are mediated by purinoceptors. These have been classified into two types, P_1 and P_2, by Burnstock & Kennedy (1985). According to recent reports, the P_2 purinoceptor types includes P_{2X}, P_{2Y}, P_{2Z} and P_{2T} subclasses. We have recently discovered that the rat megakaryocyte, the progenitor cell of the platelet, responded to extracellular ATP and ADP with an induction of K^+ current oscillation reflecting cytoplasmic Ca^{2+} oscillation (Uneyama *et al.*, 1993a,b). We have demonstrated that the purinoceptor of the megakaryocyte may be a novel subtype for the following reasons: (1) ADP was about 30 fold more potent that ATP in inducing K^+ current activation; (2) ATP-γ-S, a nonhydrolyzable analogue of ATP, had a greater effect than ATP, which excluded the possibility that ADP produced by the degradation of ATP was acting on the receptor; (3) cross-desensitization between ATP and ADP could be observed (Uneyama *et al.*, 1993b). Among the known purinoceptors, the P_{2T} type is the only one which shows higher affinity for ADP than ATP. Also, ATP acts as an antagonist at the receptor. Thus, judged by the agonist profile, the purinoceptor of the megakaryocyte may differ from other known subtypes. In this paper, we characterize further the purinoceptor on the megakaryocyte using P_2-purinoceptor antagonists.

Methods

Preparation

Adult male and female rats of Wistar strain weighing 250–350 g were anaesthetized by inhalation of an overdose of diethylether and killed by exsanguination. Femoral bones were isolated from each animal and the bone marrow was washed out with standard external solution by the use of a disposable syringe with needle. After filtration through 75 μm nylon mesh to eliminate the large mass of cells, the solution containing bone marrow cells was plated in the recording chamber (Falcon primaria culture dish, diameter 35 mm). The chamber was left at room temperature until the cells settled on the base of the chamber. Megakaryocytes could be clearly distinguished from other bone marrow cells under the phase-contrast microscope by their size (those of diameter 30–50 μm were used).

Electrical measurements

The whole-cell current measurements on megakaryocytes were mainly made with the nystatin perforated patch technique (Horn & Marty 1988; Uneyama *et al.*, 1992) at room temperature (21–24°C). When inositol 1,4,5-trisphosphate (IP_3) was introduced to the cell, the conventional patch technique was used. The current and voltage were measured by the use of a patch-clamp amplifier (List, EPC7), simultaneously recorded on a pen recorder (Sanei, RECTI-HORTZ-8K) and stored on a video cassette recorder (Mitsubishi, VH-F32) after changing the signal to a digital mode using a digital audio processor.

Solutions

The ionic composition of the standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris (hydroxymethyl)aminoethane (Tris)-OH. The internal solution for nystatin perforated patch was (in mM): KCl 150 and HEPES 2, and the pH was adjusted to 7.2 with Tris base. Nystatin was added in a final concentration of 150 μg ml^{-1} to the internal solution just before use. The internal solution for conventional whole-cell patch was (in mM): KCl 150, ATP 5, $MgCl_2$ 2 and HEPES 2, with pH adjusted to 7.2.

Drug application

Drugs were applied by the use of a rapid application method termed the 'Y-tube' method, as described elsewhere (Nakagawa *et al.*, 1990). By this technique, the solution surrounding an isolated megakaryocyte could be completely exchanged within 20 ms.

Drugs

ATP, ADP, nystatin, thrombin and reactive blues (RB) were purchased from Sigma Chemical Co. Suramin monosodium

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salt was a gift from Bayer. IP_3 was from Dojindo. 7-Chloroethyltheophylline was purchased from RBI.

Data analysis

Experimental values are presented as mean ± standard error of the mean (s.e.mean). The half-maximum inhibitory concentration (IC_{50}) and half-maximum effective concentration (EC_{50}) were obtained from the data for concentration-response curves for respective drugs. Details of the calculation were described elsewhere (Nakagawa *et al.*, 1991).

Results

Effects of suramin on ATP-induced K^+ current oscillation

By the use of nystatin perforated whole-cell patch-clamp technique, we have shown that extracellular application of ATP induced periodic K^+ current activation in rat megakaryocytes which reflected oscillation of cytoplasmic free Ca^{2+} concentration (Uneyama *et al.*, 1993a,b). ATP exerted its effect from 1 μM, and the frequency, current amplitude

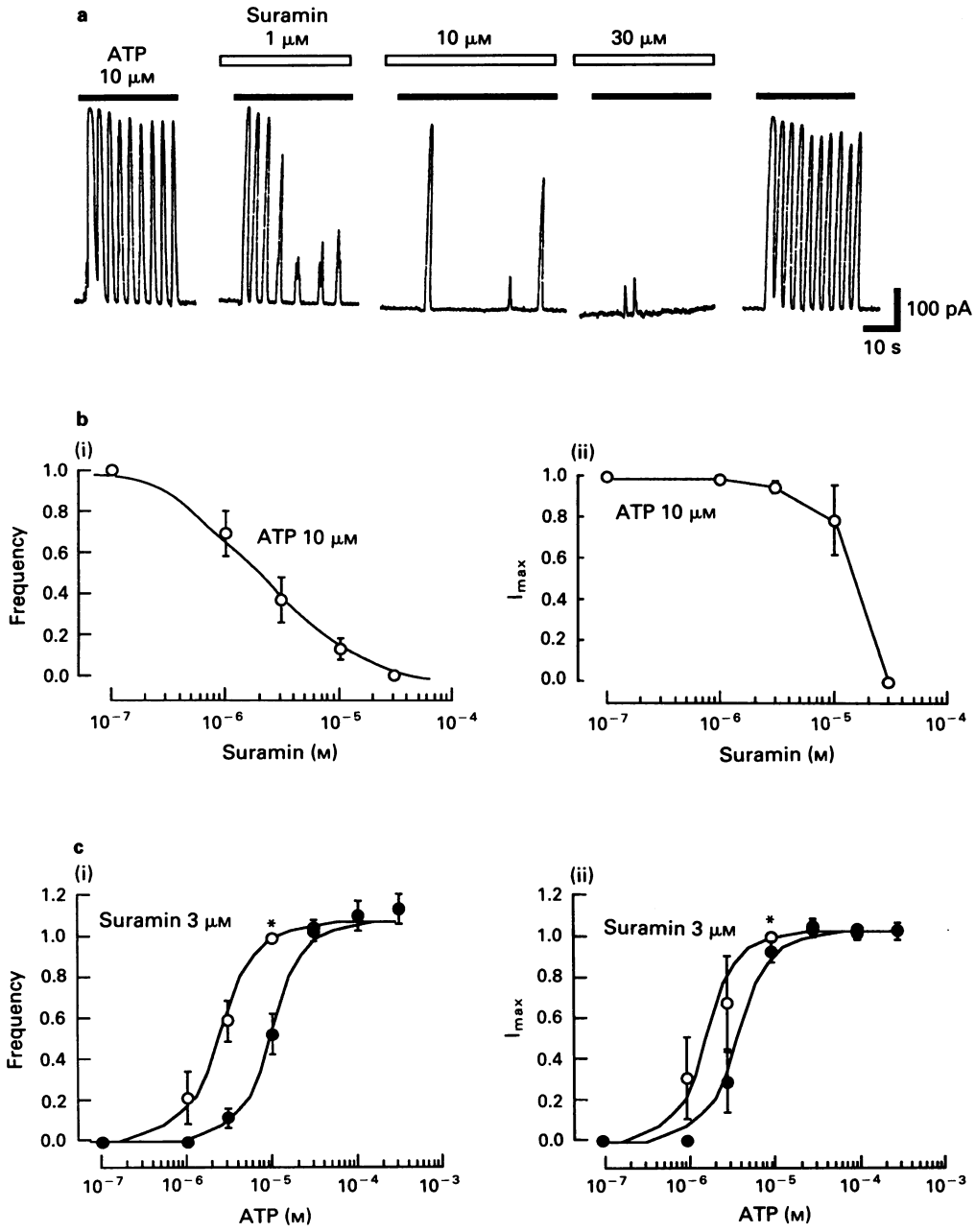

Figure 1 Effect of suramin on ATP-induced K^+ current oscillation in rat megakaryocytes. (a) Effects of 1, 10 and 30 μM suramin on 10 μM ATP-induced K^+ current. ATP was applied during the period indicated by closed bars. Suramin at each concentration was applied 2 min prior to the application of ATP. The application was repeated at least 2 min after wash out. The current traces were obtained from a single cell, and are typical of 5 separate experiments. (b) Concentration-dependency of the inhibitory effect of suramin on 10 μM ATP-induced K^+ current oscillation. The value of frequency and maximum current amplitude (I_{max}) were normalized to the value obtained by 10 μM ATP alone. Each value represents the mean ± s.e.mean of five to seven cells. (c) Concentration-response relationships of ATP with (●) or without (○) 3 μM suramin. Each value represents mean ± s.e.mean of normalized values of four or five cells. The asterisk (*) indicates the value used for normalization.

and latency of the ATP-induced K^+ current were affected. Frequency is the number of oscillatory current spikes per s, and latency is the lag period between drug application and appearance of the first current. Frequency and current amplitude increased and latency decreased when higher concentrations of agonist were applied. Figure 1a shows the effect of suramin, a P_2-antagonist, on 10 μM ATP-induced K^+ current oscillation. Suramin affected the frequency, current amplitude and latency in a concentration-dependent manner. Even when ATP was applied prior to suramin, the inhibitory effect of suramin on the ATP-induced current could be seen immediately after its application (data not shown). In addition, pretreatment of the cell with suramin for varying periods did not change the results. Then the concentration-dependency of the inhibitory action of suramin was investigated (Figure 1b). Suramin exerted its inhibitory action on 10 μM-ATP-induced oscillation from 1 μM. The frequency was more sensitive to the action of suramin than maximum current amplitude (I_{max}). This high-sensitivity of frequency is one of the general features of this oscillation as we observed previously (Uneyama *et al.*, 1993b). Thus, we used the frequency as an index of reaction intensity, and I_{max} as a reference. The estimated IC_{50} value of suramin was 2.3 ± 1.2 μM for a 10 μM ATP-induced response. In addition, the concentration-response curves of ATP shifted to a higher concentration-range without any change in the maximum ATP response in the presence of 3 μM suramin (Figure 1a). The EC_{50} values of ATP were 2.9 ± 1.0 μM and 8.8 ± 1.5 μM in the absence and presence of 3 μM suramin, respectively. These results indicated that suramin acted as an inhibitor of ATP on rat megakaryocyte purinoceptors.

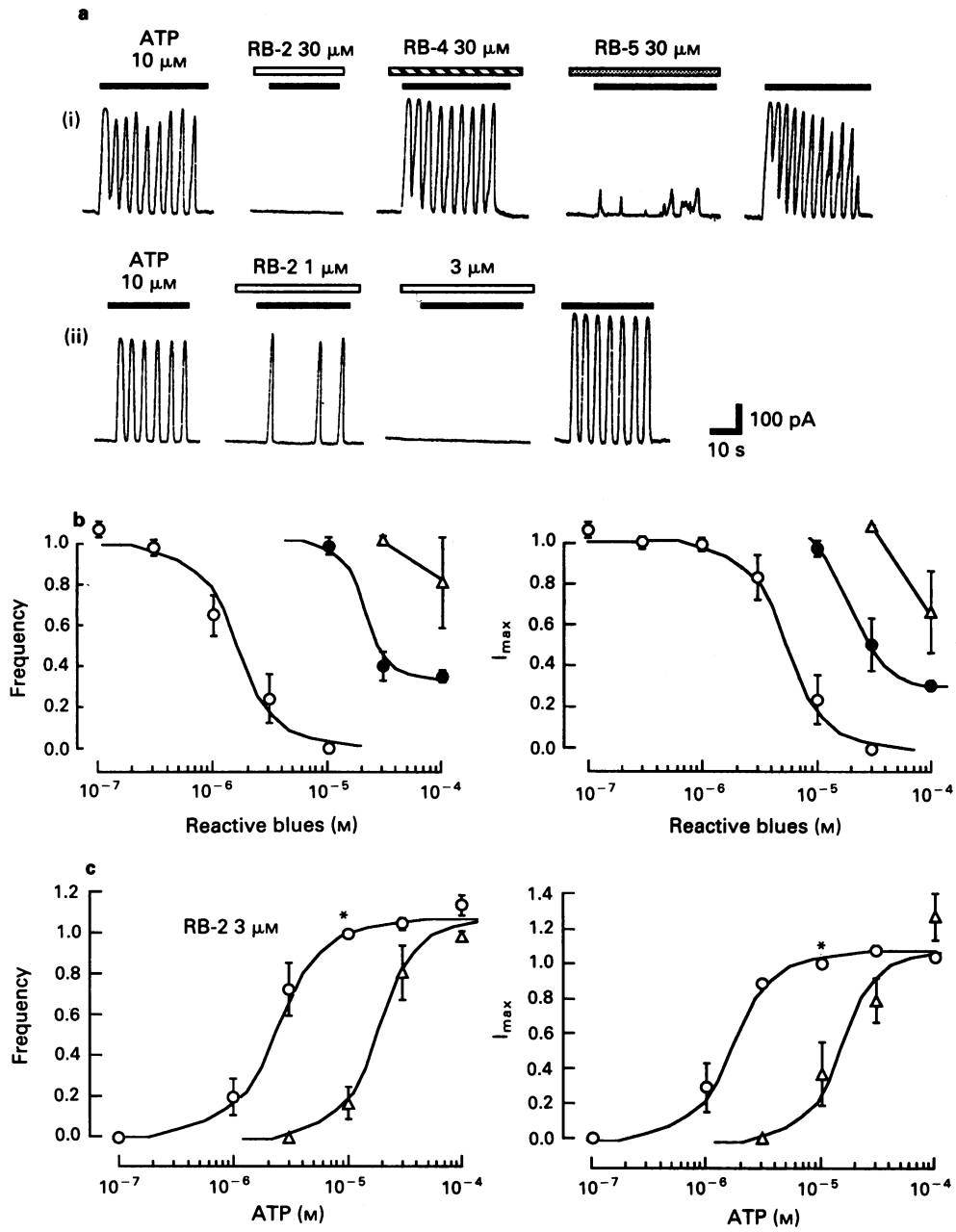

Figure 2 Effects of reactive blue (RB)s on ATP-induced K^+ current oscillation of rat megakaryocyte. (a) Effect of 30 μM RBs (i) and lower concentrations of RB-2 (ii) on 10 μM ATP-induced K^+ current oscillation. ATP was applied during the period indicated by closed horizontal bars. Each drug was applied 2 min prior to the application of ATP. Current traces in (i) and (ii) were from single cells, respectively. (b) Concentration-dependent inhibitory actions of RBs: (○) RB-2; (●) RB-5; (△) RB-4. (c) Effect of 3 μM RB-2 on the concentration-response relationships of ATP.

Effects of reactive blues on ATP-induced K^+ current oscillation

RB-2 is known as an antagonist of P_{2Y}-purinoceptors in a wide variety of cells (Burnstock & Warland, 1987). Thus, we examined the effect of RB-2 and its structural homologues such as RB-4 and RB-5 on the ATP-induced response in megakaryocytes. As shown in Figure 2a(i), 30 μM of RB-2 and RB-5 inhibited the 10 μM-ATP-induced K^+ current oscillation. RB-4 had no effect up to 30 μM. RB-2 at lower concentrations inhibited the K^+ current; even 1 μM of RB-2 showed a significant inhibitory effect on a 10 μM-ATP-induced response (Figure 2a(ii)). The inhibitory actions of RB-2 and RB-5 were fully reversible and did not depend on the pretreatment period as in the case of suramin. Next the concentration-dependency of RBs was examined (Figure 2b). RB-2 reduced the frequency of 10 μM-ATP-induced K^+ current oscillation from 0.3 μM, and the inhibitory effect was complete at 10 μM. An inhibitory effect of RB-5 was seen at >30 μM, but RB-4 had little effect even at 100 μM. Estimated IC_{50} values for 10 μM ATP were 1.3 ± 0.3 and 10.2 ± 1.1 μM for RB-2 and RB-5, respectively. Almost the same results were obtained with the effects on I_{max}. In Figure 2c, the effects of 3 μM RB-2 on the concentration-response curves of ATP are shown. The concentration-response curves of ATP were shifted to the right without reduction in the maximum response by 3 μM RB-2 in both frequency and current amplitude. The EC_{50} values of ATP in the absence and presence of 3 μM RB-2 were 2.9 ± 0.8 and 9.2 ± 2.4 μM, respectively. These results indicate that RB-2 as well as suramin is an antagonist at the purinoceptor on the megakaryocyte. 7-Chloroethyltheophylline, a nonselective antagonist for P_1-purinoceptors, had no effect on the ATP-induced response up to 100 μM (data not shown).

Effects of suramin and RB-2 on ADP-induced K^+ current oscillation

At about a 30 times lower concentration than ATP, ADP also induced K^+ current oscillation in the megakaryocyte. We examined the effect of suramin and RB-2 on ADP-induced oscillation. As shown in Figure 3, suramin also inhibited the ADP-induced oscillation. The concentration of suramin required to inhibit an ADP-induced response was almost identical to that required for ATP, though the concentration of ADP was lower than ATP. RB-2 also inhibited ADP-induced K^+ current oscillation in the same way as ATP-induced oscillation. The inhibition was fully reversible. Both in the case of suramin and RB-2, the only difference between ATP and ADP was the concentration of agonist required to induce oscillation of the same frequency. If the reaction intensity was the same, suramin and RB-2 did not distinguish ADP from ATP.

Effects of suramin and RB-2 on thrombin- and IP_3-induced responses

Thrombin, which is known as a Ca^{2+}-mobilizing agonist in the megakaryocyte (Ikeda *et al.*, 1992), also induced K^+ current oscillation through the activation of the thrombin receptor in our experimental condition (Figure 4a). As shown

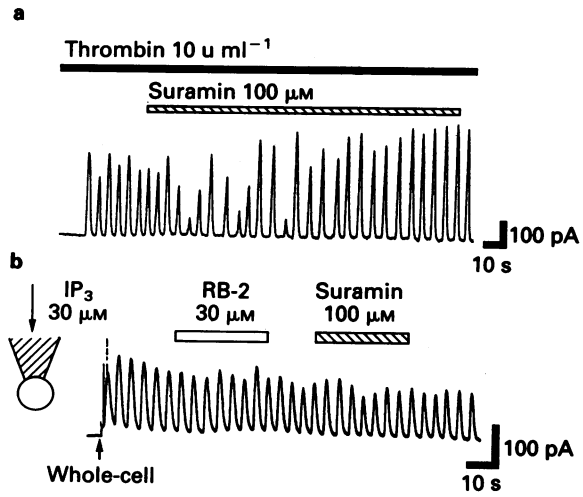

Figure 4 Effect of suramin and reactive blue-2 (RB-2) on thrombin- and inositol 1,4,5-trisphosphate (IP_3)-induced K^+ current oscillations. (a) Suramin, 100 μM was applied during a continuous application of 10 u ml^{-1} thrombin. (b), K^+ current oscillation was induced by intracellular perfusion of IP_3 by conventional whole-cell mode with the recording patch electrode filled with internal solution containing 30 μM IP_3. Then 30 μM RB-2 and 100 μM suramin were applied extracellularly during the period indicated by horizontal bars. The current traces shown in (a) and (b) are typical ones from three and four experiments, respectively.

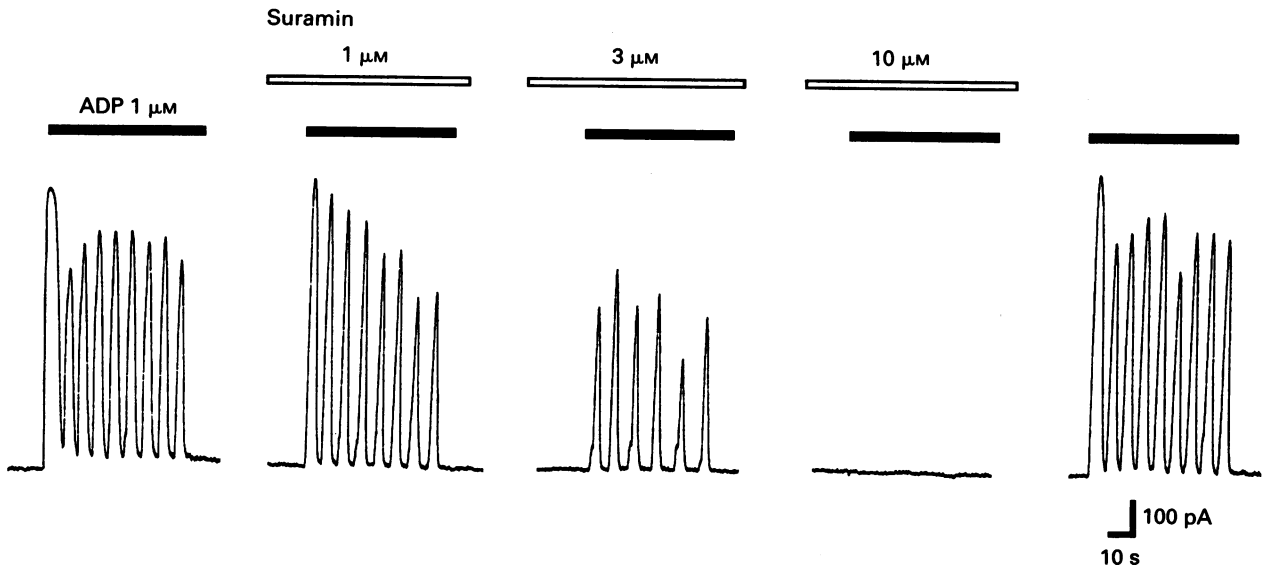

Figure 3 Effect of suramin on 1 μM ADP-induced K^+ current oscillation. Current traces were from a single cell, typical of four cells examined.

in Figure 4a, suramin at even high concentrations, did not affect the K^+ current oscillation evoked by 10 unit ml^{-1} thrombin. When suramin was applied before the application of thrombin, the result was the same. In addition, the oscillatory K^+ current was induced by intracellular application of IP_3 with whole-cell patch configuration as shown in our previous paper (Uneyama *et al.*, 1993a), and this was affected by neither suramin nor RB-2 (Figure 4b). These results clearly indicated that the inhibitory actions of suramin and RB-2 were at the receptor level.

Discussion

We show here that both suramin and RB-2 were antagonists of an apparently novel subtype of purinoceptor on the megakaryocyte. Suramin is known as a nonselective P_2-purinoceptor antagonist (Dunn & Blakeley, 1988; Hourani *et al.*, 1992) and RB-2 is known as a P_{2Y} antagonist (Burnstock & Warland, 1987). Therefore, the purinoceptor on the megakaryocyte may be one of the P_2 subtypes that resemble P_{2Y}. In addition, these compounds similarly antagonized both ATP and ADP. As the agonist selectivity of the purinoceptor on the megakaryocyte differed from previously known ones, we thought it might belong to a new subtype (Uneyama *et al.*, 1993b). However, the possibility that two types of purinoceptor (P_{2Y} and P_{2T}) coexist on the megakaryocyte could not be completely excluded. Certainly, it would be expected that the megakaryocyte contains the platelet-type (P_{2T}) purinoceptor since it is the progenitor cell of the platelet. However, the present results appear to confirm that ATP and ADP share a common receptor, and do not require separate receptors for each agonist, and agree with our previous proposal that the purinoceptor on the megakaryocyte is a novel one.

RB-2 and suramin were effective at a concentration range of 0.3–10 μM, and the IC_{50}s of these compounds were lower than the ATP concentration. As the concentrations of both ATP and antagonists were lower than reported in other experimental systems (Den Hertog *et al.*, 1989; Inoue *et al.*, 1991; Hourani *et al.*, 1992), these results did not seem to be affected by nonspecific toxic effects of these drugs. In our previous paper (Uneyama *et al.*, 1993b), we suggested that the active forms of the agonists might be ATP^{4-} and ADP^{3-}. ATP and ADP form complexes with Mg^{2+} and Ca^{2+}. The concentration of ATP^{4-} ($[ATP^{4-}]$) can be calculated from the equation; $[ATP^{4-}] = [ATP_{(total)}]/(10^{3.97}[Ca^{2+}) + 10^{4.22}[Mg^{2+}])$ (Uneyama *et al.*, 1993b). Thus, the concentration of free forms of agonists is about 1/30 in our experimental condition (2 mM Ca^{2+} and 1 mM Mg^{2+}). The low effective concentration of antagonists also suggests that ATP^{4-} and ADP^{3-} may be actual agonists.

Finally, suramin and RB-2 had no effect on thrombin-induced K^+ current oscillation of megakaryocyte indicating the inhibitory effects of suramin and RB-2 were specific for the purinoceptor-mediated response.

From all these results, it was concluded that suramin and RB-2 are antagonists at the purinoceptor on the rat megakaryocyte and that the receptor may be a new member of the P_2 class.

This work was supported by Grant-in-Aid for Scientific Research (Nos. 03304026, 04044029, 04304042 and 04404023) to N.A. from The Ministry of Education, Science and Culture, Japan.

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(Received May 17, 1993
Revised August 9, 1993
Accepted September 7, 1993)

Br. J. Pharmacol. (1994), **111**, 250–258

Modulation of cardiac L-type Ca^{2+} channels by GTPγS in response to isoprenaline, forskolin and photoreleased nucleotides

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1 Using the patch-clamp recording technique, we have investigated the effects of chronic intracellular application of guanosine thiotriphosphate (GTPγS) by cell dialysis, on the potentiation of L-type Ca^{2+} currents (I_{Ca}) by isoprenaline and forskolin and also by GTPγS and cyclic AMP released intracellularly by flash-photolysis of their caged derivatives.

2 GTPγS prevented enhancement of I_{Ca} by isoprenaline with an IC_{50} of ~10 μM and considerably reduced the ability of forskolin to increase I_{Ca}. In addition GTPγS also reduced the time-to-peak response for potentiation of I_{Ca} by forskolin. Responses to forskolin were abolished by co-dialysis of cells with the cyclic AMP antagonist, R_p-adenosine-3'-5'-mono-thionophosphate (R_p-cAMPS).

3 Photoreleased GTPγS (PR-GTPγS; ~23 μM) generally induced a biphasic increase in I_{Ca}. This response was also inhibited by chronic intracellular dialysis with GTPγS with an IC_{50} of ~1 μM.

4 Pretreatment of cells with pertussis toxin (PTX) reversed the inhibitory effect of 100 μM GTPγS on isoprenaline-induced stimulation of I_{Ca}. However, PTX pretreatment did not restore the activating action of PR-GTPγS inhibited by chronic application of GTPγS.

5 Photoreleased cyclic AMP (~5 μM; PR-cyclic AMP) increased peak I_{Ca}. This effect was inhibited by dialysis of cells with R_p-cAMPS and by stimulation of I_{Ca} by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Co-dialysis of cells with uncaged GTPγS reduced the time-to-peak for PR-cyclic AMP mediated activation of I_{Ca} but did not affect the magnitude of the response.

6 It is concluded that chronically applied GTPγS can (i) inhibit activation of I_{Ca} by isoprenaline by interacting with a PTX-sensitive guanosine nucleotide binding (G-) protein located upstream of adenylate cyclase (possibly G_i) and (ii) accelerate the response to cyclic AMP dependent phosphorylation possibly by interacting with a G-protein coupled directly to the channel.

7 In view of this diverse range of effects, care should be taken when using GTPγS to characterize G-protein-mediated events, since the resulting physiological response may be due to activation of several G-protein containing pathways.

Keywords: Calcium channels; GTP binding proteins; ventricular cells; isoprenaline; forskolin; photoreleased nucleotides

Introduction

Guanosine nucleotide binding (G-) proteins are essential intermediates in the cascade of events whereby the binding of a ligand to a receptor modulates the activity of an effector. It is now accepted that where such proteins are involved, the receptor catalyzes the binding of GTP to the G-protein, resulting in the formation of a GTP-liganded α-subunit (Gα*GTP) plus Gβγ, whereas effector modulation arises via reactions initiated by both Gα*GTP and Gβγ complexes. Important consequences of these mechanisms are receptor signal amplification and the simultaneous activation of numerous G-protein pools by a single or multiple receptors (Milligan, 1993). In cardiac tissue, enhancement of the L-type calcium current (I_{Ca}) requires coupling of β-adrenoceptors to the adenylate cyclase cascade by a stimulatory G-protein (G_s) (Trautwein & Hescheler, 1990; Kozlowski, 1992). Recently it has been proposed that G_s may also function as a membrane-delimited direct L-type Ca^{2+} channel activator in cardiac cells (Yatani *et al.*, 1987; Shuba *et al.*, 1990; Pelzer *et al.*, 1990; Kozlowski *et al.*, 1991). Further to this suggestion it has also been proposed that direct effects of G_s may act to prime L-type Ca^{2+} channels for subsequent adenosine 3':5'-cyclic monophosphate (cyclic AMP) mediated phosphorylation following β-receptor stimulation (Cavalie *et al.*, 1991). However, the significance of a direct G-protein pathway has recently been challenged by Hartzell *et al.* (1991) who suggest that a fast pathway is neither involved nor required for sympathetic regulation of L-type Ca^{2+} channels.

Guanosine thiotriphosphate (GTPγS), the slowly hydrolyzed thiophosphate analogue of GTP, has been used to demonstrate the involvement of G-proteins in modulating L-type Ca^{2+} channel function, where it has been shown, using variants of the patch-clamp recording technique (Hamill *et al.*, 1981) to have stimulatory effects (Yatani *et al.*, 1987; Shuba *et al.*, 1990; Pelzer *et al.*, 1990; Kozlowski *et al.*, 1991). This stimulatory action is a direct consequence of GTPγS binding to the α subunit of G_s, resulting in its activation and allowing it to interact either directly with L-type Ca^{2+} channels or indirectly via stimulation of adenylate cyclase and cyclic AMP production. In spite of the evidence for a stimulatory role for GTPγS there have been reports that chronic administration of high concentrations (100 to 1000 μM) of GTPγS can paradoxically prevent activation of I_{Ca} by purinoceptor (Scamps *et al.*, 1992) and histamine receptor (H_2) agonists (Hescheler *et al.*, 1987). It has been speculated that the latter effect is the result of an uncoupling of H_2 receptors from the catalytic subunit of adenylate cyclase under the influence of maximal stimulation of the stimulatory G-protein H_s by GTPγS (Hescheler *et al.*, 1987). Furthermore, Kozlowski *et al.* (1991) have also presented some evidence that high concentrations of GTPγS inhibit increases in I_{Ca} mediated by both isoprenaline and also photoreleased (PR-) GTPγS. However, the mechanisms underlying these effects are as yet unknown and consequently have prompted this investigation into the effects of chronic application of GTPγS.

We have examined in detail the inhibitory effect of GTPγS

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on increases in I_{Ca} induced by isoprenaline and forskolin, by chronic application of the nucleotide through prolonged cell dialysis. These experiments were performed in order to ascertain at which point in the signal transduction or second messenger cascade this action is exerted. In addition, parallel experiments examining the effects of chronically applied GTPγS on the stimulatory action of PR-GTPγS itself and photoreleased (PR-) cyclic AMP on I_{Ca} have been performed. Use of this technique, to increase rapidly the concentration of these nucleotides intracellularly, has enabled the kinetics of cyclic AMP and G-protein-mediated events to be determined. We show that GTPγS can prevent activation of I_{Ca} by isoprenaline, at the G-protein level, whilst simultaneously increasing the sensitivity of L-type channels to cyclic AMP-dependent phosphorylation. Some of these results have been presented in abstract form (Kozlowski *et al.*, 1992a,b).

Methods

Cell isolation

Cardiac myocytes were isolated from guinea-pig ventricles by a Langendorff perfusion with collagenase and protease according to the methods of Powell *et al.* (1980). Cells used in this study were stored in culture medium (Dulbecco's modified Eagle's medium; DMEM) supplemented with 25 mM N-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (HEPES) and 2% (v/v) serum substitute (Ultroser G, Gibco, UK), at room temperature before transferring aliquots to a small perspex bath for electrophysiological recording.

Cell incubation with pertussis toxin (PTX)

Cells were incubated for 4 h at 37°C in culture medium (see above) containing 1 μM PTX. Thereafter they were stored at room temperature (~22°C) in culture medium in the continued presence of PTX for 3–4 h. After this time, although the majority of cells were in a state of irreversible contracture, sufficient rod-shaped myocytes remained for electrophysiological recordings.

Electrophysiological recordings

L-type Ca^{2+} currents were recorded from isolated ventricular myocytes by the whole-cell configuration of the patch-clamp recording technique (Hamill *et al.*, 1981). Pipettes were pulled from borosilcate glass capillaries (Clark Electromedical, Pangbourne, UK) using a vertical puller (Narishige Ltd., Tokyo, Japan). When filled with internal solution these generally had resistances of 1–2 MΩ. I_{Ca} was evoked by use of one of two protocols. Cells were voltage clamped at −80 mV and pre-pulsed to −40 mV for 300 ms at a frequency of 0.2 Hz; thereafter the voltage was stepped to a test potential of 0 or +10 mV for 300 ms and subsequently returned to −40 mV for a further 300 ms. Alternatively, cells were voltage clamped at −40 mV and the voltage stepped to the test potential for 300 ms at a frequency of 0.2 Hz. Whole-cell currents were measured with an Axopatch 1-C patch-clamp amplifier (Foster City, CA, U.S.A.) and recorded on tape with a Sony PCM video system modified for physiological recording. Data were analysed off-line by 'VCAN' (Dempster, 1988) on a DELL 325D computer after digitizing at 2 kHz using a CED 1401 interface (Cambridge, UK).

Solutions

In the majority of experiments, cells were superfused at 34.5 to 35.5°C with a solution which contained (in mM): NaCl 144, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 5.6 and HEPES 5; pH adjusted at room temperature (~22°C) to 7.2–7.3 with NaOH (Solution A). On occasion the CsCl was replaced by KCl (Solution B). Cells were dialyzed with Solution C, contained within the pipette, which consisted of (in mM): CsCl 130, $CaCl_2$ 1, ATP-Mg 1, phosphocreatine 5, EGTA 10, HEPES 10; pH 7.2–7.3 adjusted at room temperature (free Ca^{2+} ~6 nM). Guanosine-5-(3-thiotriphosphate), 3-S-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) thio ester, triammonium salt (caged-GTPγS, 1 mM), guanosine-5-*o*-(thiotriphosphate), tetralithium salt (GTPγS, 0.1 μM to 1 mM), adenosine 3′:5′-cyclic monophosphate, P¹-(2-nitrophenyl)-ethyl ester (caged cyclic AMP, 1 mM), R_p-adenosine-3′-5′-mono-thionophosphate (R_p-cAMPS, 1 mM) were added to Solution C when required. Care was taken to protect the pipette solution from light. Forskolin (10 μM), isoprenaline (1 μM) and carbachol (10 μM) were all added to solution A or B and applied by bath perfusion (bath volume 0.5 ml, flow rate ~1.1 ml min^{-1}). EGTA (ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid), PTX, ATP-Mg, phosphocreatine and GTPγS were obtained from Sigma (Poole, UK). Caged-GTPγS was purchased from Cambridge Bioscience (Molecular Probes, Cambridge, UK) and caged cyclic AMP from Calbiochem Novabiochem (Nottingham, UK). R_p-cAMPS was obtained from Biolog, Bremen, Germany.

Flash photolysis

Photolysis of caged compounds was effected by a flash of u.v. light from a flashlamp (Hi-Tech, UK) coupled to the rear port of a Nikon Diaphot microscope (Kingston, UK) as previously described (Kozlowski *et al.*, 1991). On all occasions a single light flash was elicited 20 ms before the onset of the voltage-clamp pulse used to evoke the Ca^{2+} current. Experiments examining the effect of chronically applied GTPγS were generally initiated 10 to 15 min following formation of a stable whole-cell clamp. In all experiments at least 5 min were allowed for dialysis of the cell interior with test compounds. Changes in I_{Ca}, induced by drugs or by photoreleased nucleotides were measured relative to the curent level after 280 ms in the control pulse immediately preceding the flash (see Kozlowski *et al.*, 1991). All data in the text and tables are presented as mean ± standard error mean. Statistical significance was assessed with a Student's unpaired *t* test. When stated in the text, significance refers to a 95% confidence interval ($P<0.05$).

Efficiency of flash-photolysis

The extent of photolysis was determined using high performance liquid chromatography (h.p.l.c.) by methods similar to those described previously (Kozlowski *et al.*, 1992c). In the case of GTPγS a single flash liberated 2.3 ± 1.9% equivalent to 23 ± 19 μM ($n = 8$), while in the case of caged cyclic AMP a single flash liberated 0.46 ± 0.09% equivalent to 4.6 ± 0.9 μM ($n = 9$), a concentration which has been reported to activate half maximally L-type Ca^{2+} channels in guinea-pig ventricular myocytes (Kameyama *et al.*, 1985).

Results

Effects of sympathomimetic agents

Superfusion of isolated ventricular myocytes with 1 μM isoprenaline (a non-selective β-adrenoceptor agonist) caused, as expected, a marked increase in the amplitude of peak I_{Ca} (Figure 1a). This effect of isoprenaline on I_{Ca} was virtually abolished by chronic (10 to 15 min) intracellular application of 100 μM uncaged GTPγS (Figure 1b) added to solution C. Following cell dialysis with 1 to 1000 μM GTPγS this inhibitory effect was found to be concentration-dependent with an IC_{50} of ~10 μM (Figure 1c).

To determine whether the inhibitory effect of chronically applied GTPγS, intracellularly, was due to an interaction with signal transducing G-proteins located upstream of

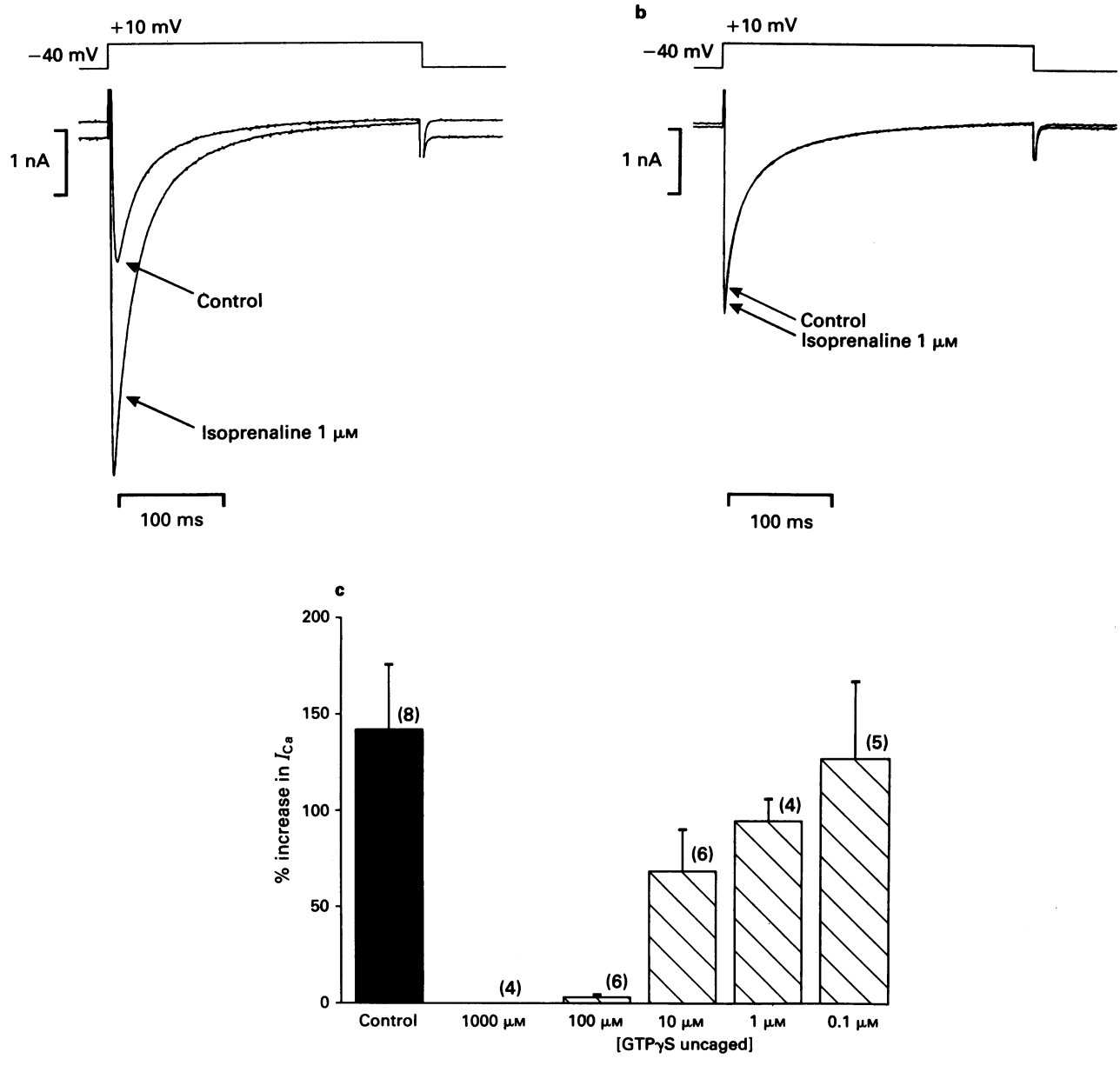

Figure 1 (a) Non leak-subtracted records of Ca^{2+} currents illustrating the effect of extracellular application of isoprenaline (1 μM) on I_{Ca}. Ca^{2+} currents were recorded from a single cardiac ventricular myocyte at a test potential of + 10 mV dialyzed with solution B and bathed in solution A. Application of isoprenaline (1 μM) markedly enhanced I_{Ca}. (b) I_{Ca} recorded under similar conditions to those described in (a) but with 100 μM GTPγS added to the pipette dialyzing solution. Under these conditions the response of I_{Ca} to isoprenaline (1 μM) was virtually abolished. (c) Graph illustrating the concentration-dependent inhibition of isoprenaline-induced enhancement of I_{Ca} by intracellularly applied GTPγS. The IC_{50} for this effect is ~10 μM. Note, where applicable the number of experiments is given in parentheses in all figures.

adenylate cyclase, the effects of extracellular application of forskolin (a direct activator of adenylate cyclase) were examined in the absence or presence of 1 mM GTPγS (note, this concentration of GTPγS totally abolished the response to isoprenaline). Under control conditions (Figure 2a) superfusion of cells with 10 μM forskolin produced a marked endhancement of I_{Ca} which was significantly reduced, but not abolished, when 1 mM GTPγS dialysed the cell interior (Figure 2b). In addition, the presence of 1 mM GTPγS intracellularly significantly reduced the time-to-peak response for enhancement of I_{Ca} from 187 ± 21 s (n = 12) to 120 ± 15 s (n = 12). To verify that the increase in I_{Ca} evoked in response to forskolin (10 μM) was mediated solely by cyclic AMP-dependent phosphorylation, the effects of forskolin (10 μM) were repeated on cells dialyzed with 1 mM R_p-cAMPS (a cyclic AMP antagonist; Van Hastert *et al.*, 1984) which prevented activation of I_{Ca} by the drug (Figure 2c). The results obtained using forskolin are summarized quantitively in Figure 2d.

Thus it appears that chronic application of uncaged GTPγS intracellularly inhibits the response to β-adrenoceptor stimulation by isoprenaline and reduces the effects of direct stimulation of adenylate cyclase by forskolin. Furthermore, uncaged GTPγS can modulate the rate at which cyclic AMP-dependent activation of I_{Ca} occurs in response to these sympathomimetic agents; with marked changes in time-to-peak response being observed in spite of gradual bath perfusion to apply these drugs.

Effects of photoreleased nucleotides

The above results prompted a further investigation into the effects of PR-GTPγS and PR-cAMP; the technique of flash photolysis being used to evoke rapid intracellular concentra-

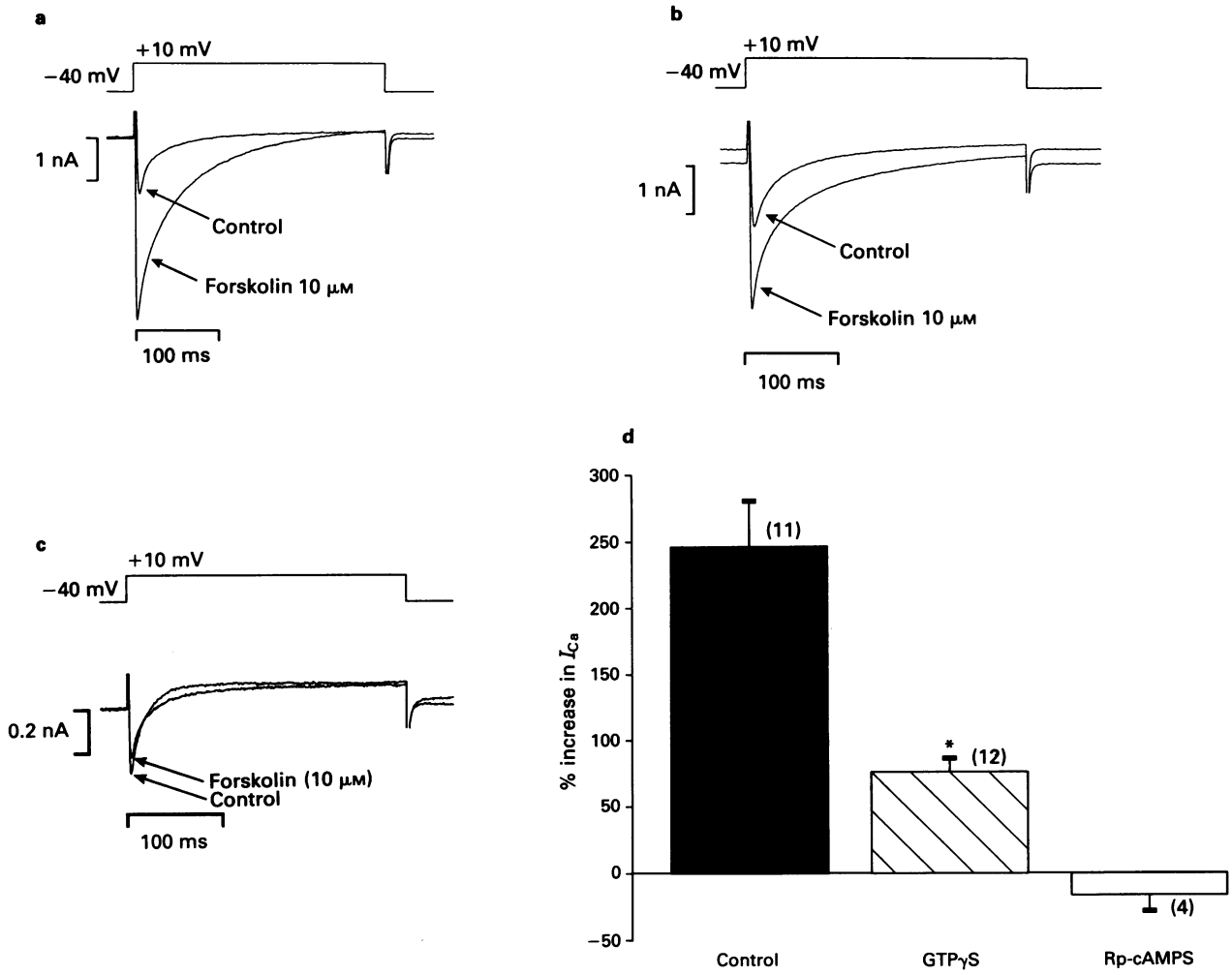

Figure 2 (a) Non leak-subtracted records of Ca^{2+} currents illustrating the effect of extracellular application of forskolin (10 μM) on I_{Ca}. Ca^{2+} currents were recorded from a single cardiac ventricular myocyte (at a test potential of + 10 mV) dialyzed with solution C and bathed in solution A. On this occasion application of forskolin increased the amplitude of I_{Ca} by ~210%. (b) I_{Ca} recorded under similar conditions to those described in (a) but with the addition of 1 mM GTPγS to the pipette dialyzing solution. Under these conditions forskolin enhanced the amplitude of I_{Ca} by ~110%. (c) I_{Ca} recorded under similar conditions to those described in (a) only in the presence of 1 mM R_p-cAMPS added to the pipette dialyzing solution. The response of I_{Ca} to forskolin (10 μM) was abolished. (d) Graph summarizing the effects of forskolin (10 μM) shown in (a), (b) and (c). Note intracellularly applied GTPγS (1 mM) significantly reduced (*) but did not abolish activation of I_{Ca} by forskolin. The responses to forskolin were abolished by R_p-cAMPS (1 mM). Note in one cell forskolin caused a 600% increase in peak I_{Ca}.

tion jumps of these nucleotides enabling an investigation into the kinetics underlying cyclic AMP and G-protein-mediated events. Initially the effects of PR-GTPγS on I_{Ca} were examined. On ~70% of the occasions tested a biphasic increase in I_{Ca} of 72.0 ± 11.0% (n = 12) with a mean time to peak of 225 ± 19 s (n = 12) was observed (for an example see Figure 3a). In the remaining ~30% of the occasions tested a smaller monophasic increase of 26.9 ± 7.1% (n = 5) with a time to peak of 46 ± 15 (n = 5) occurred (for a discussion of the mechanisms underlying these effects see Kozlowski *et al.*, 1991). Evident in these experiments was a rapid increase in I_{Ca} of 4.2 ± 0.7% (n = 17) above control 20 ms after the light flash. It should be noted that previous experiments using structurally related caged derivatives of guanosine nucleotides have revealed that neither the photolysis products nor the flash itself induce a significant, rapid change in peak I_{Ca} (Kozlowski *et al.*, 1991; 1992c). Co-dialysis of cells with 1 mM caged GTPγS in the presence of 0.1 to 100 μM uncaged GTPγS inhibited the response to PR-GTPγS (Figure 3b) in a concentration-dependent manner with an IC_{50} of ~1.0 μM (Figure 3c): a ten fold increase in sensitivity over the inhibitory effect elicited by chronic application of GTPγS on isoprenaline induced enhancement of I_{Ca}.

In contrast to the inhibitory effects of PR-GTPγS, PR-cyclic AMP produced an increase in peak I_{Ca} of 119.9 ± 24.6% with a time-to-peak of 37.8 ± 5.3 s (n = 9; Figure 4a) with no change (− 3.1 ± 1.9%; n = 9) in I_{Ca} occurring within 20 ms of the initiation of the u.v. light flash. Furthermore, the overall time course for this effect was markedly different with the response beginning to wane soon after the peak current had been reached and declining to 50% of the peak response within 108 ± 21 s (n = 9). Replacement of caged cyclic AMP with caged cyclic GMP and its subsequent photolysis (which would be expected to yield identical photoproducts to cyclic AMP) did not affect the amplitude of I_{Ca} (n = 5). The activating action of PR-cyclic AMP was markedly inhibited by co-dialysis of cells with 1 mM R_p-cAMPS (Figure 4b) with peak I_{Ca} increasing by 27 ± 5% (n = 4) after 10 s and abolished by activation of I_{Ca} with 100 μM 3-isobutyl-1-methylxanthine (IBMX) which increased I_{Ca} by 76.2 ± 27.0% (n = 3). In contrast to the inhibitory effects of GTPγS on the enhancing action of forskolin on I_{Ca},

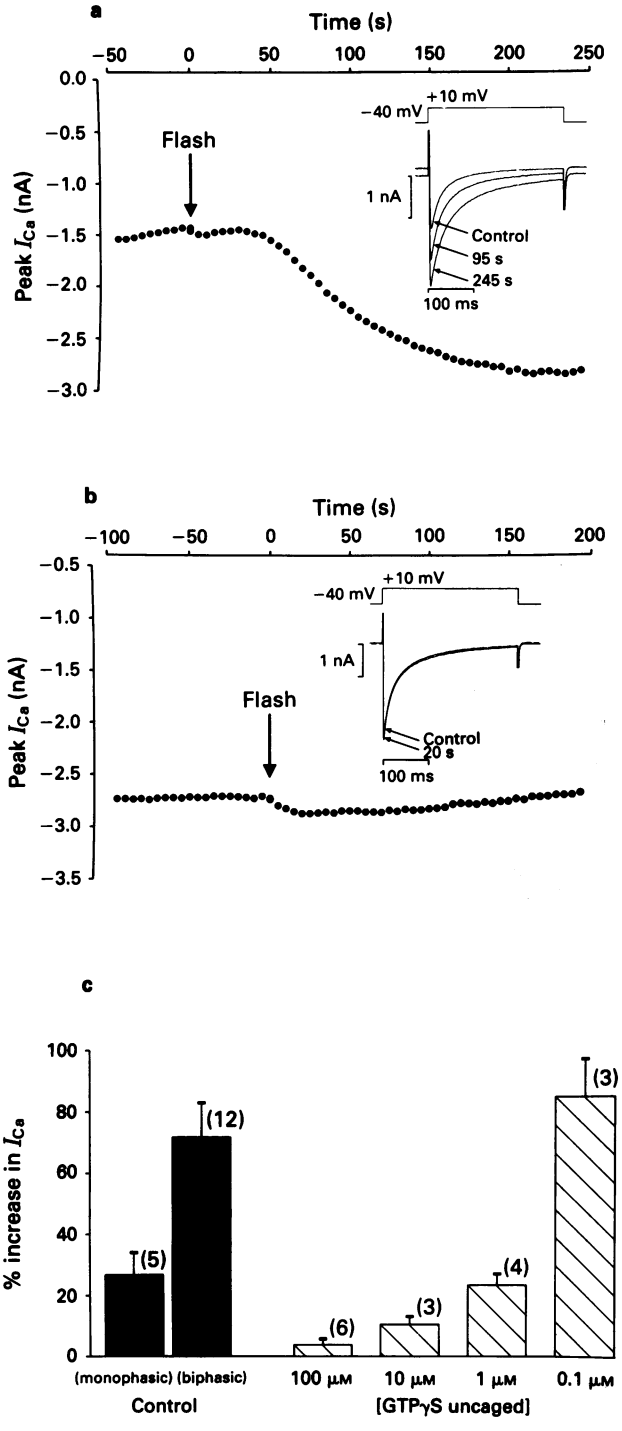

Figure 3 (a) A plot of peak I_{Ca} against time illustrating the activating effect of intracellular photorelease of caged-GTPγS. Cells were dialyzed with solution C containing 1 mM caged-GTPγS and bathed in solution B. Ca^{2+} currents were evoked at a test potential of +10mV as described in the Methods. A single u.v. light flash 20 ms before the onset of a voltage-clamp pulse induced a biphasic increase in I_{Ca} which was maximal after ~200 s. Shown in the inset are individual L-type Ca^{2+} current records taken from the time course plot at control (immediately preceding the flash), 95 and 245 s after photorelease of GTPγS. (b) A plot of peak I_{Ca} against time for inhibitory effects of GTPγS on activation of I_{Ca} by photoreleased GTPγS. Cells were dialyzed with solution C containing 1 mM caged-GTPγS and 100 μM uncaged GTPγS and bathed in solution B. I_{Ca} was evoked as described in (a). Photorelease of GTPγS under these conditions induced a small increase in I_{Ca}. Shown in the inset are individual L-type Ca^{2+} current records taken from the time course plot at control (immediately preceding the flash) and 20 s after photorelease of GTPγS. (c) Graph showing the inhibitory action of intracellularly applied GTPγS on the activating action of photoreleased GTPγS on I_{Ca}. The IC_{50} for this effect is ~1.0 μM.

chronically applied GTPγS (100 μM) did not inhibit the increase in peak I_{Ca} induced by PR-cyclic AMP (Figure 4c). However, the time-to-peak response was accelerated (Figure 4d) resulting in a significant increase in I_{Ca} of 6.1 ± 3.4% ($n = 6$) occurring within 20 ms of the light flash, compared to − 3.1 ± 1.9% ($n = 9$) in the absence of GTPγS (see above). This suggests that chronically applied GTPγS may sensitize L-type Ca^{2+} channels to cyclic AMP-dependent phosphorylation.

Effects of PTX on the responses of I_{Ca} to isoprenaline and photoreleased GTPγS

It is likely that the inhibitory effects of chronic application of GTPγS described above occur both upstream and at the level of adenylate cyclase, since enhancement of I_{Ca} in response to forskolin is much reduced by a concentration of GTPγS which abolishes the response to isoprenaline. This result could be due to the activation of an inhibitory G-protein by GTPγS, perhaps G_i, linked to adenylate cyclase. In order to test this possibility the effects of PTX on the chronic inhibitory action of GTPγS on I_{Ca} in response to isoprenaline and PR-GTPγS were examined. Isoprenaline (1 μM) applied to cells pretreated with PTX (1 μM; see Methods) and dialyzed with 100 μM GTPγS (a concentration which virtually abolished the potentiation of I_{Ca} by isoprenaline; see Figure 1) caused a marked increase in peak I_{Ca} similar in magnitude to that observed in untreated cells (Figure 5a).

It has been shown previously that I_{Ca} is unaffected by muscarinic agonists in the absence of β-adrenoceptor stimulation, but is reduced by such agents following activation of these receptors; this inhibitory effect is due to inhibition of adenylate cyclase via activation of G_i (Hescheler *et al.*, 1986). Accordingly, in order to verify the efficiency of PTX incubation the effects of carbachol (10 μM) were examined following activation of I_{Ca} by isoprenaline (50 nM) in PTX pre- and untreated cells. In PTX untreated cells, 10 μM carbachol caused a substantial inhibition (~40%) of I_{Ca} following its enhancement by isoprenaline (Figure 5b). This inhibitory effect of carbachol was significantly reduced in PTX pretreated cells (Figure 5b). Furthermore, upon dialyzing cells with GTPγS (100 μM) the response to both isoprenaline (50 nM) and carbachol (10 μM) was abolished ($n = 3$; data not shown).

In contrast to the inhibition of isoprenaline enhancement of I_{Ca} by GTPγS, the inhibitory effects of uncaged GTPγS (100 μM) on PR-GTPγS induced activation of I_{Ca} could not be restored by PTX pretreatment ($n = 7$; e.g. Figure 5c). This suggests that the underlying mechanism(s) for inhibition of β-receptor-mediated and GTPγS induced enhancement of I_{Ca} are quite different: in the case of PR-GTPγS it may depend largely upon competition for GTPγS binding sites *per se* rather than G-protein activation.

Following cell dialysis with solution C, peak I_{Ca} generally began to run-down gradually soon after the whole-cell configuration had been attained (Figure 6a). The time course of run-down generally had a bi-phasic profile similar to that reported by others (Belles *et al.*, 1988). In contrast, cells dialyzed with 10–1000 μM GTPγS showed an initial run-up of peak I_{Ca} followed by a run-down of the current. Such an effect has also been observed upon pressure assisted dialysis of cardiac myocytes with GTPγS (Shuba *et al.*, 1990). Following PTX pretreatment, in the presence of 100 μM GTPγS intracellularly, a gradual run-up of the current was observed over an identical time period. Illustrated in Figure 6b is a bar chart showing the amplitude of peak I_{Ca} after 10 to 15 min of whole-cell recording at the time of the experiment, either drug application or photolysis of the caged compound, was initiated. Although, unlike the other concentrations of GTPγS used, the amplitude of I_{Ca} in the presence of 100 μM GTPγS intracellularly was significantly increased relative to control under these conditions. Furthermore, it was also significantly smaller than the amplitude of I_{Ca} in the presence

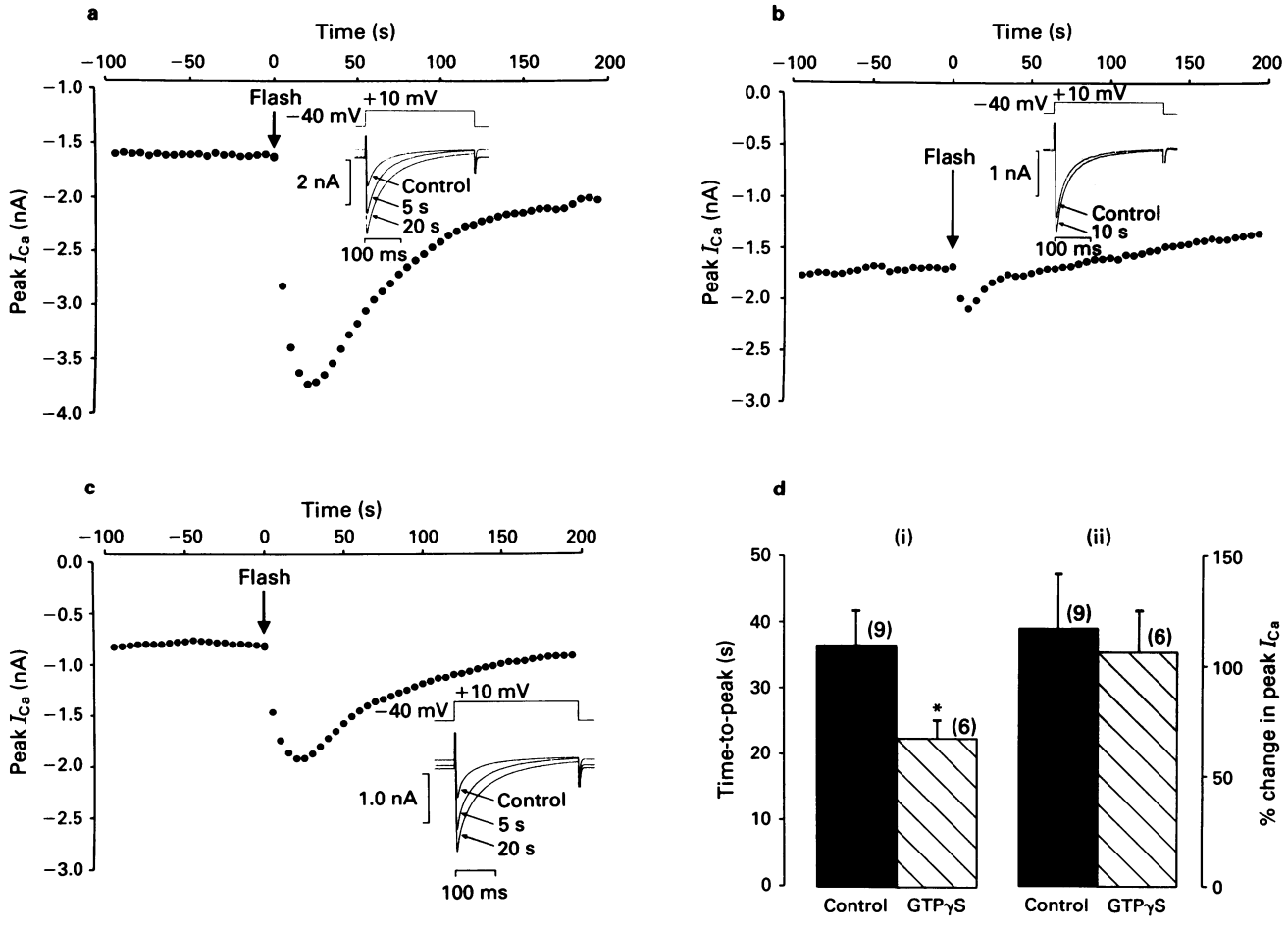

Figure 4 (a) A plot of peak I_{Ca} against time illustrating the activating effect of photoreleased cyclic AMP intracellularly. Ca^{2+} currents recorded from a single cardiac ventricular myocyte (evoked at a test potential of +10mV from a holding potential of −40 mV) dialyzed with 1 mM caged-cyclic AMP and bathed in solution A. A single light flash elicited 20 ms before the onset of a voltage-clamp pulse induced a marked increase in I_{Ca} which rapidly returned to control. Shown in the inset are individual L-type Ca^{2+} current records taken from the time course plot at control (immediately preceding the flash), 5 and 20 s after photorelease of cyclic AMP. (b) A plot of peak I_{Ca} against time for the activating effect of photorelease of caged-cyclic AMP in the presence of 1 mM R_p-cAMPS intracellularly. Conditions for recording were similar to those described in (a) with R_p-cAMPS (1 mM) added to solution B. Shown in the inset are individual L-type Ca^{2+} current records taken from the time course plot at control (immediately preceding the flash) and 10 s after photorelease of cyclic AMP. (c) A plot of peak I_{Ca} against time showing the activating effect of photorelease of caged-cyclic AMP in the presence of GTPγS. Ca^{2+} currents recorded from a single cardiac ventricular myocyte, under the conditions described in (a), co-dialyzed with 1 mM caged-cyclic AMP and 100 μM GTPγS and bathed in solution A. A single light flash elicited 20 ms before the onset of a voltage-clamp pulse induced a marked increase in peak I_{Ca} which rapidly returned to control. Shown in the inset are individual L-type Ca^{2+} current records taken from the time course plot at control (immediately preceding the flash), 5 and 20 s after photorelease of cyclic AMP. (d) Graphs illustrating the effects of intracellularly applied GTPγS (100 μM) on the activating action of photoreleased cyclic AMP on I_{Ca}. Note little difference in the magnitude of the peak response (ii) of I_{Ca} to photoreleased cyclic AMP in the presence of GTPγS (1 mM) but a significant reduction in the time-to-peak response (i).

of 10 mM cyclic AMP intracellularly or after bath application of either 1 μM isoprenaline or 10 μM forskolin.

Discussion

Rapid intracellular application of the slowly hydrolyzed GTP analogue, GTPγS, by photolysis of its caged derivative (Kozlowski *et al.*, 1991) or by pressure assisted dialysis (Shuba *et al.*, 1990; Pelzer *et al.*, 1991) is known to increase I_{Ca}. This effect is thought to involve activation of a G_s type G-protein linked both to the adenylate cyclase and directly to the L-type Ca^{2+} channel (Shuba *et al.*, 1990; Pelzer *et al.*, 1990; Kozlowski *et al.*, 1991). Since GTPγS is resistant to the intrinsic GTPase activity of the α subunit, it can, at least in cell-free patches, enhance and sustain L-type Ca^{2+} channel activity evoked by isoprenaline (Yatani *et al.*, 1987). Consequently, it would also be expected to enhance signal transduction in whole-cell recordings of I_{Ca}. Indeed low concentrations of GTPγS (< 10 μM) do potentiate activation of I_{Ca} in response to H_2 receptor stimulation by histamine (Hescheler *et al.*, 1987). In this study, however, chronic application of GTPγS prevented enhancement of I_{Ca} by isoprenaline with an IC_{50} of ~10 μM.

A simple hypothesis to account for the inhibition of the effects of isoprenaline on I_{Ca} by high concentrations of GTPγS would be that G_s (and all related G-proteins) are maximally stimulated under these conditions, thereby uncoupling the respective receptors from the amplification pathway. Such a hypothesis has been postulated for GTPγS-mediated inhibition of I_{Ca} enhancement in response to P_2 receptor stimulation (Scamps *et al.*, 1992) and the sustained potentiation of I_{Cl} by isoprenaline in guinea-pig ventricular myocytes (Hwang *et al.*, 1992). Our observations that I_{Ca} is

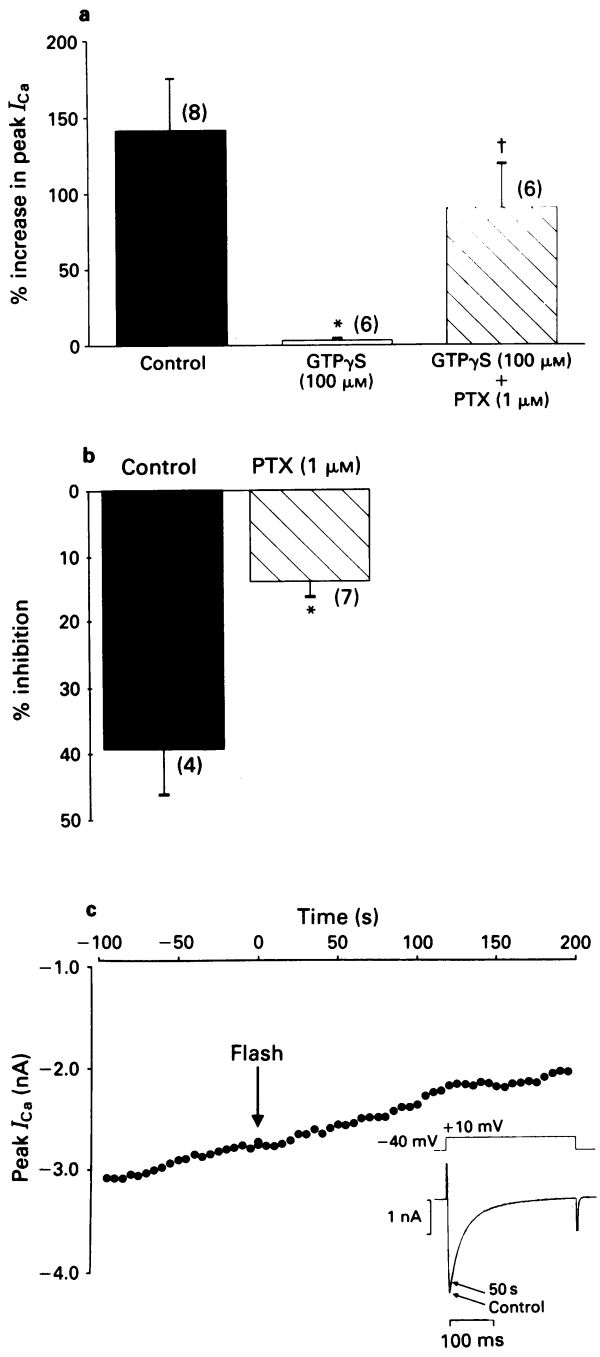

Figure 5 (a) Inhibition of isoprenaline induced activation of I_{Ca} by GTPγS. Cell dialysis with GTPγS (100 μM) produced a significant inhibition (*) of isoprenaline (1 μM)-induced enhancement of I_{Ca} (control). This inhibitory effect was reversed by pretreatment of cells with 1 μM pertussis toxin (PTX) (see Methods) and the resulting increase in I_{Ca} was significantly different to that observed in the presence of 100 μM GTPγS in PTX untreated cells (†) but not significantly different from control. (b) Graph illustrating G_i mediated inhibition of isoprenaline-induced increase of I_{Ca} as a consequence of muscarinic receptor stimulation by carbachol. Superfusion of cells with 10 μM carbachol, in the continued presence of 50 nM isoprenaline, produced a marked inhibition of I_{Ca} (control). This ability of carbachol to inhibit the effects of isoprenaline on I_{Ca} was significantly (*) reduced by pretreatment of cells with 1 μM PTX. (c) A plot of peak I_{Ca} against time for the Ca^{2+} currents recorded from a single cardiac ventricular myocyte (at a test potential of +10 mV) pretreated with PTX, dialyzed with solution B containing 1 mM caged-GTPγS, 100 mM GTPγS and bathed in solution A. A single light flash elicited 20 ms before the onset of a voltage-clamp pulse caused little change in I_{Ca}. Shown in the inset are non leak-subtracted L-type Ca^{2+}current records, taken from the time course plot shown, at control (immediately preceding the flash) and 50 s after photorelease of GTPγS.

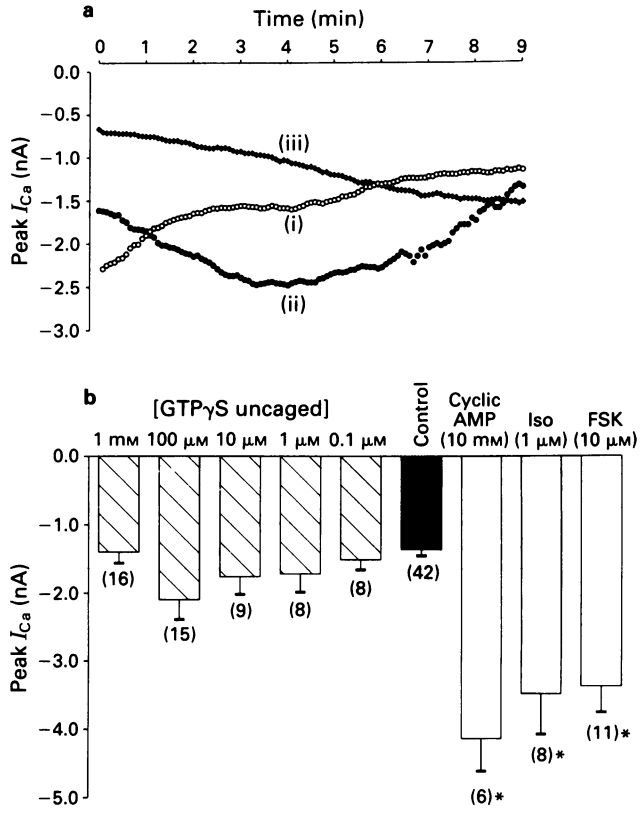

Figure 6 (a) Effects of intracellular dialysis with GTPγS on I_{Ca} recorded from voltage-clamped ventricular myocytes bathed in solution A and dialyzed with solution B in the absence and presence of GTPγS. I_{Ca} evoked from a holding potential of −40 mV at a test potential of +10 mV every 5 s. The amplitude of the current was measured relative to the current observed 280 ms following initiation of the voltage-clamp pulse. Under control conditions (○) (i) in the absence of GTPγS intracellularly, a gradual rundown of peak I_{Ca} occurred. The time course for this effect was clearly biphasic with an initial rapid phase followed by a second more gradual phase. In marked contrast, cells dialyzed with a solution containing 100 μM GTPγS (●) (ii) exhibited an initial increase in I_{Ca} followed by a pronounced rundown, while cells dialyzed with GTPγS (100 μM) following PTX pretreatment (■) (iii) exhibited a run-up of I_{Ca}. (b) Peak I_{Ca} recorded 10 to 15 min after formation of the whole-cell patch-clamp configuration under control conditions and in the presence of GTPγS (0.1 to 1000 μM) or cyclic AMP (10 mM) added to the pipette dialyzing solution (solution C). Note, the magnitude of peak I_{Ca} following stimulation by either bath application of isoprenaline (Iso; 1 μM) or forskolin (FSK; 10 μM), or intracellularly applied cyclic AMP (10 mM) was significantly different (*) from the magnitude of I_{Ca} under control conditions or in the presence of GTPγS. Data from experiments where cells were dialyzed with caged GTPγS (1 mM) or caged cyclic AMP (1 mM) have been pooled with data collected from cells dialyzed with solution C alone. The profile for run-up and run-down of I_{Ca}, or its magnitude, was unaffected by the presence of caged nucleotides intracellularly.

increased transiently in cells dialyzed with GTPγS and that the responses of I_{Ca} to both isoprenaline and carbachol are blocked in a concentration-dependent manner by GTPγS would appear to support such a notion, but a number of other observations suggest strongly that this cannot be the only mechanism involved. Firstly, it is clear that after prolonged dialysis with GTPγS the amplitude of I_{Ca} is not maximal. Secondly, care was taken to avoid the initial equilibration period following membrane rupture, so that it cannot be argued that I_{Ca} was already stimulated, thus precluding further enhancement of I_{Ca} by isoprenaline. Furthermore, since the effect of forskolin on I_{Ca} is inhibited after chronic dialysis with GTPγS it can be argued that inhibition

of adenylate cyclase occurs probably via activation of additional G-proteins by GTPγS, including G_i. Finally, this latter contention is supported by the results obtained following pretreatment of cells with PTX, which prevented the inhibitory effect of GTPγS, clearly implicating the involvement of a PTX-sensitive G-protein. It is therefore likely that an initial stimulation of G_s followed by its inhibition through activation of G_i (see Katada *et al.*, 1986) is responsible, since the response is PTX-sensitive and GTPγS would be expected to activate both G_s and G_i. In support of this notion, chronic application of GTPγS did not affect the increase in peak I_{Ca} evoked by PR-cyclic AMP. Taken together these observations suggest that the inhibitory action of chronically applied GTPγS involves activation of G_i coupled directly to adenylate cyclase.

It is unlikely that any of the mechanisms proposed above underlie the inhibitory action of chronically applied GTPγS on activation of I_{Ca} mediated by PR-GTPγS, since this effect is not sensitive to PTX. This inhibitory response, with an IC_{50} of $\sim$1 μM, is likely to be largely due to a simple competition between uncaged GTPγS and PR-GTPγS for their binding site(s). Thus as the concentration of uncaged GTPγS is increased, the number of binding sites available for interaction with photoreleased GTPγS decreases. The low concentration of uncaged GTPγS required to prevent the activating action of PR-GTPγS and externally applied isoprenaline indicates that guanosine nucleotides have a high affinity for the α subunits with maximal activation occurring at micro molar concentrations. This finding is consistent with the observations of others. For example Scamps *et al.* (1992) have shown that GTPγS markedly increases I_{Ca} at concentrations of 3 μM, while Nakajima *et al.* (1992) have shown that the EC_{50} for GTPγS-induced activation of acetylcholine-activated K^+ channels in atrial myocytes is $\sim$0.1 μM.

In addition to the inhibitory effect(s) described above, the presence of GTPγS intracellularly accelerates the response to both forskolin and PR-cyclic AMP. It is likely this is due to a sensitizing action, mediated through activation of a G_s type G-protein coupled directly to L-type Ca^{2+} channels. Such a mechanism was proposed by Cavalie *et al.* (1991) who suggested that stimulation of G_s directly coupled to the channels results in an enhanced response to cyclic AMP-dependent phosphorylation. In our experiments however, the response itself was not enhanced, but was greatly accelerated with a substantial increase in I_{Ca} occurring within 20 ms of the light flash, suggesting that PR-cyclic AMP, like photo-released GTP, (Kozlowski *et al.*, 1992c) can modulate I_{Ca} very rapidly.

In conclusion, it appears that GTPγS can evoke at least three different effects on L-type Ca^{2+} channels in cardiac ventricular myocytes. (i) Rapid or acute application of the nucleotide by flash photolysis induces L-type Ca^{2+} channel activation by stimulating G_s (see Kozlowski *et al.*, 1991 for details). (ii) Chronically applied GTPγS, by cell dialysis, inhibits isoprenaline induced activation of I_{Ca} by interacting with a PTX-sensitive binding G-protein located upstream of adenylate cyclase (probably G_i), and (iii) accelerates the response to cyclic AMP-dependent phosphorylation, possibly by interacting with a G-protein coupled directly to the L-type Ca^{2+} channel. In view of this diverse range of effects care should be taken when using GTPγS to characterize G-protein-mediated events, since the resulting physiological response may be due to activation of multiple G-protein pools. It will be of interest in the future to test whether GTP, the endogenous guanosine nucleotide, can accelerate the effects of isoprenaline on I_{Ca}. If this proves to be the case and GTP, like GTPγS, can accelerate the response to cyclic AMP-dependent processes, rapid effects due to β-receptor stimulation could be reconciled primarily with activation of G_s coupled to adenylate cyclase.

This work was supported by grants from Glaxo Group Research, The Wellcome Trust and The British Heart Foundation. R.Z.K. is a British Heart Foundation Intermediate Research Fellow and the BT Junior Research Fellow at Brasenose College, Oxford. L.J.G. is a Wellcome Prize Student. We thank Alison Gurney, Dept of Pharmacology, St Thomas' Hospital, London for her help in quantifying nucleotide release from their caged precursors.

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(Received March 3, 1993
Revised July 22, 1993
Accepted September 13, 1993)

Br. J. Pharmacol. (1994), **111**, 259–263

Prevention by phosphodiesterase inhibitors of antigen-induced contraction of guinea-pig colonic smooth muscle

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1 The ability of various phosphodiesterase (PDE) inhibitors to reduce the initial and/or late response to ovalbumin (OVA) in isolated strips of guinea-pig colonic smooth muscle from sensitized animals was examined.

2 Both the initial and late responses to OVA (0.05 mg ml^{-1}) were inhibited by the non-selective PDE inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, EC_{50} = 26.0 and 6.1 μM, respectively), and the selective inhibitors of low K_m cyclic AMP specific PDE (PDE IV), (**R**)- and (**S**)- rolipram. The (**S**)- isomer (EC_{50} = approximately 1.0 μM for both responses) was about 10 fold less potent than the (**R**)- isomer (EC_{50} = approximately 0.1 μM for both responses).

3 Zaprinast, a selective inhibitor of the cyclic GMP-specific PDE (PDE V) inhibited only the late response (EC_{50} = 1.4 μM).

4 SK&F 94120, a selective inhibitor of the cyclic GMP-inhibited low K_m cyclic AMP PDE (PDE III), inhibited the initial response (45.9 ± 11.9%, $P<0.05$) at the highest concentration tested (100 μM), and had no effect on the late response.

5 The results suggest that PDE inhibitors, especially PDE IV inhibitors, can attenuate the contractile response of guinea-pig colon to OVA.

Keywords: Phosphodiesterase inhibitors; antigen-induced contraction; guinea-pig colon; zaprinast; rolipram; SK&F 94120; 3-isobutyl-1-methyl-xanthine

Introduction

The release of inflammatory mediators such as histamine or the peptidoleukotrienes can change gastrointestinal motility (Collins *et al.*, 1989). For example, addition of antigen to isolated strips of gastrointestinal smooth muscle obtained from sensitized rat or guinea-pig, contracts both small intestinal and colonic tissue (Vermillion *et al.*, 1988; Barnette & Grous, 1992). In previous studies using sensitized guinea-pig colon, we found that antigen addition produced a biphasic contraction consisting of an initial rapid response that is blocked by the H_1-histamine receptor antagonist mepyramine, and a slower developing late contraction that is reduced by a peptidoleukotriene (LTD_4) receptor antagonist, Wy 48252, (Barnette & Grous, 1992). The combination of both an H_1-histamine receptor antagonist and an LTD_4 receptor antagonist eliminated both responses to antigen. Thus, these findings strongly suggest that antigen addition is causing the activation of an inflammatory cell, most likely the mast cell, and the release of histamine and peptidoleukotrienes (Barnette & Grous, 1992).

Although PDE isozymes have been characterized by several investigators in cardiac muscle, airway and arterial smooth muscles (Silver *et al.*, 1988; Torphy & Cieslinski, 1990; Shadid *et al.*, 1991), only recently have a limited number of studies characterizing the profile in gastrointestinal smooth muscle been reported (Silver *et al.*, 1990; Barnette *et al.*, 1993). PDE inhibitors potentiate forskolin-induced relaxation of rabbit intestinal smooth muscle (Muller & Baer, 1983) and enhance the increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) produced by forskolin in canine colonic smooth muscle (Barnette *et al.*, 1993). However, it is not known what influence PDE inhibitors have on the response to antigen in gastrointestinal smooth muscle.

The existence of at least five families of PDE isozymes is widely accepted (Torphy & Cieslinski, 1990; Nicholson *et al.*, 1991; Shahid *et al.*, 1991). Although non-selective PDE inhibitors, such as 3-isobutyl-1-methylxanthine (IBMX) have been known for some time, only recently have selective PDE inhibitors been identified, thus, giving us the pharmacological tools needed to study the effects of PDE inhibition on tissue function. The selective inhibitors for individual PDE isozymes used in this study are: (1) SK&F 94120, a selective inhibitor of cyclic GMP-inhibited PDE (PDE III); (2) (**R**)- and (**S**)- rolipram, selective inhibitors of low K_m cyclic AMP specific PDE (PDE IV); (3) zaprinast, a selective inhibitor of cyclic GMP specific PDE (PDE V).

Increases in cyclic nucleotide content produced by PDE inhibition will inhibit the release of mediators from inflammatory cells (Torphy & Undem, 1991). Activators of adenylate cyclase such as isoprenaline have been shown to inhibit the release of mast cell mediators induced by IgE stimulation (Undem *et al.*, 1988) as well as the production of oxygen free radicals from neutrophils (Nielson *et al.*, 1990). Increasing cyclic AMP via PDE inhibition will inhibit the respiratory bursts from eosinophils and neutrophils (Nielson *et al.*, 1990; Dent *et al.*, 1991) and inhibit mediator release from rat mast cells (Frossard *et al.*, 1981), human basophils (Frossard *et al.*, 1981; Peachell *et al.*, 1992) and human neutrophils (Schudt *et al.*, 1991). With their combined ability to relax smooth muscle and to inhibit inflammatory cell function, PDE inhibitors could exert a profound action on gastrointestinal motility. Therefore, the aim of this study was to examine the ability of selective PDE inhibitors to alter the response of guinea-pig colonic smooth muscle to antigen.

Methods

Tissue preparation

Male Hartley guinea-pigs (250–300 g) were sensitized to OVA by either an intramuscular injection (0.35 ml of OVA in 0.9% NaCl injected into each hindlimb), or an intraperitoneal injection of 0.5 ml of OVA (10 μg) in aluminium

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hydroxide (100 mg). No differences in tissue responsiveness to antigen between either sensitization procedure were noted. After a minimum of 14 days the sensitized animals were killed by cervical dislocation. A segment of distal colon (10–15 cm) was removed approximately 10 cm above the anus and immediately placed in oxygenated saline on ice. The segment of colon was cut along the longitudinal axis, pinned flat to a dissecting board and the mucosa removed. Eight strips of circular smooth muscle (2–3 mm by 10–15 mm) were tied with suture at either end. One end was attached to a stationary rod and the other to a Grass force-displacement transducer (FT-03) to measure isometric contractions using a multi-channel Grass polygraph (Grass Instrument Co., Quincy, MA, U.S.A.). The strips were suspended in 10 ml water jacketed tissue baths containing oxygenated Krebs buffer which was maintained at 37°C. The composition of Krebs buffer was (mM): NaCl 117.9, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and dextrose 11.1. Two grams of tension were applied to each strip which was equilibrated for a minimum of 1.5 h. The tension was readjusted during equilibration until the tissues maintained this level of tone.

Experimental protocol

After equilibration the tissues were contracted with carbachol (1.0 μM). When the carbachol-induced contractions reached a plateau, the tissues were washed for 25–30 min; no adjustments to the resting tension were made after the carbachol-induced contraction. The colonic segments were then pretreated for 20 min with either vehicle (control) or the various PDE inhibitors prior to challenge with OVA (0.05 mg ml^{-1}). After OVA challenge, a biphasic response was observed (Barnette & Grous, 1992). The initial response occurred within 2–3 min after the addition of OVA. Later responses were sustained and were calculated 15 min after the initial response. Both responses were calculated in relation to the baseline after the addition of the PDE inhibitors because addition of the PDE inhibitors decreased basal tone and phasic activity. Time-matched control tissues were run in parallel with drug-treated preparations, and control responses were calculated at the same time points as those of the PDE-treated tissues.

At the end of each experiment, tissues were contracted to carbachol (1.0 μM) and the response obtained compared to the initial carbachol contraction.

Data analysis

Both the initial and late responses to OVA were expressed as a percentage of the initial contraction produced by carbachol. The data are given as the mean ± s.e.mean ($n = 5–6$). Results from the control and treated tissues were analysed for statistical differences by a Student's paired t test with a probability value, P, less than 0.05 regarded as significant. IC_{50} values for individual compounds were calculated as 50% inhibition of the control response to antigen by linear regression.

Materials

Ovalbumin, carbachol and IBMX (3-isobutyl-1-methylxanthine) were obtained from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). SK&F 94120 [5-(4-acetamidophenyl)-pyrazin-2-(1H)-one] one zaprinast (M&B 22,948) were synthesized by Dr William Coates and colleagues (SmithKline Beecham Pharmaceuticals, Welwyn, England). (R)- and (S)-rolipram were synthesized by Dr Sigfried Christensen and colleagues at SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.) and aluminium hydroxide was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Results

Characteristics of antigen-induced contractions in guinea-pig colon have been described previously (Barnette & Grous, 1992). The initial response was blocked by an H_1-histamine receptor antagonist, while the late response was reduced by a peptidoleukotriene receptor antagonist.

All of the PDE inhibitors tested produced a concentration-dependent reduction in both basal tone and phasic activity. Pretreatment of colonic smooth muscle strips with IBMX (1.0–100 μM), a non-selective PDE inhibitor, blocked both the initial and late response to antigen challenge (0.05 mg ml^{-1} OVA, Figure 1a) with EC_{50} values of 26.0 μM and 6.1 μM, respectively. At 100 μM, IBMX significantly

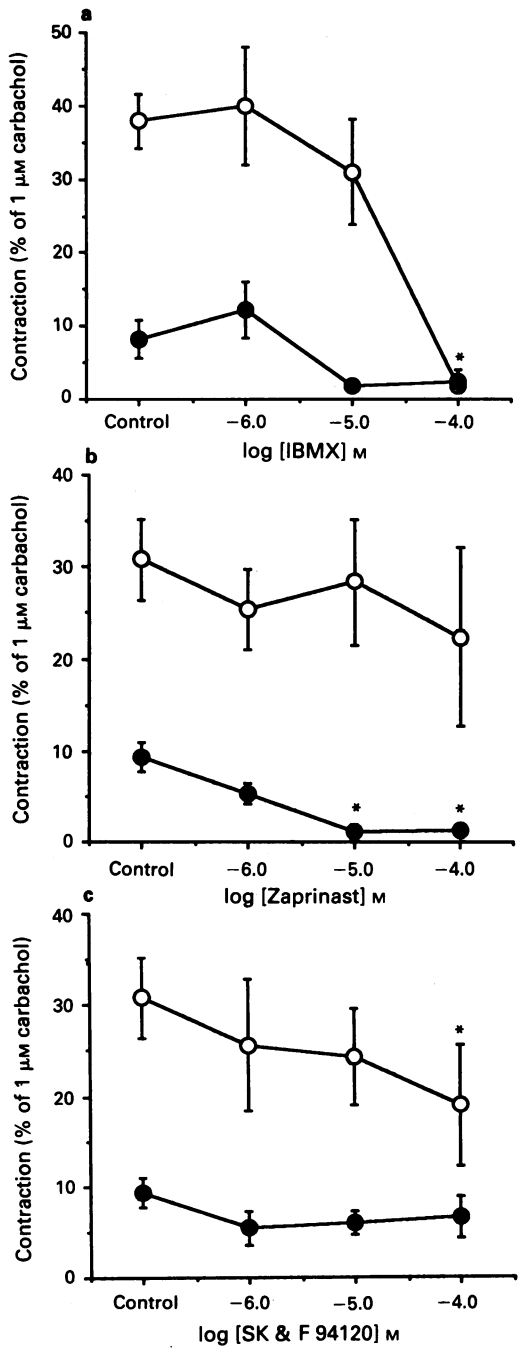

Figure 1 Effect of 3-isobutyl-1-methylxanthine (IBMX) (a) zaprinast (b) or SK&F 94120 (c) on the initial (○) and late (●) phases of ovalbumin-induced contraction of guinea-pig colonic smooth muscle. The data are the mean ± s.e.mean of 6 experiments; $*P < 0.05$.

decreased ($P<0.05$) the initial (95.5 ± 2.5%, $n=6$) and late (84.6 ± 10.1%, $n=6$) responses to OVA.

Pretreatment with zaprinast (1–100 μM), a PDE V inhibitor, did not inhibit the initial response to antigen (Figure 1b); however, it did produce a concentration-related inhibition of the late response with an EC_{50} of 1.4 μM and a maximum decrease of 88.0 ± 7.7% from the control at 10 μM ($P<0.05$).

Addition of SK&F 94120 (1.0–100 μM), a selective PDE III inhibitor, did not significantly reduce the late response to antigen (Figure 1c), and although, at a high concentration of 100 μM, SK&F 94120 significantly decreased ($P<0.05$) the initial response (45.9 ± 11.9%, $n=6$), an EC_{50} value could not be determined.

Rolipram, a PDE IV inhibitor, exists as a mixture of two enantiomers. The (R)- isomer of rolipram (0.01–3 μM) produced a concentration-dependent reduction of both the initial and late response to antigen with an EC_{50} of about 0.1 μM for both responses (Figure 2). Maximal decreases for inhibition of the initial and late responses occurred at 1 and 3 μM, respectively, and were 79.3 ± 1.9%, $n=5$ and 77.8 ± 9.8%, $n=5$, respectively. (R)-rolipram significantly decreased ($P<0.05$) the initial response at 0.1–1.0 μM, and significantly reduced ($P<0.05$) the late response at 0.1–3.0 μM. In contrast, the (S)- isomer of rolipram was approximately 10 fold less potent with respective EC_{50}'s of about 1.1 μM and 1.0 μM, for the initial and the late responses. At 1.0 and 3.0 μM, the initial response was significantly decreased ($P<0.05$), while the late response was significantly reduced ($P<0.05$) at 3.0 μM. Maximal decreases from the control were 73.1 ± 2.7, $n=5$ and 82.4 ± 11.1%, $n=5$ for the initial and late responses, respectively, both of which occurred at 3.0 μM.

To determine any non-specific relaxant effects of PDE inhibitors, all tissues were contracted to carbachol (1.0 μM) at the end of each experiment. This contraction was compared to the initial carbachol (1.0 μM) response by a paired t test and it was noted that none of the compounds examined affected the carbachol-induced contractions (data not shown).

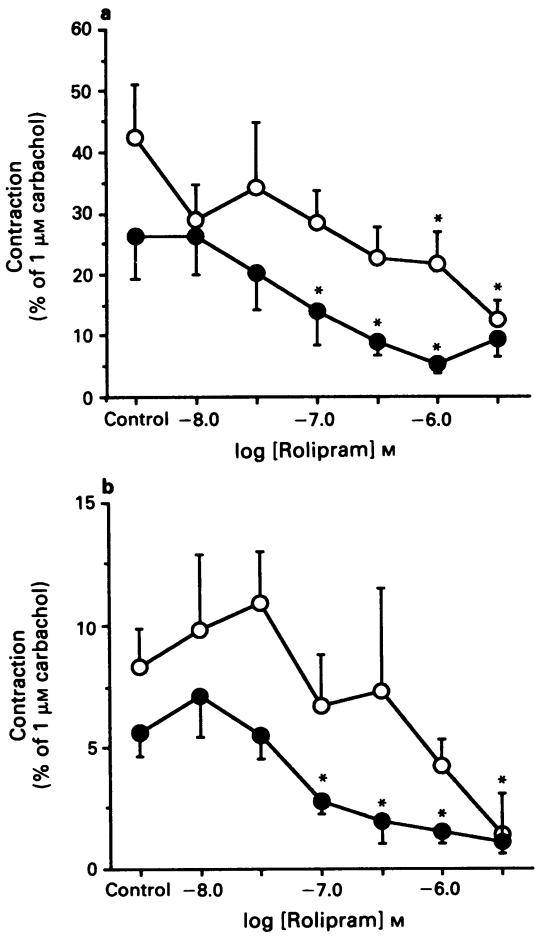

Figure 2 Effect of (R)-rolipram (●) or (S)-rolipram (○) on the initial (a) and late (b) phases of ovalbumin-induced contraction of guinea-pig colonic smooth muscle. The data are the mean ± s.e.mean of 5 experiments; $^*P<0.05$.

Discussion

Since PDE inhibitors, especially PDE IV inhibitors, have been shown to inhibit the production and release of mediators from inflammatory cells (Torphy & Undem, 1991), we were interested in determining their effects on an antigen-induced contraction of gastrointestinal smooth muscle.

In previous studies in colonic strips obtained from guinea-pigs sensitized to OVA, antigen addition produced a biphasic contraction. Moreover, the different phases of the antigen-induced response were differentially inhibited by either an H_1-histamine receptor antagonist or an LTD_4-receptor antagonist. These results suggest that the initial and late responses were caused by the release of two different inflammatory mediators, namely histamine and the peptidoleukotrienes, respectively (Barnette & Grous, 1992).

Pretreatment of colonic smooth muscle strips with IBMX, which non-selectively inhibits all PDE isozymes, inhibited both the initial and late response to antigen challenge with EC_{50} values of 26.0 μM and 6.1 μM, respectively. Although there is little information with regard to the effects of PDE inhibitors on gastrointestinal smooth muscle, IBMX was shown to potentiate the concentration-response curve to forskolin in non-sensitized rabbit small intestine, with an EC_{50} of 0.41 μM (Muller & Baer, 1983). More recently, in non-sensitized canine colonic smooth muscle in the presence of forskolin, IBMX inhibited carbachol-induced contractions with an EC_{50} of 4.0 μM (Barnette *et al.*, 1993). These values are in agreement with the ability of IBMX to inhibit all five PDE isozymes found in the canine trachealis where IC_{50} values range from 1 to 30 μM (Torphy & Cieslinski, 1990). They are also similar to results obtained with antigen-sensitized mouse mast cells (PT-18) in the presence of 0.1 μM forskolin (Torphy *et al.*, 1992a), where 5 μM IBMX produced 45 to 55% inhibition of antigen-induced LTC_4 release, and are consistent with the potency of IBMX for inhibition of PDE activity in mouse mast cells (Torphy *et al.*, 1992a).

Unlike IBMX, pretreatment with zaprinast, a PDE V inhibitor, did not inhibit the initial response to antigen; however, it did produce a concentration-related inhibition of the late response with an EC_{50} of 1.4 μM. When PDE isozymes were characterized in cardiac, arterial and airway smooth muscle in several different species the IC_{50} values for zaprinast range from 0.5 to 4.2 μM (Silver *et al.*, 1988). Zaprinast has also been reported to reduce histamine release from rat mast cells (Frossard *et al.*, 1981) but not to inhibit the release of histamine or LTC_4 from human basophils (Peachell *et al.*, 1992). Although, the PDE isozymes present in guinea-pig mast cells have not yet been characterized, guinea-pig eosinophil cyclic AMP PDE activity has been reported to be only weakly inhibited by zaprinast ($IC_{50}=35$ μM), and in these cells zaprinast is probably not acting on the PDE V isozyme (Souness *et al.*, 1991). Likewise, in non-sensitized canine colonic smooth muscle strips, the potency of zaprinast ($EC_{50}=23.1$ μM) at relaxing carbachol-induced contractions is also weak and the authors conclude that it is not acting on the PDE V isozyme (Barnette *et al.*, 1993). Overall, in the guinea-pig colon, these data suggest that zaprinast decreases or modulates antigen-induced contractions of the late response via PDE V inhibition.

Pretreatment with SK&F 94120, a selective PDE III inhibitor, did not reduce the late response to antigen. Although, the initial response was significantly decreased at the highest concentration tested (100 μM), the effects at this concentration are likely to be non-selective and not due to inhibition of PDE III. This finding is in agreement with the lack of effects of PDE III inhibitors on inflammatory cell function (Nielson *et al.*, 1990; Dent *et al.*, 1991; Souness *et al.*, 1991; Peachell *et al.*, 1992) as well as the lack of effect on bovine tracheal smooth muscle (Nicholson *et al.*, 1990) and gastrointestinal motility (Silver *et al.*, 1990). However it contrasts with results in canine colon where SK&F 94120 decreased carbachol-induced contractions (Barnette *et al.*, 1993), and also canine trachea (Torphy *et al.*, 1991b), where PDE III plays a significant role in regulating cyclic AMP hydrolysis and smooth muscle tone.

Rolipram exists as a mixture of two enantiomers. (R)-rolipram is 15 to 20 fold more potent than (S)- rolipram at binding to a site in rat brain (Schneider *et al.*, 1986), whereas, it is about 3 fold more potent at inhibiting human recombinant PDE IV catalytic activity (Torphy *et al.*, 1992b). In this study it was observed that (R)- rolipram was about 10 fold more potent than (S)- rolipram in inhibiting both the initial and late response to antigen. This effect is similar to the action of these enantiomers at the binding site in rat brain but is in marked contrast to their effect against antigen-induced contractions of guinea-pig airways, where their anti-allergic activity is associated with PDE IV catalytic activity and they lack enantiomeric selectivity (Underwood *et al.*, 1993). Although the anti-allergic activity of these enantiomers is believed to be associated with PDE IV catalytic activity, other investigators have observed a significant correlation between the displacement of rolipram binding and gastrointestinal transit (Silver *et al.*, 1990). Taken together, the results of our study suggest that the inhibitory activity of rolipram in the guinea-pig colon is associated with the rolipram binding site.

Although the site of action for these compounds is unknown, PDE inhibitors could be acting either at the level of smooth muscle or the inflammatory cell. In this study, however, PDE inhibition did not affect carbachol-induced contractions suggesting that smooth muscle is probably not the site of action. Studies in non-sensitized canine colon also support this hypothesis, since PDE inhibitors, in the absence of forskolin, did not reduce carbachol-induced contractions (Barnette *et al.*, 1993).

An alternate site of action might be the inflammatory cell itself, possibly a tissue mast cell. Inhibition of PDE, especially PDE IV, has been reported to inhibit LTC_4 and histamine release from human basophils (Peachell *et al.*, 1992), as well as superoxide anion production in human neutrophils (Schudt *et al.*, 1991) and guinea-pig eosinophils (Dent *et al.*, 1991). However, inhibition of histamine release from rat peritoneal mast cells was not affected by rolipram but was inhibited by zaprinast (Frossard *et al.*, 1981). In guinea-pig isolated airways, rolipram inhibited both antigen-induced contractions and the release of prostaglandin D_2 (PGD_2) and histamine (Underwood *et al.*, 1993). In the same study, contractions of OVA-sensitized guinea-pig tracheal smooth muscle rings produced by histamine or LTD_4 were not inhibited by rolipram suggesting it was more effective at inhibiting mediator release than smooth muscle contraction *per se*. We have previously demonstrated a biphasic response in colonic strips obtained from OVA-sensitized guinea-pigs. The initial response is mimicked by histamine and blocked by an H_1 receptor antagonist, and the late response is mimicked by LTD_4 and blocked by an LTD_4-receptor antagonist (Barnette & Grous, 1992). Since the PDE inhibitors used in this study were effective against the initial and late response to antigen in OVA-sensitized guinea-pigs, these results suggest that the compounds could be acting on inflammatory cells to reduce the release of histamine and leukotriene.

In conclusion, our findings indicate that PDE inhibitors inhibit antigen-induced contractions in guinea-pig colon, perhaps by decreasing the release of inflammatory mediators. This suggests that selective PDE inhibition may decrease the responsiveness of the gastrointestinal tract seen during inflammation, and may have a therapeutic benefit in the treatment of altered gastrointestinal motility associated with inflammatory diseases of the gastrointestinal tract.

The authors are grateful to Douglas Hay, PhD, for his expert editorial input and thank Dotti Lavan for her secretarial assistance.

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(Received June 16, 1993
Revised August 31, 1993
Accepted September 16, 1993)

Br. J. Pharmacol. (1994), **111**, 264–270

Potentiation of responses to sympathetic nerve stimulation and vasoconstrictor agents by SK&F 103829 in the feline mesenteric circulation

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1 The amplification of vasoconstrictor effects of several agonists and sympathetic nerve stimulation, caused by 5-HT_2 receptor activation, was studied in the autoperfused mesenteric circulation of anaesthetized cats. To produce long lasting and selective 5-HT_2 receptor stimulation we used SK&F 103829 (2,3,4,5 tetrahydro-8[methyl-sulphonyl]-1H3-benzazepin-7-ol methensulphonate). We assessed that SK&F 103829 was a strong contractile partial agonist in isolated preparations of rat tail artery and calf pulmonary artery.

2 The intrinsic activity of SK&F 103829 with respect to 5-hydroxytryptamine (5-HT) was 0.8 in rat tail artery and 0.6 in calf pulmonary artery. SK&F 103829-induced contractile responses were surmountably antagonized by ketanserin with a potency expected from its affinity for 5-HT_2 receptors. SK&F 103829 surmountably antagonized the effects of 5-HT in rat tail artery with a pK_p of 5.8.

3 Concentrations of SK&F 103829 causing greater than threshold constrictions enhanced vasoconstrictor responses of sympathetic nerve stimulation, noradrenaline, angiotensin II, methoxamine and α, β-methylene ATP in the mesenteric arterial bed. Increases in mesenteric arterial pressure by noradrenaline, observed in the presence of prazosin, were also potentiated by SK&F 103829.

4 Ketanserin prevented both the constrictor effect of SK&F 103829 and the SK&F 103829-evoked potentiation of the responses to noradrenaline and angiotensin II in the mesenteric arterial bed. Ketanserin, however, failed to abolish (once established) the SK&F 103829-evoked potentiation of the constrictor effects caused by both noradrenaline and angiotensin II.

5 Short lasting constrictor effects of 5-HT were reversed to dilator effects by SK&F 103829 in both the mesenteric arterial and venous bed, thereby revealing the existence of vasodilator 5-HT receptors.

6 The main finding is consistent with a sensitization of the mesenteric arterial bed to vasoconstrictor responses mediated through α_1- and α_2-adrenoceptors, angiotensin II receptors and purinoceptors by SK&F 103829-evoked activation of 5-HT_2 receptors. This property, together with the direct constrictor effect of the mesenteric arterial bed suggest that SK&F 103829 can reduce portal venous flow thereby being a useful therapeutic principle for the treatment of portal hypertension.

Keywords: Mesenteric circulation; 5-HT_2 receptors; 5-hydroxytryptamine; SK&F 103829; vasoconstrictor amplification

Introduction

Activation of vascular smooth muscle 5-HT_2 receptors by 5-hydroxytryptamine (5-HT) causes vasoconstriction through two mechanisms: (i) direct (Feniuk & Humphrey, 1989; Kaumann, 1989) and (ii) indirect, by amplifying vasoconstriction stimuli caused by a variety of agonists through their corresponding receptors (Van Nueten, 1987; De La Lande, 1989). The aim of this study was to investigate the contribution of the indirect vasonconstrictor component of 5-HT_2 receptor activation in the mesenteric circulation. The concomitant activation of several 5-HT_1-like receptor types that mediate both vasoconstriction and vasodilatation may complicate the analysis of 5-HT-induced constriction mediated through 5-HT_2 receptors. To activate 5-HT_2 receptors selectively, we chose SK&F 103829 (Figure 1), a partial agonist for 5-HT_2 receptors of the canine gastro-oesophageal sphincter (Barnette *et al.*, 1992). We verified the partial agonistic properties of SK&F 103829 on 5-HT_2 receptors of isolated preparations of rat tail artery (Kaumann & Frenken, 1988) and calf pulmonary artery (Frenken & Kaumann, 1984; 1987a). To study the effects of 5-HT_2 receptor-activation we chose a cat model of perfused mesenteric circulation (Taylor *et al.*, 1981; Taylor & Parsons, 1991). We investigated the effects of SK&F 103829 on the vasoconstriction caused by nerve stimulation, (−)-noradrenaline, methoxamine, angiotensin II and α, β-methylene ATP.

Methods

Isolated arteries

To determine the intrinsic activity, affinity and potency of SK&F 103829 for 5-HT_2 receptors we used isolated preparations of rat tail artery and calf pulmonary artery. Rat tail arteries were prepared with a method of interconnected rings (Kaumann & Frenken, 1988). Male Wistar rats (240–310 g) were pretreated with 7 mg kg^{-1} reserpine i.p. 24 h before they

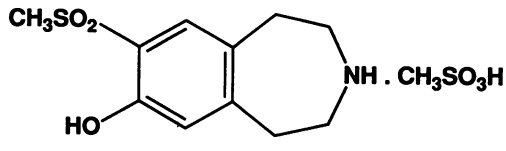

Figure 1 Structure of SK&F 103829.

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were killed. The rats were killed by a blow on the head. The ventral caudal artery was dissected in oxygenated modified Krebs solution containing (mM): Na^+ 120, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 29, HPO_4^{2-} 1, EDTA 0.04, equilibrated with 95% O_2 and 5% CO_2. The water was deionised and redistilled in glass. The artery was cleared of connective tissue. A stiff wire (Prolene No 4-0, 1.5 metric, Ethicon, Hamburg, Germany) was inserted into the artery to rub off the endothelium. From each artery 4 segments of 10 mm length were cut. To prepare strips, each of the 10 mm segments were incised 5 times to 80 to 90% of the diameter of the vessel. Incisions were cut from one side of the segments at right angles to the longitudinal axis. The spacing between the notches amounted to 1 mm. Incisions equidistant from the notches were cut into the opposite side of the segment. Gentle stretching under the dissection microscope revealed 5 interconnected rings. The interconnected rings were mounted in pairs in 50 ml organ baths (Blinks, 1965) containing the above solution supplemented with (mM): Na^+ 20, fumarate 10, pyruvate 5, L-glutamate 5, glucose 10, ascorbate 0.2 and cocaine 0.006 (Kaumann, 1983). Each tissue was tied on one end to a thread (Flexafil 6/0, J. Pfrimmer & Co., Erlangen, Germany) and clamped on the other end. The tissues were set up to contract at 37°C at a resting force of approximately 4 mN. The tissues were attached to strain gauge transducers connected through amplifiers to a polygraph.

Pulmonary arteries of calf were dissected in the local slaughterhouse and transported to the laboratory in the modified oxygenated Krebs solution. The arteries were prepared as described by Frenken & Kaumann (1984). Helicoidal strips, with the endothelium rubbed off were set up in 50 ml organ baths with a resting force of 10 mN.

Characterization of SK&F 103829 as a partial agonist

The intrinsic activity of SK&F 103829 with respect to 5-HT was obtained by determining a cumulative concentration-effect curve to 5-HT, followed by a washout period, and then a cumulative curve to SK&F 103829 in both rat tail arteries and calf pulmonary arteries. In rat tail artery a second concentration-effect curve to 5-HT was determined in the presence of the highest SK&F 103829 concentration used, (200 μM). In calf pulmonary artery, a second experiment was carried out as above but by incubating for 1 h ketanserin 100 nM after the washout of 5-HT and by determining the concentration-effect relationship to SK&F 103829 in the presence of ketanserin. It has previously been shown that successive concentration-effect curves to 5-HT in pulmonary artery reveal less than 0.3 log units changes in potency (Frenken & Kaumann, 1984). Successive concentration-effect curves to 5-HT have been shown to be reproducible in tail artery (Kaumann & Frenken, 1988). No corrections of slight changes in sensitivity of the tissues to 5-HT were made.

The equilibrium dissociation constant K_p for the SK&F 103829 – $5HT_2$ receptor complex of rat tail artery was estimated by the method of Marano & Kaumann (1976). K_p was estimated from the slope m of a weighted plot which related equieffective concentrations of 5-HT in the absence (A_2) and presence (A_3) of the partial agonist P (i.e. SK&F 103829), $A_2 = i + mA_3$, where i is the ordinate intercept. The slope m of the regression equals $m = 1 - y_p$, where the fractional $5\text{-}HT_2$ receptor occupany y_p by SK&F 103829 is given by $y_p = [P]/([P] + K_p)$. $pK_p = -\log K_p$ was calculated from (Lemoine & Kaumann, 1982):

$$\log (1/m - 1) = \log [P] - \log K_p \quad (1)$$

In vivo *experiments*

Auto perfused intestinal circulation Male or female cats (2.5–3.5 kg) were anaesthetized with pentobarbitone sodium (Sagatal) 60 mg kg^{-1}, i.p. The trachea was cannulated and systemic blood pressure (BP) recorded from a femoral artery. Blood pressure pulsations were used to trigger an instantaneous rate meter to monitor heart rate continuously. A superficial vein in the foreleg was cannulated for intravenous (i.v.) injections of drugs or additional doses of anaesthetic if required. The left carotid artery was prepared for cannulation. The abdomen was opened with a midline incision and any points where bleeding occurred were carefully ligated. The large intestine, caudal to the posterior mesenteric artery, and the duodenum, at the point where it lies alongside the single mesenteric vein, were tied with double ligatures. The posterior mesenteric artery was also ligated. The sheets of connective tissue between the intestines and the trunk of the animals were cut, ligating any small blood vessels as necessary, leaving the intestines connected to the rest of the animal by the anterior mesenteric artery and associated nerve plexus, the mesenteric vein and lymph ducts. The anterior mesenteric artery was freed from the nerve plexus and prepared for cannulation. The nerve plexus was ligated. As far as possible the lymph ducts were left intact or cut so that the contents drained into the abdominal cavity. After completion of the surgery the cats were left for about an hour to allow seepage of blood to cease and clots to form. Heparin (1000 u kg^{-1}, i.v.) was administered and the anterior mesenteric was autoperfused at constant flow (approximately 30 ml min^{-1}) with blood taken from the left carotid artery. The extracorporeal circuit was silicone-rubber tubing (internal diameter 2 mm). Under these conditions changes in perfusion pressure reflect changes in vascular resistance.

Double perfused intestinal circulation The method used has been described in detail in previous publications (Taylor *et al.*, 1981; Taylor & Parsons, 1991). Briefly the cats were set up as described for the autoperfusion experiments. In addition, a second pump, also operating at a constant volume output, removed blood from the cannulated mesenteric vein and returned it to the animal via a cannulated femoral vein. A transducer was connected into the extracorporeal circuit between the mesenteric vein and the 'venous' pump to record venous pressure. The outputs of arterial perfusion and venous pumps were carefully balanced so that the tissue was isovolaemic with a constant venous overflow pressure of around 5 mmHg. Venous constriction (decrease in capacitance) increases venous outflow pressure. Conversely venous dilatation reduces the venous outflow pressure. Previous experiments have shown that changes in perfusion pressure and venous outflow pressure reflect independent changes in arterial resistance vessels and post-capillary capacitance vessels separately.

Sympathetic nerve stimulation and drug administration In experiments designed to investigate the effects of sympathetic nerve stimulation an electrode, similar in shape to an electromagnetic flow probe, was fitted around the mesenteric artery and nerve plexus distal to the point of cannulation and nerve destruction. Transmural stimulation (25–30 V), 0.5 ms pulse duration and variable frequency (0.25–4 Hz) was applied for 45–60 s until a peak vasoconstrictor response was obtained. Intervals of 5–10 min were left between stimulation periods to allow the tissue to recover.

Vasoactive drugs, including SK&F 103829, were given directly as bolus injection into the arterial supply to the intestines through a thick-walled section of the extracorporeal circuit (viton tubing wall, thickness 3 mm), using a maximal solvent volume of 0.2 ml.

All experiments were carried out in the presence of atropine, 1 mg kg^{-1} i.v., to inhibit the parasympathetic nerves. In some experiments propranolol, 1 mg kg^{-1} i.v. plus 3 mg kg^{-1} s.c., was also given to prevent neuronally released or injected noradrenaline from stimulating pre- or postsynaptic β-adrenoceptors (Patel *et al.*, 1981).

The results are presented as means ± standard error of the mean. Some data were evaluated for differences by Student's

t test. A probability of less than 0.05 was considered significant.

Drugs used

Noradrenaline HCl (Aldrich Chem. Co.), atropine sulphate (BDH), α, β-methylene ATP (Sigma UK), propranolol HCl (ICI), angiotensin II (hypertensin) (Ciba UK), prazosin HCl (Pfizer UK), ketanserin tartrate (Janssen Belgium), SK&F 103829 and sumatriptan (SmithKline Beecham UK), methoxamine HCl and 5-hydroxytryptamine HCl (Sigma UK).

All drugs were dissolved in either twice distilled water or 0.9% NaCl. Dilutions for *in vivo* experiments were made in 0.9% NaCl. For *in vitro* experiments 5-HT was in 0.2 mM ascorbate.

Results

In vitro

Effects of SK&F 103829 in isolated arteries SK&F 103829 contracted rat tail arteries with a pEC_{50} of 5.83 ± 0.01 ($n = 4$) and an intrinsic activity of 0.77 ± 0.05 with respect to 5-HT (Figure 2). Ketanserin 100 nM caused a rightward shift of the concentration-effect curve to SK&F 103829 by 1.5 and 1.6 log units in 2 rat tail arteries (not shown). SK&F 103829 (200 μM) surmountably antagonized the effects of 5-HT (Figure 2). The $pK_p = -\log K_p$, calculated with equation (1), was 5.79 ± 0.08. The receptor occupany curve of SK&F 103829, calculated with its pK_p, matched the experimental points of the concentration-effect curve (Figure 2).

SK&F 103829 also contracted calf pulmonary arteries with a pEC_{50} of 6.02 ± 0.03 ($n = 4$) and intrinsic activity of 0.61 ± 0.06 with respect to 5-HT (Figure 3). Ketanserin 100 nM caused a 2.3 log shift of the concentration-effect curve to SK&F 103829 (Figure 3).

In vivo

Effect of SK&F 103829 33 μg kg^{-1} (1 × 10^{-7} mol kg^{-1}, i.a.) (n = 3) This dose caused only minimal changes in perfusion pressure from 78.3 ± 10.5 mmHg to 85.0 ± 11.2 mmHg. There was no significant change in heart rate (<2 beats min^{-1}) but a small increase in blood pressure was seen from 106 ± 4.9/58.3 ± 2.4 mmHg to 116.7 ± 4.4/67 ± 2.9 mmHg.

SK&F 103829 330 μg kg^{-1} (1 × 10^{-6} mol kg^{-1}, i.a.) (n = 27) This dose caused a more substantial increase in blood pressure (for example, see Figure 4) from 116 ± 2.4/58 ± 2.9 mmHg to 153 ± 4.5/90 ± 3.9 mmHg, an increase of 37 ± 2.4/32 ± 2.1 mmHg ($P < 0.001$). There was no difference between cats given atropine or atropine and propranolol. Changes in heart rate of atropine-treated cats were very variable, with some animals showing a slight bradycardia, although the mean change was a slight tachycardia from 140 ± 6.1 beats min^{-1} to 147 ± 5.5 beats min^{-1}. In cats

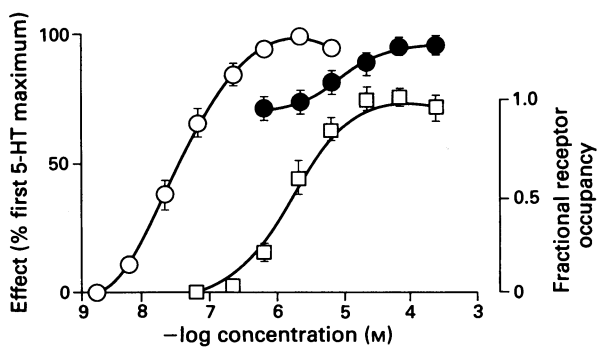

Figure 2 Rat tail artery: concentration-effect curves to 5-HT in the absence (○) and presence of SK&F 103829 (200 μM; ●) and to SK&F 103829 (□) determined between the two curves to 5-HT. Points represent mean (± s.e.mean) values of the increase in force as percentage of the maximum effect of 5-HT observed in the first curve. Results from 4 rats.

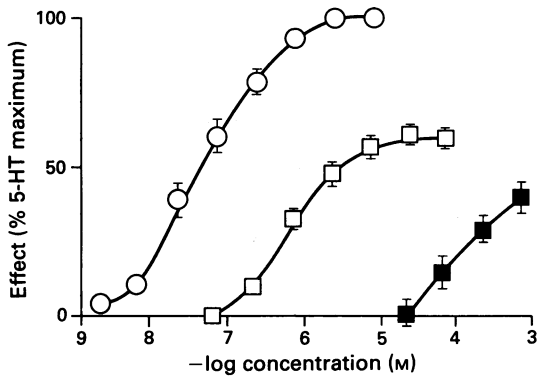

Figure 3 Calf pulmonary artery: concentration-effect curves to 5-HT (○) and SK&F 103829 in the absence (□) and presence of ketanserin (100 nM; ■). Points represent mean (± s.e.mean) values of the increase in force as percentage of maximum effect of 5-HT. Results are from 8 helicoids of 4 calves (○), 4 helicoids of 2 calves (□) and 4 helicoids of 2 calves (■).

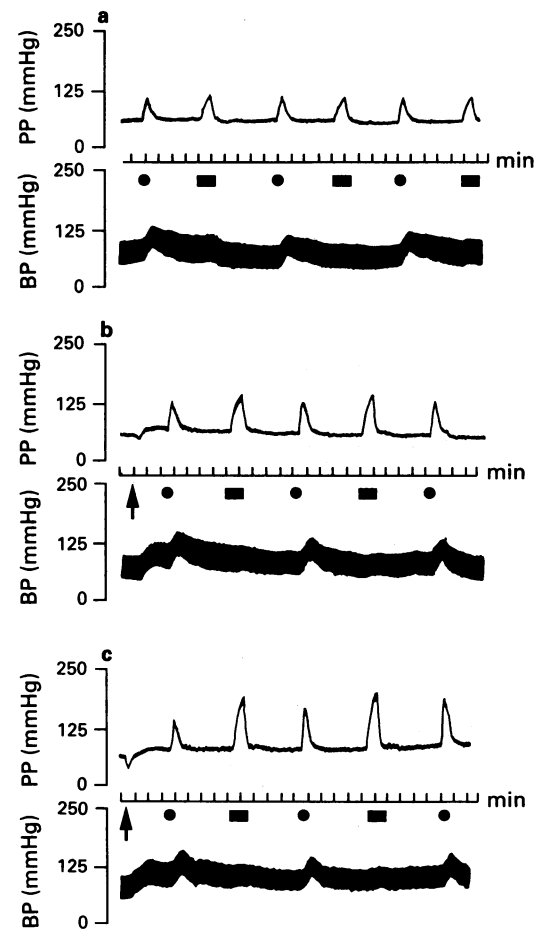

Figure 4 Autoperfused intestinal circulation of a pentobarbitone-anaesthetized cat: original tracings showing (a) control responses, over time, to noradrenaline 1 μg, i.a. (●) and sympathetic nerve stimulation 2 Hz for 60 s (■) on systemic blood pressure (BP) and intestinal perfusion pressure (PP); (b) effects of a bolus injection of SK&F 103829, 0.33 mg kg^{-1} i.a. (↑) and (c) effects of a bolus injection of SK&F 103829, 3.3 mg kg^{-1} i.a. (↑).

Table 1 Effects of SK&F 103829 on the increases in perfusion pressure (mmHg ± s.e.mean) elicited by vasoconstrictors and sympathetic nerve stimulation (N.St.) in the autoperfused intestinal circulation of pentobarbitone anaesthetised cats

Predosed with:	*Constrictor*	n	*Control response*	*Responses after SK&F 103829* 0.33 mg kg^{-1}, i.a.	3.3 mg kg^{-1}, i.a.	*%* increase*
Propranolol	N.St. 1–2 Hz	6	43.7 ± 3.8	71.2 ± 6.0 $P<0.001$	85.7 ± 8.1 $P<0.001$	96
Propranolol	Noradrenaline 1–2 μg i.a.	14	35.5 ± 3.0	51.7 ± 4.0 $P<0.001$	70.2 ± 5.2 $P<0.001$	99
Propranolol + prazosin	Noradrenaline 2–4 μg i.a.	5	23.6 ± 1.7	34.6 ± 2.6 $P<0.001$	59.2 ± 4.7 $P<0.001$	151
–	Methoxamine 30–40 μg i.a.	5	23.4 ± 3.0	39.4 ± 4.5 $P<0.002$	59.0 ± 2.8 $P<0.001$	152
–	Angiotensin II 0.1–0.2 μg i.a.	9	21.4 ± 0.9	34.9 ± 1.2 $P<0.001$	52.4 ± 2.8 $P<0.001$	145
–	α, β-methylene ATP 5–10 μg i.a.	6	28.4 ± 1.1	40.8 ± 0.9 $P<0.001$	59.8 ± 4.3 $P<0.001$	110
Propranolol + ketanserin	Noradrenaline 2–4 μg i.a.	5	32.0 ± 3.5	–	33.2 ± 6.0 NS	4
Ketanserin	Angiotensin II 0.1–0.2 μg i.a.	5	23.8 ± 3.2	–	34.0 ± 5.8 $P<0.05$	43
Propranolol	1 mg kg^{-1}, i.v. + 3 mg kg^{-1}, s.c.					
Prazosin	0.5 mg kg^{-1}, i.v.					
Ketanserin	1.0 mg kg^{-1}, i.v.					

*Maximal increase seen after SK&F 103829 3.3 mg kg^{-1}.
NS, non significant.

predosed with atropine and propranolol, the resting heart rate was low (119 ± 3.6 beats min^{-1}) and SK&F 103829 caused a small but very consistent tachycardia of 4.0 ± 0.64 beats min^{-1} ($P<0.001$). Intestinal perfusion pressure was increased from 63 ± 2.4 mmHg to 80 ± 2.8 mmHg, an increase of 17 ± 1.0 mmHg ($P<0.001$). Recovery from the effects was not complete after 30 min (Figures 4 and 6).

SK&F 103829 3.3 mg kg^{-1} (1 × 10^{-5} mol kg^{-1}, i.a.) (n = 22) This dose was given about 30 min after the previous dose at which time blood pressure (128.9 ± 4.3/71.0 ± 3.9 mmHg) and perfusion pressure (74.6 ± 3.0 mmHg) had not returned to control values. Blood pressure was further increased to 160 ± 4.9/99 ± 4.7 mmHg ($P<0.001$) following SK&F 103829 (for an example see Figure 4). There was also a marked increase in perfusion pressure (Figures 4 and 6) to 111 ± 4.3 mmHg ($P<0.001$). The effect was sustained for at least one hour. In atropine-treated cats an overall fall in heart rate was observed from 144 ± 4.7 beats min^{-1} to 125 ± 3.8 beats min^{-1}, a bradycardia of 19 ± 2.6 beats min^{-1} ($P<0.001$). In cats predosed with atropine and propranolol, SK&F 103829 did not cause a significant change in heart rate.

Augmentation of vasoconstrictor responses SK&F 103829 (33 μg kg^{-1}, i.a.) had only minimal effects on the vasoconstrictor responses (increases in perfusion pressure) to noradrenaline (1–2 μg, i.a.) or sympathetic nerve stimulation (1–2 Hz). However, 330 μg kg^{-1} i.a. of SK&F 103829 caused a significant and sustained increase in the vasoconstrictor responses to noradrenaline and sympathetic nerve stimulation (Figure 4 and Table 1). SK&F 103829 (3.3 mg kg^{-1}, i.a.) caused a further potentiation of the vasoconstrictor responses to noradrenaline and sympathetic nerve stimulation (Figure 4 and Table 1). In a second series of experiments ($n = 5$), SK&F 103829 caused a 3 fold shift to the left of the frequency-response curve to sympathetic nerve stimulation (Figure 5b). This figure also shows the relative lack of effect of sumatriptan 10 and 100 μg kg^{-1}, i.a. on the responses to sympathetic nerve stimulation. The higher dose of sumatriptan caused a slight shift to the left of the frequency-response curve but only by a factor of 1.4. Sumatriptan did not significantly alter perfusion pressure, blood pressure or heart rate.

In addition, SK&F 103829 also caused amplification of the vasoconstrictor responses to angiotensin II, methoxamine, noradrenaline (in prazosin, 0.5 mg kg^{-1} i.v., treated cats) and α, β-methylene ATP. In general, an enhancement around 100 to 150% was observed (Table 1).

Effect of ketanserin Ketanserin, (1 mg kg^{-1}, i.v.) given 30 min after the final dose (3.3 mg kg^{-1}) of SK&F 103829, was relatively ineffective in reducing the established amplification of the vasoconstrictor responses to angiotensin II (Figure 6). There was, however, a significant reduction ($P<0.005$) in resting perfusion pressure (short lasting) and in the response to noradrenaline ($P<0.05$). In contrast, predosing with ketanserin inhibited both the increases in blood pressure and perfusion pressure elicited by SK&F 103829 (Figure 6) and the amplification of the vasoconstrictor responses to noradrenaline and angiotensin II (Figure 6 and Table 1). There was a relatively greater effect on the noradrenaline responses.

Double perfusion experiments As in the previous experiments, SK&F 103829 (0.33 and 3.3 mg kg^{-1}) caused a sus-

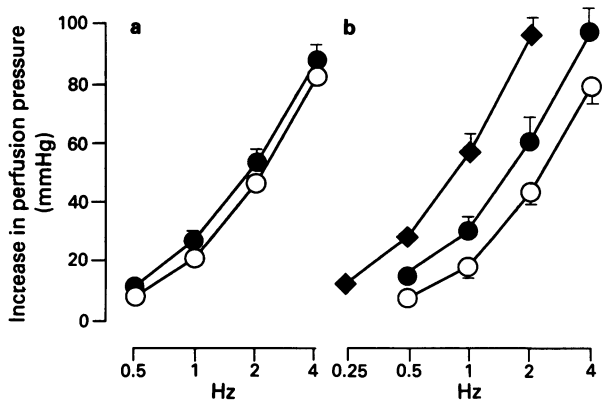

Figure 5 Autoperfused intestinal circulation of pentobarbitone anaesthetized cats: (a) increases in perfusion pressure in response to sympathetic nerve stimulation, 0.5–4 Hz (frequency-response curve) before (○, $n = 5$) and after sumatriptan 10 μg kg^{-1} i.a. (●, $n = 5$); (b) increases in perfusion pressure in response to sympathetic nerve stimulation, 0.25–4 Hz before (○, $n = 5$) and after sumatriptan 100 μg kg^{-1}, i.a. (●, $n = 5$) and SK&F 103829 3.3 mg kg^{-1}, i.a. (◆, $n = 5$). Points are mean values ± s.e.mean.

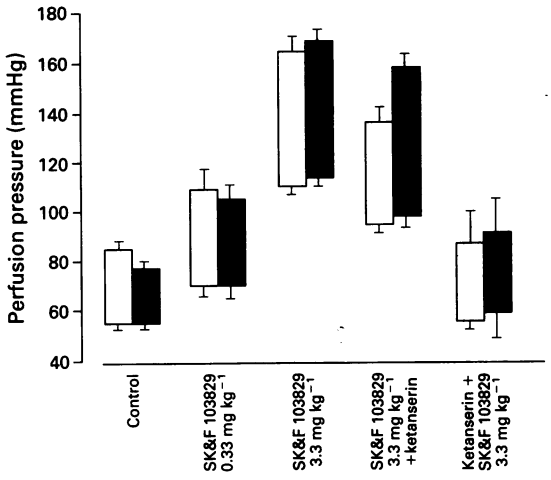

Figure 6 Autoperfused intestinal circulation of pentobarbitone anaesthetized cats: effects of SK&F 103829, 0.33 and 3.3 mg kg^{-1}, i.a. on resting intestinal perfusion pressure (represented by the bottom of each column ± s.e.mean) and on the responses to noradrenaline, 1 μg i.a. (open columns) and angiotensin II, 0.1 μg i.a. (solid columns) represented by the top of each column ± s.e.mean. The first pair of columns are the control responses to noradrenaline and angiotensin ($n = 5$). The second and third pairs show the effects of SK&F 103829 on both resting perfusion pressure and the responses to noradrenaline and angiotensin II. The fourth pair of columns show the effects of ketanserin 1 mg kg^{-1}, i.v. given 30 min after SK&F 103829 3.3 mg kg^{-1} i.a. The fifth column shows the effects of ketanserin 1 mg kg^{-1}, i.v. given 5 min before SK&F 103829, 3.3 mg kg^{-1}, i.a.

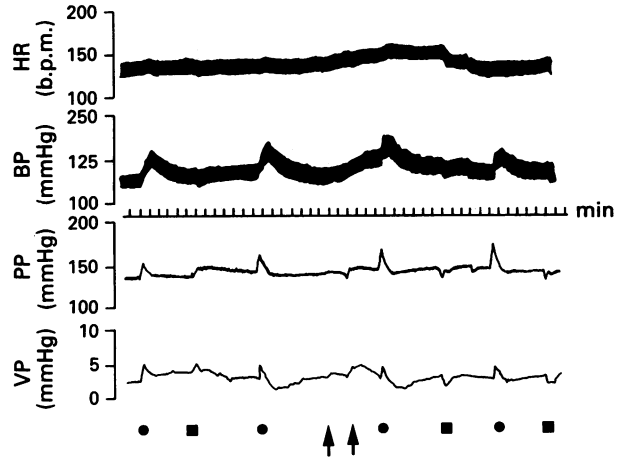

Figure 7 Double perfused intestinal circulation of a pentobarbitone anaesthetized cat: original tracing showing the effects of 5-HT 20 μg i.a. (■), noradrenaline 2 μg i.a. (●) and SK&F 103829 33 μg kg^{-1}, i.a (first arrow) and 330 μg kg^{-1} i.a. (second arrow) on heart rate (HR), systemic blood pressure (BP), intestinal perfusion pressure (PP) and venous outflow pressure (VP). Note the reversal of the effects of 5-HT after SK&F 103829. The arterial response to noradrenaline were enhanced after 5-HT and SK&F 103829 whereas the venous responses were not (see Results).

tained increase in both arterial perfusion pressure and systemic blood pressure. The effects on venous outflow pressure were variable and relatively short lasting (for example see Figure 7). Overall there was little difference between the effects of the two doses of SK&F 103829. SK&F 103829 (330 μg kg^{-1}, i.a.) increased venous outflow pressure from 4.4 ± 0.5 mmHg to 7.4 ± 0.6 mmHg, an increase of 3.0 ± 0.6 mmHg ($n = 7$, $P < 0.005$). SK&F 103829 (3.3 mg kg^{-1}, i.a.) increased venous outflow pressure from 4.1 ± 1.0 mmHg to 7.5 ± 1.1 mmHg, an increase of 3.4 ± 0.8 mmHg ($n = 5$, $P < 0.02$).

Table 2 Biphasic effects of 5-HT on arterial perfusion pressure (AP) and venous outflow pressure (VP) of the autoperfused intestinal vasculature of pentobarbitone-anaesthetized cats

Dose (μg, i.a.)	Changes in AP (mmHg) Increase	Decrease	Changes in VP (mmHg) Increase	Decrease
0.1	0	2.2 ± 1.9	0	0.2 ± 0.41
0.4	0	7.6 ± 3.8	0	0.4 ± 0.23
1.6	5.6 ± 2.6	61.0 ± 8.7	0.16	2.4 ± 0.52
6.4	15.5 ± 6.4	68.0 ± 4.3	0.92 ± 0.25	5.0 ± 0.70
25	23.3 ± 6.1	54.7 ± 3.3	2.04 ± 0.20	1.8 ± 0.80

Values are mean ± s.e.mean; $n = 5$.

The effects of 5-HT were difficult to quantify because of the complex nature of the responses. Lower doses caused biphasic changes in both perfusion pressure and venous outflow pressure. If the resting levels of pressure were low an overall increase was seen with 5-HT (Figure 7) whereas at higher resting pressure values (typically towards the end of experiments) 5-HT caused arterial and venous dilatation. The mixed effects are shown in Table 2. It was not possible to evaluate the effects of higher doses of 5-HT as contraction of gut muscle was observed causing a marked but short lasting increase in venous outflow pressure due to the squeezing of blood out of venules and small veins. In addition, high doses of 5-HT can elicit the Bezold-Jarisch reflex which results in a dramatic fall in systemic blood pressure.

SK&F 103829 inhibited the vasoconstrictor responses to 5-HT unmasking the vasodilator effects on both arterial and venous blood vessels (Figure 7). Venous constrictor responses to noradrenaline were rather variable, and difficult to quantify in these experiments, but no clear cut potentiation of the responses was seen following SK&F 103829 (Figure 7). However, as before, the arterial responses to noradrenaline were potentiated by SK&F 103829. Before SK&F 103829 the noradrenaline-evoked increase in perfusion pressure was 38.5 ± 7.0 mmHg; after 33 μg kg^{-1} SK&F 103829 it was 52.5 ± 7.9 mmHg ($P < 0.05$) and after 330 μg kg^{-1} SK&F 103829, 65.0 ± 8.7 mmHg ($P < 0.01$).

Discussion

The main finding of this work is that the partial agonist SK&F 103829 sensitizes the feline mesenteric arterial bed to a variety of vasoconstrictor stimuli. The sensitization appears to be due to 5-HT_2 receptor activation because SK&F 103829 is a 5-HT_2 receptor partial agonist *in vitro* on isolated arteries and probably also *in vivo* where it may enhance mesenteric arterial resistance.

The partial agonistic activity of SK&F 103829, elicited through 5-HT_2 receptors, was demonstrated in isolated arteries. The potent 5-HT_2 receptor-selective antagonist ketanserin (Van Nueten *et al.*, 1981) blocked SK&F 103829-induced contractions in calf pulmonary artery. Ketanserin 100 nM caused a 2.3 log unit rightward shift of the concentration-effect curve for SK&F 103829, consistent with a pK_B of 9.3 for ketanserin, an affinity estimate close to a pK_B of 9.4 for ketanserin as antagonist of the effects of 5-HT estimated in calf pulmonary artery (Frenken & Kaumann, 1984). The similarity of blocking potency of ketanserin against SK&F 103829 and 5-HT is consistent with an interaction of the three drugs with the same receptor class (5-HT_2) in calf pulmonary artery. Preliminary experiments showed that ketanserin also blocked SK&F 103829-induced contractions in a surmountable fashion in rat tail artery, consistent

with an interaction with its 5-HT_2 receptors (Frenken & Kaumann, 1987b).

As expected from a classical partial agonist, SK&F 103829 caused surmountable antagonism of the contractile effects of 5-HT in rat tail artery. The estimated pK_p for the SK&F 103829-5-HT_2 complex matched the pEC_{50} for SK&F 103829-induced contractions and its 5-HT_2 receptor occupancy curve fitted the concentration-effect data. The potency and affinity of SK&F 103829 (pEC_{50} = 5.8–6.0; pK_p = 5.8) estimated on rat tail artery and pulmonary artery agree with similar values estimated by Barnette *et al.* (1992) on isolated preparations of canine gastro-oesophageal sphincter (pEC_{50} = 6.1; pK_p = 5.9, estimated by receptor occlusion with phenoxybenzamine). In agreement with our findings on arteries with ketanserin, Barnette *et al.* (1992) also found that the contractile effects of SK&F 103829 on canine gastro-oesophageal sphincter were prevented by several 5-HT_2 receptor antagonists. Thus SK&F 103829 appears to interact with 5-HT_2 receptors in both systems. Unlike rat tail artery, however, where 5-HT produced additional contractions in the presence of a maximum effective SK&F 103829 concentration, in canine gastro-oesophageal sphincter SK&F 103829 uncovered a relaxant effect of 5-HT, presumably mediated through 5-HT_1 receptors (Barnette *et al.*, 1992).

Similarly SK&F 103829-evoked increases in mesenteric perfusion pressure and potentiation of vasoconstrictors were also inhibited by ketanserin, consistent with mediation via arteriolar 5-HT_2 receptors. The relatively greater inhibition of noradrenaline responses probably reflects the additional α_1-adrenoceptor antagonist activity of ketanserin. Prazosin did not prevent the effects of SK&F 103829. Interestingly, ketanserin was relatively ineffective against an established potentiation, of angiotensin II responses for example. This observation and the generalized nature of the augmentation for all vasoconstrictors whether acting via the inositol pathway (e.g. noradrenaline through α_1-adrenoceptors. Villalobos *et al.*, 1982) or perhaps by inhibition of adenylyl cyclase (noradrenaline in the presence of prazosin presumably through α_2-adrenoceptors, Boyer *et al.*, 1983) suggest an increase in intracellular Ca^{2+} is involved resulting from sustained stimulation of 5-HT_2 receptors. Calcium antagonists, such as nifedipine, reduce both arterial perfusion pressure and the responses to vasoconstrictors in this preparation (unpublished observations). In contrast, little or no effect is seen on either venous outflow pressure or on venous constriction elicited by noradrenaline. This may explain the apparent lack of effect of SK&F 103829 on venous constrictor responses to noradrenaline. Presumably mesenteric venules and small veins respond to acute injections of noradrenaline primarily by the release of Ca^{2+} from intracellular stores. However, venous constrictor responses to noradrenaline were variable in the experiments and further more specifically designed experiments will be needed to confirm the findings.

In agreement with a previous report (Feniuk *et al.*, 1987) sumatriptan was devoid of vascular effects in the cat mesenteric bed, suggesting a lack of 5-HT_1-like receptors that mediate constriction. Sumatriptan is known to inhibit neuronal release of vasoconstrictors in the canine saphenous vein through 5-HT_1-like receptors (Humphrey *et al.*, 1988) but did not inhibit the vasoconstrictor effect of nerve stimulation in the cat mesenteric bed. Thus neuronal 5-HT_1-like receptors appear not to play a role in the modulation of mesenteric arteriolar tone in the cat.

The 5-HT_2 receptor antagonist activity of SK&F 103829 can be demonstrated by its effects on 5-HT responses. The vasoconstrictor element of the biphasic response to 5-HT is inhibited by SK&F 103829 on both arterial and venous blood vessels. Following SK&F 103829, 5-HT elicits only a vasodilatation of both resistance and capacitance blood vessels, presumably by an effect on 5-HT_1-like receptors.

The observed effects of SK&F 103829 on the mesenteric circulation may have a bearing on the treatment of portal hypertension. The venous side of the mesenteric circulation is constricted by 5-HT through 5-HT_2 receptors, at least in the rat. 5-HT_2 receptors have not only been identified in the superior mesenteric vein of sham-operated and portal hypertensive rats (Cummings *et al.*, 1986; Kaumann *et al.*, 1988) but also in the portal collateral veins of portal hypertensive rats (Mosca *et al.*, 1992). 5-HT_2 receptor antagonists decrease high portal pressure both in portal hypertensive rats (Cummings *et al.*, 1986; Kaumann *et al.*, 1988) and patients with portal hypertension (Hadengue *et al.*, 1987; 1989; Huet *et al.*, 1988; Vorobioff *et al.*, 1989). How 5-HT_2 receptor antagonists reduce portal hypertension is unknown. One possible mechanism is that 5-HT, released from enterochromaffin cells into the portal circulation (Larsson *et al.*, 1979; Petersson *et al.*, 1979), enhance portal venous resistance by direct vasoconstriction through activation of 5-HT_2 receptors. Blockade of these 5-HT_2 receptors would not only prevent vasoconstriction but uncover vasodilatation caused by 5-HT, thereby lowering portal pressure. Thus, SK&F 103829, by blocking 5-HT_2 receptors reverted the constrictor effect of 5-HT to a dilator effect in both arteriolar and venous mesenteric vascular beds (Figure 7). Reversal of a constrictor response to a dilator response of 5-HT has previously been observed with ketanserin in the arterially perfused stomach of guinea-pig, illustrating the unmasking of vasodilator 5-HT receptor after blockade of 5-HT_2 receptors (Van Nueten *et al.*, 1981). The blockade of arteriolar 5-HT_2 receptors probably does not modulate portal pressure because free plasma 5-HT concentrations are low in arterial blood.

A potentially important factor in the genesis of portal hypertension is the hyperdynamic circulation, i.e. increased portal blood flow due to mesenteric arteriolar dilatation (Groszmann *et al.*, 1988). SK&F 103829, by causing mesenteric arteriolar constriction through a dual mechanism, would thereby be expected to reduce portal blood flow and consequently to reduce portal pressure. The dual mechanism through which SK&F 103829 constricts the feline mesenteric arteriolar bed consists of (i) direct activation of 5-HT_2 receptors, which in turn causes (ii) facilitation of a variety of vasoconstrictor stimuli mediated through α-adrenoceptors, angiotensin II receptors and purinoceptors.

We are grateful to Dr Michael Frenken for help with the experiments on isolated arteries.

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(Received May 18, 1993
Revised September 10, 1993
Accepted September 20, 1993)

Br. J. Pharmacol. (1994), **111**, 271–277

Participation of cytoskeleton in the effect of antilaminin IgG on cardiac cholinoceptors

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1 We have previously demonstrated a molecular relationship between laminin and cardiac cholinoceptors.

2 We have now explored the participation of cytoskeletal proteins in the interaction between an antilaminin IgG with cardiac cholinoceptors.

3 Antilaminin IgG, whilst it specifically reacts with laminin molecules was able to induce cardiac cholinoceptor activation; acting like an agonist, decreasing cyclic AMP concentrations, reducing heart contractility and increasing phosphoinositide turnover.

4 Antilaminin IgG also interfered with the binding of a radiolabelled muscarinic antagonist, [^{3}H]-quinuclidinyl benzilate. Colchicine and cytochalasin B, drugs that are able to prevent microfilament and microtubule polimerization, impaired the binding of antilaminin IgG to muscarinic cholinoceptors.

5 Cytochalasin B but not colchicine modified the muscarinic cholinoceptor effects mediated by regulatory G proteins (cyclic AMP and contractility) induced by antilaminin IgG.

6 It was demonstrated, by immunofluorescence, that none of these disrupting drugs altered the specific recognition of the antibody by its antigen.

7 These data indirectly suggest the participation of the cytoskeleton in the laminin and cholinergic receptor association.

Keywords: Antilaminin IgG; cholinoceptors; PI turnover; cytoskeletal proteins; heart contractility; cyclic AMP; binding assay

Introduction

It is well known that the extracellular matrix influences cell locomotion, growth and differentiation (Timpl & Dziadek, 1986; Beck *et al.*, 1990). These events are mediated by the specific interactions between matrix proteins (laminin, fibronectin, etc.) and the cell surface. Such events are accompanied by the rearrangement of the cytoskeleton. Although a great deal of information has been acquired recently on the extracellular matrix cell receptors (integrin and non-integrin receptors; Albeda & Buck, 1990), the mechanisms of the interaction among the cell receptor and cytoskeleton proteins have not been well defined.

Sugrue & Hay (1981) previously showed that laminin when added *in vitro* to the epithelial cell caused orientation of the cells' cytoskeleton. Since this interaction does not require cellular protein synthesis, it has been postulated that laminin may bind directly to the cell surface via its own receptor, which is able to interact with cytoskeletal proteins. Brown *et al.* (1983) reported that the fibrosarcoma cell surface laminin receptor protein also has the ability to bind actin with specificity and high affinity. They also postulated that this protein, 'connectin', may be a transmembrane linker that is capable of connecting laminin with actin. Moreover, Cody & Wicha (1986) proposed a model in which the configuration of laminin affects the organization of actin in the cellular cytoskeleton through the clustering of transmembrane laminin receptors. This suggests that laminin may interact with cytoskeletal proteins or cell surface receptors, serving as a functional context for cell-cell and hormone-receptor interactions.

We recently demonstrated an interaction between an antilaminin IgG with cardiac cholinoceptors (Bacman *et al.*, 1989; 1990). Concerning cardiac muscarinic cholinoceptors and laminin interactions, we have found that antilaminin IgG, whilst specifically recognizing laminin molecules, was able to induce cardiac cholinoceptor activation leading to a change in contractile parameters and other intracellualr events mediated by regulatory GTP-binding (G) proteins (Bacman *et al.*, 1990).

As the cytoskeleton modulates various membrane bound components regulating cell signal transductions (Honbech & Paul, 1986; Kupfler *et al.*, 1987), we investigated whether these proteins could be involved in the heart-cholinoceptor effect of antilaminin IgG.

Based on the above observations we hypothesized that the effects of antilaminin IgG on heart muscarinic cholinoceptors, required the participation of the cytoskeleton. If this were the case, then cytoskeletal disrupting agents should alter the antilaminin IgG effects on cholinoceptor activation mediated by regulatory G proteins.

We now show that drugs able to prevent microfilament and microtubule polymerization such as cytochalasin B and colchicine (Schliwa, 1982; Andreu & Timasheff, 1986) impair the binding of antilaminin IgG to muscarinic cholinoceptors. Cytochalasin B and not colchicine, modifies the muscarinic cholinoceptor effects mediated by regulatory G protein. These experiments, suggest that the cholinoceptor action of antilaminin IgG depends on the integrity of the cytoskeleton. However, the immunofluorescent pattern of the laminin-antilaminin reaction is not altered by the addition of cytochalasin B and/or colchicine.

Methods

Isolation of antilaminin IgG

Laminin obtained from the basement membrane matrix of an Engelbreth Holm Swarm (EHS) murine sarcoma (Gibco Research Laboratory, MD, U.S.A.) was used as antigen.

Male rabbits of approximately 2.5 kg, were injected intra-

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dermally with an emulsion prepared of equal parts of laminin (1 mg ml^{-1}) and complete Freund's adjuvant as previously described (Bacman *et al.*, 1989; 1990). Injections were repeated three times with 0.5 mg at intervals of 15 days. After 45 days post immunization animals were bled and the IgG was isolated from the rabbit antiserum as described by Bacman *et al.* (1990). IgG samples used in control experiments were obtained from preimmunized normal rabbit serum and from serum of a normal rabbit. To assess the specificity of antilaminin IgG it was preincubated with equimolar concentration of laminin during 20 min at 30 ± 0.3°C prior to performing different experiments.

Immunological characterization of IgG

Antibodies against laminin were determined by the enzyme-linked immunosorbent assay (ELISA) following the method described by Avila *et al.* (1984) and Bacman *et al.* (1990). Results of ELISA revealed an optical density of 0.8 for antilaminin IgG, and 0.01 for the IgG from the preimmune, and from the normal rabbit serum used as controls. These values were obtained with a concentration of IgG of 2.1×10^{-17} M (IgG dilution 1:640,000). To detect a cross-reaction of the antiserum with other proteins, a dot binding assay following the technique described by Hawkes *et al.* (1982) was also performed. As antigens, laminin and type IV collagen (Bethesda Res. Lab, U.S.A.), rat fibronectin (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.), and ovalbumin (Sigma) were used at a concentration of 1 mg ml^{-1}, and 1 µl of each was spotted into nitrocellulose (Schleicher & Schuell, Dassel, Germany). No cross-reaction with any of the antigens mentioned above (apart from laminin) was detected.

[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding assay

Membranes were prepared from BALB/c mice atria essentially as described by Wei & Sulakhe (1980) and Bacman *et al.* (1990). Membranes were preincubated in buffer A: Tris-HCl 5 mM, $MgCl_2$ 1 mM, sucrose 0.25 M, pH 7.8 in the presence or absence of different concentrations of normal IgG, antilaminin IgG and carbachol during 30 min at 30 ± 0.3°C with shaking. Treated membranes were then washed twice at 4°C, resuspended in buffer A and used immediately for binding assays. For cytoskeletal drug studies, membranes were first incubated for 30 min at 30 ± 0.2°C with shaking, with 5×10^{-6} M colchicine or cytochalasin B or buffer A, centrifuged at 40,000 *g* for 90 min and then with different concentrations of antilaminin IgG, normal IgG or carbachol. The pellets were resuspended in buffer A at a final concentration of 0.6 mg ml^{-1}. The membrane suspension and 0.35 nM [^{3}H]-QNB; (Dupont New England Nuclear, U.S.A.) with a specific activity of 48 Ci $mmol^{-1}$ (1 Ci = 37 GBq) was incubated with shaking, in buffer A at 25 ± 0.2°C for 60 min, in a final volume of 150 µl. The reaction was stopped with ice-cold buffer A and filtered through Whatman glass fibre filters (GF/c) under suction. After washing with 12 ml of buffer A, filters were placed in vials, dried, and counted in 8 ml scintillation cocktail (triton-toluene) with about 40% efficiency. Nonspecific binding (as measured in the presence of 1×10^{-5} M atropine) did not exceed 10%.

Cyclic AMP determination

BALB/c mice hearts were excised immediately post-decapitation. The atria were separated from the ventricles, and suspended in 1 ml of Krebs-Ringer-bicarbonate (KRB) solution. The KRB was gassed with 5% CO_2 in O_2 at 37 ± 0.3°C with 10^{-6} M carbachol, 5×10^{-7} M normal or antilaminin IgG for different times (indicated in Results) to obtain the incubation period that gave the maximum effect. The action of both normal and antilaminin IgG and carbachol, on adenosine 3':5'-cyclic monophosphate (cyclic AMP) was analysed upon atria stimulated 5 min with isoprenaline (10^{-6} M) added at the end of each incubation period. For cytoskeleton disrupting drug studies, cytochalasin B (5×10^{-6} M) and colchicine (5×10^{-6} M) were incubated with the atria for 20 min before the addition of the antibodies or carbachol plus isoprenaline. At the end of the incubation period, tissues were homogenized. Atria were preincubated in the presence of 1 mM isobutylmethyl xanthine (IBMX) in a final volume of 1 ml of KRB solution for 20 min. For a kinetic analysis of the antibodies IBMX was not used. After incubation, tissues were homogenized in 2 ml of absolute ethanol and centrifuged at 10,000 *g* for 10 min. Supernatants were collected and the pellets were rehomogenized with ethanol-water (2:1), centrifuged again as before. Supernatants (from both centrifugations) were combined and evaporated (at 55°C) to dryness. Residues were resuspended in 1 ml of 50 mM Tris-HCl (pH 7.4) containing 8 mM theophylline, 0.45 mM EDTA and 6 mM 2-mercaptoethanol. Cyclic AMP determination was developed by the competitive protein-binding assay described by Brown *et al.* (1971) using [^{3}H]-cyclic AMP as tracer.

Atria preparation for contractility

Male BALB/c mice (20–40 g) were killed by decapitation. The atria were separated from the ventricles, attached to a glass holder, and immersed in a tissue chamber containing KRB solution gassed with 5% CO_2 in O_2 and maintained at pH 7.4 and 30 ± 0.3°C. The contractile activity of paced atria in terms of *dF/dt* was recorded and assessed as previously reported (Borda *et al.*, 1984; Bacman *et al.*, 1989; 1990). Control values (= 100%) refer to *dF/dt* before the addition of drugs or normal or antilaminin IgG. The absolute value for *dF/dt* at the end of the equilibrium was 4.3 ± 0.3 g s^{-1}. After a period of equilibrium, each preparation was exposed to carbachol, normal or antilaminin IgG. Atria were exposed to antilaminin IgG for 15 min to obtain the maximal effect. For inhibition experiments, colchicine or cytochalasin B were incubated with atria for 20 min before addition of antilaminin IgG, normal IgG or carbachol.

Inositol phosphates (IPs) measurements

BALB/c mice were killed by decapitation and atria (10 mg, wet weight) were excised after allowing hearts to beat in KRB solution, gassed with 5% CO_2 in O_2, maintained at pH 7.4 and 30 ± 0.3°C. The atria were transferred to continuously oxygenated baths containing 0.5 ml of KRB with 1 µCi *myo*-[2-^{3}H]-inositol precursor ([^{3}H]-MI) (S = 15 Ci $mmol^{-1}$) from New England Nuclear. LiCl (10 mM) was added for inositol monophosphate accumulation, according to the technique described by Berridge *et al.* (1982). Tissue preparations were incubated with shaking at 37°C for 120 min. Normal, antilaminin IgG or carbachol were added during the last 30 min. When atropine, cytochalasin B or colchicine were used, they were added 60 min before the end of incubation. For measurement of total labelled IPs, water-soluble IPs were extracted after 120 min incubation following the method of Berridge *et al.* (1982). Atria, were quickly washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reactions. Then chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 2000 *g* for 15 min, and the aqueous phase of supernatants (1–2 ml) was applied to a 0.7 ml column of anion exchange resin (Bio-Rad AG 1 × 8, 100–200 mesh) suspended in 0.1 M formic acid that was previously washed with 10 mM Tris-formate pH 7.4. The resin was then washed with 20 volumes of 5 mM *myo*-inositol followed by 6 volumes of water and IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. Fractions of 1 ml were recovered and radioactivity was determined by scintillation counting. Anion-exchange chromatography revealed two peaks. The first one corresponded to the fraction of 5 mM

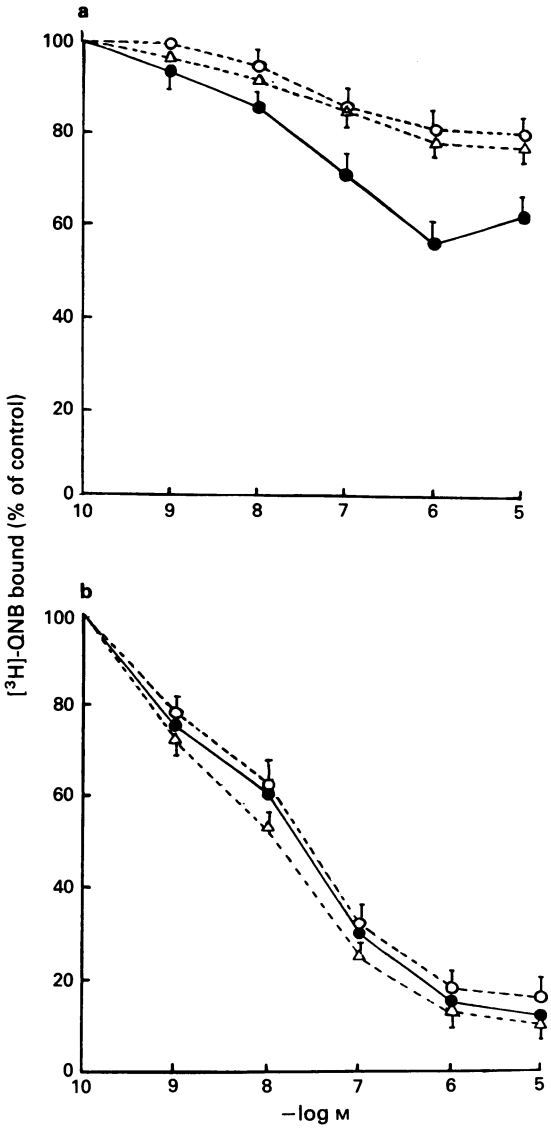

Figure 1 Effects of cytoskeletal drugs on [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) binding inhibition by increasing amount of antilaminin IgG (a) or carbachol (b). Membranes were preincubated with different concentration of antilaminin IgG or carbachol alone (●) and with 0.35 nM [^{3}H]-QNB. For cytoskeletal drugs actions, before antilaminin IgG or carbachol treatment, membranes were first treated with 5×10^{-6} M colchicine (○) or cytochalasin B (△). Control of 100% binding refers to specific radioactivity bound to atrial membranes in the absence of IgG or carbachol or to that obtained in the presence of the corresponding cytoskeletal drugs but without antibody or carbachol treatment. Means ± s.e.mean of five experiments are plotted.

myo-inositol washing that contained the removable [^{3}H]-MI. The second peak corresponded to the 1 M ammonium formate elution that contained total IPs, namely inositol triphosphate (IP3), inositol biphosphate (IP2), inositol monophosphate (IP) and inositol 1,2 cyclic monophosphate (cIP). Peak areas were determined by triangulation. Results corresponding to the 2nd peak, were expressed as a percentage of total radioactivity incorporated (1st plus 2nd peaks).

Immunofluorescence procedure

BALB/c mice were killed by decapitation. The hearts were removed and incubated at 37°C in a gassed solution of KRB in the same conditions as previously described for the contractility experiments. The preparations were incubated for 20 min with KRB alone (control) or with colchicine or cytochalasine B at a concentration of 1×10^{-5} M or 5×10^{-6} M. Then tissues were quickly frozen and cryostat sections 4–6 μm thick, were obtained. An indirect immunofluorescent technique was applied to the sections incubating them with rabbit antilaminin IgG or normal rabbit serum IgG (control) first and then with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1/10, Cappel Lab.). Antilaminin IgG and normal rabbit serum IgG were used at concentrations ranging from 1×10^{-8} M to 5×10^{-8} M. The immunofluorescent technique was also per-

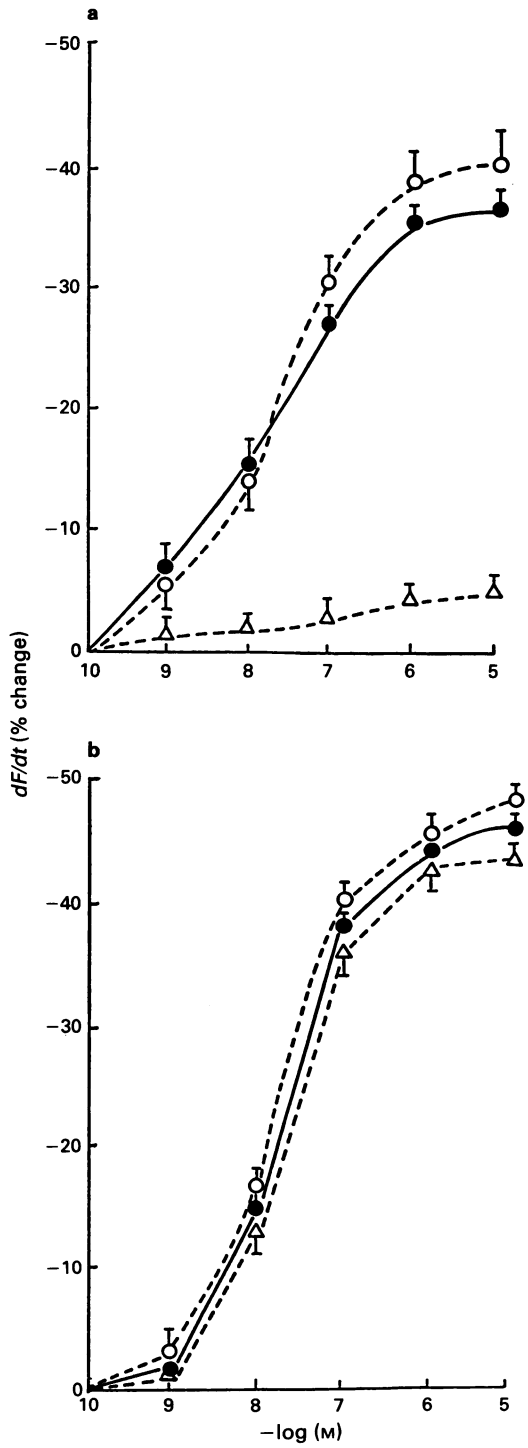

Figure 2 Influence of cytoskeletal drugs on antilaminin IgG (a) and carbachol (b) on atrial contractility. Increasing amounts of antilaminin IgG or carbachol (●) were added to the bath leaving each concentration 15 min to produce its maximal effect. Atria were exposed to 5×10^{-6} M colchicine (○) or cytochalasin B (△) before antilaminin IgG or carbachol treatment. Values are means ± s.e.mean from six experiments in each group.

formed on the sections, first incubating them with the cytoskeleton disrupting drugs for 30 min at 37°C (1×10^{-3} M or 1×10^{-5} M) and then two more steps with the unconjugated and conjugated antibodies. In all cases, sections were used unfixed or fixed for 4 min in cold acetone. Incubations with antibodies were performed at room temperature for 40 min and phosphate buffered saline was used to wash sections after each serum incubation.

Drugs and solutions

Fresh prepared solutions of the following drugs were used: carbachol, cytochalasin B, colchicine, atropine, isoprenaline, isobutylmethyl xanthine (IBMX), theophylline were from Sigma Chemical Co. The concentrations quoted in the text represent final values in incubation solutions. The salt composition of KRB was (in mM): NaCl 120.50, KCl 4.83, $CaCl_2$ 2.40, KH_2PO_4 1.21, $MgSO_4$ 1.34, $NaHCO_3$ 24.50 and glucose 5.5.

Statistical analysis

Experimental results were compared by means of a Student's t test. Differences between means were considered significant if P was equal to or less than 0.05.

Results

Effects of cytoskeletal disrupting drugs on antilaminin IgG on [^{3}H]-QNB binding to purified cardiac membranes

As can be seen in Figure 1a, there was a dose-dependent inhibition of [^{3}H]-QNB binding to mice atrial membranes when they were exposed to different concentrations of antilaminin IgG. This antilaminin IgG effect simulated the action of carbachol (Figure 1b). But while the cholinoceptor agonist, carbachol, inhibited the specific binding of [^{3}H]-QNB completely, the antibody caused only partial inhibition under these experimental conditions.

Saturation studies and Scatchard plots (Table 1) indicated that the inhibition induced by antilaminin IgG is essentially due to a decrease in number of binding sites (B_{max}) with no change in the equilibrium dissociation constant (K_d). Normal IgG affected neither the K_d nor the B_{max} (Table 1).

The effect of colchicine (5×10^{-6} M) or cytochalasin B (5×10^{-6} M) on antilaminin IgG action upon [^{3}H]-QNB binding was analysed. As can be seen in Figure 1a and Table 1, both colchicine and cytochalasin B attenuated the inhibitory action of antilaminin IgG on muscarinic cholinoceptor radioligand binding. Contrary to this, the cytoskeletal disrupting drugs were not able to alter the effect of carbachol (Figure 1b). It should be noted that neither of the two drugs modified [^{3}H]-QNB binding to cardiac membranes alone or those treated with normal IgG (Table 1).

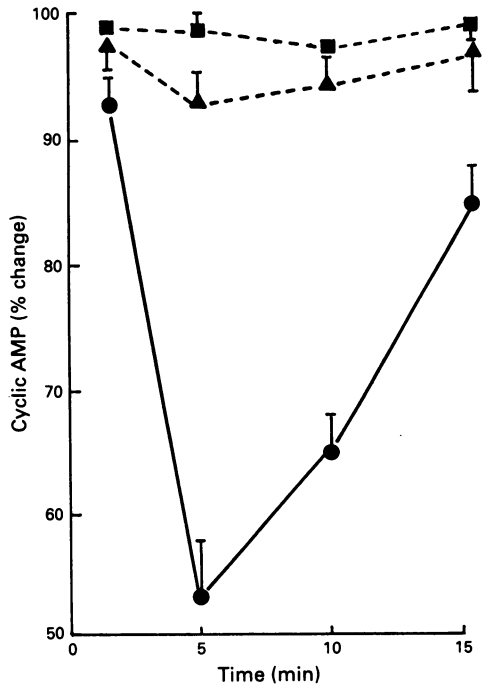

Figure 3 Time course of antilaminin IgG (●) and normal IgG (■) on isoprenaline-stimulated cyclic AMP values. Cyclic AMP levels were measured after different reaction times with 5×10^{-7} M IgGs. Inhibition studies with 5×10^{-7} M atropine (▲) were performed. Values are means ± s.e.mean of five experiments in each group performed in duplicate.

Action of cytoskeletal drugs on the muscarinic cholinoceptor effects of antilaminin IgG mediated by G regulatory proteins

The ability of antilaminin IgG to induce changes in dF/dt in mice atria is shown in Figure 2a. Antilaminin IgG induced a concentration-dependent decrease of atrial contractility. This negative inotropic effect was similar to that observed with carbachol (Figure 2b).

Table 1 Action of cytoskeletal drugs on the effect of antilaminin IgG and normal IgG upon K_d and B_{max} [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding parameters on heart membranes

	[^{3}H]-QNB binding	
Additions	K_d (nM)	B_{max} (fmol mg^{-1} protein)
None	0.22 ± 0.02	520 ± 18
Normal IgG	0.21 ± 0.02	530 ± 15
Normal IgG + cytochalasin B	0.20 ± 0.01	540 ± 20
Normal IgG + colchicine	0.23 ± 0.03	548 ± 19
Antilaminin IgG	0.19 ± 0.02	310 ± 9*
Antilaminin IgG + cytochalasin B	0.20 ± 0.03	413 ± 10**
Antilaminin IgG + colchicine	0.24 ± 0.03	422 ± 13**
Cytochalasin B	0.21 ± 0.01	550 ± 20
Colchicine	0.22 ± 0.01	540 ± 19

Cardiac membranes were or were not incubated with 5×10^{-7} M normal or antilaminin IgG or treated with 5×10^{-6} M cytochalasin B or colchicine in the presence of [^{3}H]-QNB (0.1 to 1.0 nM). The B_{max} and the K_d values were calculated from linear regression analysis. Control K_d and B_{max} values in the presence of 5×10^{-6} M cytochalasin B or colchicine alone are also shown. Means ± s.e.mean of five experiments in each group were performed by duplication.
*differ significantly between antilaminin IgG and normal IgG with $P < 0.001$ and **from antilaminin IgG + colchicine or cytochalasin B and colchicine or cytochalasin B alone with $P < 0.001$.

Table 2 Influence of cytoskeletal drugs on the effect of normal IgG upon *dF/dt* of mouse isolated atria

Additions	dF/dt ($g\,s^{-1}$)	n
None	4.5 ± 0.4	11
Normal IgG	4.8 ± 0.5	9
Normal IgG + cytochalasin B	4.4 ± 0.4	6
Normal IgG + colchicine	4.6 ± 0.3	7
Cytochalasin B	4.7 ± 0.5	12
Colchicine	4.4 ± 0.3	12

Effect of 5×10^{-7} M normal IgG on *dF/dt* of mouse atria. IgG was allowed to react for 15 min with atria suspended in KRB solution alone (none) or in the presence (during 30 min) of 5×10^{-6} M cytochalasin B or colchicine. Control *dF/dt* values of atria exposed during 45 min to cytochalasin B or colchicine (5×10^{-6} M) are also shown. Values are means ± s.e.mean. *n*: the number of preparations tested.

Table 3 Influence of cytoskeletal drugs on the effect of normal IgG on isoprenaline (Iso)-induced cyclic AMP production

Additions	*Cyclic AMP* (pmol mg^{-1} wet weight)	n
None	2.7 ± 0.9	8
Iso	27.2 ± 3.0	8
Iso + n IgG	26.2 ± 3.5	7
Iso + n IgG + colchicine	29.8 ± 3.3	6
Iso + n IgG + cytochalasin B	28.6 ± 2.9	6
Iso + colchicine	25.0 ± 3.2	5
Iso + cytochalasin B	24.9 ± 3.2	5

Effect of 5×10^{-7} M normal IgG (n IgG) on isoprenaline-induced cyclic AMP production. Control values of cyclic AMP exposed to colchicine or cytochalasin B (5×10^{-6} M) are shown. Values are means ± s.e.mean. *n*: number of preparations tested. For more details see Methods section.

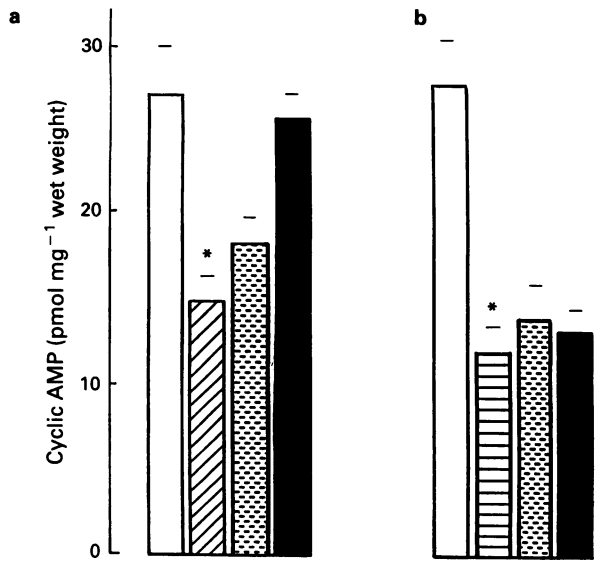

Figure 4 Effect of cytoskeletal drugs on isoprenaline-stimulated cyclic AMP production in the absence (open column) or in the presence of antilaminin IgG (a) or carbachol (b). Cyclic AMP levels were measured after 5 min of incubation with antilaminin IgG (hatched column) or carbachol (horizontally lined column). To study cytoskeletal disrupting drug actions, tissues were incubated with colchicine (stippled column) or cytochalasin B (solid column) before adding antilaminin IgG or carbachol. Values are means ± s.e.mean of five separate experiments performed in duplicate. $^*P < 0.001$ between values in absence and in presence of antilaminin IgG or carbachol.

Table 4 Action of cytoskeletal drugs on the effect of antilaminin IgG, normal IgG and carbachol upon phosphoinositide turnover

Additions	*IP formation* (% of control)	n
None	19 ± 2	5
Normal IgG	18 ± 3	6
Antilaminin IgG	68 ± 6*	7
Antilaminin IgG + atropine	17 ± 3	5
Carbachol	93 ± 9*	5
Carbachol + atropine	12 ± 2	5
Cytochalasin B	55 ± 5	7
Colchicine	59 ± 6	7

Atria were incubated for 60 min with *myo*-[2-^{3}H]-inositol and for an additional 30 min in the absence or presence of 10^{-6} M atropine or 5×10^{-6} M cytochalasin B or colchicine. Tissues were then left for a further 30 min in the absence or in the presence of 5×10^{-7} M normal or antilaminin IgG or 1×10^{-6} M carbachol. Results correspond to the percentage of the second peak related to the total radioactivity incorporated (1st + 2nd peaks). Results are means ± s.e.mean. *n*: number of experiments.
$^*P < 0.001$ versus normal IgG or none.

Cytochalasin B but not colchicine, impaired the negative inotropic effect caused by antilaminin IgG on atrial contractility (Figure 2a). In contrast, cytoskeletal disrupting drugs did not alter the negative inotropic action of carbachol (Figure 2b). Neither colchicine nor cytochalasin B modified *dF/dt* of mouse isolated atria alone or of atria treated with control IgG (Table 2).

Figure 3 shows that antilaminin IgG decreased significantly the isoprenaline-stimulated cyclic AMP production with a peak at 5 min and thereafter decreased. This effect was blunted by atropine (5×10^{-7} M) and normal IgG samples treated as antilaminin IgG did not change cyclic AMP levels (Figure 3).

The decrease in isoprenaline-stimulated cyclic AMP production by antilaminin IgG, was similar to that observed with 10^{-6} M carbachol (Figure 4). Cytochalasin B but not colchicine (both at 5×10^{-6} M) impaired the inhibitory effect of antibody upon cyclic AMP levels (Figure 4a). Neither of the two drugs modified cyclic AMP production when given alone, or in atria treated with carbachol (Figure 4b) or with normal IgG (Table 3).

As can be seen in Table 4, 5×10^{-7} M antilaminin IgG but not control IgG activated the PI turnover. This effect was also obtained with carbachol 1×10^{-6} M. Both antilaminin IgG and carbachol actions were inhibited by 5×10^{-6} M atropine, indicating muscarinic cholinoceptor participation. It was found that both colchicine and cytochalasin B modified *per se* the basal values of the PI turnover. Experiments performed to evaluate the effects of these drugs upon PI turnover in the presence of antilaminin IgG would be difficult to interpret.

Actions of cytoskeletal drugs on antilaminin IgG fixation

A continous and linear immunofluorescence (IF) surrounding the cell membranes of cardiac myoblasts from atria was observed when heart sections were incubated with antilaminin IgG (Figure 5b). No fluorescence was observed within the cells. The intensity of IF appeared to correlate with the antilaminin IgG concentration used. No fluorescence was seen in sections incubated with KRB alone or with control rabbit serum IgG (Figure 5a). Results were similar in fixed or unfixed sections but a better tissue preservation was obtained in fixed tissues and a lower background in unfixed sections.

The immunofluorescent pattern observed did not change when atrial preparations or heart sections were incubated

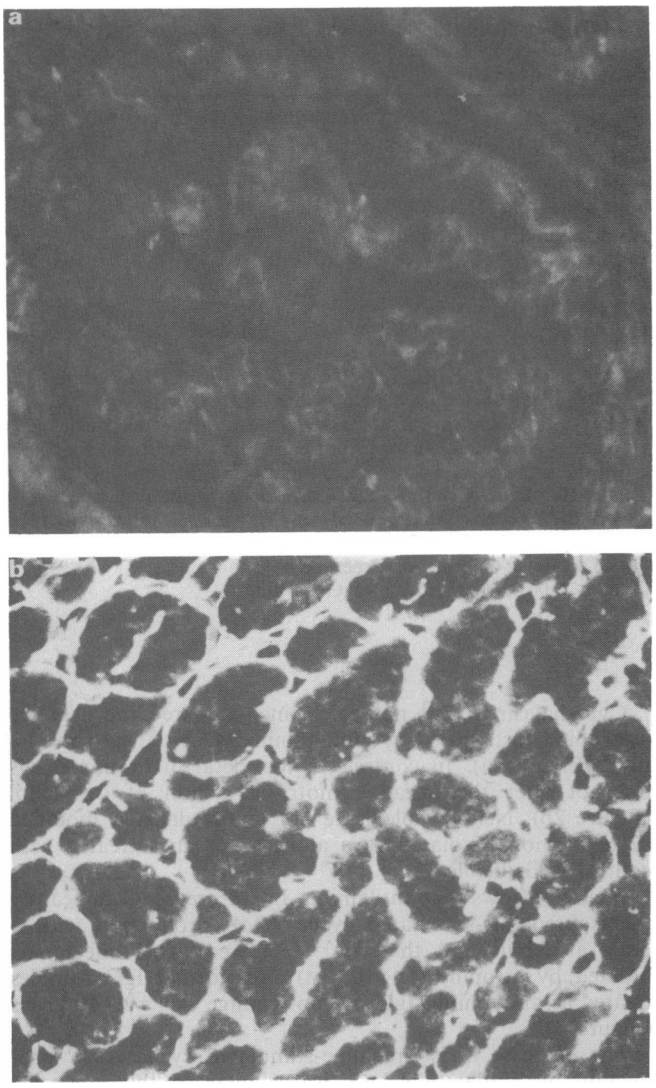

Figure 5 Indirect immunofluorescent technique performed on cryostat sections of mouse heart tissues. (a) Incubation with a normal rabbit IgG used as control. No fluorescence is observed. (b) Incubation with rabbit antilaminin IgG (1×10^{-6} M). A bright, continuous and linear immunofluorescence is observed surrounding the cardiac muscle cells.

with cytochalasin B or colchicine prior to the antilaminin IgG.

Discussion

We have previously shown that antilaminin IgG binding to laminin in heart tissue, may interfere with cholinoceptors and may activate a transduction system common to the one linked to the muscarinic cholinoceptor and thus, may induce a cholinoceptor biological response (Bacman *et al.*, 1989; 1990). The cholinoceptor effects of antilaminin IgG were prevented by preincubating the antibody with the specific antigen laminin.

It has been reported that a factor obtained from the basal lamina-rich extract of the *Torpedo california* electric organ, similar to the molecules concentrated at the neuromuscular junction basement membrane, caused acetylcholine receptor aggregation and formation of patches of acetylcholinesterase, since monoclonal antibodies against this factor blocked and immunoprecipitated the acetylcholine receptor-aggregating activity (Fallon *et al.*, 1985; Wallace *et al.*, 1985). Only laminin caused the acetylcholine receptor movement when it was applied to cultured myotubes (Vogel *et al.*, 1983). On the other hand, it has been suggested that tubulin forms complexes with specific regulatory G proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades (Wang *et al.*, 1990). Additionally, some of the cholinoceptor-mediated cellular transmembrane signals are associated with regulatory G proteins (Martin *et al.*, 1986; Taylor & Marrit, 1986).

We have here shown that effects of antilaminin IgG on muscarinic cholinoceptor radioligand binding were abrogated by cytoskeletal inhibitors, colchicine and cytochalasin B. Conversely, these agents did not alter carbachol binding. These results suggest that during the antilaminin IgG and cholinoceptor interactions a plasma membrane micro-redistribution takes place, and probably that this phenomenon requires intact microtubules and microfilaments. The inhibition of the polymerization of the cytoskeletal actin has additional effects on the production of muscarinic intracellular signals induced by antilaminin IgG associated with regulatory G protein. Surprisingly, the treatment with cytochalasin B but not colchicine, impaired selectively antilaminin IgG action on both, heart contractility and cyclic AMP production. These data suggest that cytochalasin B binding protein, perhaps actin, but not tubulin, is involved in the transduction system induced by antilaminin IgG coupled to heart muscarinic cholinoceptors mediated by regulatory G protein.

Olson *et al.* (1991) demonstrated that the *in vitro* organization of intestinal epithelial cells on a basement membrane into multicellular structures requires specific interactions between laminin receptors and intact actin microfilaments. The integrity of microtubules was not required for this pattern formation. Shaw *et al.* (1990) demonstrated that the activation-dependent adhesion of macrophages to laminin is mediated by the $\alpha 6\beta 1$ integrin.

Disruption of the actin network with cytoskeleton inhibitors abolishes macrophage adhesion to laminin (Mercurio & Shaw, 1988). This interaction appears to involve the anchoring of $\alpha 6\beta 1$ integrin to both extracellular laminin and the actin cytoskeleton process that may be facilitated by protein kinase C phosphorylation of the $\alpha 6$ subunit.

On the other hand, previous reports demonstrated a relation betwen cytoskeletal proteins and membrane-bound enzymes, especially between tubulin and adenylate cyclase in various systems (Gemsa *et al.*, 1977; Rudolph *et al.*, 1977; Puck, 1977; Insel & Kennedy, 1978; Hagmann & Fishman, 1980). It has also been suggested that tubulin itself is a type of GTP-binding protein as long as it exerts biological functions through protein-protein interactions, which are modulated by nucleotide binding protein (Hughes, 1983; Sternlicht *et al.*, 1987).

The effect of cytoskeletal disrupting agents was not due to the drugs modification *per se* either of binding or of biological effect, as demonstrated by the fact that there were no changes in binding, cyclic AMP production and heart contractility parameters, when they were pretreated with these drugs. In the case of the PI turnover experiments, the results are difficult to explain since colchicine and cytochalasin B alone, modified the PI turnover. The molecular interactions occurring between the cytoskeletal proteins, regulatory G proteins and PI turnover have not been determined. Recently, evidence for a functional association between the lymphoma membrane cytoskeleton and the 41 kDa (Gi α-like protein), that increased the IP formation and surface Thy-1 receptors microdistribution, was observed (Bourgninon *et al.*, 1990).

Finally, the fact that colchicine and cytochalasin B did not change the characteristic immunofluorescent pattern obtained when heart sections were incubated with antilaminin IgG, suggests that this pattern is basically an expression of the interaction between the antibody and the extracellular laminin that surrounds the myoblast cell membrane. These

results could also suggest that cytoskeleton proteins are not involved in the antibody-laminin cell receptor interaction.

The discrepancy between the [^{3}H]-QNB binding displacement where carbachol has a greater maximal effect than antilaminin IgG (Figure 1) and the negative inotropic potency where the two agents were similar (Figure 2), could be explained by the fact that, the antibody unlike carbachol, interfered with the labelled antagonist in a noncompetitive fashion; while they may share common signal transduction pathways inducing muscarinic cholinoceptor biological responses. Moreover, the binding and the biological effect triggered by the full cholinoceptor agonist, carbachol, were not altered by the drugs that prevent microfilament and microtubule polymerization; the agonist-receptor interaction and the transducing of signals, suggest that cytoskeletal proteins are not involved.

The present data, are consistent with the existence of some linkage between muscarinic cholinoceptor and laminin receptors, in which the integrity of the cytoskeleton is required.

This work was supported by Grant PID-BID CONICET 0352. We thank Mrs Elvita Vannucchi and Mrs Tania G. Borda for excellent technical assistance and typing the manuscript, respectively.

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(Received February 2, 1993
Revised September 15, 1993
Accepted September 20, 1993)

Br. J. Pharmacol. (1994), **111**, 278–282

Differential vasodilator properties of KRN2391, cromakalim, nitroglycerin and nifedipine in rabbit isolated femoral artery and vein

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1 The selectivity for artery and vein of KRN2391, cromakalim, nitroglycerin and nifedipine was examined in isolated femoral artery and vein preparations of the rabbit.

2 All drugs produced a concentration-dependent relaxation in both femoral artery and vein.

3 Nitroglycerin was more potent in femoral vein than in femoral artery at all concentrations. The EC_{50} value obtained in the vein was about 14 times smaller than that obtained in artery.

4 Cromakalim and nifedipine were almost equipotent on both vascular preparations. Cromakalim at the highest concentration (10^{-5} M) produced 88 and 78% relaxation in femoral artery and vein, respectively. The maximum relaxation induced by nifedipine (10^{-6} M) was less than 50% in both preparations.

5 KRN2391 was active at a lower concentration in the vein than in the artery and its maximum relaxation at 10^{-5} M was about 90% in both preparations.

6 Glibenclamide (10^{-6} M) inhibited the vasorelaxation caused by KRN2391 in both artery and vein. Methylene blue (10^{-5} M) also inhibited the relaxant action of KRN2391 but this action was slight in the artery.

7 These results suggest that KRN2391 and nitroglycerin are more potent in the vein than in the artery and cromakalim and nifedipine are equipotent in both. It is considered that the relaxation induced by low concentrations of KRN2391 reflects predominantly its action as a nitrate and that at high concentrations it acts as a K^+ channel opener in addition to its nitrate action. The different vascular selectivities of these drugs are thought to relate to the differences in their mechanisms of action in vascular smooth muscle.

Keywords: KRN2391; nitroglycerin; cromakalim; nifedipine; rabbit femoral artery; rabbit femoral vein

Introduction

KRN2391, N-cyano-*N'*-(2-nitroxyethyl)-3-pyridinecarboximidamide monomethansulphonate, is a new vasodilator which has a nitrate moiety in its chemical structure. With respect to its mechanism of action, it has been reported to possess both a K^+ channel opening action and a nitrate action (Kashiwabara *et al.*, 1991; Okada *et al.*,1991; Ishibashi *et al.*, 1992). In *in vivo* experiments on anaesthetized dogs, KRN2391 produced a preferential increase in coronary blood flow in comparison with other peripheral blood flows (Ogawa *et al.*, 1993). In addition, KRN2391 has been reported to induce an increase in oxygen supply through increases in coronary blood flow and decreases in myocardial oxygen consumption in dogs (Ogawa *et al.*,1992). Thus, KRN2391 is thought to be a vasodilator selective for the coronary vascular bed with both a K^+ channel opening action and a nitrate action. Recently, Kasai *et al.* (1993) have shown a potent antivasoconstrictor effect of KRN2391 in porcine isolated coronary arteries contracted by noradrenaline, acetylcholine, 5-hydroxytryptamine, thromboxane A_2 (using a stable analogue U46619) and endothelin-1 which are considered to be the cause of coronary vasospasm (Vanhoutte & Houston, 1985). From these profiles of KRN2391, it is possible that it could be useful in therapy of ischaemic heart disease such as angina pectoris.

At present, nitrates and Ca^{2+} channel blockers are extensively used as antianginal drugs. Although the coronary dilator actions of nitrates and Ca^{2+} channel blockers contribute to their beneficial effects, the influences of these drugs on preload or afterload also play an important role in their antianginal effects. The finding that vasodilators produce either a reduction in preload or afterload is associated with their selectivity for vein or artery, i.e. vasodilators selective for vein and venules such as nitroglycerin produce a reduction in preload (Gotanda *et al.*, 1989), while vasodilators relatively selective for artery such as Ca^{2+} channel blockers produce a potent reduction in afterload (Sakanashi *et al.*, 1986; 1988). Therefore, a study concerning the selectivity of KRN2391 on vein and artery was considered to be important in analysing its effect on systemic circulation *in vivo*. In the present study, we examined the properties of KRN2391 compared with those of nitroglycerin and nifedipine in femoral artery and vein of the rabbit. Cromakalim, a K^+ channel opener (Weir & Weston, 1986; Coldwell & Howlett, 1987), was also examined because a K^+ channel opening action partly contributes to the vasodilator mechanism of KRN2391.

Methods

Preparations

Femoral arteries and veins were excised from male rabbits (New Zealand White, 2.7–3.5 kg) that had been anaesthetized with ether, and killed by exsanguination. Immediately after excision, these vessels were placed in Krebs-Ringer solution of the following composition (mM): NaCl 112, KCl 4.7, $CaCl_2$ 2.2, $NaHCO_3$ 25, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 14. Adherent fat and connective tissue were cleaned from the vessels, and rings of tissues (each 3 mm long) were prepared. The endothelium was removed by rubbing the intimal surface

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with a stainless steel rod. Each preparation was mounted in an organ bath filled with 10 ml Krebs-Ringer solution. This was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Isometric tension was recorded on a pen-writing recorder (FBR-252A, TOA Electronics, Tokyo, Japan) via a transducer (TB-611T, Nihon Koden, Tokyo, Japan). An initial resting tension of 0.5 g and 0.2 g was applied to the arterial and venous preparations, respectively. These resting tensions were chosen by referring to Nakajima & Nosaka (1983). After an initial equilibration period of 120 min, the preparations were contracted by 10^{-6} and 10^{-5} M noradrenaline in arteries and veins, respectively. These concentrations of noradrenaline were determined as those producing submaximum contractions (see Results). When the preparations had reached stable tension, each test drug was then added noncumulatively to the organ bath according to the method of Nakajima & Nosaka (1983). Glibenclamide (10^{-6} M) and methylene blue (10^{-5} M) were added to the bath 20 min before the addition of noradrenaline when their effects on KRN2391-induced relaxation were to be evaluated.

Drugs

KRN2391 and cromakalim were synthesized at Kirin Brewery Pharmaceutical Research Laboratory. (−)-Noradrenaline bitartrate and papaverine hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Glibenclamide, methylene blue and nifedipine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitroglycerin was used from commercially available ampoules (Millisrol, Nippon Kayaku, Tokyo, Japan). Cromakalim (5×10^{-3} M) and nifedipine (10^{-2} M) were dissolved in dimethyl sulphoxide, and glibenclamide was prepared in dimethyl sulphoxide/10% $KHCO_3$/twice distilled water (10:5:85). These solutions were diluted in twice distilled water. All other drugs were dissolved in twice distilled water.

Statistical analysis

Data are presented as mean ± s.e.mean. Relaxation responses caused by the test drugs are expressed as a percentage of the maximum relaxation attainable at the end of each experiment by the addition to the bath of 10^{-4} M papaverine. Differences were evaluated for significance ($P<0.05$) by Student's t test for unpaired data for single comparisons. The concentration of a drug which caused a 50% response (EC_{50} value) with 95% confidence limits was calculated by the probit method.

Results

Figure 1 shows concentration-response curves for noradrenaline in the femoral artery and vein. The effect of noradrenaline was more potent in the femoral artery than in the vein. The EC_{50} value was $7.18 \pm 0.08 \times 10^{-8}$ M in artery ($n = 6$) and $7.75 \pm 3.53 \times 10^{-7}$ M in vein ($n = 7$). The concentrations of noradrenaline chosen to induce submaximum contractions were 10^{-6} M and 10^{-5} M in femoral artery and vein, respectively. The developed tension produced by these concentrations of noradrenaline was 4.8 ± 0.33 g in the artery ($n = 6$) and 0.42 ± 0.07 g in the vein ($n = 7$).

Figure 2 shows concentration-response curves for the test drugs in the femoral artery and vein. Nitroglycerin over the concentration range 10^{-9}–10^{-5} M produced a concentration-dependent relaxation in femoral artery and vein (Figure 2a). The threshold concentration of nitroglycerin was lower in the vein than in the artery. The activity of nitroglycerin was greater in the vein than in the artery and at 10^{-7} and 10^{-6} M, the % relexation value in the vein was significantly greater than that in the artery. Cromakalim (10^{-7}–10^{-5} M) and nifedipine (10^{-10}–10^{-6} M) also produced a concentration-dependent relaxation in the femoral artery and vein (Figure 2b and c). Cromakalim showed similar activity in the artery and vein at 10^{-7} M but at 10^{-6} M the % relaxation value was significantly greater in artery than that in vein. KRN2391 studied over the concentration-range 10^{-9}–10^{-5} M produced relaxation in femoral artery and vein in a concentration-dependent manner (Figure 2d). At 10^{-8} and 10^{-7} M, the vasodilator activity of KRN2391 was significantly greater in the vein than in the artery.

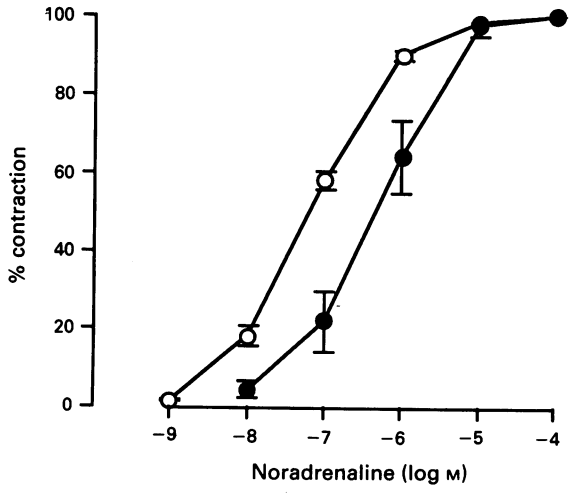

Figure 1 Concentration-response curves for noradrenaline in rabbit isolated femoral artery (○) and vein (●). Ordinate scale: % contraction expressed as a percentage of the response at 10^{-4} M noradrenaline. Each value is presented as a mean ± s.e.mean derived from 6–7 experiments.

Table 1 summarizes the maximum relaxation of each drug in artery and vein. The maximum response of each drug was not different between artery and vein.

Table 2 shows the EC_{50} value of each drug. The EC_{50} values of KRN2391 and nitroglycerin in artery were greater than those in vein. In contrast, the value of cromakalim in artery was smaller than that in vein. Because the relaxation at 10^{-6} M nifedipine did not reach 50% in both vascular preparations (Figure 2c and Table 1), the EC_{50} values of nifedipine could not be calculated.

Figure 3 shows the effects of glibenclamide (10^{-6} M) and methylene blue (10^{-5} M) on the KRN2391-induced relaxation in the femoral artery and vein. Table 2 also shows the EC_{50} values in the presence of glibenclamide or methylene blue. Glibenclamide inhibited the vasorelaxation caused by KRN2391 and the concentration-response curves similarly shifted to the right both in artery and vein. The EC_{50} values in the presence of glibenclamide were about 11 times greater than those in the absence of glibenclamide in both vascular preparations. Methylene blue also antagonized the relaxant action of KRN2391. The concentration-response curve in the vein shifted to the right at all concentrations of KRN2391, but the action of KRN2391 obtained in the artery was clearly inhibited by methylene blue only at 10^{-6} M KRN2391. The EC_{50} values with methylene blue were 2.7 and 6.4 times greater than those without methylene blue in artery and vein, respectively.

Discussion

In the present study, at low concentrations, KRN2391 produced a greater vasodilatation in the femoral vein than in the femoral artery, whereas, at high concentrations it produced equal vasodilatation in artery and vein. Since the vasorelaxant activity of KRN2391 is reported to be based on both a nitrate action and a K^+ channel opening action (Kashiwabara *et al.*, 1991; Okada *et al.*, 1991; Ishibashi *et al.*, 1992), the properties of KRN2391 observed in the present

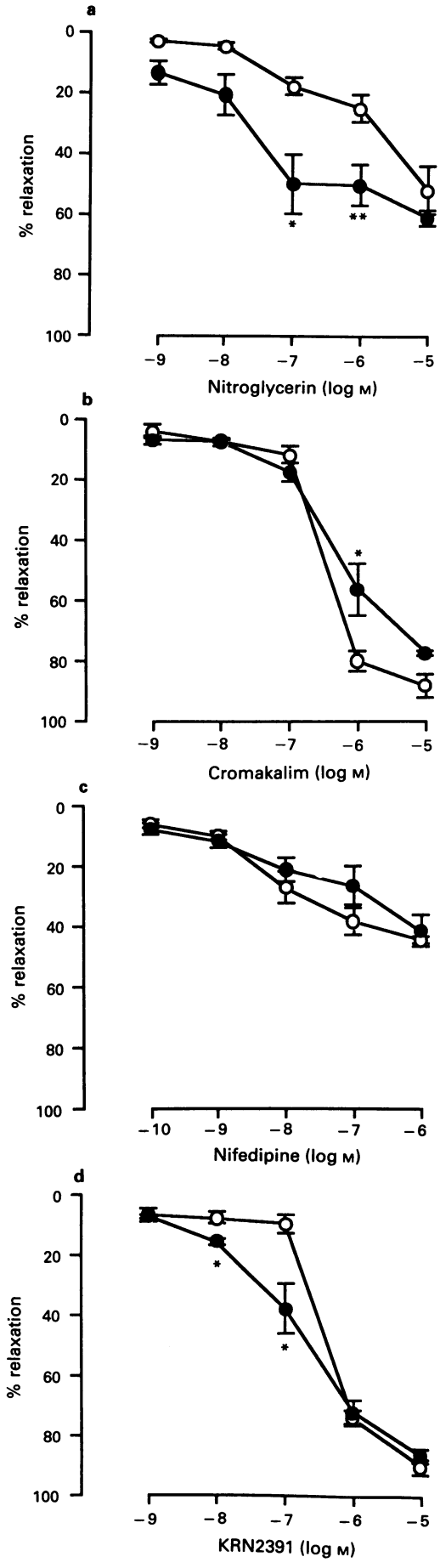

Figure 2 Effects of nitroglycerin (a), cromakalim (b), nifedipine (c) and KRN2391 (d) on the contractions induced by noradrenaline in femoral artery (○) and vein (●). Ordinate scale: % relaxation expressed as a percentage of the response at 10^{-4} M papaverine. Each value is presented as a mean ± s.e.mean derived from 4–7 experiments. $*P<0.05$, $**P<0.01$: significant difference from the corresponding value for the artery.

Table 1 The maximum relaxation induced by KRN2391, nitroglycerin, cromakalim and nifedipine against contractions produced by noradrenaline in isolated femoral artery and vein of the rabbit

	Maximum relaxation (%)	
	Artery	*Vein*
Nitroglycerin (10^{-5} M)	51.73 ± 7.67	60.78 ± 2.51
Cromakalim (10^{-5} M)	88.20 ± 3.77	77.50 ± 0.65
Nifedipine (10^{-6} M)	44.48 ± 0.92	41.58 ± 5.28
KRN2391 (10^{-5} M)	90.18 ± 2.58	86.50 ± 2.26

Each value is the mean ± s.e.mean of 4–7 experiments.

Table 2 EC_{50} values for the relaxation effects of KRN2391, nitroglycerin and cromakalim against contractions induced by noradrenaline

	EC_{50} (10^{-7} M)	
	Artery	*Vein*
Nitroglycerin	111 (48.6–361)	7.95 (3.59–21.8)
Cromakalim	3.81 (2.65–5.56)	8.65 (5.40–14.7)
KRN2391	4.02 (2.74–6.00)	2.10 (1.37–3.27)
KRN2391 + glibenclamide	43.6 (31.0–62.0)	24.5 (16.4–36.9)
KRN2391 + methylene blue	10.7 (7.45–15.4)	13.4 (8.11–22.5)

Each value is calculated by the probit method from the results of 4–8 experiments.
The numbers in parentheses indicate 95% confidence limits.

study appear to reflect this dual mechanism of action. The experiments with blocking agents provided evidence of these properties of KRN2391. Vasorelaxation induced by KRN2391 in both artery and vein was antagonized by glibenclamide, a pharmacological antagonist of K^+ channel openers (Cavero *et al.*, 1989; Eltze, 1989). On the other hand, methylene blue, an inhibitor of soluble guanylate cyclase (Grutter *et al.*, 1981), inhibited the response of the vein more than that of the artery. Therefore, it is considered that the relaxation induced by low concentrations reflects predominantly its action as a nitrate and that at high concentrations reflects its actions as a K^+ channel opener in addition to a nitrate action.

Nitroglycerin produced more potent vasodilatation in the femoral vein than in the femoral artery. These results with nitroglycerin are in good agreement with those of earlier studies in similar preparations from the same species (Nakajima & Nosaka, 1983; Toyoda *et al.*, 1986). The guanosine 3′:5′-cyclic monophosphate (cyclic GMP) increased by activating guanylate cyclase acts as a mediator of nitrates including nitroglycerin (Kukovetz *et al.*, 1979; Itoh *et al.*, 1985). The next step is thought to involve hyperpolarization of cell membranes (Ito *et al.*, 1978), inhibition of Ca^{2+} influx

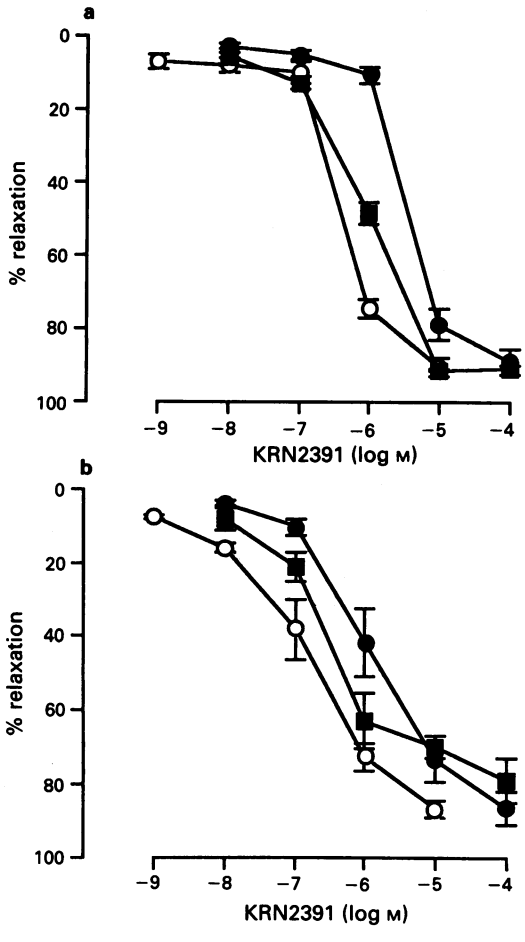

Figure 3 Effects of glibenclamide (10^{-6} M, ●) and methylene blue (10^{-5} M, ■) on KRN2391-induced relaxation of rabbit femoral artery (a) and vein (b) precontracted with noradrenaline. The control concentration-response curves (○) are the same as those in Figure 2d. Ordinate scale: % relaxation expressed as a percentage of the response to 10^{-4} M papaverine. Each value is presented as a mean ± s.e.mean derived from 4–8 experiments.

(Thorens & Haeusler, 1979), an increase in Ca^{2+} extrusion (Itoh *et al.*, 1985), Ca^{2+} sequestration (Imai & Kitazawa, 1981) and a reduction in the amount of myosin light chain phosphorylation (Murad, 1986). The difference in development of these processes may be associated with the different sensitivity to nitroglycerin between artery and vein. Axelsson *et al.* (1982) also proposed that nitrates, including nitroglycerin, induce vasodilatation by interacting with the sulphydryl group on a nitrate 'receptor'. Therefore, another possible explanation of the different sensitivity may be that there is a difference in the quantity of sulphydryl groups between artery and vein.

The present study showed that cromakalim at high concentrations produced more potent vasodilatation of the femoral artery than of the femoral vein. K^+ channel openers such as cromakalim, are though to act by increasing the outward K^+ current by opening K^+ channels and thereby causing hyperpolarization of cell membranes (Cook, 1988). This hyper polarization induces a reduction in intracellular free Ca^{2+} through the inhibition of voltage-dependent Ca^{2+} channels and receptor-operated Ca^{2+} channels (Cook, 1988). Recently, in addition to these mechanisms, K^+ channel openers have been reported to reduce the release of Ca^{2+} from intracellular stores through inhibition of inositol-1,4,5-trisphosphate production (Ito *et al.*, 1991). There may be a difference in the number of K^+ channels between the femoral artery and vein.

The relaxation induced by nifedipine was less than 50% even at its maximum concentration in femoral artery and vein. In contrast, KRN2391 and cromakalim produced about 78–90% relaxation in both preparations. These results indicate that the remaining contraction by noradrenaline in the presence of nifedipine is due to Ca^{2+} release from intracellular storage sites. As mentioned above, the vasodilator mechanism of K^+ channel openers is thought to be based on the inhibitions of extracellular Ca^{2+} influx and release of Ca^{2+} from intracellular storage sites, triggered by hyperpolarization of the cell membranes. Therefore, KRN2391 and cromakalim seem to be able to inhibit the remaining contraction which cannot be inhibited by nifedipine. Nitroglycerin also produced less than 60% relaxation even at 10^{-5} M. However, nitroglycerin-induced relaxation did not reach a plateau in the femoral artery and vein. In the present study, we did not examine the vasorelaxant activity of nitroglycerin at higher concentrations because a higher concentration of nitroglycerin could not be obtained. If more than 10^{-5} M nitroglycerin is used, it may induce further relaxation in femoral artery and vein.

In summary, the present results suggest that the vasorelaxant activity of nitroglycerin was greater in the femoral vein than in the femoral artery, while cromakalim and nifedipine were equipotent in both vascular smooth muscle preparations. In addition, the properties of KRN2391 at low concentrations are similar to those of nitroglycerin but at high concentrations its effects seem to reflect a combination of actions of nitroglycerin and cromakalim. The differences in vasorelaxant activity of KRN2391, nitroglycerin, cromakalim and nifedipine between the femoral artery and vein are thought to be based on their different mechanisms of action in vascular smooth muscle.

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(Received June 1, 1993
Revised September 8, 1993
Accepted September 20, 1993)

Br. J. Pharmacol. (1994), **111**, 283–287

Extracellular pancuronium affects sodium current in chick embryo sensory neurones

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1 The action of pancuronium on transmembrane sodium conductance was investigated in dorsal root ganglion neurones of chick embryos. The Na^+ current was measured by use of the patch-clamp technique in whole-cell configuration.

2 Externally perfused pancuronium (50 μM to 1 mM) reversibly inhibited the current by a fast mechanism of action. Inhibition was concentration-dependent (with a half-effective dose of 170 μM) but not voltage-dependent.

3 The activation and inactivation kinetics of the Na^+ current were estimated in pancuronium and in control solution by fitting experimental data with a Hodgkin-Huxley theoretical model.

4 The activation time constant τ_m, at negative membrane voltages, was larger in the presence of pancuronium than in the control. In contrast, the inactivation time constant τ_h was smaller during drug perfusion at membrane voltages < -10 mV. The steady-state inactivation h_∞ was not affected by pancuronium.

5 These results suggest that pancuronium may reduce the sodium current by interacting with the sodium channels in both the resting and open states.

Keywords: Pancuronium; muscle relaxants; sodium channel; dorsal root ganglion; patch-clamp; chick embryo; sensory neurones

Introduction

Pancuronium bromide, $C_{35}H_{60}Br_2N_2O_4$, is a *bis*-quaternary ammonium compound with the steroidal chemical structure shown in Figure 1 (Savage *et al.*, 1971).

Pancuronium is a non-depolarizing neuromuscular blocking agent developed more than twenty years ago (Baird & Reid, 1967; Buckett *et al.*, 1967). Pancuronium is still widely administered in clinical environments to provide muscle relaxation both during surgical operations and in critically ill subjects in Intensive Care Units. To patients needing long-term mechanical ventilation, huge doses (1 mg kg^{-1} body weight, twenty times as large as the average dose administered to surgical patients) are given for days, sometimes for weeks (Agoston *et al.*, 1990).

Like other non-depolarizing muscle relaxants, pancuronium is known to inhibit neuromuscular transmission by competing with acetylcholine for binding sites on nicotinic receptors (Bowman, 1990). Moreover, Yeh & Narahashi (1977) showed that additional mechanisms of action can be considered. These authors observed that internally perfused pancuronium inhibited the sodium current of voltage-clamped squid axons. This compound was quite ineffective if applied to the extracellular site of the axon and did not exhibit any notable action on potassium membrane conductance. Since the interaction of the drug with sodium conductance in cell membranes is a pharmacologically and clinically important phenomenon, it seemed appropriate to check on this interaction: (a) in a different neuronal preparation, i.e. the dorsal root ganglion (DRG) sensory neurones of chick embryos; (b) under different experimental conditions, perfusing pancuronium from the external side of the cell membrane; (c) by employing a different electrophysiological technique (the patch-clamp technique in whole-cell configuration).

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Methods

The experiments were performed on primary culture neurones from DRG of 10-day old chick embryos, with the approval of the *ad hoc* Committees on Animal Research at our institutions. Sensory neurones were grown according to the procedure described elsewhere (Barde *et al.*, 1980; Carbone & Lux, 1984). Experiments were performed from 4 h to 4 days after plating.

The external and internal solutions were chosen such as to eliminate potassium and calcium currents. The composition of the external solution was as follows (mM): NaCl 120, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 20, HEPES 10 and $CdCl_2$ 1. The electrode filling solution contained (mM): CsCl 120, TEACl 20, EGTA 10, HEPES 10 and $MgCl_2$ 2. Solutions were adjusted to pH 7.3 with the addition of NaOH (external solution) or CsOH (internal solution), and the osmolarity was adjusted to 300 mosm kg^{-1} by adding mannitol.

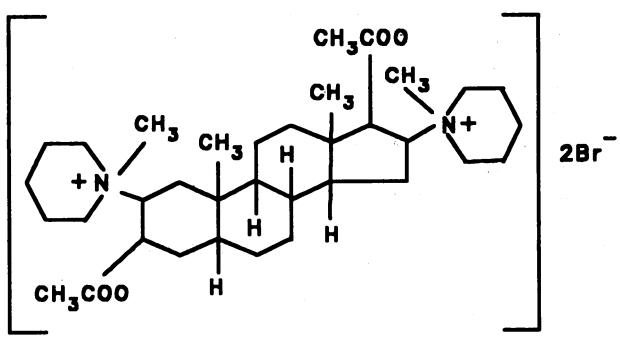

Figure 1 Chemical structure of pancuronium bromide: 1,1'-(3α,17β-dihydroxy-2β-5α-androstan-2β,16β-ylene) bis[1-methylpiperidinium] dibromide diacetate. Molecular weight 732.7. Percentage composition: C 57.39, H 8.25, Br 21.81, N 3.82, O 8.73. Pancuronium dissolves in 30 parts chloroform, one part water at 20°C.

Pancuronium bromide for clinical use (Pavulon, from N.V. Organon, Oss, Holland) was applied by means of a perfusion system working at a flow-rate of 1 ml min^{-1}. This system allowed a full change of solution in less than 0.5 s via a blunt glass pipette held close to the cell with a micromanipulator. Control experiments were performed with pure pancuronium bromide, supplied by Sigma Co., St. Louis, MO, U.S.A., but no difference was ever observed.

The experimental setup has been described previously (Nobile *et al.*, 1990). Sodium current measurements were made at room temperature (20 to 22°C) by using the patch-clamp technique in whole-cell configuration (Hamill *et al.*, 1981). Membrane currents were evoked by voltage steps of 10 mV, 40 ms long, from −40 to 60 mV, at a holding potential (V_H) of −60 mV or −80 mV, and recorded by use of an L/M EPC-7 patch-clamp amplifier (List Medical Electronics, Darmstad, Germany). Special care was exercised in compensating for access resistance to improve the accuracy of voltage control and the response time-constant of the amplifier. Large electrodes with low series resistance (2 to 3 MΩ) were used. The iterative increase in the positive-feedback series-resistance compensation circuit and an accurate tuning of capacitance compensation allowed us to reach a good compromise with the tendency of the amplifier to oscillate at about 75 to 80% compensation. Finally, measurements where a time-dependent change in the access resistance was suspected, were discarded. Passive current components were corrected by using the P/4 stimulation protocol from a V_H of −120 mV. Experimental data were digitized at 16 bit by employing a digital audio processor (SONY PCM 701 ES), and stored in a video tape recorder (SONY SL HF 100). Data were retrieved from the recorder, filtered at 3 kHz with an 8-pole Bessel filter, and then acquired with a personal computer (IBM PC AT) for further analyses. The electrophysiological data-processing software, VCAN VI-1, was kindly provided by Dr J. Dempster, Dept. of Physiology and Pharmacology, University of Strathclyde, Glasgow, Scotland.

Results

The time-course of a family of whole-cell currents, under control conditions and during perfusion with 150 μM pancuronium, is shown in Figure 2. Currents were evoked in a voltage-clamped neurone held at −60 mV V_H by step depolarizations from −30 to 10 mV. The external solution contained Na^+ (120 mM), Ca^{2+} (2 mM), and Cd^{2+} (1 mM), and the internal solution contained Cs^+ (120 mM) and TEA (20 mM). A fast transient inward current was present at each applied depolarizing potential, with a maximum peak amplitude between −10 and 0 mV. By contrast, a low, long-lasting, residual outward current was observed at membrane depolarizations more positive than −20 mV. In the absence of Ca^{2+} and K^+ currents, the fast transient disappeared in the presence of 3 μM tetrodotoxin (TTX) in the bath, or when the external sodium was replaced with choline. These effects, together with the fast activation and inactivation rates and with the voltage range of excitation, indicated that the inward current was carried by Na^+ ions. The Na^+ current was inhibited by pancuronium at each applied potential, and the peak amplitude was reduced to about 60% of its previous value. Interestingly, the residual outward current was affected by the presence of pancuronium, as well.

At V_H = −60 mV, the presence of the Na^+ channel ultra-slow inactivation process must be taken into account (Carbone & Lux, 1986; Ruben *et al.*, 1992). For this reason, the same experiments were performed on cells held at V_H = −80 mV: the percentages of Na^+ current inhibition due to pancuronium action turned out to be quite comparable in both cases. Therefore, the −60 mV value of V_H was preferred in the following experiments, as the preparation showed a better stability and a longer life.

The current-voltage relationships in control conditions, in the presence of 250 μM pancuronium, and after wash-out of the drug are shown in Figure 3. I_{peak} values were measured by using 10 mV depolarizing steps from −40 to 60 mV, at V_H = −60 mV, and averaged over seven neurones. The current recovery was complete after wash-out with control solution. In Figure 4, the ratios between Na^+ current in pancuronium and in control conditions are plotted versus depolarization. The Na^+ current inhibition by pancuronium is not voltage-dependent except for very large depolarizations, where the outward current prevails.

The inhibitory effect of pancuronium on the Na^+ current was concentration-dependent. Concentrations between 50 μM and 1 mM were tested: in every case, the observed inhibition of the Na^+ current amplitude was completely reversible. Similar results were obtained in 65 DRG neurones. The dose-response relation is shown in Figure 5. The ratio of the peak current with and without pancuronium (I/I_{max}), was measured at six different concentrations by using depolarizing voltage pulses to −10 mV from a holding potential of

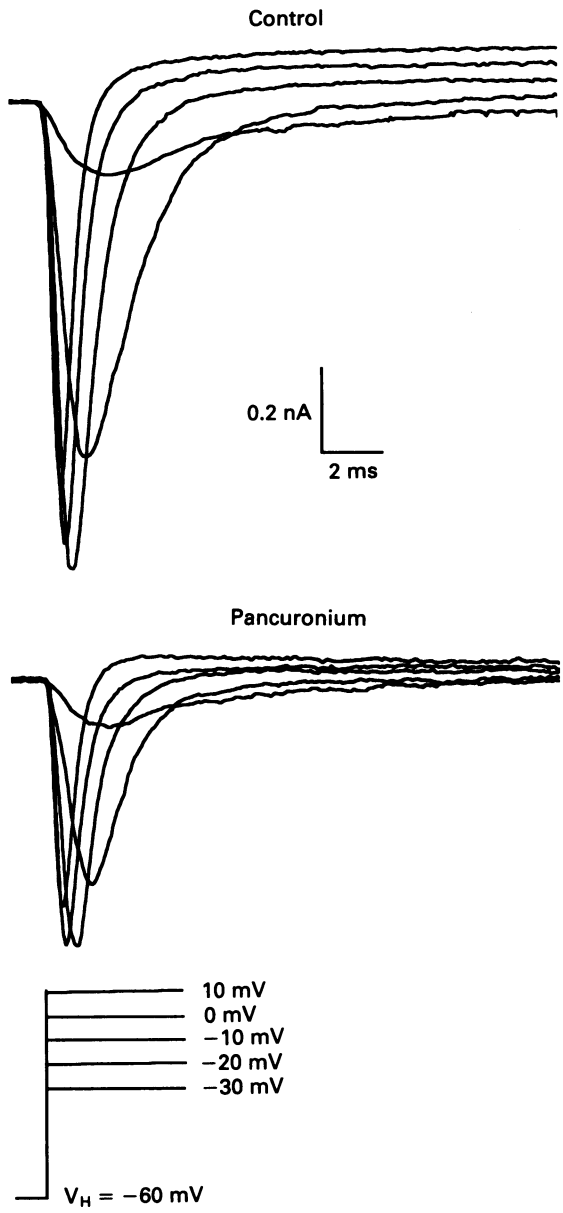

Figure 2 Whole-cell currents evoked by depolarizing steps to indicated values in control conditions and during perfusion with 150 μM pancuronium (V_H = −60 mV). After removal of Ca^{2+} and K^+, a transient inward Na^+ current, showing a maximum between −10 and 0 mV, is evoked. A low, long-lasting outward current is still present.

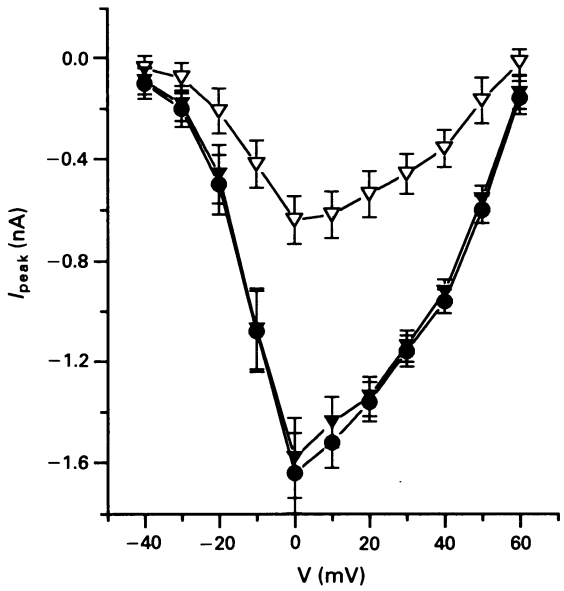

Figure 3 Current-voltage relationship in control conditions (●), during the perfusion of 250 μM pancuronium (▽), and after washout with control solution (▼); 10 mV depolarizing steps from −40 mV to 60 mV (V_H = −60 mV) were used. Data were averaged over seven neurones. Mean values ± s.e.mean are shown.

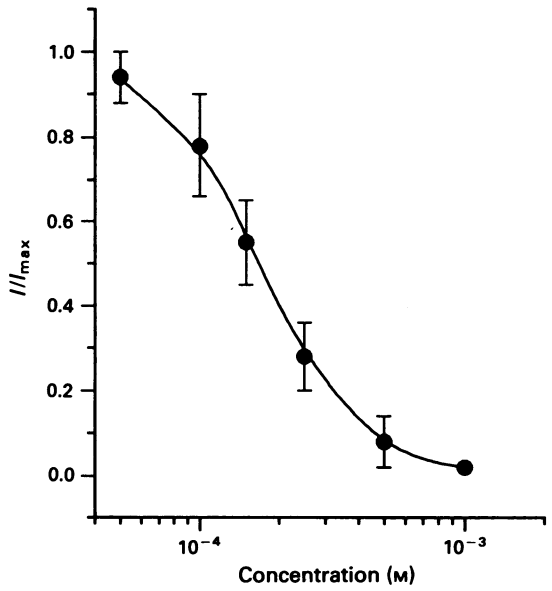

Figure 5 Pancuronium dose-response curve. Depolarizing pulses to −10 mV (V_H = −60 mV) were used to measure the ratio of the peak current with and without pancuronium. Values of I/I_{max} were averaged over ten cells, and the s.e.means are shown. The experimental points were best approximated with the equation shown in the text. The half-effective dose was 170 μM.

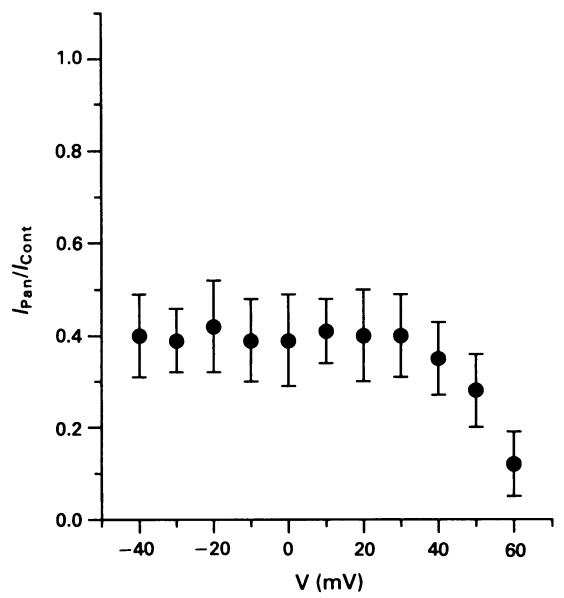

Figure 4 Voltage-dependence of Na^+ current inhibition. Peak current ratios in pancuronium and in the control have been calculated from the same data as in Figure 3. Mean values ± s.e.mean are shown.

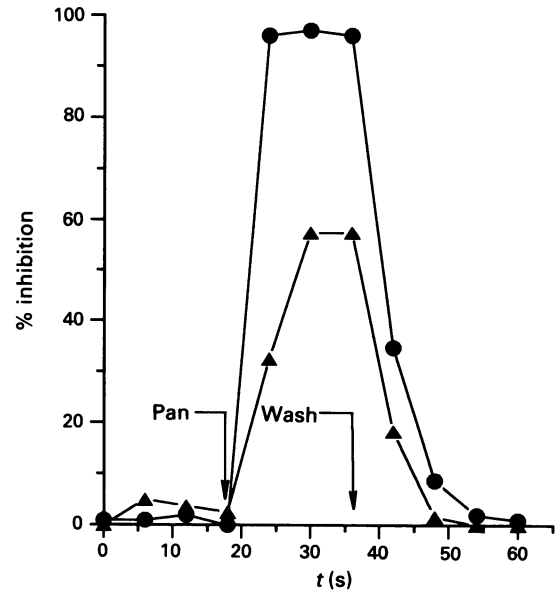

Figure 6 Time-course of Na^+ current inhibition and recovery. Currents were elicited by repetitive (period = 6 s) depolarizing pulses to −20 mV, 40 ms long. At t = 18 s, pancuronium 500 μM (●) or 150 μM (▲) was applied. After the steady-state of drug action was reached, the cell was washed with drug-free solution (t = 36 s). T = 22°C.

−60 mV. At a 50 μM concentration, the ratio I/I_{max} was close to one, while at 1 mM the current was fully blocked. Experimental data were fitted according to the equation:

$$I/I_{max} = 1 - [C^n/(C^n + K_I^n)]$$

where C is the pancuronium concentration, K_I is the concentration value producing a half-inhibition of current and n is the Hill coefficient. The best fit gave K_I = 170 μM and n = 2.1, suggesting that two pancuronium molecules must bind to a site in order to inhibit the Na^+ current.

The time-dependence of Na^+ current inhibition and recovery at different pancuronium concentrations are shown in Figure 6: depolarizing voltage pulses of 40 ms duration from V_H = −60 mV to −20 mV, were employed. Pulses were repeated every 6 s, and pancuronium was perfused during stimulation. At 500 μM pancuronium, the steady-state inhibition value was reached very rapidly (≤6 s, for 95% inhibition), at the first pulse after perfusion. The lack of block accumulation with repetitive stimuli suggests that inhibition is not use-dependent at this frequency and concentration. Similar results were obtained at higher concentrations. At concentrations lower than 500 μM, the inhibition time-course

was relatively slower (about 12 s to 60% steady-state block, at 150 μM). An almost complete recovery was then reached in a slightly longer time after wash-out (≈15 s), at each tested concentration.

The activation and inactivation kinetics of the Na^+ current were estimated, in the absence and in the presence of pancuronium, by fitting experimental data with a simplified Hodgkin-Huxley model:

$$I_{Na} = I_{max}[1 - \exp(-t/\tau_m)]^3 \times [h_\infty - (h_\infty - 1)\exp(-t/\tau_h)]$$

where τ_m and τ_h are the activation and inactivation time constants respectively, and h_∞ is the steady-state inactivation. The resulting values of τ_m and τ_h were voltage-dependent, with larger values at more negative membrane potentials. The voltage dependence of τ_m is shown in Figure 7a, in control conditions, during drug perfusion and after recovery. The activation time constant in the tested voltage range (−30 to 0 mV) was significantly larger in the presence of pancuronium than in control or recovery conditions. The difference became negligible at positive membrane potentials. The voltage dependence of τ_h is shown in Figure 7b. Unlike τ_m, τ_h showed a significant reduction during perfusion with the drug at membrane potentials more negative than −10 mV.

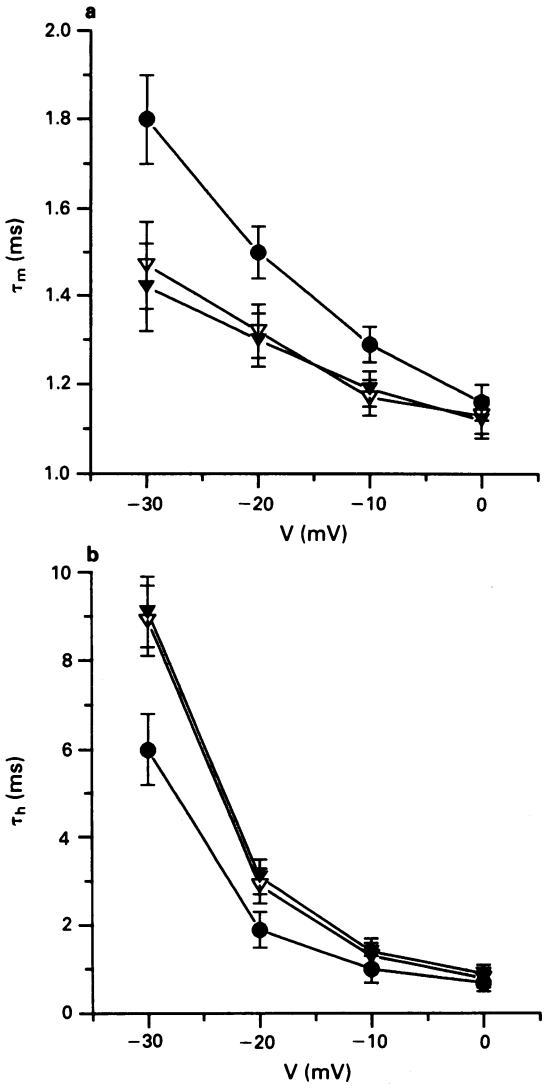

Figure 7 Voltage-dependence of activation and inactivation time-constants in control solution (▼), in pancuronium (●) and after recovery (▽). (a) τ_m is larger in pancuronium than in the control at membrane potentials <0 mV; (b) the inactivation time-constant τ_h is reduced by pancuronium at membrane potentials < −10 mV. Data were averaged over six cells; s.e.means are shown.

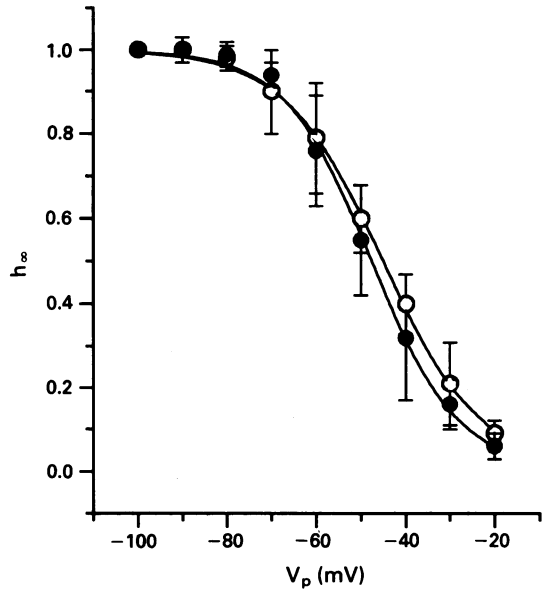

Figure 8 Effects of pancuronium on steady-state Na^+ inactivation (h_∞) at different voltages. The peak of the Na^+ current was measured during a 30 ms long depolarizing test pulse to 0 mV, following 70 ms long conditioning prepulses to various potentials (V_p). Current values, averaged over five cells and normalized to the value at −100 mV, were fitted by a Boltzmann equation (see text). Differences between control (○) and pancuronium (●) are not significant. s.e.means are shown. $V_H = -80$ mV.

The steady-state Na^+ inactivation was measured in control conditions and in presence of pancuronium. The percentage of non-inactivated sodium current (h_∞) was measured at 0 mV, after a 70 ms long conditioning prepulse ranging between −100 mV and −20 mV. The voltage-dependence of h_∞, both under control conditions and during perfusion with 250 μM pancuronium, is shown in Figure 8. Experimental data were fitted by a smooth curve derived from the Boltzmann relation:

$$h_\infty = 1/[1 + \exp((V_p - V_{\frac{1}{2}})/k)]$$

where V_p is the prepulse value, $V_{\frac{1}{2}}$ is the potential of half-inactivation and k is the slope of the curve. The best fit gave $V_{\frac{1}{2}} = -45$ mV, $k = 11$ mV in control and $V_{\frac{1}{2}} = -47$ mV, $k = 10$ mV in pancuronium, indicating that pancuronium does not affect the steady-state inactivation of Na^+ current.

Discussion

The present results show that, when externally perfused, pancuronium bromide selectively affects the amplitude of the tetrodotoxin-sensitive sodium current in chick embryo DRG sensory neurones. Na^+ current inhibition is voltage-independent and concentration-dependent.

Some interesting differences between our data and results obtained by Yeh & Narahashi (1977) on squid giant axons should be considered.

Pancuronium strongly affects sodium conductance in externally perfused DRG neurones, but interacts with squid axon Na^+ channels only by internal perfusion. A similar effect has been reported for the general anaesthetic ketamine: Shrivastav (1977) found that ketamine blocks the Na^+ current very efficiently when applied intracellularly to squid axons. However, the same drug in frog isolated nodes of Ranvier inhibits Na^+ currents when externally perfused (Benoit *et al.*, 1986). These authors suggest a direct action of ketamine from the outside of the nodal membrane.

The activation time-constant, τ_m, of the Na^+ current is affected by pancuronium in DRG neurones, and becomes larger during drug perfusion than in control conditions. This effect was not observed in squid axon membranes. On the contrary, the Na^+ current inactivation seems to be accelerated by pancuronium in both preparations, due to an apparent reduction in the inactivation time-constant, τ_h. Yeh & Narahashi (1977) inferred that, in squid axons, pancuronium binds to a site inside the pore of the open channels, blocking the Na^+ flux and reducing the number of conducting channels. Therefore, the decrease in current amplitude during membrane depolarizations, is due to both the real Na^+ channel inactivation and the channel block by pancuronium. However, in DRG neurones, pancuronium affects the sodium current activation mechanism, suggesting the possibility of interaction between pancuronium and Na^+ channels also at rest.

In order to explain these differences, two hypotheses could be put forward.

The presence of an external binding site for pancuronium in DRG Na^+ channels might account for the effects of the drug during external perfusion. Appreciable structural differences were observed by Sato & Matsumoto (1992) among Na^+ channels of squid and vertebrates. These authors found a subunit amino-acid sequence of squid Na channel considerably shorter (25%) than those of the three main rat brain α-subunits. Moreover, a similar effect was observed for the 'delayed rectifier' K^+ channel of vertebrate neurones: unlike the other classes of K^+ channels, the delayed rectifier is blocked by the quaternary ammonium tetraethylammonium (TEA) through an external binding site. This inhibition is voltage-independent but it does not modify the K^+ current kinetics (Hille, 1967).

Difficulties encountered by the drug in diffusing from the external to the cytoplasmic side of the squid axon membrane could be another possibility. Pancuronium ionization is highly insensitive to changes in pH, due to $pK_a > 13$ (Bertrand & Concina, 1980). This molecule bears positive charges also at physiological pH values, and cannot cross lipid membranes easily in spite of the lipophilic androstane moiety. Differences in cell membrane compositions (glia sheet thickness, presence of peculiar membrane surface components, surface charge density and distribution) might therefore facilitate or hinder pancuronium permeation. Hille (1992) reported the possibility of quaternary ammonium compounds reaching their binding site in a pore through a hydrophobic pathway. This allows the drug binding and unbinding even to closed Na^+ channels. According to this view, pancuronium, permeating the DRG cell membrane, may in part reach its binding site through the hydrophobic pathway before the Na^+ channel opening. The channel activation, therefore, might be apparently delayed by the rate-limiting process of drug dissociation, as observed for lipophilic steroids like deoxycholate (Wu *et al.*, 1980).

Interestingly, the observed residual outward current is also affected by pancuronium. The nature of this current is not clear: it might be carried by Cs^{2+} ions crossing potassium channels insensitive to TEA. The possibility of a pancuronium interaction with potassium channels was tested in DRG neurones perfused with TEA-free solutions. Inhibition of the K^+ current was observed in the same range of drug concentrations affecting the Na^+ current (data not shown). These results, too, differ from squid axon data (Yeh & Narahashi, 1977), according to which some inhibitory effect on the K^+ current was observed only at very high concentrations. We think that it should be interesting to investigate also the effects of pancuronium on neuronal potassium conductance.

In conclusion, pancuronium reduces sodium conductance when externally perfused in chick embryo DRG sensory neurones. Na^+ current inhibition might enhance the neuromuscular blocking action of pancuronium, but it might also be a critical side-effect in clinical applications. Both these possibilities are of great interest to Intensive Care Unit patients, to whom long-term muscle relaxation is provided by large doses of pancuronium.

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(Received August 4, 1993
Accepted September 20, 1993)

Br. J. Pharmacol. (1994), **111**, 288–294

Stimulating action of KW-5139 (Leu[13]-motilin) on gastrointestinal motility in the rabbit

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1 The gastrointestinal motor stimulating action of the motilin analogue, KW-5139 (Leu[13]-motilin), was investigated both in the anaesthetized rabbit and in rabbit isolated smooth muscle tissues.

2 KW-5139 (0.3–10 $\mu g\,kg^{-1}$, i.v.) produced motor stimulating actions in the gastric antrum, ileum and descending colon, the excitatory responses of which were initiated at the same time but declined with different time courses. The rank order of the excitatory response was: descending colon $\geqslant$ gastric antrum $>>$ ileum.

3 Atropine (1–3 $mg\,kg^{-1}$, i.v.) or naloxone (1 $mg\,kg^{-1}$, i.v.) completely suppressed the excitatory response to KW-5139 in the gastric antrum, but only partially attenuated that in the descending colon. This suggests that the mechanism of the excitatory response is different in the gastric antrum and the descending colon, and that cholinergic neural pathway is involved in the response of the gastric antrum.

4 KW-5139 (0.1 nM–1 μM) caused concentration-dependent contractions of the gastric antrum, duodenum, jejunum, ileum and the descending colon *in vitro*. In the rabbit intestine, the contractile response to KW-5139 was strongest in the duodenum and weakest in the ileum.

5 The contractile response to KW-5139 in the intestinal segments were not affected by tetrodotoxin, but were decreased by verapamil, or pretreatment with a high concentration of porcine motilin, confirming the involvement of motilin receptors in the response to KW-5139.

6 The present results suggest that the rabbit is a suitable species for the investigation of motilin on gut motility, because of the high responsiveness of the descending colon as well as the upper gastrointestinal tract.

Keywords: Motilin; rabbit gastrointestinal tract *in vivo*; motor stimulating action

Introduction

Motilin, a 22 amino acid polypeptide isolated from the mucosae of upper small intestine of the hog (Brown *et al.*, 1971; 1973), stimulates mammalian gastrointestinal (GI) motility in *in vivo* and *in vitro* experiments, although the mechanism of the stimulating action seems to differ among species. Motilin appears to stimulate motility of the stomach and small intestine of the dog by excitation of the cholinergic neurones (Inatomi *et al.*, 1989; Itoh, 1990; Itoh *et al.*, 1991; Kitazawa *et al.*, 1992), whereas this peptide produces contraction of the stomach, pylorus and the duodenum of the rabbit and man by direct action on the smooth muscle cells (Segawa *et al.*, 1976; Adachi *et al.*, 1981; Poitras *et al.*, 1987; Lüdtke *et al.*, 1989; Satoh *et al.*, 1990).

Motilin given intravenously to the dog initiates a contractile response, the period of which is similar to that of the interdigestive migrating motor complex (Itoh *et al.*, 1976; Lee *et al.*, 1983; Itoh, 1990), while the peptide does not affect isolated strips of dog GI tissues (Segawa *et al.*, 1976; Poitras *et al.*, 1987). Therefore, it was impossible to compare the response of motilin between *in vivo* and *in vitro* experiments. Isolated rabbit and human GI smooth muscle have been shown to exhibit high contractile responsiveness (Segawa *et al.*, 1976; Adachi *et al.*, 1981; Lüdtke *et al.*, 1989; Satoh *et al.*, 1990) and high affinity binding sites (Bormans *et al.*, 1986; Depoortere *et al.*, 1991) to motilin, and, thus, the duodenal strip of the rabbit was used for a biological assay of motilin-like peptides (Vogel & Brown, 1990). Recently, the amino acid sequence of rabbit motilin has been established and the physiological significance of motilin in GI motility has been proposed (Banfield *et al.*, 1992). Our previous study demonstrated that KW-5139 (Leu[13]-motilin), an analogue of porcine motilin, produces a contraction of rabbit isolated duodenum by stimulation of both the smooth muscle and cholinergic neurones (Kitazawa *et al.*, 1993).

In spite of the high responsiveness and frequent usage of rabbit tissue in motilin studies, few *in vivo* studies concerning the effect of motilin on rabbit GI motility have been published (Fox, 1990). The present study was undertaken to determine the effect of KW-5139 on the rabbit GI motility in both *in vivo* and *in vitro* experiments. Preliminary results of this study have been presented in abstract form (Ishii *et al.*, 1992).

Methods

In vivo *study*

Male rabbits (Japan White strain), weighing 2.3–3.3 kg, were anaesthetized with urethane (1.3 $g\,kg^{-1}$, i.p.). After anaesthesia had been induced, the trachea was cannulated to facilitate respiration. Polyethylene cannulae were inserted into the left carotid artery for measurement of systemic blood pressure through a pressure transducer (MPU-0.5, Nihon Kohden), and into the right ear vein for systemic application of KW-5139 and the other drugs. The abdominal cavity was opened by midline incision, and the gastric antrum, ileum and the descending colon were exposed. To measure GI motility, intraluminal pressure changes were detected by rubber balloons, inserted into the gastric antrum, ileum, or the descending colon through a small incision in the GI wall. The measurement of the motility was performed usually in

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two different GI regions (gastric antrum and ileum, or gastric antrum and descending colon) of each animal at the same time. The balloons were filled with distilled water (23–24°C, pressure applied to the balloon was usually set at 10 cmH_2O) and connected to a pressure tranducer equipped with an ink-writing polygraph (RM-6200, Nihon Kohden). The animals were allowed to equilibrate for 60 min at which time steady contractile activity and blood pressure were established. Mean blood pressure of the urethane-anaesthetized rabbit was 92 ± 5.5 mmHg ($n = 6$). Each dose of KW-5139 (0.1–10 $\mu g\ kg^{-1}$), atropine, pyrilamine or naloxone was dissolved in saline, and administered into the right ear vein. The actions evoked on the GI motility and blood pressure were recorded. When KW-5139 was applied at 60 min intervals, we could obtain the reproducible mechanical response over 8–9 h. For quantitative analysis of GI motility, the motor index was calculated by the measurement of the area surrounded by the contraction waves and baseline during the 10 min period, before and after administration of KW-5139, and used for comparison of regional responsiveness. The increase in motility induced by KW-5139 was calculated by the following equation: Increase in motility (%) = 100 × motor index after KW-5139 stimulation/motor index before KW-5139 stimulation.

In vitro *study*

White male rabbits were anaesthetized with Na pentobarbitone (30 $mg\ kg^{-1}$, i.v.) and exsanguinated. After a midline incision, the antrum of the stomach, duodenum, jejunum, ileum and the descending colon were surgically isolated. Both longitudinal and circular muscle layers (20 × 2 mm) were prepared from the gastric antrum. These smooth muscle layers and intestinal segments (20–30 mm) of duodenum, jejunum, ileum and descending colon were suspended separately, each in an organ bath (40 ml), containing Tyrode solution continuously bubbled with a gas mixture of 95% O_2–5% CO_2. Bathing Tyrode solution was kept at 28 ± 1°C in accordance with the study of Adachi *et al.* (1981) to prevent excessive spontaneous contractions. The mechanical activity of the preparation was measured with an isotonic transducer (TD-112S, Nihon Kohden) and recorded on an ink-writing recorder. The initial load was set at 1 g for gastric longitudinal and circular muscles or 4 g for intestinal segments. These preparations were rinsed with a fresh nutrient solution every 15 min, and were allowed to equilibrate for 60 min to establish a steady baseline and spontaneous contractile activity. Prior to the addition of KW-5139, each smooth muscle preparation was subjected to 3 or 4 stimulations with acetylcholine (ACh, 100 μM) for 30 s to stabilize the contractility. The concentration-dependent contractions were determined after cumulative addition of KW-5139 (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM, 1 μM) in the bath at 2 min cycles. The amplitude of the maximum contractile response during each 2 min cycle was determined and expressed as a percentage of the response to 100 μM ACh, which was sufficient to produce the maximum contraction in each preparation. EC_{50} values were calculated from the concentration-response curves and expressed as geometric means with 95% confidence limits in parentheses.

Drugs

Substances used in the present experiments were as follows, acetylcholine chloride (Ovisot, Daiichi Seiyaku), atropine sulphate (Nakarai), pyrilamine maleate (Sigma), naloxone hydrochloride (Sigma), tetrodotoxin (Sigma), verapamil hydrochloride (Sigma), porcine-motilin (Peninsula Laboratories Inc.). KW-5139 (Leu^{13}-motilin, Kyowa Hakko Kogyo Co. Ltd.) was produced by recombinant DNA techniques (Miyashita *et al.*, 1988). The drugs and motilin peptides were dissolved in saline or distilled water.

Statistics

All data, except for EC_{50} values, are expressed as means ± s.e.mean. The significance of difference was determined by

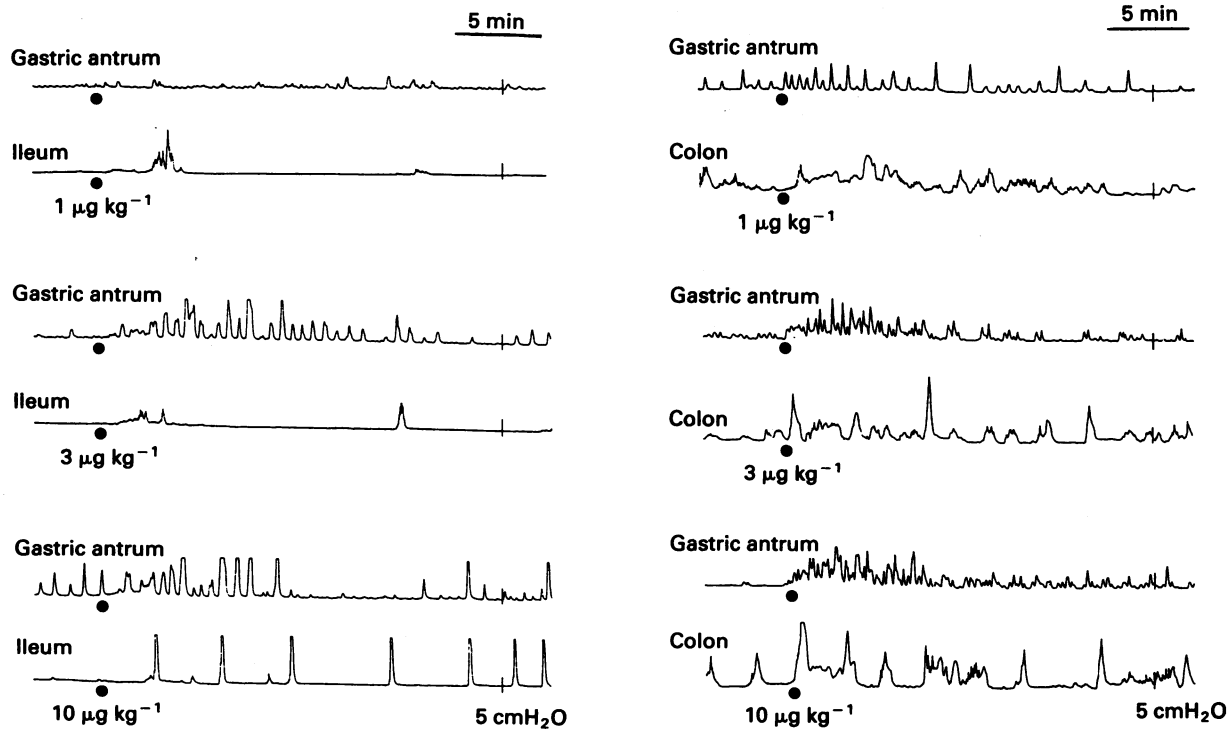

Figure 1 Typical effects of three increasing doses of KW-5139 on the motility of gastric antrum, ileum and descending colon of the anaesthetized rabbit. Each dose of KW-5139 (1–10 $\mu g\ kg^{-1}$) was administered intravenously at the point indicated by (●).

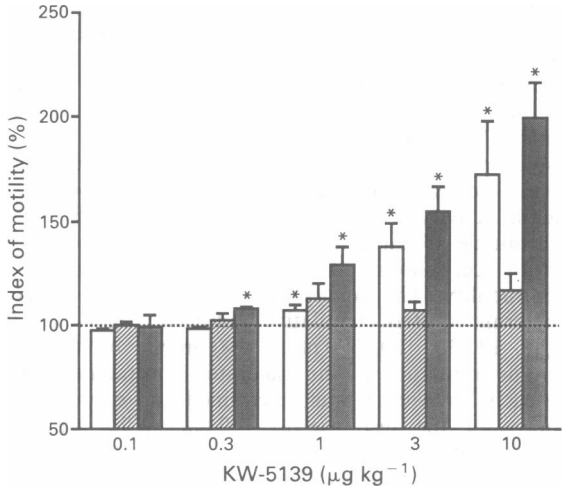

Figure 2 Dose-related gastrointestinal motor stimulating action induced by KW-5139 in the anaesthetized rabbit. Effects of KW-5139, given i.v., on the motility of the gastric antrum (open columns), ileum (hatched columns) and the descending colon (stippled columns) are illustrated. Ordinate indicates an index of motility (%) for 10 min after administration of KW-5139 (control = 100%, see Methods). Columns represent the means with s.e.mean of 4–8 animals. *Significantly different from the motor index before stimulation with KW-5139 (paired t test).

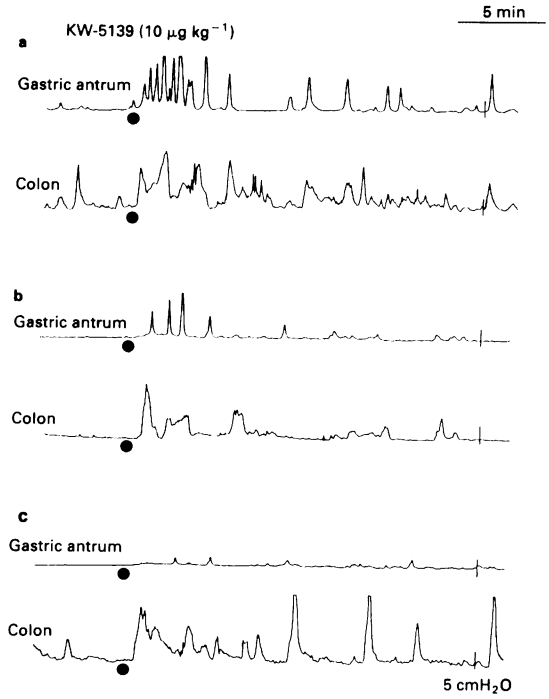

Figure 3 Effects of atropine on the excitatory response of gastric antrum and descending colon to KW-5139 in the anaesthetized rabbit. (a) Control mechanical response induced by KW-5139 (10 μg kg^{-1}, i.v.). After treatment with atropine (b, 1 mg kg^{-1}, i.v.; c, 3 mg kg^{-1}, i.v.) for 7 min, a reduction in motor stimulating action induced by KW-5139 was observed. KW-5139 was administered intravenously at the point indicated by (●).

a paired t test or Sign-Wilcoxon test, and P values less than 0.05 were considered significant.

Results

Effect on gastrointestinal motility in vivo

The lowest dose (0.1 μg kg^{-1}) of KW-5139 given intravenously did not produce any GI motility changes; however, doses of 0.3 μg kg^{-1} or higher produced a motor stimulating action in the gastric antrum, ileum and the descending colon which occurred almost at the same time (Figures 1 and 2). The systemic blood pressure did not change significantly at any dose (data not shown). Excitatory responses produced in the gastric antrum and the descending colon appeared rapidly after the application of KW-5139 (within 10–20 s), and lasted for about 15–20 min, but the response in the ileum was less notable and short-living compared with that in other two regions. The excitatory responses in the gastric antrum were, somewhat different from those in the descending colon. KW-5139 induced mainly a phasic contraction in the antrum, while, in the descending colon, the peptide induced both a tonic and phasic response, the latter being superimposed on the former (Figure 1). Figure 2 shows the dose-response relationships of the GI motor stimulating action of KW-5139 in the three GI regions. The rank order of the excitatory response was: descending colon $\geqslant$ gastric antrum $>>$ ileum. The doses of KW-5139 required to increase spontaneous motility by 50% were 3.2 ± 1.4 μg kg^{-1}, i.v. ($n = 4$) for the descending colon and 4.6 ± 1.4 μg kg^{-1}, i.v. ($n = 5$) for the gastric antrum, respectively. On the other hand, the action on the ileum induced by KW-5139 (10 μg kg^{-1}, i.v.) was only 17 ± 8% ($n = 4$) of the spontaneous motility.

To examine the mechanisms underlying the excitatory response to KW-5139, the effects of some autonomic drugs were determined. Atropine (1 or 3 mg kg^{-1}, i.v.) almost completely suppressed the gastric contraction but only partly suppressed the colonic contraction induced by KW-5139 (10 μg kg^{-1}) (Figure 3). The inhibition by atropine (3 mg kg^{-1}) of the motility in the gastric antrum (90 ± 5.7%, $n = 4$) was significantly ($P < 0.05$) greater than that in descending colon (55 ± 6.6%, $n = 4$). Naloxone (1 mg kg^{-1}, i.v.) significantly ($P < 0.05$) suppressed the gastric response to KW-5139 (10 μg kg^{-1}) (inhibition; 62 ± 6.8%, $n = 5$) without markedly decreasing the colonic response (inhibition; 15 ± 8.5%, $n = 5$) (Figure 4a). Since histamine is known to contract the rabbit GI smooth muscle, the effect of pyrilamine (1 mg kg^{-1}, i.v.) on the mechanical response to KW-5139 was tested. As shown in Figure 4b, pyrilamine did not markedly decrease the gastric and colonic excitatory responses induced by KW-5139 (10 μg kg^{-1}). Relative excitatory responses after treatment with pyrilamine were 105 ± 15% ($n = 3$) in the gastric antrum, and 84 ± 2.4% ($n = 3$) in the descending colon, respectively.

Effect on isolated smooth muscle preparations

KW-5139, applied in the organ bath, caused contraction of the gastric antrum (both longitudinal and circular muscle), duodenum, jejunum, ileum and the descending colon in a concentration-dependent manner with different sensitivity from region to region (Figure 5). The minimum effective concentrations were 1 nM for duodenum, 3 nM for jejunum and descending colon, and 10 nM for gastric circular and longitudinal muscle, and ileum, respectively. The EC_{50} values (95% confidence limits) and maximum amplitudes of the contraction (expressed as a % of the 100 μM ACh-induced response) were 100 (44–242) nM and 21 ± 6.8% ($n = 9$) (longitudinal muscle of the gastric antrum), 28(21–36) nM and 64 ± 14.8% ($n = 7$) (circular muscle of the gastric antrum), 4.2 (3.0–5.4) nM and 88 ± 7.1% ($n = 6$) (duodenum), 5.5 (4.4–6.9) nM and 77 ± 7.5% ($n = 5$) (jejunum), 12 (3.7–39) nM and 13 ± 1.8% ($n = 8$) (ileum), 20 (13–28) nM and 42 ± 6.6% (n = 7) (descending colon), respectively. These results indicate that the circular muscle is more sensitive to

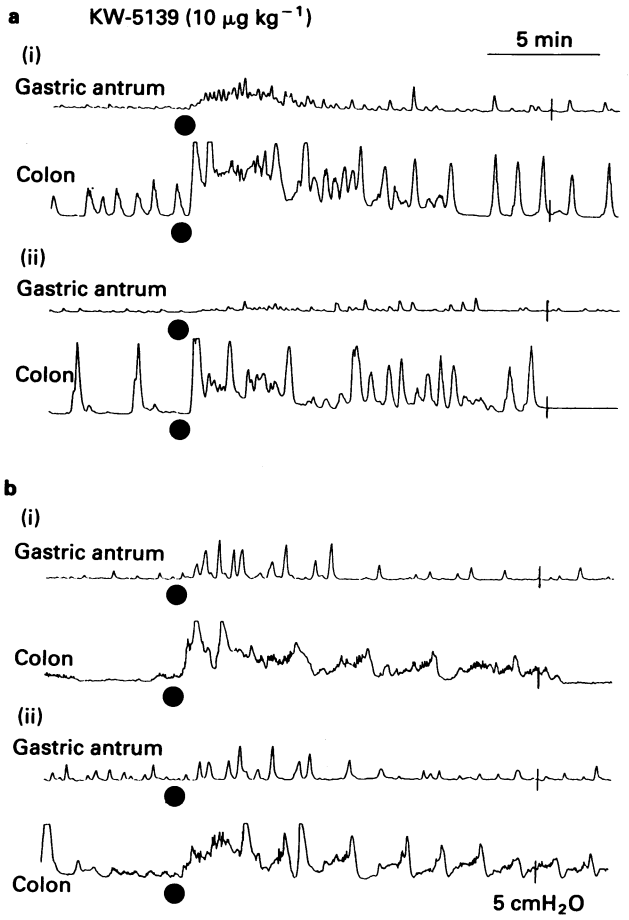

Figure 4 Effects of naloxone and pyrilamine on the excitatory response of gastric antrum and descending colon induced by KW-5139 in the anaesthetized rabbit. (i) Control mechanical responses induced by KW-5139 (10 μg kg^{-1}, i.v.). After pretreatment with naloxone (a (ii), 1 mg kg^{-1}, i.v.) or pyrilamine (b (ii), 1 mg kg^{-1}, i.v.) for 10 min, motor stimulating action induced by KW-5139 was observed. KW-5139 was applied intravenously at the point indicated by (●).

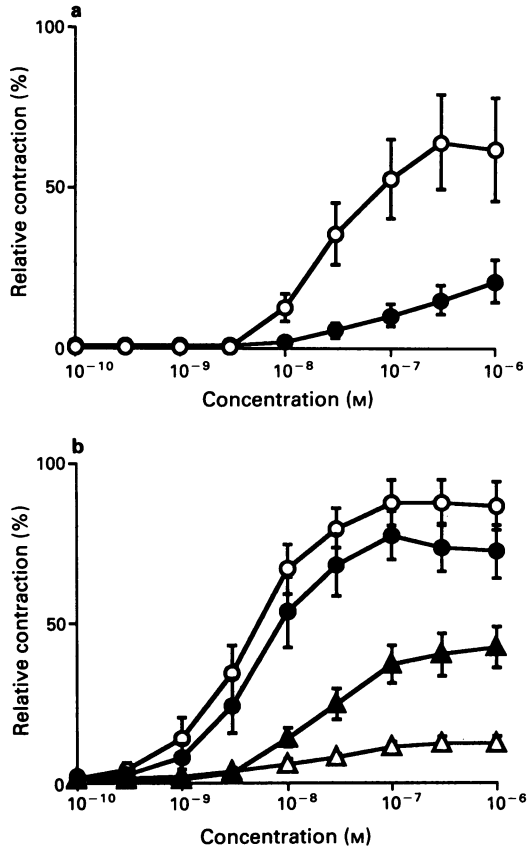

Figure 5 Concentration-contraction relationships of KW-5139 in isolated smooth muscle preparations of the rabbit gastrointestinal tract. (a) Concentration-response curves for longitudinal (●) and circular (○) muscle of the gastric antrum. (b) Concentration-response curves for duodenum (○), jejunum (●), ileum (△) and descending colon (▲). Relative contraction was expressed as a % of the amplitude of the acetylcholine (100 μM)-induced response in each region. Points represent the means ± s.e.mean of 5–6 experiments.

KW-5139 than the longitudinal muscle in the gastric antrum, that the duodenum is the most sensitive and that the ileum is the least sensitive of the intestinal smooth muscle preparations tested.

Figure 6 represents a comparison of the reproducibility of the contractile response to KW-5139 in rabbit GI smooth muscles. After cumulative application of KW-5139 at 2 min cycles to obtain the first concentration-response curve, the preparations were rinsed 4–5 times with fresh Tyrode solution, equilibrated for 1 h, and then subjected to another cumulative application of KW-5139 for the second concentration-response curve. We could obtain reproducible concentration-related contractions in the duodenum, ileum and the descending colon at 1 h interval applications; however, in the gastric circular muscle, the amplitude of the contractile response was significantly suppressed at the second cumulative application without causing a notable change of EC_{50}. The EC_{50} value (95% confidence limits) and the maximum contractions were 26 (19–34) nM and 79 ± 19.2% ($n = 3$) in the first application, and 44 (12–77) nM and 15 ± 12.6% ($n = 3$) in the second, respectively. In the gastric longitudinal muscle, the maximum contraction was also suppressed at the second application (data not shown).

Pharmacological properties of the response to KW-5139 were examined in the duodenum (upper intestine) and the descending colon (lower intestine) segments, which showed relatively high responsiveness to KW-5139 and good reproducibility of the response. Tetrodotoxin (1 μM) did not markedly decrease the initial phasic contractile response to KW-5139 (10–100 nM), in agreement with previous studies (Depoortere *et al.*, 1991; Kitazawa *et al.*, 1993). In contrast, pretreatment with verapamil (1 μM) for 30 min diminished the spontaneous contractility and significantly ($P < 0.05$) decreased the KW-5139-induced contraction. Relative amplitudes after verapamil were 15.4 ± 5.9% ($n = 5$) for the duodenum (10 nM KW-5139) and 36.0 ± 6.7% ($n = 6$) for the descending colon (100 nM KW-5139), respectively. These results indicate that KW-5139 acts directly on the intestinal smooth muscle to produce the phasic response, and that external Ca^{2+} plays an important role in the contractile response.

Effects of long-lasting exposure to motilin peptide (motilin peptide-induced desensitization) on the response to KW-5139 were examined in the duodenum and the descending colon to clarify whether or not KW-5139 acts on the motilin receptor. After observing the control contractile responses to KW-5139, porcine motilin and ACh, the preparation was markedly contracted by exposure to a high concentration (10 μM) of KW-5139 or porcine motilin for 10 min. The evoked contraction, sustained during the presence of KW-5139 in the bath, returned to the basal level in 20–40 min after repeated rinses with fresh Tyrode solution. Thereafter, the contractions induced by the test stimulants were

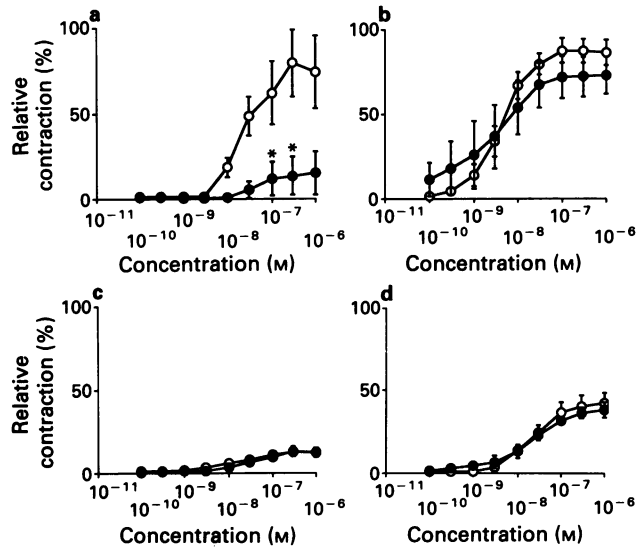

Figure 6 Reproducibility of the contractile response to KW-5139 in four different regions of the gastrointestinal tract of the rabbit. KW-5139 was cumulatively applied during 2 min cycles and the first concentration-response curve was obtained (○) in circular muscle of gastric antrum (a), and segments of duodenum (b), ileum (c) and descending colon (d). After 3–4 rinses with fresh Tyrode solution and equilibrium for 1 h, KW-5139 was cumulatively applied again and the second concentration-response relationship was established (●). Relative contraction was expressed as a % of the amplitude of acetylcholine (100 μM)-induced contraction. *Significantly different from the correspondent control value (○) (paired *t* test). Points represent the means ± s.e.mean of 3–7 experiments.

examined. As shown in Figure 7a and Table 1, pretreatment with KW-5139 (10 μM for 10 min) in the duodenum significantly decreased the contractile responses to KW-5139 (10 nM) and porcine motilin (10 nM) without inhibiting the contraction induced by ACh (100 μM). In addition, pretreatment with porcine motilin (10 μM for 10 min) also significantly decreased the contraction induced by KW-5139 and porcine motilin but did not change the response to 100 μM ACh (Figure 7b, Table 1). Similarly, effects of pretreatment with motilin peptides on the response to KW-5139, porcine motilin and ACh were examined in the descending colon. Continuous exposure to motilin peptides caused marked transient contractions, which were slightly different from that in the duodenum, and the elevated tonus easily returned to the baseline level by repeated rinses. Pretreatment with KW-5139 or porcine motilin (10 μM for 10 min) significantly decreased the responsiveness to KW-5139 and porcine motilin. However, in both cases, ACh (100 μM)-induced contractions were not affected (Figure 7c, d, Table 1).

Discussion

From the present results of the *in vitro* mechanical study, it is assumed that KW-5139 acted on the motilin receptor and caused the contraction of the rabbit GI tissue. Evidence for this assumption is as follows: first, the difference in sensitivity to KW-5139 among the regions of the small intestine was similar to that to porcine motilin (Satoh *et al.*, 1990) and second, the pharmacological properties of the contraction induced by KW-5139 (tetrodotoxin-resistance and verapamil-sensitiveness) were consistent with those of porcine motilin

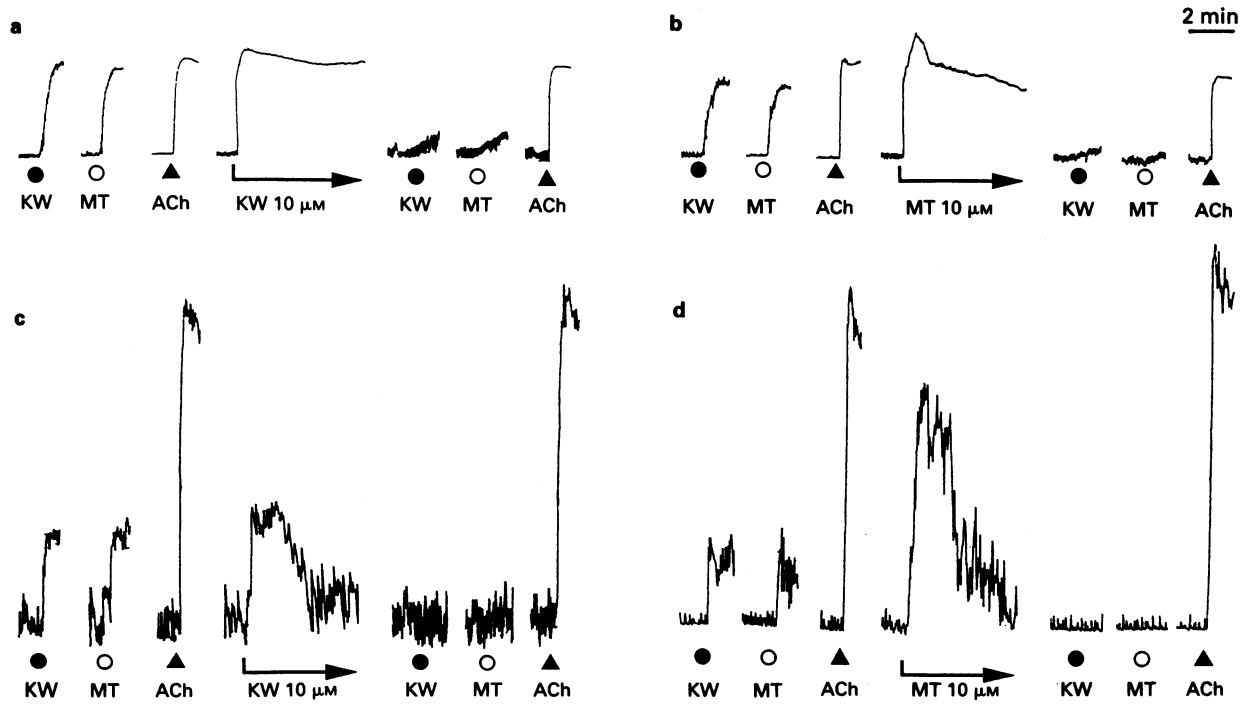

Figure 7 Typical effects of pretreatment with a high concentration of KW-5139 or porcine motilin on the contractile response to KW-5139, porcine motilin, and acetylcholine in the rabbit duodenum and the descending colon. (a, b) The contractile responses to KW-5139 (10 nM, KW, ●) and porcine motilin (10 nM, MT, ○) in the duodenum were significantly decreased after the pretreatment with KW-5139 (10 μM for 10 min, a) or porcine motilin (10 μM for 10 min, b). (c, d) The contractile responses to KW-5139 (100 nM, KW, ●) and porcine motilin (100 nM, MT, ○) in the descending colon were also selectively decreased after pretreatment with KW-5139 (10 μM for 10 min, c) or porcine motilin (10 μM for 10 min, d). On the other hand, the contractile response to acetylcholine (ACh, 100 μM, ▲) was not decreased in any conditions.

Table 1 Contractile responses to KW-5139, porcine motilin (MT) and acetylcholine (ACh) before and after treatment with a high concentration of KW-5139 or porcine motilin in the rabbit isolated duodenum and descending colon

		Amplitude of contractile response					
		Before treatment			*After treatment*		
Duodenum		ACh (100 μM)	KW-5139 (10 nM)	MT (10 nM)	ACh (100 μM)	KW-5139 (10 nM)	MT (10 nM)
KW-5139	10 μM	100	92.0 ± 10.2	90.7 ± 10.5	110.4 ± 9.3	16.4 ± 4.2*	19.2 ± 4.7*
MT	10 μM	100	86.5 ± 17.7	74.0 ± 10.6	95.7 ± 6.7	7.2 ± 1.9*	10.8 ± 3.2*
Colon		ACh (100 μM)	KW-5139 (100 nM)	MT (100 nM)	ACh (100 μM)	KW-5139 (100 nM)	MT (100 nM)
KW-5139	10 μM	100	22.5 ± 5.4	15.0 ± 3.4	116.8 ± 7.0	1.1 ± 1.1*	0.8 ± 0.6*
MT	10 μM	100	33.2 ± 14.6	40.2 ± 12.9	114.4 ± 8.5	0.0 ± 0.0*	1.0 ± 1.0*

Each preparation was treated with 10 μM KW-5139 or porcine motilin for 10 min as shown in Figure 7. The amplitude of the contractile response is expressed as a percentage of the contractile response to 100 μM ACh before the treatment. Values are means ± s.e.means of 5–6 experiments. *$P<0.05$, significantly decreased from the response to the agent before treatment (Sign-Wilcoxon test).

(Adachi *et al.*, 1981; Satoh *et al.*, 1990). Third, cross-desensitization between KW-5139- and porcine motilin-induced contraction occurred, as was the case with the ACh release induced by both peptides (Kitazawa *et al.*, 1993).

The dog has been used as a good experimental species for the study of the action of motilin *in vivo* (Itoh, 1990). It is well known that motilin administered intravenously in dogs causes a series of strong contractions or electrical activity changes corresponding to phase III of the interdigestive migrating motor complex in the gastric antrum and the duodenum without causing simultaneous motility changes in the small intestine, and that the contractions produced migrate through the small intestine towards the terminal ileum (Itoh *et al.*, 1976; Wingate *et al.*, 1976; Matsumoto *et al.*, 1986; Inatomi *et al.*, 1989; Itoh, 1990). The observations in man with motilin are in agreement with the results in dogs (Janssens *et al.*, 1983).

In the present study, KW-5139 produced simultaneous excitatory responses of the gastric antrum, ileum and the descending colon of the anaesthetized rabbit. The dose of KW-5139 required to produce the response in this species (0.3–10 μg kg^{-1}, i.v.) was slightly higher than that in the dog (Kitazawa *et al.*, 1992). There was considerable difference in the sensitivity of the intestinal regions to KW-5139 between the rabbit and the dog. The order of the excitatory response in the *in vivo* study (descending colon ≥ gastric antrum >> ileum) was similar to that of the amplitude of the maximum contraction in the *in vitro* study (gastric antrum (circular muscle) > descending colon >> ileum). The higher responsiveness of the gastric antrum than the ileum might be due to the regional difference in the density of motilin receptors, which is high in the stomach and decreases aborally toward the terminal ileum in the rabbit (Bormans *et al.*, 1986).

There have been controversial results concerning the effect of motilin on colonic motility in the dog. Motilin did not evoke an excitatory response of the colon (Itoh *et al.*, 1976; Matsumoto *et al.*, 1986; Itoh, 1990), while motilin produced the colonic motor complex (Bickel & Belz, 1988). In contrast, the present results as well as the previous mechanical studies on the rabbit colon *in vitro* (Satoh *et al.*, 1990; Depoortere *et al.*, 1991; Hasler *et al.*, 1992), have demonstrated that motilin obviously stimulates motor activity of the rabbit colon. Moreover, radioligand binding studies in the rabbit have demonstrated that the density of motilin receptors in the colon was comparable to that in the upper GI tract (Depoortere *et al.*, 1991; Hasler *et al.*, 1992). From these results, motilin is considered to act not only on the upper GI tract but also on the colon of the rabbit, and thus, this species seems to be useful to examine the effect of motilin on colonic motility. The physiological significance of motilin in the rabbit GI function has not yet been clarified. Recently, however, the presence of rabbit motilin, different from porcine motilin in amino-acid positions 8-16-20-22, was demonstrated in the duodenal mucosa (Banfield *et al.*, 1992). Therefore, it is likely that motilin regulates the motility of both upper and lower GI tract of the rabbit.

Fox *et al.* (1983) and Inatomi *et al.* (1989) found that the contractile responses of the canine stomach to motilin were almost completely blocked by treatment with atropine or tetrodotoxin, and suggested that the action of motilin depends on the activation of cholinergic neurones. In contrast, naloxone was ineffective on the motilin-induced contractile response of the canine stomach (Inatomi *et al.*, 1989). We found that the contractile response of gastric antrum in rabbits induced by KW-5139 was markedly decreased by atropine and naloxone. These findings suggest that a neural pathway involving acetylcholine and opiate peptide is required for the action of KW-5139 in the rabbit gastric antrum. However, it was impossible to evaluate further the pharmacological properties of the KW-5139-induced contraction of the gastric antrum *in vitro*, because the gastric contraction was not reproducible even with application at intervals of 60 min, which is quite different from those of the other regions. This phenomenon might reflect the difference of contractile mechanisms (e.g., receptor or receptor-coupled signal transduction) between gastric and intestinal tissues; however, clarification of the difference was beyond the scope of the present experiments.

In contrast to the case with the gastric antrum, the colonic motor stimulating action of KW-5139 *in vivo* was partly (about 50%) decreased by atropine and not affected by naloxone. In the descending colon, a non-cholinergic mechanism in addition to the cholinergic pathway thus seems to play a role in the excitatory action of KW-5139. One possible mechanism of the non-cholinergic response is the direct action on the smooth muscle cells, as was demonstrated in isolated smooth muscle segments (this study, Adachi *et al.*, 1981; Satoh *et al.*, 1990; Depoortere *et al.*, 1991; Kitazawa *et al.*, 1993) and isolated smooth muscle cells (Hasler *et al.*, 1992). On the other hand, electrical field stimulation of the rabbit colon elicits an atropine-resistant contraction (Koelbel *et al.*, 1989). Moreover, Fox *et al.* (1984, 1991) showed that motilin stimulated the motility of the canine small intestine through non-cholinergic neural mechanisms including the decrease of vasoactive intestinal polypeptide release. Therefore, the possibility that motilin stimulates the non-cholinergic neural pathway in the rabbit colon cannot be excluded completely.

In summary, our present study *in vivo* demonstrates the stimulation of GI motility by motilin in the rabbit. Because of the high sensitivity to motilin, especially of the colon, the rabbit is considered to be a useful species to evaluate the response of the GI tract to motilin.

The authors wish to thank M. Kawaguchi and M. Watanabe for skilful technical assistance and Dr A. Karasawa for careful comments on the manuscript.

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(Received June 1, 1993
Revised September 2, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 295–301

Thermodynamic analysis of agonist and antagonist binding to the chicken brain melatonin receptor

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1 The binding of 2-[^{125}I]-iodomelatonin to chicken brain membranes, and the inhibition of binding by melatonin, N-acetyltryptamine and luzindole, were examined at temperatures between 4°C and 37°C.

2 At all temperatures studied, the binding affinity (K_d or K_i) for 2-[^{125}I]-iodomelatonin, melatonin (both agonists) and, to a lesser extent, N-acetyltryptamine (a partial agonist) was reduced by inclusion of guanosine triphosphate (GTP, 1 mM) in the assay. GTP did not affect the K_i for luzindole, a melatonin receptor antagonist.

3 The maximal density of binding sites (B_{max}) was not affected by temperature but the K_d showed a peak at 21°C with lower values at both higher and lower temperatures giving curvilinear van't Hoff plots ($\ln K_A$ vs 1/temperature).

4 Derived changes in entropy ($\Delta S°$) and enthalpy ($\Delta H°$) of binding for all of the melatonin ligands decreased as temperature increased.

5 The affinity, and thus the free energy of binding, $\Delta G°$, of these ligands at the melatonin receptor have identical values at several temperatures yet at these temperatures $\Delta S°$ and $\Delta H°$ were very different, implying that more than one intermolecular force must be involved in the binding of ligand and receptor.

6 Conceivably, the large positive $\Delta S°$ observed at low temperatures, perhaps as a result of hydrophobic interactions, is compensated by a corresponding, but opposite, change in enthalpy at higher temperatures. However, it is not clear what type of binding force(s) would show such a temperature-dependence.

7 These studies suggest that caution must be exercised in the molecular interpretation of derived measures of $\Delta S°$ and $\Delta H°$ obtained from direct measurements of $\Delta G°$.

Keywords: Thermodynamics; melatonin; 2-[^{125}I]-iodomelatonin binding; G-protein coupling

Introduction

In recent years, a number of studies have examined the thermodynamic changes which take place on binding of various neurotransmitters to their specific receptors. In a landmark study on β-adrenoceptors, Weiland *et al.* (1979) observed that agonist binding was enthalpy-driven whereas antagonist binding was entropy-driven. They proposed that enthalpy-driven agonist binding represents a conformational change in the receptor which leads to a biological response, while the entropy increase occurring upon antagonist binding reflects a simple binding event with no transfer of information. Similar thermodynamic changes occur on binding of benzodiazepine receptor agonists and antagonists (Mohler & Richards, 1981). This interpretation has been criticised as it has been pointed out that an agonist-induced conformation change in the receptor is not the only (or likely) cause of the observed negative changes in entropy induced by agonists (Miklavic *et al.*, 1990). Furthermore, it has not been possible to generalise these findings to other receptors. For example, binding of agonists at the dopamine D_2 receptor may be accompanied by positive or negative changes in entropy, and the binding of D_2 antagonists may be entropy or enthalpy-driven (Kilpatrick *et al.*, 1986). Similarly the binding of different muscarinic antagonists can be either entropy or enthalpy-driven (Gies *et al.*, 1986). Nevertheless, thermodynamic analysis may provide information on the inter-molecular forces which are likely to be involved in the binding of ligands to receptors (Testa *et al.*, 1987; Waelbroeck *et al.*, 1993) as the expected signs of changes in entropy and enthalpy accompanying the various individual kinds of interactions that may take place (hydrophobic association, van der Waals, H-bond formation etc.) are known (Ross & Subramanian, 1981).

High affinity, G-protein-coupled binding sites for the pineal hormone, melatonin (5-methoxy N-acetyltryptamine) have been identified in the brain of several species by use of both membrane binding assays and *in vitro* autoradiographic techniques (Morgan & Williams, 1989; Krause & Dubocovich, 1991). Activation of these binding sites has been shown to mediate several biological responses; inhibition of [^{3}H]-dopamine release *in vitro* from neuronal retina (Dubocovich, 1983), aggregation of pigment granules in the melanophores of *Xenopus laevis* (Sugden, 1991), inhibition of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) synthesis in pars tuberalis (Morgan *et al.*, 1989; Carlson *et al.*, 1989) and inhibition of gonadotrophin-releasing hormone stimulation of luteinizing hormone release and second messenger responses in neonatal rat pituitary cells (Vanecek & Vollrath, 1989; Vanecek & Klein, 1992). Melatonin, secreted into the circulation from the pineal gland, is known to regulate seasonal changes in various aspects of physiology in photoperiodic species (Bartness & Goldman, 1989) and has been implicated in the mechanisms which regulate circadian rhythms (Armstrong, 1989). Melatonin synthesized locally within the eye regulates various aspects of retinal physiology (Besharse *et al.*, 1988).

In the present study, the agonist 2-[^{125}I]-iodomelatonin has been used to investigate the temperature-dependency of binding to chicken brain melatonin sites. The thermodynamic changes induced on binding of 2-[^{125}I]-iodomelatonin and melatonin, which are agonists, N-acetyltryptamine, a putative partial agonist (Dubocovich, 1985), and, luzindole (2-benzyl *N*-acetyltryptamine), an antagonist (Dubocovich, 1988) have been compared.

Methods

Preparation of chicken brain membranes

Chickens (*Gallus domesticus*, White Leghorn) were obtained from Orchard Farms (Buckinghamshire) at 1 day of age and were housed under a diurnal lighting cycle (12:12 L:D, lights on at 06 h 00 min) until killed between 14 h 00 min and 15 h 00 min at 3 months of age. The whole brain was removed, frozen in liquid nitrogen and stored at − 70°C. Each brain was homogenized in 20 vols of Tris-HCl (50 mM, pH 7.4) containing phenylmethylsulphonylfluoride (PMSF, 1 mM), leupeptin (50 $\mu g\ ml^{-1}$) and EGTA (1 mM). The homogenate was centrifuged (35,000 *g*, 30 min, 4°C). The pellet was re-homogenized in the same buffer and centrifuged for a second time. The final membrane pellet was resuspended in Tris-HCl and aliquots frozen at − 70°C until used.

2-[^{125}I]-iodomelatonin binding to chicken brain membrane preparations

For saturation analysis of 2-[^{125}I]-iodomelatonin binding to chicken membranes aliquots (70 μg) of membranes were incubated with 8 concentrations (6 to 1100 pM) of 2-[^{125}I]-iodomelatonin. Nonspecific binding was defined in the presence of non-radioactive melatonin (1 μM). Competition assays to determine IC_{50} values of melatonin, N-acetyltryptamine and luzindole were done on duplicate samples using a concentration of 2-[^{125}I]-iodomelatonin between 70 and 105 pM and at least six concentrations of each competing drug. For the time-course experiments, duplicate aliquots of membranes were incubated with 2-[^{125}I]-iodomelatonin (50 to 80 pM) for varying times (0–24 h) at 4, 13, 21, 29 and 37°C. From these experiments, incubation times of 24 h, 4 h, 90 min, 60 min and 30 min were chosen at 4, 13, 21, 29 and 37°C in order to ensure that equilibrium was reached. Reactions were terminated by the addition of 2 ml of ice-cold Tris-HCl buffer to each tube and immediate filtration through glass fibre filters (GF/C; Whatman Ltd., Maidstone, Kent). Each tube was then rinsed with a further 2 ml of buffer and each filter was washed (2 × 5 ml) and counted in a LKB 1282 compugamma CS counter.

Data analysis

Data from saturation experiments were analysed by non-linear regression analysis using the ENZFITTER programme (Leatherbarrow, 1987) and the equation

$$B = B_{max} * F/(K_d + F)$$

where B = the concentration of ligand bound to the receptor, F = the concentration of free ligand, K_d = the equilibrium dissociation constant and B_{max} = the maximal concentration of binding sites.

IC_{50} values were determined in competition assays using the ALLFIT programme (De Lean *et al.*, 1978) with the four parameter logistic equation

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

where X and Y are the concentration of the competing compound and percentage inhibition of 2-[^{125}I]-iodomelatonin binding respectively, and A is the maximal binding (in the absence of competitor), B is the slope factor, C is the IC_{50} and D is the minimal binding (nonspecific binding). Inhibition constants (K_i) were then calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Thermodynamic analysis

The Gibb's free energy change of binding ($\Delta G°$, kcal mol^{-1}) was calculated from the equation:

$$\Delta G° = -RT\ln K_A \qquad (1)$$

where R is the gas constant (1.99 cal/mol deg^{-1}), T is the temperature in degrees Kelvin, and K_A is the reciprocal of the equilibrium dissociation constant (K_d) or inhibition constant (K_i). As the van't Hoff plots for each of the compounds examined were curvilinear we used the procedure of Edelhoch & Osborne (1976) and fitted a second degree polynomial to the graph of $\Delta G°$ against temperature (°K). From the equation

$$\Delta G° = -RT\ln K_A = A + BT + CT^2 \qquad (2)$$

three constants were obtained. Values of the standard free enthalpy change $\Delta H°$ (Kcal mol^{-1}) and standard entropy change $\Delta S°$ (cal/mol $°K^{-1}$) were then obtained by use of the following equations:

$$\Delta H° = \frac{d(\Delta G°/T)}{d(1/T)} = A - CT^2 \qquad (3)$$

$$\Delta S° = \frac{d\Delta G°}{dT} = -B - 2CT \qquad (4)$$

Protein determination

Protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Materials

2-[^{125}I]-iodomelatonin (2200 Ci $mmol^{-1}$) was purchased from DuPont, Stevenage, Herts. Unlabelled 2-iodomelatonin was from Research Biochemicals Inc., Natick, MA, U.S.A. Melatonin and GTP were from Sigma Chemical Co., Poole, Dorset. N-acetyltryptamine was synthesized as described previously (Sugden, 1991) and luzindole (2-benzyl N-acetyltryptamine) was obtained from Dr S.M. Reppert, Boston, U.S.A. All drugs were dissolved in methanol at a stock concentration of 10 mM and stored at − 30°C.

Results

Control experiments for temperature dependence of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membrane preparations

Thermodynamic analysis of binding data requires that the system studied is truly at equilibrium. The time-course of association of 2-[^{125}I]-iodomelatonin (50 to 80 pM) to chicken

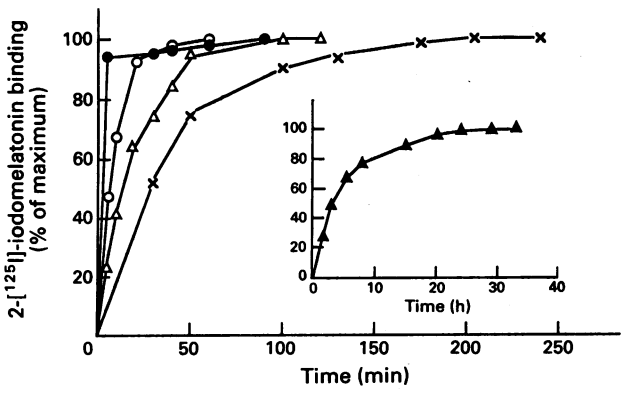

Figure 1 Kinetics of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membranes at different temperatures. The concentration of 2-[^{125}I]-iodomelatonin used was 50 to 80 pM and melatonin (1 μM) was used to define specific binding. The temperatures used were 4°C (▲, inset), 13°C (X), 21°C (Δ), 29°C (○) and 37°C (●). Each point represents the mean of duplicate determinations which differed by $< 10\%$.

Table 1 Changes in K_d and B_{max} of 2-[^{125}I]-iodomelatonin binding to chicken brain membranes with temperature

	Temperature (°C)				
	4	13	21	29	37
K_d (pM)	64.6 ± 2.9	41.5 ± 1.3	20.2 ± 1.1	50.5 ± 4.6	103.2 ± 5.9
B_{max} (fmol mg^{-1} protein)	27.4 ± 0.7	25.8 ± 0.4	27.6 ± 0.4	25.1 ± 0.4	26.2 ± 0.7

K_d and B_{max} were calculated using non-linear regression analysis from data obtained in saturation experiments. Data given are the mean ± s.e.mean of 4 to 6 determinations. One way analysis of variance indicated that K_d values, but not B_{max}, varied significantly with temperature ($P<0.05$).

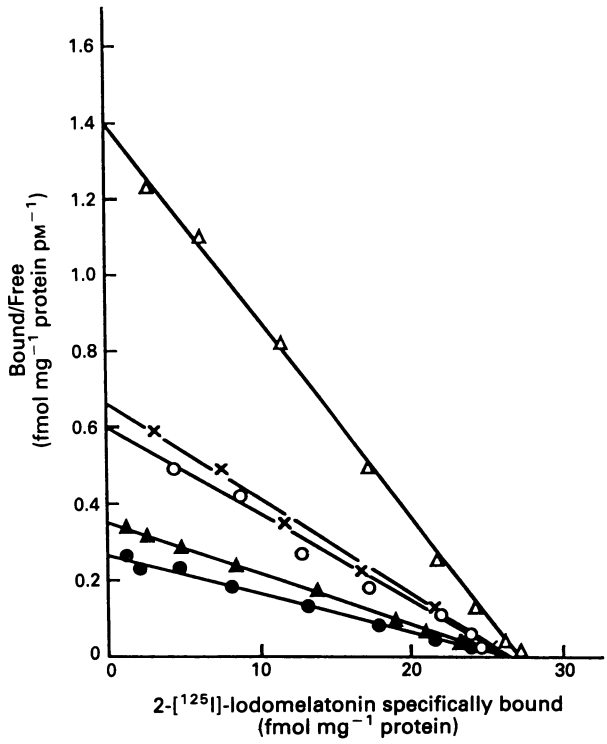

Figure 2 Scatchard analysis of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membranes at different temperatures. Each line shows a representative Scatchard plot at 4°C (▲), 13°C (X), 21°C (△), 29°C (○) and 37°C (●). Specific binding was defined using melatonin (1 μM).

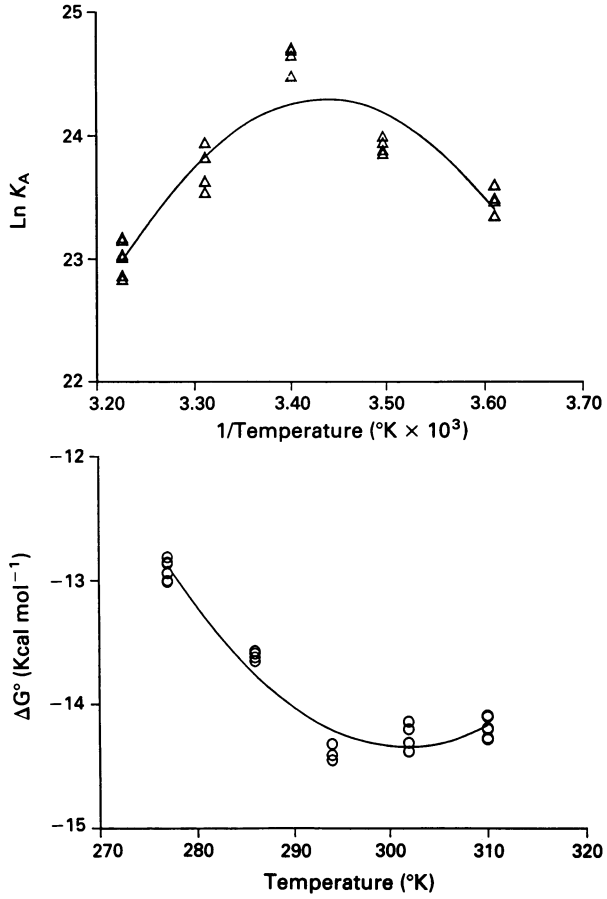

Figure 3 (a) van't Hoff plot of the dependence of the K_A for 2-[^{125}I]-iodomelatonin binding on temperature. Each triangle represents a K_A value ($K_A = K_d^{-1}$) from an individual saturation experiment. Data were fitted using a second degree polynomial equation. (b) Dependence of the standard Gibbs free energy change (ΔG°) on temperature for binding of 2-[^{125}I]-iodomelatonin to chicken brain membranes. Each circle represents ΔG° calculated from an individual saturation curve. Data were fitted with a second degree polynomial equation and the constants A, B and C were obtained and used in the equations given in Methods to calculate changes in enthalpy (ΔH°) and entropy (ΔS°).

brain membranes was measured at 4, 13, 21, 29 and 37°C to ensure equilibrium (Figure 1). The rate of association increased as the temperature of incubation increased. The following incubation times which allow apparent equilibrium at each temperature were chosen for saturation and competition studies: 30 min at 37°C, 60 min at 29°C, 90 min at 21°C, 4 h at 13°C, and 24 h at 4°C.

Changes in affinity with temperature were reversible and not an artifact of the longer times required to reach equilibrium at the lower temperature. This was clear since the affinity of the receptor for 2-[^{125}I]-iodomelatonin measured at 37°C, after preincubation of membranes at 4°C for 24 h, was the same as its affinity after the 37°C incubation alone; similarly the affinity for 2-[^{125}I]-iodomelatonin at 4°C was not changed by prior incubation at 37°C for 30 min (data not shown).

Changes in affinity are not a reflection of changes in the pH of the Tris-HCl incubation buffer as separate buffers were used which were adjusted to pH 7.4 at each incubation temperature. Furthermore, K_d and B_{max} values were not significantly different when assays were done at pH 6.5 or 8.5 (data not shown).

The effect of temperature on specific 2-[^{125}I]-iodomelatonin binding to chicken brain membrane preparations

Non-linear regression analysis of saturation studies of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membranes showed no significant change in apparent B_{max} values with temperature (Table 1; Figure 2). K_d values did vary significantly with temperature (Table 1). The apparent affinity of 2-[^{125}I]-iodomelatonin (K_d) was greatest at 21°C; at 4°C, 13°C, 29°C and 37°C the apparent affinity was reduced. At each of the temperatures tested Hill coefficients (n_H) were

Table 2 Thermodynamic parameters for 2-[^{125}I]-iodomelatonin binding in chicken brain membranes

	Temperature (°C)				
	4	13	21	29	37
$\Delta G°$ (Kcal mol^{-1})	− 12.94 ± 0.03	− 13.60 ± 0.02	− 14.41 ± 0.03	− 14.26 ± 0.05	− 14.19 ± 0.03
$\Delta H°$ (Kcal mol^{-1})	+ 23.5	+ 9.2	− 4.0	− 17.6	− 31.5
$\Delta S°$ (cal/mol deg^{-1})	+ 132.7	+ 81.6	+ 36.2	− 9.3	− 54.7

$\Delta G°$, standard Gibbs free energy of binding; $\Delta H°$ standard free enthalpy change; $\Delta S°$ standard free entropy change. $\Delta G°$ was calculated using the equation $\Delta G° = -RT\ln K_A$ where R is the gas constant (1.99 cal/mol deg^{-1}) and T is the temperature in °K, and K_A = 1/mean ± s.e.mean K_d obtained in saturation studies. Data given are the mean ± s.e.mean of 4 to 6 determinations. One way ANOVA indicated that $\Delta G°$ varied significantly with temperature.

Table 3 Effect of temperature and GTP (1 mM) on inhibition constant (K_i) values of melatonin analogues binding to chicken brain membranes

Compound	*Conditions*	K_i (nM) 4°C	21°C	37°C
Melatonin	Control	0.33 ± 0.03	0.07 ± 0.004	0.76 ± 0.04
	+ GTP	1.85 ± 0.23	0.71 ± 0.10	2.80 ± 0.39
N-acetyltryptamine	Control	2200 ± 101	280 ± 14	2160 ± 110
	+ GTP	3070 ± 237	1030 ± 78	2460 ± 180
Luzindole	Control	2220 ± 270	573 ± 72	3130 ± 369
	+ GTP	1550 ± 122	581 ± 44	1850 ± 140

K_i values were determined from IC_{50} values obtained in a single competition experiment using the Cheng-Prusoff equation (1973). Errors given are the standard errors of the computer derived estimates.

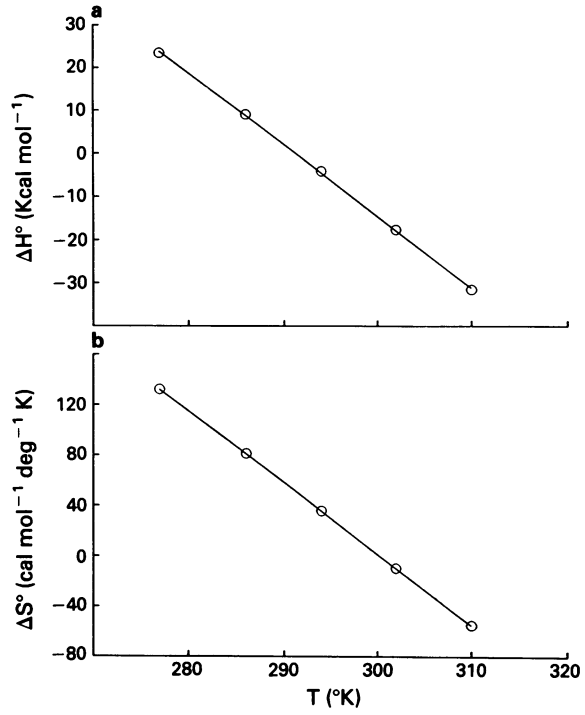

Figure 4 Changes in the thermodynamic parameters of 2-[^{125}I]-iodomelatonin binding with temperature in chicken brain membranes. The standard free enthalpy change ($\Delta H°$, a) and the standard free entropy change ($\Delta S°$, b) were calculated from the curve of $\Delta G°$ against temperature (Figure 3) as described in Methods.

close to unity (0.99–1.06) and Scatchard plots were linear, suggesting a single class of binding sites.

Thermodynamic parameters of 2-[^{125}I]-iodomelatonin binding

The van't Hoff plot for 2-[^{125}I]-iodomelatonin binding was clearly not linear (Figure 3a). The curve of $\Delta G°$ against temperature (Figure 3b) fitted well ($r = 0.989$) to a second order polynomial equation allowing the thermodynamic parameters $\Delta H°$ and $\Delta S°$ to be calculated by the method of Edelhoch & Osborne (1976). Both $\Delta H°$ and $\Delta S°$, calculated from the curve of $\Delta G°$ against temperature as described in Methods, varied over the temperature range studied (Figure 4; Table 2). Large positive changes in entropy and enthalpy were found at the lower temperatures (<21°C). An alternative analysis of $\Delta G°$ against temperature as two straight lines, the first over the temperature range 4 to 21°C and the second from 21 to 37°C, is unsatisfactory because only 5 temperatures were studied, and the inflection occurs in the centre of the temperature range.

Temperature-dependence and effect of GTP on affinity of melatonin, N-acetyltryptamine and luzindole

The K_d values obtained for 2-[^{125}I]-iodomelatonin in saturation experiments at 4°C, 21°C and 37°C were used to calculate K_i values for melatonin, N-acetyltryptamine and luzindole using the Cheng-Prusoff equation and IC_{50} values obtained in competition experiments at these temperatures (Table 3). All three compounds inhibited 2-[^{125}I]-iodomelatonin binding completely with pseudo Hill coefficients close to unity (0.81–1.09). K_A values varied with temperature (Figure 5). $\Delta H°$ and $\Delta S°$ were calculated from the curves of $\Delta G°$ against temperature. As for 2-[^{125}I]-iodomelatonin, positive changes in entropy and enthalpy were found at 4°C, but these thermodynamic parameters became negative at 37°C (Table 4).

As expected, guanosine triphosphate (GTP, 1 mM) decreased the affinity of melatonin for the 2-[^{125}I]-iodomelatonin binding site. Preliminary experiments indicated that GTP was equipotent with GTPτS, a non-hydrolysable GTP analogue, and that a higher concentration of GTP (10 mM) did not shift melatonin affinity more than 1 mM GTP at either 4, 21 or 37°C (data not shown). This finding agrees with previous results on 2-[^{125}I]-iodomelatonin binding in chicken retina which show that the inhibitory effect of 10 mM GTP was not significantly greater than 1 mM GTP (Chong & Sugden, 1991). The inhibitory effect of GTP was clearly seen at each of the temperatures studied (4, 21 and 37°C) (Figure 5; Table 3). The affinity of luzindole, reported to be a melatonin receptor antagonist (Dubocovich, 1988), was not reduced by

Table 4 Effect of GTP on the thermodynamic parameters of the binding interactions of melatonin analogues with the 2-[^{125}I]-iodomelatonin binding site of chicken brain membranes.

		Thermodynamic parameters at:-								
		4°C			21°C			37°C		
Compound	*Conditions*	ΔG°	ΔH°	ΔS°	ΔG°	ΔH°	ΔS°	ΔG°	ΔH°	ΔS°
Melatonin	Control	− 12.08 ± 0.07	+ 36.3	+ 174.5	− 13.73 ± 0.08	− 6.9	+ 23.4	− 12.91 ± 0.14	− 49.8	− 118.9
	+ GTP	− 11.08 ± 0.07	+ 21.0	+ 116.2	− 12.32 ± 0.09	− 3.5	+ 30.1	− 12.15 ± 0.09	− 28.0	− 50.9
N-acetyltryptamine	Control	− 7.18 ± 0.03	+ 40.4	+ 172.0	− 8.83 ± 0.03	− 2.4	+ 22.0	− 8.05 ± 0.03	− 45.0	− 119.3
	+ GTP	− 7.00 ± 0.04	+ 20.3	+ 98.5	− 8.06 ± 0.05	− 0.04	+ 27.3	− 7.97 ± 0.05	− 20.3	− 39.7
Luzindole	Control	− 7.18 ± 0.07	+ 28.4	+ 128.7	− 8.41 ± 0.08	− 3.7	+ 16.3	− 7.82 ± 0.08	− 35.6	− 89.5
	+ GTP	− 7.38 ± 0.04	+ 20.3	+ 99.7	− 8.40 ± 0.05	− 2.2	+ 20.9	− 8.14 ± 0.05	− 24.6	− 53.2

Units are: ΔG°, Kcal mol^{-1}; ΔH°, Kcal mol^{-1}; ΔS°, cal/mol deg^{-1}. Errors on ΔG° are calculated from the standard errors of the estimates of IC_{50} values.

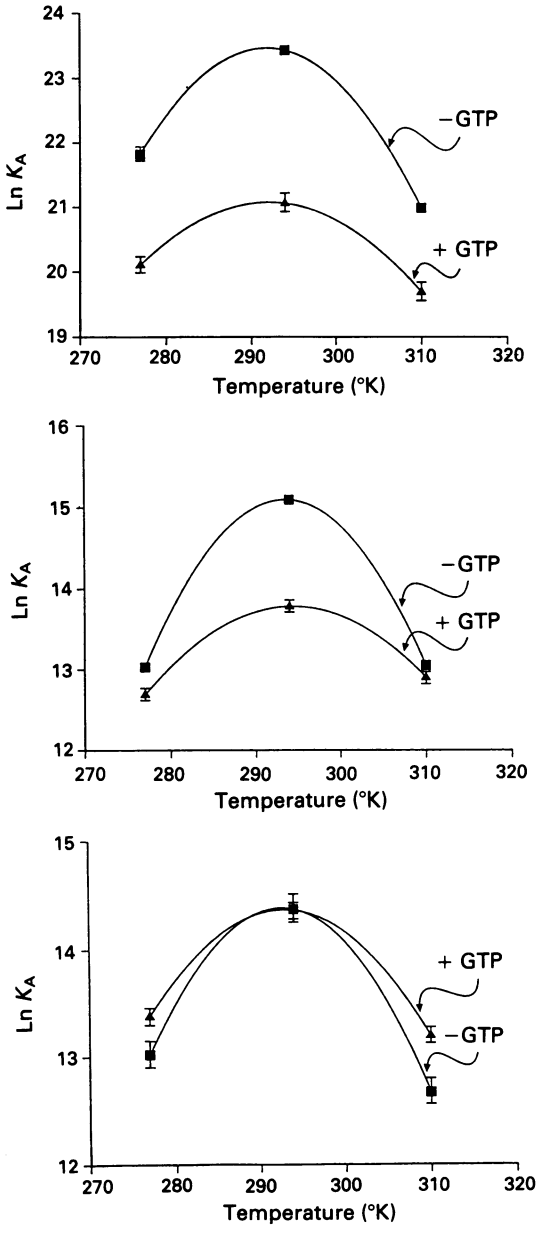

Figure 5 van't Hoff plots of the dependence of K_A on temperature for melatonin (a), N-acetyltryptamine (b) and luzindole (c) in chicken brain membranes in the absence (■) or presence (▲) of GTP (1 mM). K_A values were calculated from K_i values obtained in competition experiments using 2-[^{125}I]-iodomelatonin (70 to 105 pM). Data were fitted with a second order polynomial equation and ΔH° and ΔS° calculated as described in Methods.

GTP at any of the temperatures studied (Figure 5; Table 3). N-acetyltryptamine, which has been reported to be a partial agonist at the melatonin receptor (Dubocovich, 1985; Sugden, 1991), did show some shift in affinity with GTP but not as dramatic a shift as melatonin, a full agonist at the receptor.

Discussion

A number of studies have now reported specific binding of 2-[^{125}I]-iodomelatonin in brain, pituitary and retinal tissue using both radioreceptor assays and *in vitro* autoradiography (Morgan & Williams, 1989; Krause & Dubocovich, 1991). The 2-[^{125}I]-iodomelatonin binding site has a high affinity for its endogenous ligand, melatonin, suggesting that it is physiologically relevant to the range of melatonin concentrations normally found in the circulation, and has a unique pharmacology quite distinct from that of 5-hydroxytryptamine and other neurotransmitters (Sugden & Chong, 1991). In addition, the pharmacological and biochemical characteristics of the site appear to be very similar in marsupial and eutherian mammals, birds and reptiles suggesting that the melatonin receptor has been well-conserved through evolution (Rivkees *et al.*, 1989; Sugden & Chong, 1991; Paterson *et al.*, 1992).

In the present study, the affinity of 2-[^{125}I]-iodomelatonin clearly changed with temperature. The highest affinity was seen at 21°C with a reduced affinity both at lower (4°C and 13°C) and higher (29°C and 37°C) temperatures. This is in contrast to a recent study (Dubocovich, 1991) which reported that 2-[^{125}I]-iodomelatonin binding to chicken brain and retinal membranes had a substantially lower affinity at 0°C (K_d ~340–440 pM) than at 25°C and 37°C (K_d ~60–100 pM). In the study by Dubocovich (1991), binding at 0°C was allowed only 5 h to reach equilibrium. In our study a much longer period was required to reach equilibrium at this temperature (Figure 1) so that it seems likely that the results obtained at 0°C in Dubocovich's study have underestimated the true affinity of 2-[^{125}I]-iodomelatonin.

The binding affinity of melatonin, N-acetyltryptamine and luzindole to the 2-[^{125}I]-iodomelatonin binding site was also temperature-dependent (Figure 5). Like 2-[^{125}I]-iodomelatonin, each of these compounds showed the highest affinity at 21°C and lower affinities at 4°C and 37°C. For melatonin, an agonist, GTP reduced affinity at each temperature by 3.5 to 11 fold. For N-acetyltryptamine, a putative partial agonist, GTP also shifted affinity but to a lesser extent (1.1 to 3.7 fold). The melatonin receptor antagonist, luzindole (Dubocovich, 1988), did not show any reduction in affinity with GTP at any temperature (ratio K_d (+ GTP)/K_d(-GTP) = 0.6 to 1.0). GTP was as potent as GTPγS, a stable analogue of GTP which is resistant to metabolism, in inhibiting 2-[^{125}I]-

iodomelatonin binding in chicken brain membranes (Chong & Sugden, unpublished data) suggesting that the differences in the GTP-shift observed at different temperatures do not reflect the metabolism of GTP.

Calculation of the thermodynamic parameters ΔH° and ΔS° for all four compounds used in these studies reveals that as temperature increases there are reductions in both enthalpy and entropy of binding. Because binding was studied only at 5 temperatures, analysis of the data as two straight lines is unsatisfactory. However, it is possible that the inflection in the van't Hoff plot at around 21°C may, in fact, represent a 'break' which could be explained by a phase transition in the lipid bilayer containing the receptor, as has been suggested for [^{3}H]-flunitrazepam binding to rat brain benzodiazepine receptors (Speth *et al.*, 1979). Whether a phase transition of the lipid bilayer can account for the curvilinear van't Hoff plot can only be resolved by further determinations of 2-[^{125}I]-iodomelatonin binding affinity at temperatures between 13 and 29°C. It would also be interesting to determine if an Arrhenius plot obtained by measuring the dissociation rate constant (K_{off}) at different temperatures showed a sudden break indicative of a phase transition. There are several other examples of curvilinear van't Hoff plots for ligand-receptor-binding in the literature (Gies *et al.*, 1986; Mei *et al.*, 1987; Borea *et al.*, 1988). In one case, the hypothesis that a phase transition in the lipid bilayer might be responsible for the break in the van't Hoff plot was ruled out as an Arrhenius plot was linear (Waelbroeck *et al.*, 1985).

In previous thermodynamic studies of protein-ligand association, positive changes in entropy have generally been interpreted to reflect a decrease in the local ordering of water molecules adsorbed on both the ligand and receptor (Kilpatrick *et al.*, 1986; Gies *et al.*, 1986; Ross & Subramanian, 1981) when ligand and receptor associate. However, as pointed out by Miklavic *et al.* (1990) the fundamental properties of water would suggest that this type of hydrophobic binding would be strongly temperature-dependent. Direct experimental evidence confirms that this is the case for model hydrophobic interactions (Abraham & Matteoli, 1988). In such model systems, curvilinear van't Hoff plots are obtained and ΔH° and ΔS° do change with temperature. In the present experiments, ΔH° and ΔS° also changed with temperature, consistent with the interpretation that a hydrophobic interaction may drive the binding of melatonin ligands to the receptor.

However, for 2-[^{125}I]-iodomelatonin binding to the melatonin receptor this interpretation is not entirely satisfactory and cannot completely explain the observed changes in affinity with temperature. The affinity, and free energy of binding, ΔG°, have equal values at a number of temperatures within the range studied (4° to 37°C). For example, for 2-[^{125}I]-iodomelatonin the data in Figure 3 indicate that at 4°C and 33°C the affinity of the ligand for the receptor is equal. Yet at these two temperatures ΔS° is markedly different; at 4°C, ΔS° is $+133$ cal/mol deg^{-1}, while at 33°C ΔS° (determined from Figure 4) is -31 cal/mol deg^{-1}. At 4°C, it could be argued that the large positive change in ΔS°, perhaps as a result of hydrophobic interaction, may drive binding of ligand and receptor. At 33°C this cannot be the case as ΔS° is negative. Nevertheless, the affinity of the ligand for the receptor is identical at both temperatures. Thus at 33°C some other intermolecular interaction must compensate for the reduction in hydrophobic binding. Clearly, more than one factor must be involved in the binding of 2-[^{125}I]-iodomelatonin to its receptor so that, in this particular case, the negative change of entropy with increasing temperature is compensated by a corresponding, but opposite, change in enthalpy with temperature. What type of binding force(s) would show this difference in temperature dependence is not clear.

An additional complication is that in the absence of GTP, 2-[^{125}I]-iodomelatonin binds not to the isolated receptor protein but probably to a melatonin receptor-G protein-GDP complex, the so-called ternary complex. The contribution of these additional components to the measured free energy of binding, and whether this contribution changes with temperature, is not known. However, the changes in affinity and ΔG° with temperature are qualitatively similar for both the agonists investigated (melatonin, 2-[^{125}I]-iodomelatonin) and for the antagonist, luzindole. In this latter case, binding to the receptor would not be complicated by interactions with the G protein-GDP complex. Thus it may be that the contribution of the binding forces arising from these elements of the ternary complex is minor.

Clearly care must be taken not to overinterpret the changes in ΔH° and ΔS°; the original experimental measurements of binding constants lead directly to the determination of ΔG° and it is the value of the Gibb's free energy which determines the extent of binding. The van't Hoff plot allows an easy numerical partitioning of ΔG° between ΔH° and ΔS°. However, care must be taken in associating these derived changes in enthalpy and entropy with the particular molecular events involved in ligand-receptor binding. Melatonin, by contrast with many neurotransmitter amines, does not have highly favourable sites for hydrogen or ionic bond formation. In comparison to its metabolic precursor, 5-hydroxytryptamine, the amine is transformed to an amide and the hydroxyl group methylated. This may account for the inflection of the van't Hoff plots occurring in the observed temperature range. The data presented here show that caution must be exercised in the molecular interpretation of thermodynamic parameters derived from ΔG° (Raffa & Porreca, 1989).

We would like to thank Drs Peter Garratt and Michael Ewing, Chemistry Department, University College London for their helpful advice on the interpretation of the thermodynamic data and Dr Steve Reppert for providing a sample of luzindole.

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(Received August 19, 1993
Revised September 20, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 302–310

Interaction of tolbutamide and cytosolic nucleotides in controlling the ATP-sensitive K^+ channel in mouse β-cells

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1 In mouse pancreatic β-cells the role of cytosolic nucleotides in the regulation of the sulphonylurea sensitivity of the adenosine 5′-triphosphate-sensitive K^+ channel (K_{ATP}-channel) was examined. Patch-clamp experiments with excised inside-out membrane patches were carried out using an experimental protocol favouring phosphorylation of membrane proteins.

2 In the absence of Mg^{2+}, the K_{ATP}-channel-inhibiting potency of cytosolic nucleotides decreased in the order ATP = adenosine 5′-O-(3-thiotriphosphate) (ATPγS) > adenosine 5′-diphosphate (ADP) > adenosine 5′-O-(2-thiodiphosphate) (ADPβS) = adenylyl-imidodiphosphate (AMP-PNP) > 2′-deoxyadenosine 5′-triphosphate (dATP) > uridine 5′-triphosphate (UTP) > 2′-deoxyadenosine 5′-diphosphate (dADP) > guanosine 5′-triphosphate (GTP) > guanosine 5′-diphosphate (GDP) > uridine 5′-diphosphate (UDP).

3 In the presence of Mg^{2+}, the inhibitory potency of cytosolic nucleotides decreased in the order ATPγS > ATP > AMP-PNP > ADPβS > dATP > UTP. In the presence of Mg^{2+}, the K_{ATP}-channels were activated by dADP, GTP, GDP and UDP.

4 Tolbutamide inhibited the K_{ATP}-channels not only in the presence but also in the prolonged absence of Mg^{2+}. In nucleotide-free solutions, the potency of tolbutamide was very low. When about half of the K_{ATP}-channel activity was inhibited by ATP, AMP-PNP, ADPβS or ADP (absence of Mg^{2+}), the potency of tolbutamide was increased.

5 Tolbutamide (100 μM) slightly enhanced the channel-inhibiting potency of AMP-PNP and inhibited the channel-activating effect of MgGDP in a non-competitive manner.

6 Channel activation by MgGDP (0.5 mM) competitively antagonized the inhibitory responses to AMP-PNP (1 μM–1 mM). This effect of GDP was neutralized by tolbutamide (100 μM).

7 The stimulatory effect of 0.5 mM MgGDP was neutralized by 200 μM AMP-PNP. Under these conditions the potency of tolbutamide was much higher than in the presence of 0.5 mM MgGDP alone or in the absence of any nucleotides.

8 dADP (0.3–1 mM) increased the potency of tolbutamide. Additional application of 200 μM AMP-PNP caused a further increase in the potency of tolbutamide.

9 In conclusion, in the simultaneous presence of inhibitory and stimulatory nucleotides, binding of sulphonylureas to their receptor causes direct inhibition of channel activity, non-competitive inhibition of the action of stimulatory nucleotides and interruption of the competitive interaction between stimulatory and inhibitory nucleotides. The latter effect increases the proportion of K_{ATP}- channels staying in the nucleotide-blocked state. In addition, this state potentiates the direct effect of sulphonylureas.

Keywords: ATP-sensitive K^+ channel; pancreatic β-cell; tolbutamide; ATP; AMP-PNP; MgADP; MgGDP

Introduction

Initiation of insulin release requires increase in the cytosolic Ca^{2+} concentration of the pancreatic β-cell. Enhancement of Ca^{2+} influx across the plasma membrane is induced by depolarization of the β-cell to the threshold potential at which voltage-dependent Ca^{2+} channels are activated (for reviews see Henquin & Meissner, 1984; Matthews, 1985). Glucose and other insulin-releasing fuels depolarize the β-cell by raising the adenosine 5′-triphosphate (ATP) and lowering the adenosine 5′-diphosphate (ADP) concentration at the cytosolic face of the β-cell membrane and thereby inhibiting a distinct K^+ channel (K_{ATP}-channel) (Ashcroft *et al.*, 1984; Cook & Hales, 1984; for reviews see Ashcroft & Rorsman, 1991; Dunne & Petersen, 1991). The K_{ATP}-channel is controlled by at least three separate sites for cytosolic nucleotides: (1) ATP and some structurally related nucleotides inhibit the K_{ATP}-channel both in the absence and presence of Mg^{2+}. (2) The Mg complexes of ADP (MgADP) and some other nucleoside diphosphates increase the activity of the K_{ATP}-channel. (3) The Mg complex of ATP (MgATP) slows considerably the rapid decline of K_{ATP}-channel activity (channel run-down) observed in excised membrane patches in the absence of ATP (Ohno-Shosaku *et al.*, 1987). As run-down is also reduced in Mg^{2+}-free cytosol-like solution (Kozlowski & Ashford, 1990), it may involve a Mg^{2+}-dependent protein dephosphorylation and MgATP might act by serving as substrate for one or several protein kinases closely associated with the β-cell membrane.

Sulphonylureas (e.g. tolbutamide) inhibit and diazoxide activates the K_{ATP}-channel by interaction with separate binding sites in the β-cell plasma membrane (Sturgess *et al.*, 1985; Trube *et al.*, 1986; for reviews see Ashford, 1990; Ashcroft & Rorsman, 1991; Dunne & Petersen, 1991; Panten *et al.*, 1992; Ashcroft & Ashcroft, 1992). Binding studies and patch-clamp experiments suggest that the binding sites for these drugs are not identical with the sites mediating regulation of the K_{ATP}-channel by cytosolic nucleotides (Schwanstecher *et al.*, 1992b,c,e). Phosphorylation of the K_{ATP}-channel and/or regulatory proteins appears to be involved in the mechanism of action of diazoxide (Kozlowski *et al.*, 1989; Dunne, 1989). Moreover, diazoxide is only effective when the

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site for stimulatory nucleoside diphosphates is occupied (Schwanstecher *et al.*, 1992c; Larsson *et al.*, 1993). There is also evidence that an intracellular regulatory protein, which binds Mg-purine nucleotides, controls the effect of diazoxide (Kozlowski & Ashford, 1992). The potency of sulphonylureas is much lower in excised membrane patches than in intact β-cells, unless the internal side of the plasma membrane is exposed to ADP in the presence of Mg^{2+} (Zünkler *et al.*, 1988; Panten *et al.*, 1990). Test solutions supplemented with ADP and Mg^{2+} contain both MgADP which activates the channel and free ADP which inhibits the channel, but less effectively than free ATP. Studies with nucleotides structurally related to ADP suggest that enhancement of the sulphonylurea sensitivity is due to the combined actions of cytosolic nucleotides at the aforementioned inhibitory and stimulatory nucleotide receptors (Schwanstecher *et al.*, 1992d). However, it is unclear why these nucleotide actions enhance the sulphonylurea sensitivity. Moreover, the situation has been complicated by the finding that even 5 mM tolbutamide did not induce complete block of K_{ATP}-channels in the absence of any nucleotides (Schwanstecher *et al.*, 1992c).

We have therefore performed patch-clamp experiments with excised inside-out membrane patches in order to elucidate further the role of cytosolic nucleotides in the regulation of the sulphonylurea sensitivity of the K_{ATP}-channel.

Methods

Isolation and culture of pancreatic β-cells

Pancreatic β-cells were isolated from male albino mice (NMRI, 11–15 weeks old, fed *ad libitum*) and cultured for 1–4 days as previously described (Panten *et al.*, 1990). RPMI 1640 tissue culture medium was supplemented with 10 mM D-glucose.

Electrophysiological recording and analysis

The inside-out configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record currents flowing through K_{ATP}-channels as previously described (Panten *et al.*, 1990). All experiments were performed at room temperature (20–22°C). The bath was perfused continuously at 2 ml min^{-1}. Pipettes were pulled from borosilicate glass and had resistances between 3 and 8 MΩ when filled with pipette solution. Unless stated otherwise in the Results section, the membrane potential was clamped at − 50 mV. Inward membrane K^+ currents are indicated as downward deflections in all current traces.

With the exception of the experiments in Figure 7, the cytosplasmic face of the membrane patch was exposed for 45 s periods to intracellular solution which contained Mg^{2+} and 1 mM ATP and was applied directly to the patch from an U-shaped polythene capillary (Fenwick *et al.*, 1982; Ohno-Shosaku *et al.*, 1987). These 45 s periods alternated with 15 s periods of intracellular solution (with or without Mg^{2+}) which was supplemented with or without test substances (various nucleotides and/or drugs) and was applied to the patch by bath perfusion (experimental designs shown in Figures 1 and 4). Before and after application of test substance-containing bath solution, there were periods during which the same solution was applied except that the test substances were omitted. The mean of the amplitudes of the current responses (current amplitudes) during application of test substances was normalized to the mean current amplitude during nucleotide- and drug-free control periods in each single experiment. The single-channel current amplitudes of the K_{ATP}-channels were not changed by tolbutamide (1 μM–20 mM), diazoxide (300 μM) or the tested nucleotide concentrations.

Recordings were made with an LM-EPC 7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Current signals were stored on magnetic tape (Store 4, Racal Recorders, Hythe, UK) at 1 7/8 in s^{-1} (bandwidth 0.5 kHz, − 3 dB point). For analysis, taped data were digitized at 2 kHz using an Axolab 1100 computer interface (Axon Instruments, Foster City, CA, U.S.A.) and stored in a microcomputer. Analysis of the data was performed with the computer programme, pCLAMP 5.5.1 (Axon Instruments). For Figures 1, 4 and 7 taped data were replayed into a chart recorder (220, Gould, Cleveland, OH, U.S.A.).

Chemicals and solutions

Tolbutamide, Na_2-2′-deoxyadenosine 5′-diphosphate (dADP) and Na_2-2′-deoxyadenosine 5′-triphosphate (dATP) were obtained from Sigma (St. Louis, MO, U.S.A.). Li_4-adenosine 5′-O-(2-thiodiphosphate) (ADPβS), Na_2-ATP, Li_4-adenylyl-imidodiphosphate (AMP-PNP), Li_4-adenosine 5′-O-(3-thiotriphosphate) (ATPγS), Li_2-guanosine 5′-diphosphate (GDP), Li_2-guanosine 5′-triphosphate (GTP), K_2-uridine 5′-diphosphate (UDP) and Na_3-uridine 5′-triphosphate (UTP) were from Boehringer (Mannheim, Germany). All other chemicals were obtained from the sources described elsewhere (Panten *et al.*, 1989). Stock solutions of tolbutamide and diazoxide were prepared daily in 50–100 mM KOH.

Unless stated otherwise, the solution at the cytoplasmic side of the membrane (intracellular solution) contained (concentrations in mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid (EGTA) 10 and 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid (HEPES) 5 (titrated to pH 7.15 with KOH) (free $[Ca^{2+}] = 0.05$ μM). The free Mg^{2+} concentration was held close to 0.7 mM by adding appropriate amounts of $MgCl_2$ to the nucleotide-containing solutions. The required amounts of $MgCl_2$ and the composition of the solutions for pH 7.15 were calculated with a computer programme (Fabiato, 1988) except that some stability constants of the programme were exchanged as detailed previously (Schwanstecher *et al.*, 1992d). In addition, the stability constants for ADP were also used for UDP and those for ATP were also used for dATP, UTP and GTP. Addition of total concentrations of nucleoside triphosphates as high as 100 μM to the intracellular solution caused only negligible reduction in calculated free Mg^{2+} concentration. Therefore, we assumed that the applied ATPγS concentrations (up to 100 μM) did not alter the free Mg^{2+} concentration, particularly since the stability constants for Mg^{2+} complexes of ATP and ADP decrease when sulphur replaces oxygen (Pecoraro *et al.*, 1984; Schwanstecher *et al.*, 1992d). All experiments of Figures 1, 2 and 6 began with application of normal intracellular solution (with 0.7 mM free Mg^{2+}) during the 15 s periods. Subsequently the intracellular solution applied during the 15 s periods contained no $MgCl_2$ and was supplemented with 1 mM EDTA (Mg-free intracellular solution). The addition of 1 mM EDTA reduced free $[Ca^{2+}]$ to 0.04 μM. After addition of 1 mM Na_2-ATP, the intracellular solution was also used for filling the U-shaped polythene capillary of the aforementioned microflow system (Ohno-Shosaku *et al.*, 1987). In the experiments of Figure 7 the capillary of the microflow system contained intracellular solution from which $MgCl_2$ was omitted and which was supplemented with 1 mM EDTA and 1 mM Na_2-ATP. Unless stated otherwise in the Results section, the pipette solution contained (in mM): KCl 146, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10 (titrated to pH 7.40 with KOH).

The pH of all solutions was determined after adding EDTA, tolbutamide, diazoxide or any nucleotides and was readjusted if necessary. Tolbutamide and diazoxide were completely dissolved at the highest concentrations used (20 mM and 0.3 mM, respectively).

Treatment of results

Values are presented as mean ± s.e.mean. Significances were calculated by the two-tailed U-test of Wilcoxon and of Mann and Whitney. $P<0.05$ was considered significant. The concentration-inhibition relationships for tolbutamide or nucleotides (Figures 2, 3, 5–9, 11) were analysed by fitting the function

$$(1) \qquad E = a\left(1 - \frac{[A]^n}{EC_{50}^n + [A]^n}\right) + b$$

to the experimental data by a non-linear least-squares routine where a = maximal current amplitude (normalized) in the absence of test substance, b = normalized current amplitude in the presence of maximally effective concentrations of test substance, E = normalized current amplitude in the presence of test substance, [A] = concentrations of test substance, EC_{50} = half-maximally effective concentration and n = slope parameter (Hill coefficient). Relations between nucleotide concentrations and stimulatory effects (Figures 3, 10) were analysed by fitting the function

$$(2) \qquad E = a\left(\frac{[A]^n}{EC_{50}^n + [A]^n}\right) + b$$

to the experimental data where a = normalized current amplitude in the presence of a maximally effective concentration of stimulatory nucleotide, b = normalized current amplitude in bath solution free of stimulatory nucleotide, E = normalized current amplitude in the presence of stimulatory nucleotide, [A] = concentration of stimulatory nucleotide, EC_{50} = half-maximally effective concentration and n = slope parameter (Hill coefficient).

Results

It has been previously observed that AMP-PNP is less potent at the inhibitory site of the K_{ATP}-channel in the absence than in the presence of Mg^{2+} (Ashcroft & Kakei, 1989; Schwanstecher *et al.*, 1992d). To test whether this effect of Mg^{2+} reflects a general phenomenon being valid for all inhibitory nucleotides, we examined the concentration-dependent effects of several nucleotides both in the absence and in the presence of Mg^{2+}. Figure 1 shows the typical design of our inside-out experiments. In order to slow the rundown of channel activity, the cytoplasmic face of the membrane patch was exposed for 45 s periods to an intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ATP, alternating with 15 s periods serving as test or control periods. The example in Figure 1 demonstrates that addition of 0.1 or 0.3 mM dATP inhibited the K_{ATP}-channel activity in the absence of Mg^{2+} by 30 or 66%, respectively. Similar experiments in the absence (Figure 2, Table 1) and in the presence of Mg^{2+} (Figure 3, Table 1) revealed that ATPγS, ADPβS and dATP were less potent channel inhibitors in the absence than in the presence of Mg^{2+} whereas ATP was slightly less inhibitory in the presence than in the absence of Mg^{2+}. The presence of Mg^{2+} caused a great decrease in potency of UTP and channel activation by GDP, UDP and GTP (Figure 3, Table 1).

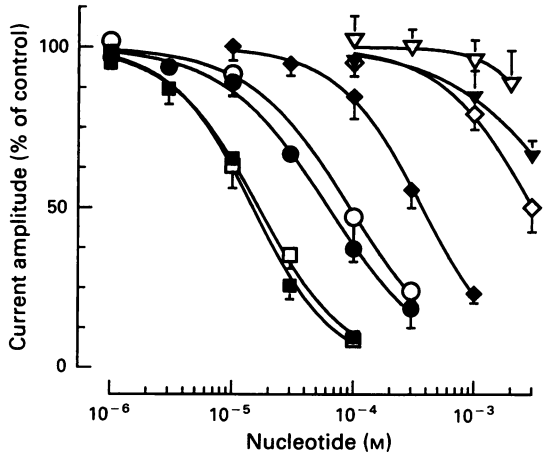

Figure 2 Inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells by nucleotides in the absence of Mg^{2+}. Using the experimental design shown in Figure 1, the mean current amplitude during 15 s periods of exposure to Mg-free intracellular solution containing the indicated concentrations of nucleotide (logarithmic scale) was normalized to the mean current amplitude during 15 s periods of exposure to control solution (Mg-free intracellular solution) before and after application of nucleotide in each single experiment. The curves through the current amplitude data points were fitted as described in the Methods section. (■) ATP; (□) ATPγS; (●) ADPβS; (○) dATP; (◆) UTP; (◇) GTP; (▼) GDP; (▽) UDP. Symbols indicate the mean with s.e.mean (when larger than symbols). For abbreviations, see text.

We then examined the effects of channel-inhibiting nucleotides on the potency of tolbutamide in an intracellular solution with 0.7 mM free Mg^{2+}. Figure 4 shows a typical experiment with some channel run-down which was observed in most of the experiments of this series. AMP-PNP (30 μM) inhibited the activity of the K_{ATP}-channels by 50%. Further addition of 100 μM tolbutamide decreased the channel activity to 17% of the activity in control solution (nucleotide- and tolbutamide-free). When the K_{ATP} channels were inhibited by 30 μM AMP-PNP, 50 μM ADPβS or 20 μM ATP, 20 mM tolbutamide caused a reduction of channel activity to 1.4 ± 0.2, 3.7 ± 2.4 or 3.2 ± 0.7%, respectively, of the activity in control solution ($n = 3-5$; Figure 5). These effects of 20 mM tolbutamide were not significantly different from its effect in the absence of any nucleotides (reduction to 1.4 ± 0.6% of control activity, $n = 5$; Figure 5), suggesting that maximally effective tolbutamide concentrations induce complete channel block under these conditions. However, the half-maximally effective concentrations (EC_{50}) of tolbutamide

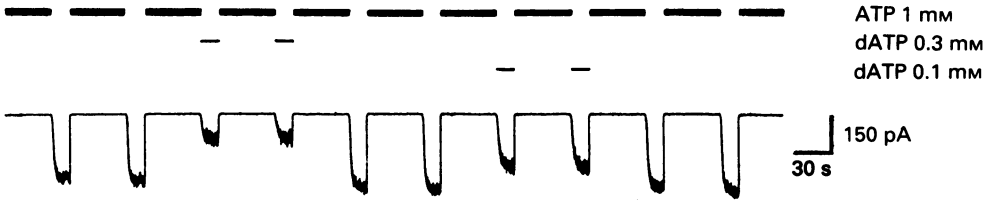

Figure 1 Inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells by dATP in the absence of Mg^{2+}. The uppermost horizontal bars indicate application of intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ATP for 45 s by the capillary of the microflow system. The other horizontal bars above the current trace indicate application of Mg-free intracellular solution with dATP for 15 s by the bath. The 15 s periods of application of nucleotide- and Mg-free intracellular solution to the bath represent the control periods.

Table 1 Effects of nucleotides on opening activity of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells

Nucleotide	EC_{50} (μM) absence of Mg^{2+}	Hill coefficient absence of Mg^{2+}	n	EC_{50} (μM) presence of Mg^{2+}	Hill coefficient presence of Mg^{2+}	n
ATP	14.8	1.3	(10)	18.8	1.2	(4–16)
ATPγS	16.5	1.2	(5–6)	11.6	1.1	(4–5)
ADPβS	61.9	1.0	(3–4)	43.6	1.0	(6)
dATP	92.8	1.0	(3–4)	85.9	1.1	(3–6)
UTP	369.0	1.2	(3–9)	1684.0	1.3	(4–12)
GTP	3048.0	1.2	(5–6)	423.0[a]	3.1[a]	(3–4)
GDP	>3000.0		(3–4)	34.0[a]	1.2[a]	(8–21)
UDP	>2000.0		(4–7)	114.0[a]	1.4[a]	(3–16)

Data were obtained in the experiments shown in Figures 2 and 3. Unless stated otherwise data refer to inhibitory effects. Number of experiments (n) in parentheses. [a]Channel activation. For abbreviations, see text.

were lower in the presence than in the absence of these inhibitory nucleotides (Figure 5, Table 2).

In accordance with previous results (Ashcroft & Rorsman, 1991), absence of Mg^{2+} in the intracellular solution induced activation of the K_{ATP}-channels (Figure 6). When the K_{ATP}-channels were inhibited by 100 μM ADP under these conditions, 20 mM tolbutamide caused a reduction of channel activity to 3.6 ± 0.9% ($n = 3$) of the activity in control solution (nucleotide-and tolbutamide-free intracellular solution with 0.7 mM free Mg^{2+}) (Figure 6), suggesting complete channel block by maximally effective tolbutamide concentrations in the absence of Mg^{2+}. In a Mg-free intracellular solution, the EC_{50} value for tolbutamide was lower in the presence than in the absence of 100 μM ADP (Figure 6, Table 2).

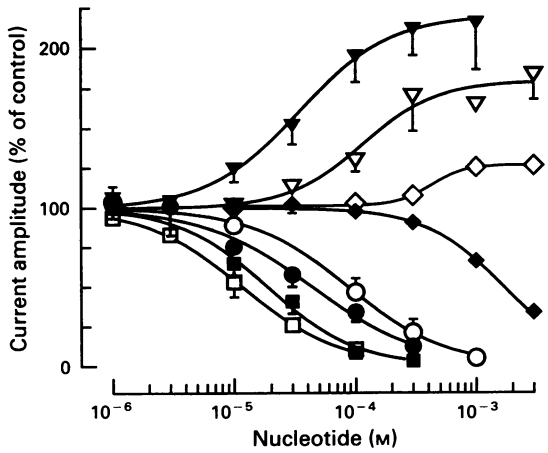

Figure 3 Activation and inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells by nucleotides in the presence of Mg^{2+}. Using an experimental design similar to that in Figure 1, the mean current amplitude during 15 s periods of exposure to intracellular solution (with 0.7 mM free Mg^{2+}) containing the indicated total concentrations of nucleotide (logarithmic scale) was normalized to the mean current amplitude during 15 s periods of exposure to control solution (intracellular solution with 0.7 mM free Mg^{2+}) before and after application of nucleotide in each single experiment. The curves through the current amplitude data points were fitted as described in the Methods section. (■) ATP; (□) ATPγS; (●) ADPβS; (○) dATP; (◆) UTP; (◇) GTP; (▼) GDP; (▽) UDP. Symbols indicate the mean and the vertical lines the s.e.mean (when larger than symbols). For abbreviations, see text.

Evidence has been presented that the binding properties of the sulphonylurea receptor are modulated by protein phosphorylation (Schwanstecher *et al.*, 1991). Therefore, we wanted to see whether tolbutamide is effective during prolonged inhibition of protein kinase reactions. As these reactions require MgATP as substrate, we removed Mg^{2+} not only from the intracellular solution perfusing the bath but also from the intracellular solution applied to the inside of the patch membrane by the capillary of the microflow system. A typical experiment illustrated in Figure 7a shows that channel run-down was usually not observed in the experiments of this series. The base-line for the recording was set by exposing the patch membrane for 5 s periods to Mg-free intracellular solution with 1 mM ATP and thereby inducing complete block of the K_{ATP}-channels. The channel activity decreased within 5 s after the start of application of 100 μM tolbutamide and rose again within 25 s after changing back to tolbutamide-free solution. Channel activity in the new steady state induced by 100 μM tolbutamide amounted to 58.1% of the activity in the absence of tolbutamide. Similar kinetics as shown in Figure 7a were observed in each single experiment when testing 10 μM–5 mM tolbutamide. Assuming complete channel block at maximally effective tolbutamide concentrations, the EC_{50} value for tolbutamide was 45.3 μM (Hill coefficient = 0.34; $n = 10–17$; Figure 7b).

To test whether the potency of tolbutamide depends on the extracellular K^+ concentration or the direction of the ATP-sensitive K^+ currents, experiments with a pipette solution containing 5.6 mM K^+ (appropriate replacement of KCl by NaCl in the pipette solution, membrane potential clamped at 0 mV) were performed, using the experimental design applied for determination of the potency of tolbutamide in the

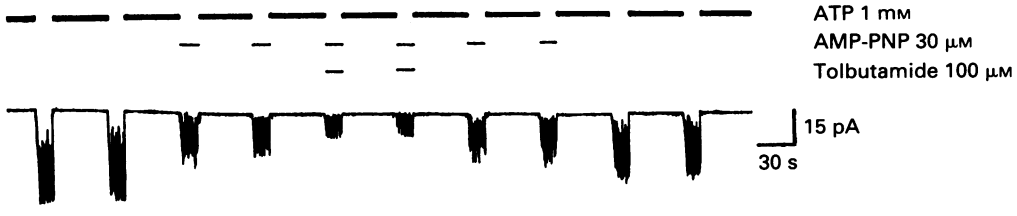

Figure 4 Effect of AMP-PNP on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The uppermost horizontal bars indicate application of intracellular solution containing 0.7 mM free Mg^{2+} and 1 mM ATP for 45 s by the capillary of the microflow system. The other horizontal bars above the current trace indicate application of intracellular solution containing 0.7 mM free Mg^{2+} and 30 μM AMP-PNP (with or without 100 μM tolbutamide) for 15 s by the bath. The 15 s periods of application of nucleotide- and tolbutamide-free intracellular solution (with 0.7 mM free Mg^{2+}) by the bath represent the control periods. For abbreviations, see text.

absence of any nucleotides, but in the presence of 0.7 mM free Mg^{2+} (Figure 5). Under these conditions 5 mM tolbutamide caused a reduction of channel activity to 12.8 ± 4.2% ($n = 6$) of the activity in the control solution (nucleotide- and tolbutamide-free), and the EC_{50} value for tolbutamide was 123.2 μM (Hill coefficient = 0.39; $n = 6-9$; data not shown in a figure). This EC_{50} value was even higher than that observed in the presence of 150 mM K^+ in the pipette solution (78.9 μM; Table 2).

The role of channel-activating nucleotides in the control of the effectiveness of sulphonylureas was examined with GDP and dADP. When the K_{ATP}-channels were activated by 1 mM

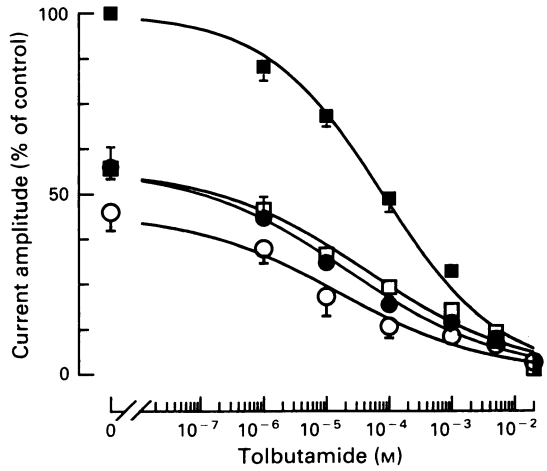

Figure 5 Effects of inhibitory nucleotides on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and tolbutamide concentration (logarithmic scale) during 15 s periods of intracellular solution (with 0.7 mM free Mg^{2+}) containing no nucleotide (■), 30 μM AMP-PNP (□), 50 μM ADPβS (●) or 20 μM ATP (○). Using the experimental design shown in Figure 4, the mean current amplitude during application of tolbutamide was normalized to the mean current amplitude during 15 s periods of exposure to control solution (intracellular solution with 0.7 mM free Mg^{2+}) before and after application of tolbutamide or nucleotide alone in each single experiment. The curves through the current amplitude data points were fitted as described in the Methods section. Symbols indicate the mean and the vertical lines the s.e.mean (when larger than symbols).

Table 2 Effects of nucleotides on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells

Nucleotide	*EC_{50} (μM) for tolbutamide*	*Hill coefficient*	*Number of experiments*
None	78.9	0.46	5–8
None[a]	167.6	0.43	3–18
AMP-PNP (30 μM)	44.2	0.35	4–13
ADPβS (50 μM)	21.7	0.36	3–9
ATP (20 μM)	15.9	0.36	3–18
ADP (100 μM)[a]	74.1	0.36	3–6
GDP (1 mM)	101.4	0.44	3–16
GDP (1 mM) + AMP-PNP (30 μM)	104.9	0.43	3–8
GDP (1 mM) + AMP-PNP (200 μM)	4.6	0.76	5–7
dADP (1.0 mM)	24.5	0.62	3–11
dADP (0.3 mM)	22.7	0.49	4–11
dADP (0.3 mM) + AMP-PNP (30 μM)	26.9	0.52	3–13
dADP (0.3 mM) + AMP-PNP (200 μM)	3.4	0.92	5–7

Data were obtained in the experiments shown in Figures 5, 6, 8 and 9. Unless stated otherwise, 0.7 mM free Mg^{2+} was present.[a] Mg-free intracellular solution was applied by bath perfusion. For abbreviations, see text.

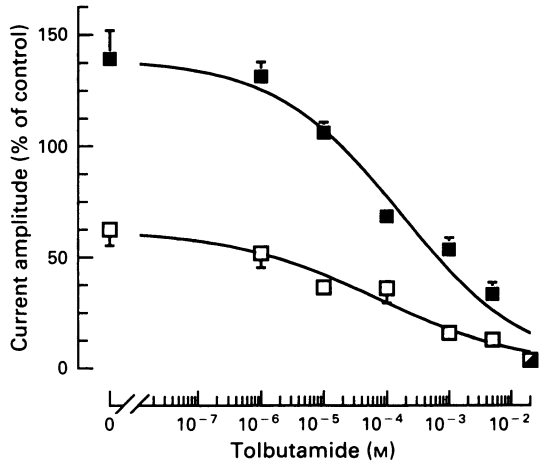

Figure 6 Effects of ADP in the absence of Mg^{2+} on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and tolbutamide concentration (logarithmic scale) during 15 s periods of exposure to Mg-free intracellular solution containing no nucleotide (■) or 100 μM ADP (□). Using an experimental design similar to that in Figure 4, the mean current amplitude during application of tolbutamide was normalized to the mean current amplitude during 15 s periods of exposure to control solution (intracellular solution with 0.7 mM free Mg^{2+}) before and after application of Mg-free solutions in each single experiment. The curves through the current amplitude data points were fitted as described in the Methods section. Symbols indicate the mean with s.e.mean (when larger than symbols).

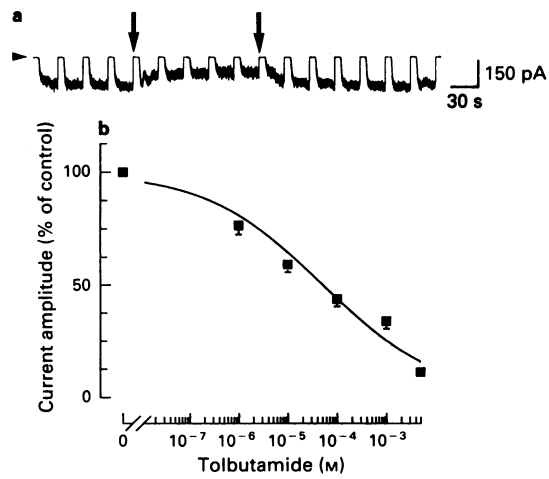

Figure 7 Effects of prolonged removal of Mg^{2+} on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. Mg-free intracellular solution was applied both during 20 s periods by bath perfusion and during 5 s periods by the capillary of the microflow system. (a) Current trace obtained from an inside-out patch exposed for 5 s periods to 1 mM ATP, alternating with 20 s periods with tolbutamide or without test substance. The trace starts about 15 min after switching to bath perfusion with Mg-free intracellular solution and 10 min after establishing the inside-out configuration. The arrows above the trace indicate the start of bath exchange when switching from control solution (no test substance) to solution with 100 μM tolbutamide and then back to control solution. The horizontal arrowhead indicates the base-line (all K_{ATP}-channels closed by 1 mM ATP). (b) Relationship between normalized current amplitude and tolbutamide concentration (logarithmic scale). Using the experimental design shown in (a), the mean current amplitude during the last three 20 s periods of exposure to tolbutamide was normalized to the mean current amplitude during 20 s periods of exposure to control solution (last three periods before the left arrow and third to fifth period after the right arrow). The curves through the current amplitude data points were fitted as described in the Methods section. Symbols indicate the mean with s.e.mean (when larger than symbols).

GDP, 20 mM tolbutamide caused a reduction of channel activity to 16.3 ± 1.1 ($n = 3$) of the activity in control solution (nucleotide- and tolbutamide-free) (Figure 8). Assuming complete channel block by maximally effective tolbutamide concentrations in the presence of 1 mM GDP, the EC_{50} value for tolbutamide was only slightly higher in the presence of 1 mM GDP than in its absence (Table 2). However, in conjunction with 0.3 mM diazoxide, 1 mM GDP strongly antagonized the channel-inhibiting effect of tolbutamide (Figure 8). On the other hand, neutralization of the stimulatory effect of 1 mM GDP by 200 μM AMP-PNP enhanced the potency of tolbutamide by about 17 fold as compared to its potency in the absence of any nucleotides

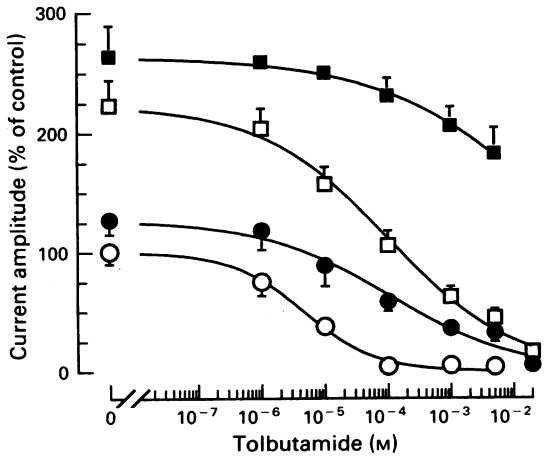

Figure 8 Effects of GDP on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and tolbutamide concentration (logarithmic scale) during 15 s periods of intracellular solution (with 0.7 mM free Mg^{2+}) containing 1 mM GDP (□), 1 mM GDP + 0.3 mM diazoxide (■), 1 mM GDP + 30 μM AMP-PNP (●) or 1 mM GDP + 200 μM AMP-PNP (○). Experimental design, normalization of the current amplitudes and curve fitting were similar to those in Figure 5, except that curve (■) was fitted by eye. Symbols indicate the mean with s.e.mean (when larger than symbols).

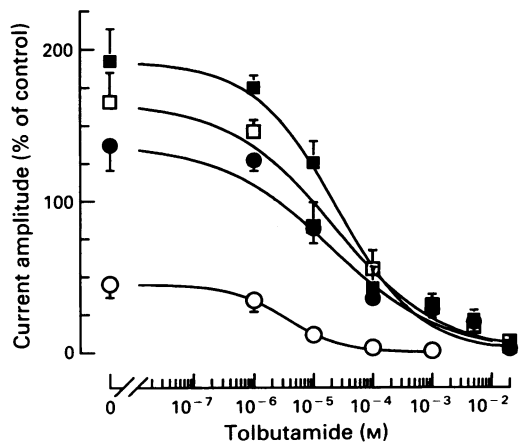

Figure 9 Effects of dADP on tolbutamide-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and tolbutamide concentrations (logarithmic scale) during 15 s periods of intracellular solution (with 0.7 mM free Mg^{2+}) containing 1 mM dADP (■), 0.3 mM dADP (□), 0.3 mM dADP + 30 μM AMP-PNP (●), or 0.3 mM dADP + 200 μM AMP-PNP (○). Experimental design, normalization of the current amplitudes and curve fitting were similar to those in Figure 5. Symbols indicate the mean and the vertical lines the s.e.mean (when larger than symbols). For abbreviations, see text.

(Figure 8, Table 2). A 30 μM concentration of AMP-PNP was insufficient to affect the potency of tolbutamide observed in the presence of 1 mM GDP (Figure 8, Table 2).

When the K_{ATP}-channels were activated by 1 mM dADP, 20 mM tolbutamide caused a reduction of channel activity to 7.3 ± 2.5% ($n = 3$) of the activity in the control solution (nucleotide- and tolbutamide-free) (Figure 9), suggesting complete channel block by maximally effective tolbutamide concentrations in the presence of 1 mM dADP. The EC_{50} value for tolbutamide was much lower in the presence of 1 mM dADP than in its absence (Table 2). A 0.3 mM concentration of dADP induced less pronounced channel activation than 1 mM dADP, but was sufficient to produce a decrease in the EC_{50} value for tolbutamide (Figure 9, Table 2). The combined effects of 0.3 mM dADP and 200 μM AMP-PNP caused inhibition of channel activity and enhanced the potency of tolbutamide by about 23 fold as compared to its potency in the absence of any nucleotides (Figure 9, Table 2). A 30 μM concentration of AMP-PNP was insufficient to affect the potency of tolbutamide observed in the presence of 0.3 mM dADP (Figure 9, Table 2).

Figure 10 shows the concentration-dependent effects of GDP on K_{ATP}-channel activity in the presence of an AMP-PNP or tolbutamide concentration inducing about 50% channel inhibition in the absence of GDP. AMP-PNP (30 μM) and tolbutamide (100 μM) increased the EC_{50} value for GDP (compared to control; see Table 1) to 225 μM (Hill coefficient = 0.93; $n = 5-7$) and 232 μM (Hill coefficient = 2.69; $n = 4-6$), respectively. However, the maximum effect of GDP was reduced by tolbutamide (100 μM), but not by AMP-PNP (30 μM).

In order to characterize further the mode of interaction between GDP on the one hand and AMP-PNP and tolbutamide on the other hand, concentration-response curves for AMP-PNP were obtained (Figure 11). In the absence of any other test-substance, AMP-PNP inhibited the K_{ATP}-channel activity half-maximally at 30 μM (dashed line in Figure 11; taken from Schwanstecher *et al.*, 1992d). A tolbutamide concentration (100 μM) decreasing the channel activity by 52 ± 5% enhanced the potency of AMP-PNP

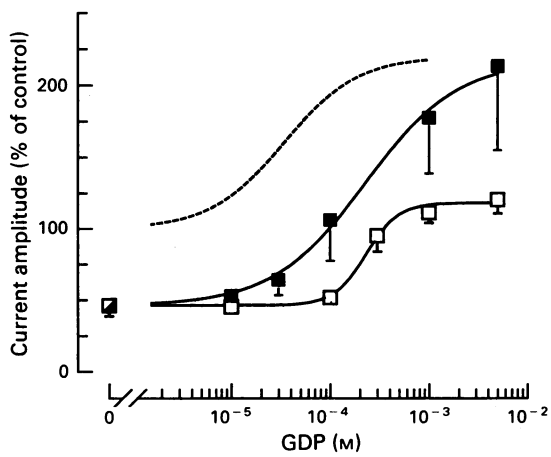

Figure 10 Effects of AMP-PNP and tolbutamide on GDP-induced activation of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and GDP concentrations (logarithmic scale) during 15 s periods of intracellular solution (with 0.7 mM free Mg^{2+}) containing 30 μM AMP-PNP (■) or 100 μM tolbutamide (□). Experimental design, normalization of the current amplitudes (control periods free of nucleotides and tolbutamide) and curve fitting were similar to those in Figure 5. Symbols indicate the mean with s.e.mean (when larger than symbols). To facilitate comparison, the corresponding concentration-dependent effects of GDP in the absence of tolbutamide and AMP-PNP are shown as a dashed line (taken from Figure 3). For abbreviations, see text.

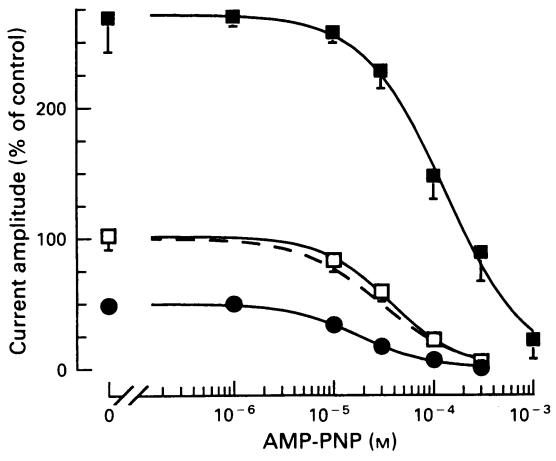

Figure 11 Effects of tolbutamide on AMP-PNP-induced inhibition of K_{ATP}-channels in inside-out patches of mouse pancreatic β-cells. The curves represent the relationships between normalized current amplitudes and AMP-PNP concentration (logarithmic scale) during 15 s periods of intracellular solution (with 0.7 mM free Mg^{2+}) containing 1 mM GDP (■), 1 mM GDP + 0.1 mM tolbutamide (□) or 0.1 mM tolbutamide (●). Experimental design, normalization of the current amplitudes (control periods free of nucleotides and tolbutamide) and curve fitting were similar to those in Figure 5. Symbols indicate the mean and the vertical lines the s.e.mean (when larger than symbols). To facilitate comparison, the corresponding concentration-dependent effects of AMP-PNP in the absence of GDP and tolbutamide are shown as a dashed line (data taken from Figure 3 in Schwanstecher *et al.*, 1992d). For abbreviations, see text.

(EC_{50} = 18 μM; Hill coefficient = 1.24; n = 3–4). Channel activation by 1 mM GDP strongly antagonized the responses to AMP-PNP over the whole range of tested concentrations; the EC_{50} value for AMP-PNP was 131 μM (Hill coefficient = 1.07; n = 5–16). This effect of 1 mM GDP was neutralized by 100 μM tolbutamide; the EC_{50} value for AMP-PNP was 36 μM (Hill coefficient = 1.26; n = 4–6).

Discussion

In the inside-out experiments of the present study, a maximally effective concentration of MgATP was usually present as substrate for protein kinases except for 15 s test periods. This experimental protocol favoured phosphorylation of the K_{ATP}-channels and/or regulatory proteins and strongly reduced channel run-down (Ohno-Shosaku *et al.*, 1987). Under these conditions the order of inhibitory effectiveness of cytosolic nucleotides in Mg^{2+}-free solution was ATP = ATPγS > ADP > ADPβS = AMP-PNP > dATP > UTP > dADP > GTP > GDP > UDP (Figure 2; for ADP, dADP and AMP-PNP see Panten *et al.*, 1990; Schwanstecher *et al.*, 1992d). Neglecting minor variations, this order is consistent with previous findings in β-cells, skeletal muscle and cardiac muscle (Spruce *et al.*, 1987; Lederer & Nichols, 1989; Ashcroft & Kakei, 1989). Variations in half-maximally inhibitory ATP concentrations (15 μM, Figure 2; 4 μM, Ashcroft & Kakei, 1989) probably reflect the different methods used to make allowance for channel run-down. Unlike non-hydrolysable ATP and ADP analogues (ATPγS, AMP-PNP, AMP-PCP, ADPβS), ATP was less inhibitory in the presence than in the absence of Mg^{2+} (see Results: Ashcroft & Kakei, 1989). It is therefore conceivable that enzymatic degradation of MgATP in the inside-out membrane and the attached bleb of cytoplasm led to underestimation of the potency of MgATP (Schwanstecher *et al.*, 1992c).

In contrast to a previous interpretation (Schwanstecher *et al.*, 1992d), there was no evidence for activation of K_{ATP}-channels by the Mg complex of the non-hydrolysable ADP analogue ADPβS. As has been reported for cardiac cells (Tung & Kurachi, 1991), MgUDP induced opening of K_{ATP}-channels in β-cells, though less effectively than MgGDP. Channel activation by MgGDP and MgdADP was less pronounced than in previous investigations (Schwanstecher *et al.*, 1992c,d). One possible explanation could be variation in cytoplasmic constituents involved in the mechanism of channel activation by nucleoside diphosphates (Bokvist *et al.*, 1991). The concentration-response curves for GTP and UTP in the presence of Mg^{2+} could reflect a combination of channel inhibition and relief of channel inhibition by enzymatically formed MgGDP and MgUDP.

In inside-out patches exposed to nucleotide-free solutions, tolbutamide concentrations higher than 20 mM were required for complete block of K_{ATP}-channels (Figure 5). In previous studies the tolbutamide concentrations were probably not high enough to induce complete channel closure (Zünkler *et al.*, 1988; Sturgess *et al.*, 1988; Schwanstecher *et al.*, 1992c). However, it is unclear whether all effects of millimolar tolbutamide concentrations resulted from occupation of specific sulphonylurea receptors. When inhibiting about half of the channel activity by ATP, AMP-PNP, ADPβS or ADP (in the absence of Mg^{2+}), the potency of tolbutamide was increased. This increase seems to reflect a reciprocal interaction between channel-inhibiting nucleotides and tolbutamide since tolbutamide slightly enhanced the potency of AMP-PNP. The previous observation that ADPβS (100 μM) enhanced the effectiveness of tolbutamide (100 μM) (Schwanstecher *et al.*, 1992d) is in accordance with the present observations. The failure to detect similar effects for ADP (50–100 μM in the absence of Mg^{2+}) and AMP-PNP (200 μM) (Panten *et al.*, 1990; Schwanstecher *et al.*, 1992d) has probably been due to the shapes of the concentration-response curves for tolbutamide (Figure 6) and the difficulty in determining the percentage of channel inhibition induced by a single tolbutamide concentration at rather low channel activity. Far too low channel activity also prevented reliable determination of the potency of tolbutamide in conjunction with inhibitory nucleotide concentrations higher than those in the presented experiments. Presumably the potency of tolbutamide is highest in the presence of near maximally inhibitory concentrations of nucleotides.

In test solutions supplemented with Mg^{2+} and GDP, dADP or ADP, about half of the nucleotide is present as Mg complex. As 0.5 mM free GDP inhibits the K_{ATP}-channel activity by less than 10% (Figure 2), the responses to GDP (up to 1 mM total concentration) in our Mg^{2+}-containing test solutions must have been produced almost exclusively by MgGDP. Channel activation by 0.5 mM MgGDP strongly reduces the inhibitory potency of AMP-PNP, but weakly reduces that of tolbutamide (Figures 8 and 11). On the other hand, both AMP-PNP and tolbutamide diminish the stimulatory potency of MgGDP (Figure 10). However, the maximum effect of MgGDP is decreased by tolbutamide, but not by AMP-PNP (Figure 10). These findings suggest competitive and non-competitive inhibition of the action of MgGDP by AMP-PNP and tolbutamide, respectively. Evidence has been presented that the K_{ATP}-channel is controlled via four separate binding sites for inhibitory nucleotides, stimulatory nucleotides, sulphonylureas and diazoxide (Schwanstecher *et al.*, 1992c). When the stimulatory effect of MgGDP is neutralized by applying an appropriate concentration of AMP-PNP, a proportion of the K_{ATP}-channels stays in the AMP-PNP-blocked state. In this situation tolbutamide decreases channel activity both directly and indirectly by non-competitive inhibition of the action of MgGDP. In addition, it is assumed that tolbutamide binding interrupts the competitive antagonism between MgGDP and AMP-PNP and thereby enhances the proportion of the AMP-PNP-blocked state. This view is consistent with the finding that sensitization of MgGDP-induced channel activation by diazoxide (0.3 mM) strongly reduces the potency of

tolbutamide (Figure 8). Occupation of the binding site for diazoxide inhibits binding of glibenclamide to both the membrane-bound and the solubilized sulphonylurea receptor (Schwanstecher *et al.*, 1992a).

At a concentration of 0.5 mM, MgdADP is probably as effective a channel activator as MgGDP, and both are maximally effective (see Results; Schwanstecher *et al.*, 1992d). However, the action of dADP at the inhibitory site is stronger than that of GDP. Free dADP (0.5 mM) inhibits the K_{ATP}-channel activity by 40% (Schwanstecher *et al.*, 1992d). This effect can explain that dADP (1 mM total concentration), but not GDP (1 mM total concentration) enhances the potency of tolbutamide (Figures 8 and 9; Schwanstecher *et al.*, 1992d). In conjunction with 200 μM AMP-PNP, dADP (0.3 mM total concentration) leads to an EC_{50} value for tolbutamide of 3.4 μM. This high potency is probably brought about by the additive effects of 200 μM AMP-PNP and 0.15 mM free dADP at the receptor site for inhibitory nucleotides. The action of ADP at the inhibitory site is even more potent than that of dADP. Free ADP causes 50 and 90% inhibition of K_{ATP}-channel activity at 0.05 and 0.5 mM, respectively (Panten *et al.*, 1990). In the presence of Mg^{2+} and 1 mM total ADP, the channel activity is similar to that observed in the absence of any nucleotides (Zünkler *et al.*, 1988; Schwanstecher *et al.*, 1992c), indicating neutralization of the inhibitory effect of free ADP (0.5 mM) by the stimulatory effect of MgADP (0.5 mM). Under these conditions the EC_{50} value for tolbutamide is 4.0 μM (Schwanstecher *et al.*, 1992c). A nearly identical EC_{50} value (4.6 μM) is obtained for tolbutamide in the presence of 200 μM AMP-PNP and 0.5 mM MgGDP (Figure 8). This AMP-PNP concentration induces the same percentage of channel inhibition as 0.5 mM free ADP (Schwanstecher *et al.*, 1992d). As the above EC_{50} values are similar to the EC_{50} values in intact β-cells (2.2–2.5 μM; Gillis *et al.*, 1989; Panten *et al.*, 1990), the potency of sulphonylureas in intact β-cells, too, appears to be controlled by stimulatory and inhibitory nucleotides.

The mechanisms discussed so far do not explain why the potency of tolbutamide for K_{ATP}-channel inhibition is so low in the absence of cytosolic nucleotides (EC_{50} = 79 μM). In microsomal membranes from pancreatic islets, the K_D value for tolbutamide binding is 12–15 μM in the absence of nucleotides and 32 μM in the presence of 0.1 mM MgATP (Panten *et al.*, 1989; Schwanstecher *et al.*, 1992e). These findings and additional evidence suggest that the binding affinity of the sulphonylurea receptor is decreased by protein phosphorylation (for reviews see Ashcroft & Ashcroft, 1992; Panten *et al.*, 1992). It is therefore conceivable that the low potency of tolbutamide for K_{ATP}-channel inhibition is due to phosphorylation of the sulphonylurea receptor. However, rapid alteration of phosphorylation of the sulphonylurea receptor does not appear to be necessary for the mechanism of action of sulphonylureas. Presumably prolonged removal of Mg^{2+} inhibits protein phosphorylation, but does not prevent strong and rapidly reversible responses to tolbutamide (see Results).

In conclusion, the present study reveals a complex mechanism leading to enhanced potency of sulphonylureas in the presence of certain cytosolic nucleotides. Firstly, channel-inhibiting nucleotides increase the potency of sulphonylureas in a reciprocal manner. Secondly, sulphonylureas inhibit the effect of channel-stimulatory nucleotides by a non-competitive mechanism, whereas inhibitory nucleotides competitively antagonize stimulatory nucleotides. This competitive interaction is interrupted by sulphonylureas. Therefore, in the simultaneous presence of stimulatory and inhibitory nucleotides, application of sulphonylureas enhances the proportion of K_{ATP}-channels staying in the nucleotide-blocked state.

This work was supported by the Deutsche Forschungsgemeinschaft (Pa 229/5-1).

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(Received February 19, 1993
Revised September 1, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1994), **111**, 311–317

Effect of a peptide inhibitor of protein kinase C on G-protein-mediated increase in myofilament Ca^{2+}-sensitivity in rabbit arterial skinned muscle

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1 To investigate the role of protein kinase C in the increase mediated by guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins) in the sensitivity of the contractile proteins to Ca^{2+} in vascular smooth muscle, the effect of a novel peptide inhibitor of protein kinase C (PKC_{19-36}) on Ca^{2+}-induced contraction and myosin light chain (MLC) phosphorylation was studied in the presence and absence of guanosine 5'-O-(3-thiotriphosphate) ($GTP_\gamma S$) in β-escin-skinned smooth muscle strips of rabbit mesenteric artery. For comparison, the effects were also observed of PKC_{19-36} on the action of phorbol 12,13-dibutylate (PDBu, an activator of PKC) on the two Ca^{2+}-induced responses.

2 In β-escin-skinned strips treated with ionomycin, Ca^{2+} (0.1–3 μM) concentration-dependently produced contraction in parallel with an increase in MLC-phosphorylation. $GTP_\gamma S$ (10 μM) and PDBu (0.1 μM) each shifted both the Ca^{2+}-force and Ca^{2+}-MLC-phosphorylation relationships to the left without a significant change in either maximum response. The relationship between force and MLC-phosphorylation was not modified by either $GTP_\gamma S$ or PDBu, indicating that the sensitivity of MLC-phosphorylation to Ca^{2+} is enhanced by both $GTP_\gamma S$ and PDBu.

3 PKC_{19-36} itself modified neither the contraction nor MLC-phosphorylation induced by Ca^{2+} but it did block the PDBu-induced enhancement of these two Ca^{2+}-induced responses. By contrast, PKC_{19-36} did not modify the $GTP_\gamma S$-induced enhancement of the two Ca^{2+}-induced responses. Guanosine 5'-O-(2-thiodiphosphate) ($GDP_\beta S$) attenuated the $GTP_\gamma S$-induced enhancement of the Ca^{2+}-induced contraction.

4 These results suggest that $GTP_\gamma S$ increases Ca^{2+}-induced MLC-phosphorylation through the activation of a PKC-independent mechanism and thus causes an increase in the sensitivity of the contractile proteins to Ca^{2+} in β-escin-skinned smooth muscle of rabbit mesenteric artery.

Keywords: G-proteins; protein kinase C; myosin phosphorylation; vascular smooth muscle

Introduction

It is generally believed that a Ca^{2+}-mobilizing hormone (agonist) binds to its receptor and promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) through activation of heterotrimeric (αβγ) G-proteins, and that this is achieved by catalysing the replacement of the guanosine 5'-diphosphate (GDP) bound to the α-subunit by guanosine 5'-triphosphate (GTP) (Bourne *et al.*, 1990). Following the hydrolysis of PIP_2, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are produced: the former releases Ca^{2+} from the cellular storage sites and the latter activates protein kinase C (PKC). It is also known that treatment with GTP or guanosine 5'-O-(3-thiotriphosphate) ($GTP_\gamma S$) increases the contraction, phosphorylation of myosin light chain (MLC) and unloaded shortening velocity induced by low concentrations of Ca^{2+} through activation of unidentified G-proteins in chemically skinned smooth muscle (Nishimura *et al.*, 1988; Fujiwara *et al.*, 1989; Kitazawa *et al.*, 1989; Itoh *et al.*, 1992; Kubota *et al.*, 1992). It was recently suggested that agonists acting together with GTP or $GTP_\gamma S$ increase MLC-phosphorylation through inhibition of MLC-phosphatases (Kitazawa *et al.*, 1991; Kubota *et al.*, 1992), but the precise mechanism for this is not well understood.

It has been reported that $GTP_\gamma S$ itself activates heterotrimic G-proteins and thus increases the activity of phospholipase C, causing production of IP_3 in vascular smooth muscle (Sasaguri *et al.*, 1985). This suggests that, following application of $GTP_\gamma S$, DAG (a co-product of IP_3 via hydrolysis of PIP_2) may also be produced, causing activation of PKC in vascular smooth muscle. In saponin-skinned smooth muscle, we have previously reported that 12-O-tetradecanoyl-13-acetate (TPA), a potent activator of PKC, increased the contraction, MLC-phosphorylation and unloaded shortening velocity in the presence of 0.3 μM Ca^{2+} (Itoh *et al.*, 1986b; Fujiwara *et al.*, 1988). Similar effects of phorbol esters on Ca^{2+}-induced contraction were also reported in Triton X-100-treated skinned vascular smooth muscle (Chatterjee & Tejada, 1986). These results suggest that PKC, activated by an application of $GTP_\gamma S$, may play a primary role in the $GTP_\gamma S$-induced enhancement of Ca^{2+}-induced contractile responses in vascular smooth muscle. Indeed, Nishimura *et al.* (1988) reported that H-7, an inhibitor of protein kinase C, inhibited the $GTP_\gamma S$-induced enhancement of the Ca^{2+}-induced contraction, and suggested that PKC may play an important role in the $GTP_\gamma S$-induced Ca^{2+}-sensitization in vascular tissue. However, since H-7 inhibits PKC at its ATP-binding site, a region with a high degree of sequence homology in most kinases (Hidaka *et al.*, 1984; Edelman *et al.*, 1987), this agent may have some non-specific actions, especially at high concentrations, such as inhibition of guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase, adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase and MLC-kinase (Hidaka *et al.*, 1984). In preliminary experiments, on β-escin-skinned smooth muscle, we found that H-7, at concentrations over 10 μM, concentration-dependently inhibited both the contraction and MLC-phosphorylation induced by 10 μM Ca^{2+} with no change in the relationship between force and MLC-phosphorylation, suggesting that high concentrations of H-7 may inhibit MLC-kinase as well as PKC. Thus, the contribution of PKC to the $GTP_\gamma S$-induced enhancement of Ca^{2+}-induced contraction needs further examination, using more specific PKC inhibitors.

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PKC_{19-36}, which corresponds to the autoinhibitory region of PKC, has been recently introduced and shown to inhibit selectively PKC (House & Kemp, 1987; Gong *et al.*, 1992). In order to investigate the contribution of PKC to the mechanisms by which G-proteins mediate an enhancement of the contraction induced by Ca^{2+}, we studied the effects of PKC_{19-36} on Ca^{2+}-induced contraction and MLC-phosphorylation in ionomycin-treated, β-escin-skinned smooth muscle. For comparison, we also studied the effects of PKC_{19-36} on the action of PDBu (an activator of PKC) on the same Ca^{2+}-induced responses.

Methods

Male albino rabbits, weighing 1.9–2.5 kg, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then exsanguinated. A segment of the third branch of the mesenteric artery distributing to the region of the ileum (diameter around 70 μm) was excised immediately and cleaned by removal of connective tissue in Krebs solution at room temperature. Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The fine circularly-cut muscle strip (0.2–0.3 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) was transferred to a chamber of 0.1 ml volume and mounted horizontally, the resting force being adjusted to obtain a maximal contraction in 128 mM K^+.

Experiment on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were made using β-escin (Kobayashi *et al.*, 1989; Itoh *et al.*, 1992). The methods used for skinning and the composition of the solutions have been described elsewhere (Itoh *et al.*, 1986b). Briefly, skinned muscle preparations were obtained by treatment with β-escin (30 μM) in Ca^{2+}-free solution containing EGTA and adenosine 5'-triphosphate (ATP) ('relaxing solution') for 25 min. When the Ca^{2+}-force relationship was to be determined, the concentration of EGTA in the solution was 4 mM and 1 μM ionomycin was applied to avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle. To prevent deterioration of the Ca^{2+}-induced contraction, 0.1 μM calmodulin was applied throughout the experiments, as described previously (Itoh *et al.*, 1986a). Various concentrations of Ca^{2+} were applied cumulatively from low to high concentration. The amplitude of contraction induced by each of the various concentrations of Ca^{2+} was normalized with respect to that induced by 10 μM Ca^{2+} in the same strip.

Measurements of myosin light chain phosphorylation

Muscle strips (0.2–0.3 mm length, 1.2 mm width, 0.02–0.03 mm thickness) were suspended in a relaxing solution for over 10 min. Then, the tissues were skinned with a relaxing solution containing 30 μM β-escin with 1 μM ionomycin for 25 min and washed again with the relaxing solution (containing no β-escin). Each skinned muscle strip was then suspended in solutions containing various concentrations of Ca^{2+} for 15 min in the presence or absence of either 10 μM $GTP_\gamma S$ or 0.1 μM PDBu. The strips were quick-frozen with 10% trichloroacetic acid (TCA) in acetone-dry ice containing 10 mM dithiothreitol and allowed to reach room temperature. The strips were then washed three times with acetone to remove residual TCA. Protein, including myosin light chain (MLC), was extracted in a lysing solution containing 8 M urea, 20 mM tris(hydroxymethyl)aminomethane (Tris), 23 mM glycine, 10 mM dithiothreitol, 0.004% bromophenol blue and saturated sucrose (pH 8.6) (Persechini *et al.*, 1986).

Nonphosphorylated, monophosphorylated and diphosphorylated forms of MLC were separated by urea/glycerol-polyacrylamide gel electrophoresis followed by electrophoretic transfer of the proteins to nitrocellulose paper and quantitation of the relative amounts of each form by an immunoblot procedure, as reported previously (Persechini *et al.*, 1986). Antibody against bovine tracheal MLC was used as a first antibody and biotin-labelled goat anti-rabbit IgG (Histofine, Seikagaku Kogyo, Japan) as a second antibody. Alkaline phosphatase-labelled streptavidine was then applied. Regions containing MLC were visualized as dark blue bands after incubation with the colour development reagents, 5-bromo-4-chloro-3-indolyl phosphate toluidine and *p*-nitroblue tetrazolium chrolide (Alkaline phosphatase substrate kit, Vector Laboratories, CA, U.S.A.). Relative amounts of nonphosphorylated, monophosphorylated and diphosphorylated MLC were quantified densitometrically by scanning the blot using a chromatoscanner (CS-930, Shimadzu, Japan). The phosphorylation of MLC was expressed in mol PO_4 mol^{-1} MLC.

Calculation of Hill coefficient

The slope of the concentration-response relationship for the effect of Ca^{2+} on force and MLC-phosphorylation is shown by the Hill coefficient (n) and mid-point position ($pK = (-\log K)$, where K is the dissociation constant). These parameters were obtained by fitting the data points for each curve to eqn.(1) by a non-linear least-squares method.

$$F/F_0 = (C/K)^n/[1 + (C/K)^n] \quad (1)$$

Where C represents the concentration of Ca^{2+}, F is the amplitude of contraction at any given concentration of Ca^{2+}, and F_0 is the maximum response evoked by 10 μM Ca^{2+} expressed as a relative force of 1.0.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134, glucose 11.5. The concentration of K^+ was modified by replacing NaCl with KCl, isosmotically. The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH maintained at 7.3–7.4.

For experiments on skinned muscle, the composition of the 'relaxing solution' was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N*-*N*'-*bis*-(2-ethanesulphonic acid) (PIPES), 5.1 mM $Mg(methanesulphonate)_2$, 5.2 mM adenosine 5'-triphosphate (ATP), 5 mM phosphocreatine and 4 mM ethyleneglycol-*bis*-(β-aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA). Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(methanesulphonate)_2$ to 4 mM EGTA, based on the calculation reported previously (Itoh *et al.*, 1986a). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were EGTA, PIPES and HEPES (Dojin, Japan), PKC_{19-36} (Peninsula Laboratories), calmodulin, β-escin and $GTP_\gamma S$ (Sigma) and ionomycin (free acid; Calbiochem). The antibody against bovine tracheal MLC was provided by Dr J.T. Stull (Department of Physiology and Pharmacology, University of Texas Southwestern Medical Center, TX, U.S.A.).

Statistics

The values recorded were expressed as mean ± s.d. and statistical significance determined by a paired or unpaired Student's *t* test. Probabilities less than 5% ($P < 0.05$) were considered significant.

Results

$GTP_\gamma S$ and PDBu both increase the sensitivity of contractile proteins to Ca^{2+} in β-escin-skinned smooth muscle

The effects were observed of $GTP_\gamma S$ and PDBu on the contraction and phosphorylation of myosin light chain (MLC) induced by various concentrations of Ca^{2+} in ionomycin-, β-escin-skinned smooth muscle strips (Figure 1). In the skinned muscle, the minimum concentration of Ca^{2+} needed to produce contraction was 0.1 μM and the maximum contraction was obtained at 3 μM Ca^{2+}. Figure 1a shows the effects of applying 10 μM $GTP_\gamma S$ or 0.1 μM PDBu on contractions induced by various concentrations of Ca^{2+} (0.03–10 μM). $GTP_\gamma S$ and PDBu each shifted the Ca^{2+}-force-relation curve to the left with no change in the maximum amplitude of contraction induced by 10 μM Ca^{2+}. The threshold concentration of Ca^{2+} needed to produce contraction was slightly lower in the presence of either $GTP_\gamma S$ (0.03 μM) or PDBu (0.03 μM) than in control (0.1 μM). The concentration of Ca^{2+} required for half-maximum force (ED_{50}) was 0.61 ± 0.18 μM in control ($n = 12$), 0.18 ± 0.13 μM in the presence of $GTP_\gamma S$ ($n = 6$) and 0.14 ± 0.07 μM in PDBu ($n = 6$) and the ED_{50} values obtained in the presence of $GTP_\gamma S$ or PDBu were significantly smaller than that in the corresponding control ($P < 0.05$). The Hill coefficient was 2.2 ± 0.3 in control, 1.3 ± 0.2 in the presence of $GTP_\gamma S$ and 1.6 ± 0.3 in PDBu.

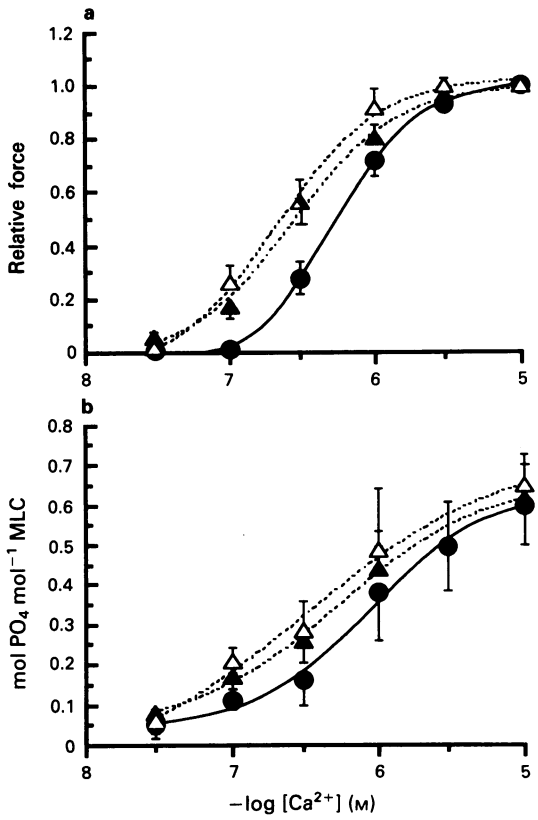

Figure 1 Effects of $GTP_\gamma S$ and PDBu on relationships between Ca^{2+}-force (a) and Ca^{2+}-myosin light chain (MLC) phosphorylation (b) in β-escin-skinned smooth muscle strips. Ca^{2+} at any given concentration (0.03–10 μM) was applied for 15 min in a relaxing solution containing 4 mM EGTA in the absence (●) or presence of either 10 μM $GTP_\gamma S$ (▲) or 0.1 μM PDBu (△). In (a), the maximum amplitude of contraction induced by 10 μM Ca^{2+} was normalized as a relative force of 1.0 for each strip. The curves for the effects of Ca^{2+} on force and MLC-phosphorylation were obtained by fitting the data points to eqn.(1) by a non-linear least-squares method (see Methods). Results shown are each the mean of 6–12 observations with s.d. For abbreviations, see text.

Figure 1b shows the effects of applying 10 μM $GTP_\gamma S$ or 0.1 μM PDBu on the MLC-phosphorylation induced by various concentrations of Ca^{2+} (0.03–10 μM). In Ca^{2+}-free solution containing 4 mM EGTA, MLC-phosphorylation was 0.04 ± 0.04 mol PO_4 mol^{-1} MLC ($n = 6$) and Ca^{2+} (0.03–10 μM) concentration-dependently increased the MLC-phosphorylation. Under these conditions, Ca^{2+} (0.03–10 μM) mainly increased the monophosphorylated form of MLC (over 95%) whether in the presence or absence of $GTP_\gamma S$ or PDBu. $GTP_\gamma S$ and PDBu each shifted the relationship between Ca^{2+} and MLC-phosphorylation (monophosphorylated form) to the left without affecting the maximum level of MLC-phosphorylation obtained with 10 μM Ca^{2+}. The ED_{50} values of Ca^{2+} for monophoshorylated form of MLC were 0.88 ± 0.06 μM in control ($n = 6$), 0.53 ± 0.08 μM in the presence of $GTP_\gamma S$ ($n = 6$) and 0.31 ± 0.08 μM in PDBu ($n = 6$). The ED_{50} value obtained in the presence of $GTP_\gamma S$ or PDBu was significantly smaller than that in control ($P < 0.05$).

Figure 2 shows the relationship between relative force and the monophosphorylated form of MLC in the presence of various concentrations of Ca^{2+} with and without $GTP_\gamma S$ or PDBu. Neither $GTP_\gamma S$ nor PDBu changed this relationship.

$GTP_\gamma S$ increases Ca^{2+}-induced contraction and MLC-phosphorylation by a PKC-independent mechanism

PKC_{19-36}, a synthetic peptide corresponding to autoinhibitory domain of PKC, inhibits PKC with a K_i value of 0.2 μM when assayed with a synthetic peptide, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, as a substrate (House & Kemp, 1987).

PKC_{19-36} (0.1 mM) modified neither the contraction (1.1 ± 0.2 times control) nor the MLC-phosphorylation induced by 0.3 μM Ca^{2+} alone. The MLC-phosphorylation induced by 0.3 μM Ca^{2+} was 0.16 ± 0.06 mol PO_4 mol^{-1} MLC in control ($n = 6$) and 0.17 ± 0.03 mol PO_4 mol^{-1} MLC in the presence of 0.1 mM PKC_{19-36} ($n = 4$). The effects of PKC_{19-36} were then observed on the contraction induced by 0.3 μM Ca^{2+} in the presence of either 10 μM $GTP_\gamma S$ or 0.1 μM PDBu (Figure 3). PKC_{19-36} (10–100 μM) concentration-dependently attenuated the PDBu-induced enhancement of

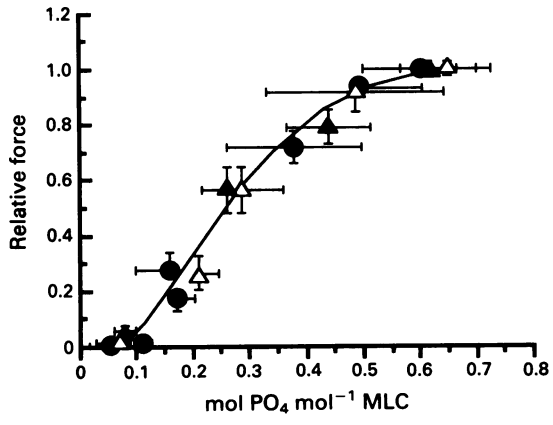

Figure 2 The relationship between relative force and myosin light chain (MLC) phosphorylation in the presence of various concentrations of Ca^{2+} (0.03–10 μM) with and without either $GTP_\gamma S$ or PDBu in β-escin-skinned smooth muscle strips: (●) control; (▲) in the presence of 10 μM $GTP_\gamma S$; (△) in 0.1 μM PDBu. The curve was obtained by fitting the data points to eqn.(1) by a non-linear least-squares method according to the equation: $F/F_0 = (C/K)^n/[1 + (C/K)^n]$. n, Hill coefficient; K, dissociation constant. C and F/F_0 represent MLC-phosphorylation (mol PO_4 mol^{-1} MLC) and relative force, respectively. The fitted parameters of n and K were 2.5 and 0.28 mol PO_4 mol^{-1} MLC, respectively ($r = 0.99$). The data shown in Figure 1 were used for this analysis. Results shown are each the mean of 4–8 observations with s.d. For abbreviations, see text.

the Ca^{2+}-induced contraction and, at 0.1 mM blocked the action of PDBu (Figure 3b). The PKC peptide (0.1 mM) also blocked the PDBu-induced enhancement of the MLC-phosphorylation induced by 0.3 μM Ca^{2+} (Figure 3c). By contrast, PKC_{19-36} failed to modify the $GTP_\gamma S$-induced enhancement of either contraction or MLC-phosphorylation induced by 0.3 μM Ca^{2+} (Figures 3 and 4). PKC_{19-36} did not seem to change the relationship between relative force and MLC-phosphorylation in the presence of either $GTP_\gamma S$ or PDBu (Figure 4).

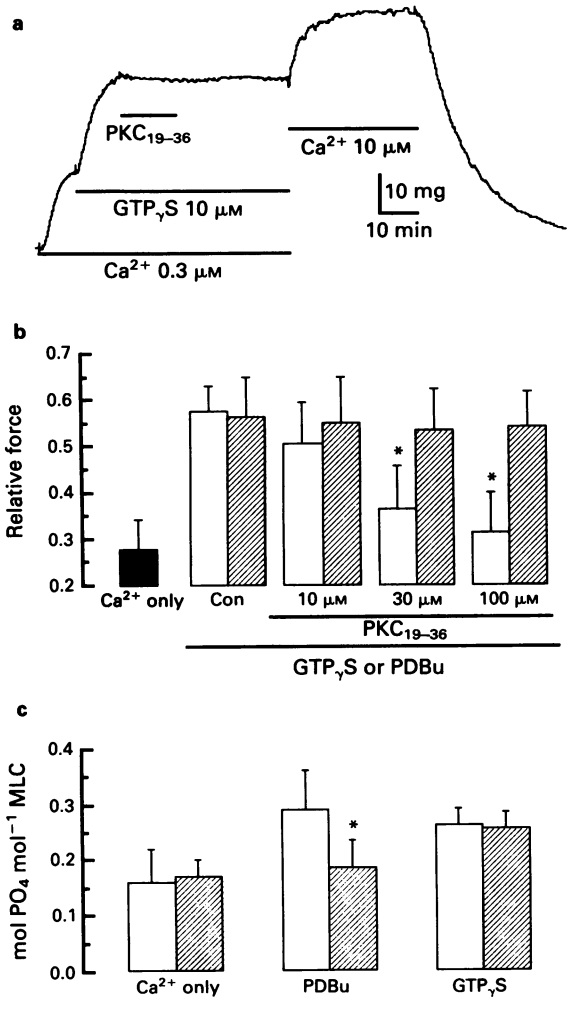

Figure 3 Effects of a protein kinase C inhibitor, PKC_{19-36}, on contraction and myosin light chain (MLC) phosphorylation induced by 0.3 μM Ca^{2+} in the presence of $GTP_\gamma S$ or PDBu in β-escin-skinned smooth muscle strips. (a) Ca^{2+} (0.3 μM) was applied first. Then, $GTP_\gamma S$ (10 μM) was applied on the steady state of contraction induced by 0.3 μM Ca^{2+}. Subsequently, PKC_{19-36} (0.1 mM) was applied during the tonic phase of the contraction induced by Ca^{2+} in the presence of $GTP_\gamma S$. Following washout of PKC_{19-36} by a solution containing Ca^{2+} with $GTP_\gamma S$, 10 μM Ca^{2+} was finally applied to obtain the maximum Ca^{2+}-induced contraction for each strip. Each agent was applied as indicated by the bar. (b) Concentration-dependent effects of PKC_{19-36} on contraction induced by 0.3 μM Ca^{2+} in the presence of 10 μM $GTP_\gamma S$ or 0.1 μM PDBu. Solid column, the contraction induced by 0.3 μM Ca^{2+} alone; open column, in the presence of 0.1 μM PDBu; hatched column, in 10 μM $GTP_\gamma S$. PKC_{19-36} was applied cumulatively from low to high concentration on the steady state contraction induced by 0.3 μM Ca^{2+} in the presence of either PDBu or $GTP_\gamma S$. The maximum amplitude of contraction induced by 10 μM Ca^{2+} was normalized as a relative force of 1.0 for each strip. (c) Effects of PKC_{19-36} on MLC-phosphorylation induced by 0.3 μM Ca^{2+} in the presence or absence of either 10 μM $GTP_\gamma S$ or 0.1 μM PDBu. Open column, control; hatched column, in the presence of 0.1 mM PKC_{19-36}. Results shown are each the mean of 4 observations with s.d. *Significant difference from the corresponding control. For abbreviations, see text.

$GDP_\beta S$ (1 mM) slightly inhibited the contraction induced by 0.3 μM Ca^{2+} and greatly attenuated the $GTP_\gamma S$-induced enhancement of this contraction (Figure 5a and b). Neomycin interacts with polyphosphoinositides and inhibits the activation of phospholipase C (Cockcroft & Gomperts, 1985; Kobayashi *et al.*, 1989), but at 0.1 mM it did not modify the contraction induced by 0.3 μM Ca^{2+} in the presence or absence of $GTP_\gamma S$ (Figure 5c). The extent of the $GTP_\gamma S$-induced enhancement of the Ca^{2+}-induced contraction was 2.2 ± 0.2 times control in the presence of neomycin ($n = 4$) and 2.2 ± 0.3 times control in its absence ($n = 4$).

The effects of 1 μM PDBu and 30 μM $GTP_\gamma S$ were observed on the contraction induced by 0.3 μM Ca^{2+} in the presence of either 0.1 μM PDBu or 30 μM $GTP_\gamma S$. PDBu (1 μM) did not modify the amplitude of Ca^{2+}-induced contraction in the presence of either 0.1 μM PDBu (1.03 ± 0.04 times control, $n = 3$) or 30 μM $GTP_\gamma S$ (1.04 ± 0.05 times control, $n = 4$). While, $GTP_\gamma S$ slightly enhanced the amplitude of Ca^{2+}-induced contraction in the presence of PDBu (1.27 ± 0.08 times control, $n = 4$). The maximum amplitude of contraction induced by 0.3 μM Ca^{2+} in the presence of 30 μM $GTP_\gamma S$ (2.98 ± 0.42 times the amplitude of contraction induced by 0.3 μM Ca^{2+} alone, $n = 3$) was similar to that obtained in the presence of $GTP_\gamma S$ with PDBu (2.78 ± 0.38 times the amplitude of contraction induced by 0.3 μM Ca^{2+} alone, $n = 4$).

Discussion

Myosin light chain (MLC) phosphorylation by the Ca^{2+}-calmodulin-MLC-kinase complex plays a pivotal role in the initiation of smooth muscle contraction (Hartshorne, 1987; Itoh *et al.*, 1989). In the last few years, many reports have indicated that agonists increase the sensitivity of the contractile proteins to Ca^{2+} in intact and skinned vascular smooth muscle (Morgan & Morgan, 1984; Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; Rembold, 1990; Hori *et al.*, 1992). Agonist-induced Ca^{2+}-sensitization of the contractile proteins requires GTP and is inhibited by $GDP_\beta S$ in chemically skinned smooth muscle, suggesting that G-proteins may play a role in this sensitization (Nishimura *et al.*, 1988; Kitazawa *et*

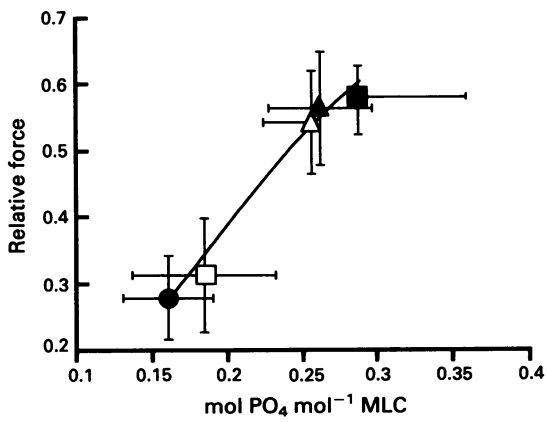

Figure 4 Effects of PKC_{19-36} on the relationship between relative force and myosin light chain (MLC) phosphorylation in the presence and absence of either $GTP_\gamma S$ or PDBu in β-escin-skinned smooth muscle strips. (●) Ca^{2+} (0.3 μM) alone; (△ ▲) in the presence of 10 μM $GTP_\gamma S$ without (▲) or with (△) 0.1 mM PKC_{19-36}; (□ ■) in 0.1 μM PDBu without (■) or with (□) 0.1 mM PKC_{19-36}. The data shown in Figure 3 were used for this analysis and the curve was obtained by fitting the data points by a non-linear least-squares method using the parameters obtained in Figure 2 ($r = 0.98$). The maximum amplitude of contraction induced by 10 μM Ca^{2+} was normalized as a relative force of 1.0 for each strip. Results shown are each the mean of 4 observations with s.d. For abbreviations, see text.

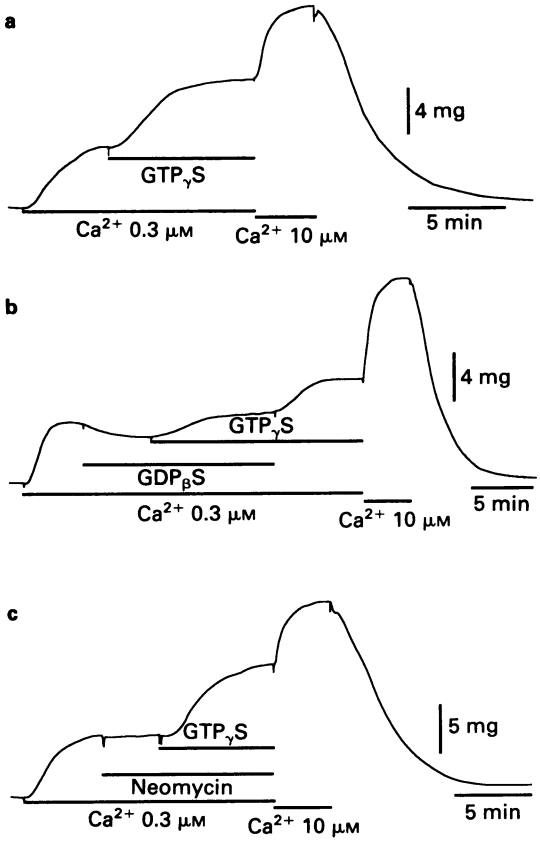

Figure 5 Effects of $GDP_\beta S$ and neomycin on contraction induced by 0.3 μM Ca^{2+} in the presence of $GTP_\gamma S$ in β-escin-skinned smooth muscle strips. After the muscle strip had been skinned by an application of 30 μM β-escin for 25 min, 0.3 μM Ca^{2+} was applied. In (a) 10 μM $GTP_\gamma S$ was then applied on the steady state of the contraction induced by 0.3 μM Ca^{2+}. Finally, 10 μM Ca^{2+} was applied to obtain the maximum Ca^{2+}-induced contraction for that strip. In (b) $GDP_\beta S$ (1 mM) was present for 8 min before application of 10 μM $GTP_\gamma S$ in a solution containing 0.3 μM Ca^{2+}. $GTP_\gamma S$ was then applied in a solution containing 0.3 μM Ca^{2+} with or without $GDP_\beta S$. In (c) 0.1 mM neomycin was applied on the Ca^{2+}-induced steady state contraction and 10 μM $GTP_\gamma S$ then applied in the presence of 0.3 μM Ca^{2+} and neomycin. Each agent was applied as indicated by the bar. The results shown are typical traces and were reproducible in another 2 strips. For abbreviations, see text.

al., 1989). It was recently hypothesized that, in smooth muscle, agonists acting together with GTP or $GTP_\gamma S$ inhibit MLC-phosphatases through the activation of unidentified G-proteins and thus enhance Ca^{2+}-induced MLC-phosphorylation, causing an increase in the amplitude of the Ca^{2+}-induced contraction (Kitazawa *et al.*, 1991; Kubota *et al.*, 1992). In the present experiments on β-escin-skinned smooth muscle, $GTP_\gamma S$ increased both the contraction and the MLC-phosphorylation induced by low concentrations of Ca^{2+} (0.03–1 μM), but not by the highest concentration of Ca^{2+} (10 μM), and this action of $GTP_\gamma S$ was attenuated by $GDP_\beta S$. Since the relationship between force and MLC-phosphorylation was not modified by $GTP_\gamma S$, these results suggest that $GTP_\gamma S$ activates G-proteins and consequently increases the MLC-phosphorylation that occurs in response to Ca^{2+}, thus causing the increase in the Ca^{2+}-induced contraction in β-escin-skinned smooth muscle of the rabbit mesenteric artery.

It is known that agonists induce hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) through activation of heterotrimeric G-proteins, leading to generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The former contributes to the agonist-induced Ca^{2+} release from the Ca^{2+}-storage sites and the latter activates protein kinase C (PKC). For the following reasons it seems likely that $GTP_\gamma S$ activates PKC through the production of DAG and then increases the sensitivity of the contractile proteins to Ca^{2+}: (1) $GTP_\gamma S$ itself produces hydrolysis of PIP_2 through activation of heterotrimeric G-proteins in vascular smooth muscle (Sasaguri *et al.*, 1985). (2) Phorbol esters (TPA and PDBu), activators of PKC (Nishizuka, 1984), increase the contraction, MLC-phosphorylation and unloaded shortening velocity in the presence of 0.3 μM Ca^{2+} (Chatterjee & Tejada, 1986; Itoh *et al.*, 1986b; Fujiwara *et al.*, 1988). (3) PDBu shifts both the Ca^{2+}-force and Ca^{2+}-MLC-phosphorylation curves to the left in β-escin-skinned muscle strips (in the present experiments). In the present experiments, PKC_{19-36} (a novel peptide inhibitor of PKC, House & Kemp, 1987) itself modified neither the contraction nor the MLC-phosphorylation induced by 0.3 μM Ca^{2+}, but it completely inhibited the PDBu-induced enhancement of both the Ca^{2+}-induced responses, suggesting that PKC_{19-36} selectively inhibits PKC, but not MLC-kinase in β-escin-skinned smooth muscle. Under our experimental conditions, PKC_{19-36} failed to modify the $GTP_\gamma S$-induced enhancement of either the Ca^{2+}-induced contraction or MLC-phosphorylation, suggesting that PKC may not play a primary role in the $GTP_\gamma S$-induced enhancement of responses induced by Ca^{2+} in β-escin-skinned smooth muscle of the rabbit mesenteric artery. This hypothesis is also supported by the finding that neomycin did not modify the $GTP_\gamma S$-induced enhancement of the contraction induced by 0.3 μM Ca^{2+} in the skinned strips. Since neomycin interacts with polyphosphoinositides and inhibits the production of both IP_3 and DAG through its inhibitory action on phospholipase C (Cockcroft & Gomperts, 1985; Kobayashi *et al.*, 1989), this agent would be expected to inhibit the $GTP_\gamma S$-action if PKC activated by the synthesized DAG played a role in the $GTP_\gamma S$-induced response in the skinned muscles. Indeed, in preliminary experiments, we found that under the same experimental conditions, neomycin completely blocked the endothelin-1-induced increase in the sensitivity of the contractile proteins to Ca^{2+}. However, we cannot deny the possibility that our results with β-escin-skinned smooth muscle may underestimate the role of PKC in the $GTP_\gamma S$-induced enhancement of the Ca^{2+}-induced responses if some unidentified proteins that contribute to the action of $GTP_\gamma S$ leak out from skinned muscle cells.

$GTP_\gamma S$ slightly enhanced the contraction induced by 0.3 μM Ca^{2+} in the presence of PDBu, but PDBu did not modify the Ca^{2+}-induced contraction in the presence of $GTP_\gamma S$. The maximum amplitude of Ca^{2+}-induced contraction in the presence of 30 μM $GTP_\gamma S$ was similar to that in the presence of $GTP_\gamma S$ with PDBu, indicating that $GTP_\gamma S$ is a more potent stimulant than PDBu in enhancing the Ca^{2+}-induced contraction and there is no possible additivity on the actions between PDBu and $GTP_\gamma S$. These results suggest that PDBu and $GTP_\gamma S$ may act on common targets through activations of different signalling pathways.

There are two major superfamilies of G-proteins involved in signal transduction: one is a superfamily of heterotrimeric G-proteins acting as transducers for membrane receptors and the other is the superfamily including ras p21 and its related small G-proteins (Bourne *et al.*, 1990). It was recently suggested that small G-proteins, like rho p21 (Hirata *et al.*, 1992) and/or ras p21 (Pfitzer & Satoh, 1993), may contribute to the agonist-induced increase in the sensitivity of the contractile proteins to Ca^{2+}. Since neomycin did not modify the $GTP_\gamma S$-induced enhancement of the Ca^{2+}-induced contraction in β-escin-skinned muscles, the present results may also suggest that small G-proteins, rather than heterotrimeric G-proteins, contribute to the $GTP_\gamma S$-induced increase in myofilament Ca^{2+}-sensitivity in skinned smooth muscle.

Recently, Steusloff *et al.* (1993) reported that in α-toxin-skinned longitudinal smooth muscle of the guinea-pig ileum, carbachol together with GTP enhances the contraction induced by submaximal concentration of Ca^{2+} with corresponding

increase in tyrosine phosphorylation of unidentified proteins. The result suggests that the other serine/threonine kinases, such as mitogen-activated protein (MAP) kinase, may also play a role on $GTP_\gamma S$-induced enhancement of Ca^{2+}-induced contraction. This remains to be clarified.

The phosphorylation of heavy meromyosin by PKC does not stimulate actin-activated myosin MgATPase activity *in vitro*, but rather reduces the rate of phosphorylation of MLC by MLC-kinase causing a decrease in the actin-activated MgATPase activity of heavy meromyosin which has been pre-phosphorylated by MLC-kinase (Nishikawa *et al.*, 1984). In glycerin-treated porcine carotid artery, the phosphorylation of MLC by PKC neither provokes contraction nor modifies the contraction induced by Ca^{2+}-calmodulin (Sutton & Haeberle, 1990), suggesting that MLC-phosphorylation by PKC inhibits rather than enhances Ca^{2+}-induced smooth muscle contraction. In the present experiments, PDBu enhanced both the Ca^{2+}-induced contraction and MLC-phosphorylation without a change in the relationship between force and MLC-phosphorylation in β-escin-skinned muscle. Under the conditions of our experiments, Ca^{2+} (0.03–1 μM) increased the monophosphorylated form of MLC in a concentration-dependent manner. Further, a peptide inhibitor of PKC, PKC_{19-36}, inhibited the PDBu-induced enhancement of both the Ca^{2+}-induced contraction and MLC-phosphorylation without a change in their relationship. Furthermore, *in vitro* phosphorylation of MLC-kinase by PKC causes a decrease in its affinity for the Ca^{2+}-calmodulin complex (Ikebe *et al.*, 1985). Although we did not examine whether or not PKC directly phosphorylates MLC, all these results suggest that PKC may indirectly activate the phosphorylation of MLC by MLC-kinase, possibly through an inhibition of MLC-phosphatases.

It was recently hypothesized that PKC may increase Ca^{2+}-induced contractions though a phosphorylation of caldesmon (Walsh, 1990) and/or calponin (Nakamura *et al.*, 1993) which are thin-filament-linked regulatory proteins in smooth muscle. However, we found that PDBu enhanced the Ca^{2+}-induced contraction in parallel with an increase in the phosphorylation of MLC. Thus, the present result does not support the above hypothesis, although we cannot deny the possibility of a minor contribution of these thin-filament-linked regulator proteins to the PDBu-induced response.

In conclusion, $GTP_\gamma S$ increases the phosphorylation of MLC induced by lower concentrations of Ca^{2+} through the activation of a PKC-independent mechanism and thus enhances the Ca^{2+}-induced contraction.

We thank Dr R.J. Timms for the language editing. We thank Dr J.T. Stull (Univ. of Texas, Dallas, TX, U.S.A.) for providing an antibody against bovine tracheal myosin light chain. This work was partly supported by a Grant-In-Aid from the Ministry of Education of Japan.

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(Received June 28, 1993
Revised September 7, 1993
Accepted September 23, 1993)

Br. J. Pharmacol. (1994), **111**, 318–324

Differential effects of centrally-active antihypertensives on 5-HT_{1A} receptors in rat dorso-lateral septum, rat hippocampus and guinea-pig hippocampus

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1 The electrophysiological responses elicited by 5-hydroxytryptamine$_{1A}$- (5-HT_{1A}) receptor agonists in rat and guinea-pig CA1 pyramidal neurones and rat dorso-lateral septal neurones were compared *in vitro* by use of conventional intracellular recording techniques.

2 In the presence of 1 μM tetrodotoxin (TTX), to prevent indirect effects, 5-HT, N,N-dipropyl-5-carboxamidotryptamine (DP-5-CT) and 8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT) hyperpolarized the neurones from rat and guinea-pig brain.

3 The hypotensive drug flesinoxan, a selective 5-HT_{1A} receptor agonist, hyperpolarized neurones in all three areas tested; however, another hypotensive agent with high affinity at 5-HT_{1A}-receptors, 5-methyl-urapidil, hyperpolarized only the neurones in rat hippocampus and septum.

4 In guinea-pig hippocampal neurones, 5-methyl-urapidil behaved as a 5-HT_{1A}-receptor antagonist.

5 The relative efficacies (5-HT = 1) of DP-5-CT, 8-OH-DPAT, flesinoxan and 5-methyl-urapidil at the three sites were: rat hippocampus, 1.09, 0.7, 0.5 and 0.24; rat septum, 0.88, 0.69, 0.82 and 0.7; guinea-pig hippocampus, 1.0, 0.69, 0.89 and 0, respectively.

6 It is concluded that the hypotensive agents flesinoxan and 5-methyl-urapidil appear to have different efficacies at 5-HT_{1A} receptors located in different regions of the rodent brain. Whether these regional and species differences arise from receptor plurality or variability in intracellular transduction mechanisms remains to be elucidated.

Keywords: 5-Hydroxytryptamine (5-HT); 5-HT_{1A}-receptors; N,N-dipropyl 5-carboxamidotryptamine (DP-5-CT); 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT); flesinoxan; 5-methyl-urapidil; hippocampus; lateral septum

Introduction

Six subtypes of 5-hydroxytryptamine$_1$ (5-HT_1) type receptors have been identified in the mammalian central nervous system (Humphrey *et al.*, 1993) and of these, the 5-HT_{1A} receptors have been studied in most detail. Upon activation by 5-HT or synthetic agonists, these G-protein-coupled receptors decrease the activity of adenylate cyclase (DeVivo & Maayani, 1986) and inhibit neuronal activity by increasing membrane conductance to potassium ions (Andrade & Nicoll, 1987a; Colino & Halliwell, 1987). The pharmacology of 5-HT_{1A} receptors is of particular interest since it appears that the anxiolytic drug, buspirone, and the antihypertensive agents, urapidil and flesinoxan, may exert all or part of their beneficial effects via these receptors (see Taylor, 1988; and Saxena & Villalon, 1990, for an overview).

Exactly how and where these compounds act is unknown, but it seems likely that their anxiolytic actions are due to modulation of the activity of 5-HT containing neurones in the raphe nuclei and/or via actions on 5-HT_{1A} receptors located on neurones in the limbic system (Traber & Glaser, 1987). This hypothesis has received considerable support from the results of electrophysiological studies showing that buspirone and other putative anxiolytics such as ipsapirone, gepirone and MDL 73,005EF, do indeed have agonist, partial agonist or antagonist effects at 5-HT_{1A}-receptors on raphe, hippocampal and septal neurones (Andrade & Nicoll, 1987b; Sprouse & Aghajanian, 1987; van den Hooff & Galvan, 1992). In addition, long-term changes in limbic system function have been observed following chronic treatment with anxiolytics of this type (Blier & de Montigny, 1990).

Interest in the anti-hypertensive actions of compounds acting at 5-HT_{1A} receptors arose following the discovery that the selective 5-HT_{1A} receptor ligand, 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT; Gozlan *et al.*, 1983) lowers blood pressure in the rat and the cat via an action on the central nervous system (Fozard & Ramage, 1984; Gradin *et al.*, 1985; Fozard *et al.*, 1987). Two other compounds have been identified that appear to act in such a novel manner, urapidil (Sanders & Jurna, 1985; Ramage, 1986; Sanders *et al.*, 1990) and flesinoxan (Wouters *et al.*, 1988). Whilst urapidil itself had a relatively low affinity for 5-HT_{1A} receptors (Fozard & Mir, 1987), 5-methyl-urapidil and flesinoxan have affinities in the low nanomolar range and are potent hypotensive agents in animals (Gross *et al.*, 1987; Mandal *et al.*, 1990; Wouters *et al.*, 1988). More recently, it has been reported that flesinoxan also has anxiolytic and perhaps even anti-depressant activity (Olivier *et al.*, 1991; Schipper *et al.*, 1991).

Although there have been several detailed descriptions of the actions of these antihypertensives on the cardiovascular system and the behaviour of animals (Ramage 1990; Schipper *et al.*, 1991), there are little data available regarding their actions on single neurones in the mammalian brain. The present experiments were therefore performed to compare the actions of the 5-HT_{1A}-receptor agonists 5-HT, N,N-dipropyl-5-carboxamido-tryptamine (DP-5-CT) and 8-OH-DPAT with those of flesinoxan and 5-methyl-urapidil. Preliminary accounts of some of these results have been presented to the Deutsche Gesellschaft für Pharmakologie und Toxikologie (Boeijinga *et al.*, 1992; Leishman & Galvan, 1993).

Methods

Slice preparation

Male Sprague-Dawley rats and albino guinea-pigs (both species 100–300 g; Charles River, France) were decapitated

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and the brains removed and cooled for 1 min in Krebs solution at 2–4°C. The hippocampi were isolated from the surrounding brain tissue and 400 μm slices were cut on a McIlwain tissue chopper and stored prior to recording in a small chamber filled with oxygenated Krebs solution (22–26°C). To prepare septal slices, a block of rat forebrain tissue containing the septum was mounted on a small stage and frontal slices (ca. 400 μm) were cut with a Vibroslice (Campden Instruments, London). The slices were stored in a small holding chamber filled with oxygenated Krebs solution at 36°C.

For recording purposes, slices were transferred to a perspex recording chamber, where they were sandwiched between two nylon nets and continuously superfused (6–8 ml min^{-1}) with a modified Krebs solution at 34°C. The composition of the modified Krebs solution was (mM): NaCl 119, KCl 3.0, NaH_2PO_4 1.2, $MgCl_2.6H_2O$ 1.2, $CaCl_2.2H_2O$ 2.0, $NaHCO_3$ 25 and D-glucose 10; all solutions were continuously gassed with 95% O_2/5% CO_2. Compounds were applied in fixed concentrations via the superfusate; due to the design of the perfusion system, there was a delay of about 30–40 s before the new solution arrived in the recording chamber.

Intracellular recording

Intracellular recordings were made with glass microelectrodes filled with 3 M KCl (70 to 100 MΩ). Electrical signals were amplified with a bridge balance amplifier, monitored on a digital oscilloscope and recorded on a chart recorder and a personal computer running pClamp software (Axon Instruments, Foster City, CA, U.S.A.). The neurones included in this study exhibited stable resting membrane potentials more negative than − 55 mV and membrane resistances greater than 80 MΩ for septal neurones and ⩾ 20 MΩ for hippocampal neurones. In addition, cells not exhibiting a 'robust' response to 5-HT were not further studied. In practice this meant that the minimum acceptable membrane potential changes were as follows (applied concentrations of 5-HT in parentheses): rat septum ⩾ 7 mV (10 μM), rat and guinea-pig hippocampus, both ⩾ 4 mV (30 μM). In all of the experiments described, tetrodotoxin (TTX; 1 μM) was included in the perfusion medium to block action potential-dependent neurotransmitter release and thus preclude indirect effects of the compounds under study.

Analysis of the results

The response to an agonist was defined as the maximal change in membrane potential attained during superfusion (1–10 min) of the compound. Unless otherwise stated, all numerical data are given as the mean ± s.e.mean. 2-tailed, paired and unpaired *t* tests or ANOVA were calculated as necessary using the programme Instat (Graphpad Software, San Diego, CA, U.S.A.).

Materials

Drug sources were as follows: 5-hydroxytryptamine creatinine sulphate complex and tetrodotoxin (Sigma, France), flesinoxan (Duphar, The Netherlands), 5-methyl-urapidil, N,N-dipropyl-5-carboxamidotryptamine maleate and (±)-8-hydroxy-2(di-n-propylamino)tetralin HBr (Reseach Biochemicals Incorporated, Massachusetts, U.S.A.).

Results

The results described are based on stable intracellular recordings from 77 neurones located in the dorsolateral part of the septum, 51 rat CA1 pyramidal neurones and 44 guinea–pig CA1 pryamidal neurones. The mean resting membrane potential and membrane resistance of these cells are shown in Table 1. The guinea-pig hippocampal neurones had a significantly more negative membrane potential than rat hippocampal CA1 neurones and rat septal neurones had a higher membrane resistance than the hippocampal neurones of both species.

Actions of 5-HT

Bath application of 5-HT (0.3–100 μM) hyperpolarized the membrane potential and reduced the membrane resistance in a concentration-dependent manner in all three brain areas tested (Figures 1 and 2a). The observed hyperpolarizations peaked within about 1–1.5 min and the membrane potential repolarized towards control levels following return to drug-free Krebs solution. The maximum hyperpolarizations and changes in membrane resistance induced by 5-HT are shown in Table 1 as are the reversal potentials measured from current-voltage relationships constructed before and during the application of 5-HT. 5-HT-induced hyperpolarizations in rat septal neurones were significantly larger than those observed in either rat or guinea-pig hippocampal CA1 pyramidal cells. The estimated reversal potentials of between

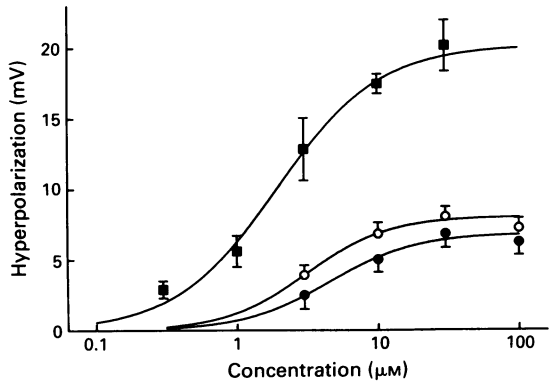

Figure 1 Concentration-response curves for 5-hydroxytryptamine (5-HT) measured in neurones in the rat dorso-lateral septal nucleus (■; number of determinations in different cells, $n = 7–66$), the rat CA1 area (●; $n = 8–17$) and the guinea-pig CA1 area (○; $n = 4–6$). The applied concentrations (μM) are on the abscissa scale and the ordinate scale shows the hyperpolarization in mV (mean and s.e.mean shown).

Table 1 Passive membrane properties and the changes induced by 5-hydroxytryptamine (5-HT) in rat and guinea-pig hippocampal CA1 neurones and rat dorso-lateral septal neurones *in vitro*

	Control solution		*5-HT containing solution*		
	Em (mV)	*Rm (MΩ)*	*ΔEm (mV)*	*ΔRm (%)*	*Erev (mV)*
Rat hippocampus CA1	− 71 ± 5* (51)	41 ± 10* (51)	− 7 ± 0.5* (34)	30 ± 1* (8)	− 84 ± 3* (6)
Guinea-pig hippocampus CA1	− 77 ± 4*† (44)	50 ± 11* (44)	− 6.5 ± 0.5* (44)	30 ± 1* (34)	− 97 ± 1† (7)
Rat septum	− 66 ± 5 (77)	136 ± 55 (77)	− 20 ± 2 (8)	72 ± 4 (8)	− 98 ± 2 (4)

Resting membrane potentials (Em) and membrane resistances (Rm) of 3 types of limbic neurones measured in control Krebs solution (means ± s.d.; number of cells in parentheses). The changes (ΔEm and ΔRm) as well as the reversal potentials (Erev) measured during superfusion of 30 μM 5-HT are given as means ± s.e.means. *$P<0.01$ compared to rat septum. †$P<0.01$ compared to rat hippocampus; one way ANOVA, Bonferroni corrected *P* values.

– 84 and – 98 mV are consistent with the hypothesis that the underlying conductance change during the action of 5-HT is due to the opening of potassium channels. A contribution from a change in chloride ion conductance cannot be excluded, however under the present recording conditions (employing 3 M KCl-filled microelectrodes), this would be most unlikely (Newberry & Nicoll, 1985). The complete concentration-response relationships are shown in Figure 1 and the estimated EC_{50} values for the pooled data were 1.9, 3.1 and 4.5 μM, in rat septal, rat hippocampal and guinea-pig hippocampal neurones, respectively.

The responses to 5-HT in rat and guinea-pig CA1 pyramidal neurones differed significantly in their time-courses, particularly in the rates of repolarization (see Figure 2a). The time to 50% recovery in rat hippocampal neurones was 79 ± 6 s ($n = 25$) and 308 ± 16 s ($n = 25$) in guinea-pig hippocampal neurones ($P < 0.01$, unpaired 2-tailed t test). In the rat hippocampus the 5-HT-induced hyperpolarizations were often (19 out of 41 cells) followed by a depolarization and an increase in membrane resistance (Figure 2a; Andrade & Nicoll, 1987a; Colino & Halliwell, 1987). However, in the guinea-pig CA1 neurones, no such changes in membrane potential and resistance were observed ($n = 33$).

Actions of synthetic 5-HT_{1A} agonists

In rat and guinea-pig hippocampus, DP-5-CT, 8-OH-DPAT, flesinoxan and 5-methyl-urapidil were applied to separate groups of neurones at 3 or 30 μM. This technique was adopted as (1) the hyperpolarizations were only slowly reversible and (2) the slow onset of action and the relatively small maximum response ($\leqslant 10$ mV) rendered cumulative drug addition impracticable. In rat septal neurones, full concentration-response relationships were determined by cumulative addition of agonists.

Figure 2b, c illustrates examples of the effects of DP-5-CT and 8-OH-DPAT in rat and guinea-pig CA1 neurones. Each compound induced a hyperpolarization and a reduction in membrane resistance, which were poorly reversible. The onset of action of 8-OH-DPAT was clearly slower than that of DP-5-CT or 5-HT. The antihypertensive agents, flesinoxan and 5-methyl-urapidil, hyperpolarized rat septal and hippocampal neurones; however, only flesinoxan hyperpolarized guinea-pig CA1 neurones (Figure 3). The maximum hyperpolarizations measured during application of DP-5-CT, 8-OH-DPAT, flesinoxan and 5-methyl-urapidil to the three types of limbic neurones are shown in Table 2. Reversal potentials were estimated from current-voltage relationships constructed before and during superfusion with the various agonists. The values obtained (– 84 to – 106 mV; Table 2) are similar to those found during the action of 5-HT (Table 1) and suggest that the synthetic agonists also increase membrane conductance for potassium ions.

In the guinea-pig hippocampus, application of 3 μM ($n = 5$) and 30 μM ($n = 5$) 5-methyl-urapidil failed to elicit a hyperpolarization or a reduction in membrane resistance, indicating that in these cells this compound has no important agonist activity. Indeed, further experiments demonstrated that 5-methyl-urapidil (3 μM) antagonized the action of 5-HT (Figure 4). Control hyperpolarizations elicited by 30 μM 5-HT were – 5.6 ± 0.6 mV and this was reduced to – 1.6 ± 0.7 mV ($n = 4$; $P < 0.01$, paired, 2-tailed t test) following superfusion with 3 μM 5-methyl-urapidil.

Figure 5 illustrates the concentration-response relationships for flesinoxan and 5-methyl-urapidil in rat dorso-lateral septal neurones. The estimated EC_{50} values from the pooled data for these compounds are 25 and 45 nM, respectively. In 2 of the 9 cells responsive to 5-HT, application of 10 μM 5-methyl-urapidil was without effect on the membrane potential; these cells were not incluced in the curves shown in Figure 5.

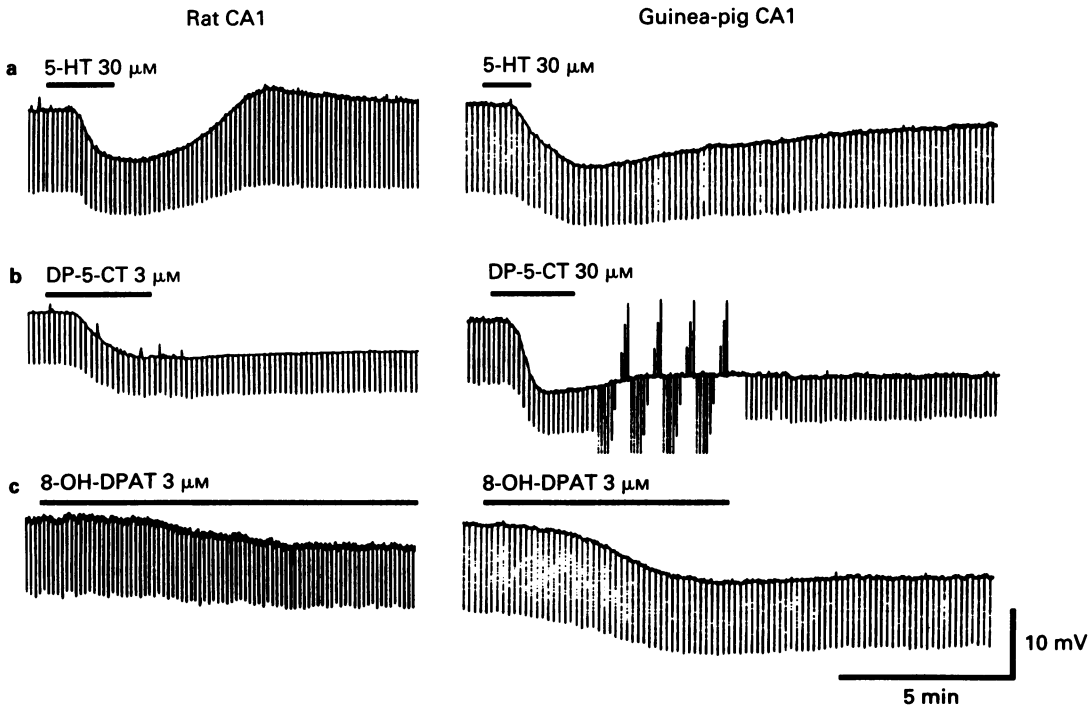

Figure 2 Comparison of the actions of (a) 30 μM 5-hydroxytryptamine (5-HT), (b) 3 and 30 μM N,N-dipropyl-5-carboxamidotryptamine (DP-5-CT) and (c) 3 μM 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in different neurones in the rat (left column) and in the guinea-pig (right column) hippocampal CA1 area. Bath application of the compounds (horizontal bars) hyperpolarized the membrane potential and decreased membrane resistance. The transient downward deflections are membrane responses to intracellular injection of constant current pulses (300 ms, 0.1 Hz, 0.15–0.4 nA). The 30–40 s delay in the perfusion line (see methods) has not been corrected for, in this and all subsequent figures.

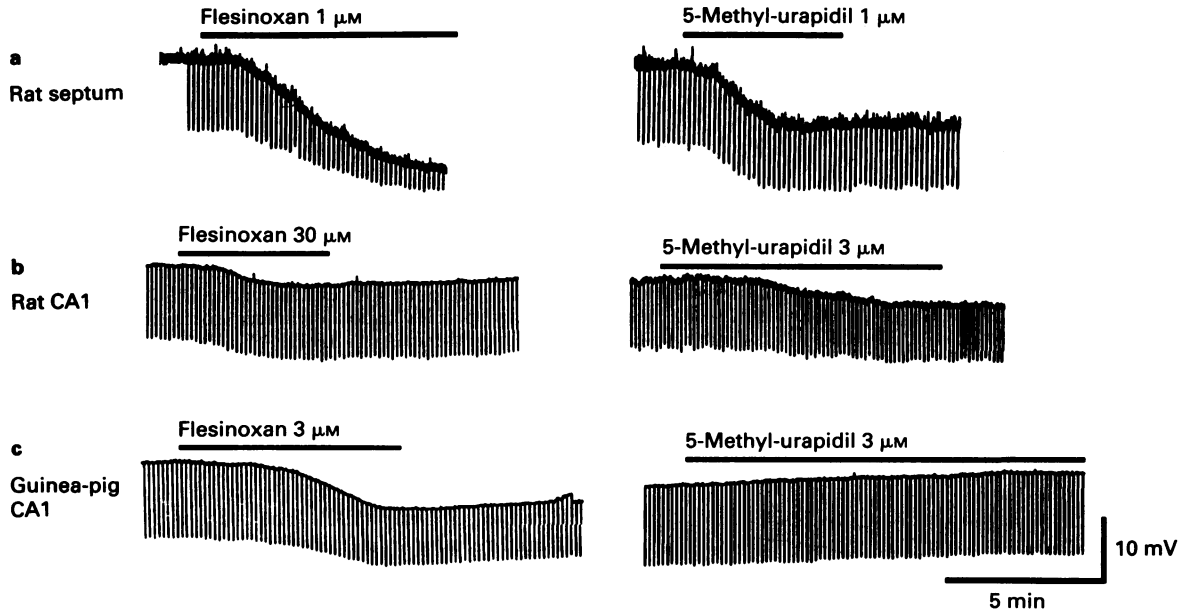

Figure 3 Comparison of the actions of flesinoxan (1–30 μM) and 5-methyl-urapidil (1–3 μM) on the membrane potential and membrane resistance of neurones in (a) the rat dorso-lateral septal nucleus, (b) the rat CA1 hippocampal area and (c) the guinea-pig CA1 hippocampal area. Note that 5-methyl-urapidil was without effect on the guinea-pig hippocampal neurone.

Table 2 Maximal hyperpolarizations evoked by N,N-dipropyl-5-carboxamido tryptamine (DP-5-CT), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), flesinoxan and 5-methyl-urapidil in rat and guinea-pig hippocampal CA1 neurones and rat dorso-lateral septal neurones

	DP-5-CT		*8-OH-DPAT*		*Flesinoxan*		*5-Methyl-urapidil*	
	ΔEm	*Erev*	*ΔEm*	*Erev*	*ΔEm*	*Erev*	*ΔEm*	*Erev*
Rat hippocampus CA1	−7.5 ± 1.3 (6)	−92 ± 4 (3)	−4.9 ± 0.4 (4)	−8.9 (1)	−3.3 ± 0.6 (7)	−93 ± 8 (3)	−1.8 ± 0.3 (9)	−8.4 (1)
Guinea-pig hippocampus CA1	−6.4 ± 1.7 (30)	−100 ± 4 (5)	−5.2 ± 0.7 (9)	−99 ± 7 (3)	−7.1 ± 0.6 (5)	−104 ± 2 (3)	No effect (10)	
Rat septum	−17.5 ± 1.4 (10)*	−106 (1)	−13.8 ± 0.6 (8)*	ND	−16.4 ± 1.4 (5)	−104 (2)	−14 ± 1.2 (5)	ND

Changes in membrane potential (ΔEm) and reversal potentials (Erev) are given as means ± s.e.means, number of cells in parentheses; ND = not determined. The concentrations of agonists applied to the rat hippocampus, the guinea-pig hippocampus and the rat septum were as follows, DP-5-CT: 3, 30 and 1 μM; 8-OH-DPAT: all 3 μM; flesinoxan: 30, 30 and 1 μM and 5-methyl-urapidil: 30, 30 and 10 μM, respectively. *Data taken from van den Hooff & Galvan (1992).

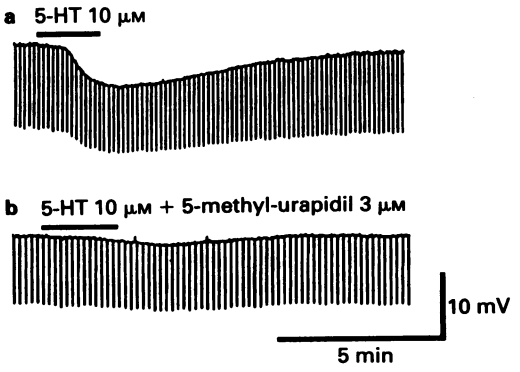

Figure 4 (a) A hyperpolarization evoked by 10 μM 5-hydroxytryptamine (5-HT) in a single guinea-pig hippocampal CA1 neurone. (b) The response recorded from the same cell after 60 min superfusion with 3 μM 5-methyl-urapidil; the 5-HT-induced hyperpolarization was blocked. The electronic potentials were elicited by constant current pulses (− 0.25 nA, 250 ms, 0.1 Hz). Note that the response of this cell to 5-HT in control solution was slightly larger than average (see text and Table 1).

5-Methyl-urapidil is a potent ligand at 5-HT$_{1A}$ receptors and is also an effective α_1-adrenoceptor antagonist (Hanft & Gross, 1989). In order to exclude the possibility that the hyperpolarizations elicited by this compound were due to α_1-antagonist activity, the actions of the α_1-receptor antagonist, prazosin, were tested in the septal neurones. In 4 cells, a 5–8 min application of 3 μM prazosin failed to evoke a significant change in membrane potential (− 0.3 ± 0.2 mV).

Relative efficacies of 5-HT$_{1A}$ ligands

The concentration-response relationships for the hyperpolarizations elicited by 5-HT demonstrate that 30 μM appears to be a just-maximal concentration in hippocampal and septal neurones. To determine the efficacies of the various 5-HT$_{1A}$-receptor agonists, hippocampal neurones were first exposed to 30 μM 5-HT and then, following complete recovery, to a 3 μM concentration of synthetic agonist and in a separate series of experiments to 30 μM 5-HT followed by 30 μM synthetic agonist. The largest hyperpolarization induced by the synthetic agonist was taken as the maximum provided that (a) it was not significantly different from

Table 3 Relative efficacies of 5-HT_{1A}-receptor agonists measured in three types of rodent limbic neurones *in vitro*

	DP-5-CT	*8-OH-DPAT*	*Flesinoxan*	*5-Methyl-urapidil*
Rat hippocampus CA1	1.09 ± 0.07 (6)*	0.7 ± 0.08 (4)	0.5 ± 0.11 (7)	0.24 ± 0.04 (9)
Guinea-pig hippocampus CA1	1.0 ± 0 (4)*	0.69 ± 0.07 (9)	0.89 ± 0.08 (6)*	0 (5)
Rat septum	0.88†	0.69	0.82†	0.7

Relative efficacies were estimated in single cells in the rat and guinea-pig hippocampus and the results are expressed as the means ± s.e.means (n). Results from the rat septum were estimated from mean concentration-response curves. *$P > 0.05$ compared to mean of 1 (One-sample Student's t test) and †$P > 0.05$ mean maximum hyperpolarization compared to mean maximum 5-HT hyperpolarization (ANOVA). DP-5CT, N,N-dipropyl-5-carboxamido tryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propyl-amino)tetralin.

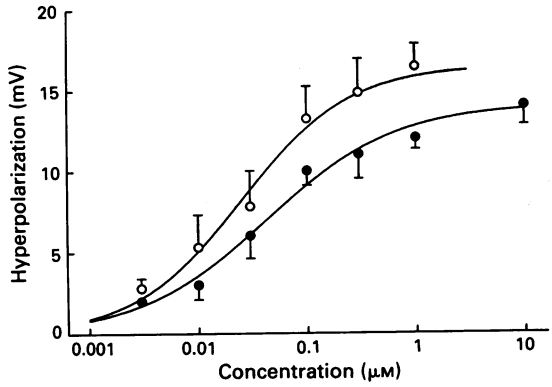

Figure 5 Concentration-response curves for flesinoxan (○; $n = 4-5$) and 5-methyl-urapidil (●; $n = 3-7$) measured in the rat dorso-lateral septal nucleus. Ordinates and abscissae are as in Figure 1.

the maximum 5-HT response or (b) the hyperpolarization to the higher concentration of agonists was not significantly larger than that to the lower concentration. The relative amplitudes of the 'maximum' hyperpolarizations allowed an estimate of the relative efficacies of the synthetic ligands. In the case of septal neurones the intrinsic activity was determined from the concentration-response curves.

The relative efficacies of the various agonists are shown in Table 3. These estimates show that DP-5-CT is a full agonist and 8-OH-DPAT a strong partial agonist in all three regions. Flesinoxan was effectively a full agonist in the rat septum and the guinea-pig hippocampus but exhibited reduced efficacy in the rat hippocampus. 5-Methyl urapidil behaved as a strong partial agonist in the rat septum, a weak partial agonist in the rat hippocampus and as an antagonist in the guinea-pig hippocampus.

Discussion

The aim of the present experiments was to compare the action of 5-HT and several hypotensive, 5-HT_{1A}-receptor agonists on single neurones in three different brain regions under identical experimental conditions.

5-HT hyperpolarized the three types of neurone under study most probably via an increase in potassium conductance as has been reported previously (Andrade & Nicoll, 1987a; Colino & Halliwell, 1987; Joëls *et al.*, 1987). The hypotensive agents, flesinoxan and 5-methyl-urapidil, also induced concentration-dependent hyperpolarizations of rat septal and rat hippocampal neurones that reversed at potentials close to the predicted potassium equilibrium potential. The time course of the responses to these compounds was similar to that of the known 5-HT_{1A}-receptor agonists, 8-OH-DPAT and DP-5-CT (Andrade & Nicoll, 1987b; van den Hooff & Galvan, 1992). The reasons for the observed prolonged recovery phase are unknown; however, it was interesting to note that in guinea-pig hippocampal neurones, the recovery from 5-HT itself took significantly longer than that observed after activation of 5-HT_{1A}-receptors in rat hippocampus (Andrade & Nicoll, 1987a; Beck *et al.*, 1992), rat septum (Joëls *et al.*, 1987; van den Hooff & Galvan, 1992) and rat dorsal raphe nucleus (Sprouse & Aghajanian, 1987).

In the septal nucleus, the rank order of potency estimated from concentration-response curves was flesinoxan > 5-methyl-urapidil > 5-HT. This is in good agreement with the rank order of affinities of these compounds for 5-HT_{1A}-receptors obtained from radioligland binding studies. The reported pIC_{50} or K_i values are 8.8, 8.4 and 8.0, respectively (Gross *et al.*, 1987; Wouters *et al.*, 1988). In addition, radioligand binding experiments have demonstrated that with the exception of 5-methyl-urapidil, which has high affinity for α_1-adrenoceptors, the 4 synthetic agonists under study are relatively selective for 5-HT_{1A}-receptors (Gross *et al.*, 1987; Wouters *et al.*, 1988; Hoyer, 1991). Since the α_1-adrenoceptor antagonist, prazosin, did not mimic the action of 5-HT on rat septal neurones, it can be reasonably concluded that all of these compounds were acting on the same receptor subtype, namely 5-HT_{1A}-receptors. The availability of potent and selective antagonists at 5-HT_{1A}-receptors, such as WAY 100135 (Fletcher *et al.*, 1993) will possibly allow more precise characterization of these agonists in the future.

The actions of compounds with high affinity for 5-HT_{1A}-receptors has been shown to differ, depending on the neurones tested (Gartside *et al.*, 1990) or even on the level of receptor expression in constructed cell systems (Boddeke *et al.*, 1992). For example, the partial agonists, buspirone, ipsapirone and MDL 73,005EF showed changes in efficacy (relative to 5-HT) from 0 to 80% depending on the level of receptor expression in a cell line expressing human 5-HT_{1A}-receptors (Boddeke *et al.*, 1992). The present experiments also sought to resolve the question of whether these compounds exhibit differences in relative efficacy because (1) there are differences in receptor reserve (Meller *et al.*, 1990), (2) there are differences in coupling efficiency to second messenger systems (Fargin *et al.*, 1989) or (3) there are intrinsic differences in the receptors.

The present results show that in the neurones studied here, 8-OH-DPAT behaved as a strong partial agonist in all three cell types and DP-5-CT was a full agonist in both hippocampal neurones and probably a full agonist in the rat septum. However, the estimates of intrinsic activity show that flesinoxan had a higher efficacy in the guinea-pig hippocampus than in the rat hippocampus, whereas 5-methyl-urapidil showed the reverse behaviour. Furthermore, in the case of 5-methyl-urapidil, the efficacy in the guinea-pig hippocampus was so low as to render the compound an antagonist. It is unclear, at present, how these results could be accounted for by changes in receptor reserve or differences in coupling

efficiency to second messengers, which should affect both compounds in a similar manner. Thus, the actions of flesinoxan and 5-methyl-urapidil tend to support the hypothesis that the guinea-pig and rat hippocampal 5-HT$_{1A}$-receptors are not identical. The human (Fargin *et al.*, 1988) and rat (Albert *et al.*, 1990) 5-HT$_{1A}$-receptors have already been cloned and sequenced, but to the best of our knowledge, this had not been done with guinea-pig tissue. Since it has already been shown that several 5-HT receptors exhibit small sequence differences between species leading to significant changes in pharmacology (Hartig *et al.*, 1992), it would be of great interest to see if this type of interspecies variation can account for the present findings.

8-OH-DPAT, flesinoxan and 5-methyl-urapidil all lower blood pressure in experimental animals (Fozard & Ramage, 1984; Wouters *et al.*, 1988; Mandal *et al.*, 1991), and the structually-related compound, urapidil, is an effective antihypertensive drug in man (Schook *et al.*, 1989). The hypotensive actions of these compounds have been postulated to arise from an action at brain stem neurones containing 5-HT$_{1A}$ receptors (Fozard & Mir, 1987; Gillis *et al.*, 1987). The present results show that 8-OH-DPAT, flesinoxan and 5-methyl-urapidil have direct, potent inhibitory actions on mammalian central neurones mediated via 5-HT$_{1A}$-receptors. If these compounds also hyperpolarize the membrane potential of brain stem medullary neurones, the resulting decrease of tonic drive to spinal preganglionic sympathetic neurones (Ramage, 1990) could contribute to their hypotensive actions.

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(Received July 19, 1993
Revised September 6, 1993
Accepted September 23, 1993)

Br. J. Pharmacol. (1994), **111**, 325–331

Effects of N^G-nitro-L-arginine methyl ester on regional haemodynamic responses to $MgSO_4$ in conscious rats

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1 We assessed regional haemodynamic responses to the vasodilator, $MgSO_4$, in the absence and presence of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), in conscious chronically instrumented Long Evans rats ($n = 9$).

2 $MgSO_4$ (loading dose 220 μmol kg^{-1} min^{-1} for 7 min, maintenance dose 56 μmol kg^{-1} min^{-1} for 7 min), alone, caused slight bradycardia and hypotension accompanied by reductions in renal and mesenteric flows, but a marked hyperaemic vasodilatation in the hindquarters (flow, Δ 54 ± 6%, vascular conductance, Δ 77 ± 5%).

3 L-NAME (183 nmol kg^{-1} min^{-1}) caused hypertension (29 ± 2 mmHg) accompanied by bradycardia (− 51 ± 6 beats min^{-1}) and reductions in flow and vascular conductance in the renal (− 18 ± 4% and − 35 ± 3%, respectively), mesenteric (− 35 ± 3% and − 49 ± 3%, respectively), and hindquarters (− 26 ± 3% and − 42 ± 3%, respectively) vascular beds. In the presence of L-NAME, the hypotensive and bradycardic effects of $MgSO_4$ were still apparent, but its hindquarters hyperaemic vasodilator effect was significantly attenuated.

4 In order to determine if the inhibitory action of L-NAME on the hindquarters hyperaemic vasodilator action of $MgSO_4$ was a non-specific effect, due to the change in baseline conditions caused by L-NAME, we also examined responses to $MgSO_4$ in the presence of endothelin-1 (12.5 pmol kg^{-1} min^{-1}) or angiotensin II (50 pmol kg^{-1} min^{-1}). In the presence of either peptide, the overall effects of $MgSO_4$ on hindquarters flow and vascular conductance were unchanged.

5 In a separate experiment ($n = 8$) we determined that the inhibitory effect of L-NAME on the hyperaemic vasodilator response to $MgSO_4$ was prevented by L-arginine, and also demonstrated that the β_2-adrenoceptor antagonist, ICI 118551, caused significant inhibition of the hindquarters haemodynamic effects of $MgSO_4$.

6 We conclude that the hindquarters haemodynamic effects of $MgSO_4$ in conscious rats involve a substantial L-NAME-sensitive component which depends on activation of β_2-adrenoceptors, probably as a consequence of adrenal medullary adrenaline release.

Keywords: $MgSO_4$; vasodilatation; nitric oxide; N^G-nitro-L-arginine methyl ester; β_2-adrenoceptors

Introduction

Magnesium (Mg^{2+}) can cause relaxation of vascular smooth muscle *in vitro* and vasodilatation *in vivo* (see Altura & Altura, 1985, for review). However, in normotensive rats, infusion of Mg^{2+} has been claimed to cause no change in heart rate, blood pressure or regional blood flow, other than in the heart (DiPette *et al.*, 1987). This latter observation is of interest in the light of recent reports of the beneficial effects of Mg^{2+} in myocardial infarction (Teo *et al.*, 1991; Woods *et al.*, 1992; Horner, 1992).

Some *in vitro* evidence indicates that Mg^{2+} influences endothelium-derived-relaxing-factor (EDRF) release but the effect seems to be one whereby elevation of plasma Mg^{2+} would cause inhibition of EDRF release (Ku & Ann, 1991; Zhang *et al.*, 1992); hence, the latter would not be expected to be involved in the vasodilator effects of Mg^{2+}. However, any possible involvement of EDRF in the visceral haemodynamic action of Mg^{2+} has not been investigated *in vivo*.

There is convincing support for the assertion that a major EDRF is nitric oxide (NO), which is produced through the action of the enzyme, NO synthase (see Moncada *et al.*, 1991; Gardiner & Bennett, 1993, for review). Various analogues of L-arginine, including N^G-nitro-L-arginine methyl ester (L-NAME), inhibit the production of NO, and thus any influence of L-NAME on responses to Mg^{2+} *in vivo* might indicate an involvement of NO. Therefore, in the present work we determined the regional (renal, mesenteric and hindquarters) haemodynamic responses to Mg^{2+} in conscious rats, in the absence and presence of L-NAME. However, the latter has marked cardiovascular effects itself, causing hypertension and widespread regional vasoconstriction (Gardiner *et al.*, 1990c). Since it is feasible that these changes in baseline status would affect responses to Mg^{2+}, we also compared the effects of Mg^{2+} in the absence and presence of two other substances that cause hypertension and vasoconstriction, namely endothelin-1 (ET-1) and angiotensin II (AII).

There is some evidence that L-NAME can act as a muscarinic receptor antagonist (Buxton *et al.*, 1993), so in a secondary experiment we assessed the ability of L-arginine to prevent the inhibitory effects of L-NAME on haemodynamic responses to $MgSO_4$. Finally, because $MgSO_4$ caused prominent hyperaemic vasodilatation in the hindquarters (see Results), and because activation of β_2-adrenoceptors has similar effects in this vascular bed (Gardiner *et al.*, 1991b,c; 1992), we investigated the influence of the β_2-adrenoceptor antagonist, ICI 118551 (Bilski *et al.*, 1983) on haemodynamic responses to $MgSO_4$.

Methods

A group of nine male, Long Evans rats (350–450 g) were used in the primary study. Animals were anaesthetized (sodium methohexitone, 60 mg kg^{-1}, i.p., supplemented as required) and had pulsed Doppler probes (Haywood *et al.*, 1981)

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implanted to allow monitoring of renal, mesenteric and hindquarters blood flows (Gardiner *et al.*, 1990c,d). At least 7 days after probe implantation, animals were briefly anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) for the placement of intravascular catheters in the abdominal aorta (via the caudal artery) to monitor systemic arterial blood pressure and in the right jugular vein to administer $MgSO_4$ and vasoconstrictor substances. Animals were then left to recover for at least 24 h before experiments were begun, and the protocols were run over the following 4 days. The experiments were as follows:-

Effect of $MgSO_4$ alone

Animals were given an intravenous infusion of $MgSO_4$ as a loading dose (220 μmol kg^{-1} min^{-1} for 7 min) followed by a maintenance dose of 56 μmol kg^{-1} min^{-1} for 7 min. This dose schedule for $MgSO_4$ was based on a previous study in which plasma Mg^{2+} levels rose from 0.58 ± 0.01 mmol l^{-1} to 3.8 ± 0.13 mmol l^{-1} at the end of infusion, accompanied by a reduction of -10 ± 2 mmHg in mean arterial blood pressure (Kemp *et al.*, 1993). However, it is not likely that a steady state was reached for the effects of $MgSO_4$ (see Results).

Effect of $MgSO_4$ in the presence of L-NAME

L-NAME (183 mmol kg^{-1} min^{-1}) (Gardiner & Bennett, 1992) was infused continuously and after 60 min, when haemodynamic changes had stabilised, $MgSO_4$ was infused for 14 min (as above).

Effect of $MgSO_4$ in the presence of ET-1

ET-1 (12.5 pmol kg^{-1} min^{-1}) was infused continuously and after 20 min, when haemodynamic changes had stabilized, $MgSO_4$ was infused for 14 min (as above). The dose of ET-1 was chosen to have similar haemodynamic effects to L-NAME, although it was not possible to match the hindquarters vasoconstrictor responses (see Results).

Effect of $MgSO_4$ in the presence of AII

AII (50 pmol kg^{-1} min^{-1}) was infused and after 20 min, when haemodynamic changes had stabilized, $MgSO_4$ was infused for 14 min (as above). The dose of AII was chosen to have mesenteric and hindquarters vasoconstrictor effects similar to those of L-NAME, but it was not possible to match the pressor and the renal vasoconstrictor actions of AII to those of L-NAME under these circumstances. Only one experiment per day was performed on each animal and, over the first 3 days, animals received $MgSO_4$ alone, or $MgSO_4$ in the presence of ET-1 or AII. The order in which these experiments were run was randomized; however, L-NAME was always given on the fourth day because of its long-lasting effects.

One blood sample was taken during the last minute of the $MgSO_4$ infusion for determination of the plasma Mg^{2+} concentration. Plasma Mg^{2+} levels were measured on a Kodak Ektachem 700X (Department of Clinical Chemistry, University Hospital, Nottingham), for which the lowest limit of detection was 0.08 mmol l^{-1} and co-efficients of variation on standards of 0.86 and 1.97 mmol l^{-1} Mg^{2+} were less than 2.5%.

Influence of L-arginine on the effects of $MgSO_4$ in the presence of L-NAME

In a secondary experiment, animals ($n = 8$) were given $MgSO_4$ alone (as above). At least 3 h later a primed infusion of L-arginine was begun (1.42 mmol kg^{-1} bolus, 1.42 mmol kg^{-1} h^{-1} infusion) 20 min before L-NAME which was administered for 60 min prior to $MgSO_4$ (as above).

Effect of $MgSO_4$ in the presence of ICI 118551

Prior to the experiments above, and on a separate experimental day, the same animals were given $MgSO_4$ alone or $MgSO_4$ 60 min after primed infusion of ICI 118551 (670 nmol kg^{-1} bolus, 335 nmol kg^{-1} h^{-1} infusion).

Throughout the experiments, continuous recordings were made of heart rate (HR) mean arterial blood pressure (MAP) and renal, mesenteric and hindquarters Doppler shift signals, both phasic and mean (using a modified Crystal Biotech VF-1 system) (Gardiner *et al.*, 1990b). Measurements (averaged over 20 s) were made immediately before infusion of L-NAME, ET-1 or AII, and in each animal these values were the baseline to which all the subsequent changes were referred. Sixty minutes after the start of L-NAME infusion, or 20 min after the start of ET-1, or AII infusion, cardiovascular variables were measured again to give steady-state responses to L-NAME, ET-1 or AII, respectively. Thereafter, the loading dose of $MgSO_4$ was given over 7 min and measurements were made at the end of this period, and again at the end of the subsequent 7 min period during which the maintenance dose of $MgSO_4$ was given.

In the experiment in which $MgSO_4$ was given alone, measurements were made 20 min before (i.e., baseline), and immediately before the start of $MgSO_4$ infusion (i.e., control value), and at the end of the infusion of the loading dose and of the maintenance dose of $MgSO_4$ (as above).

Percentage changes in mean Doppler shift signals were taken as indices of flow changes (Haywood *et al.*, 1981), and mean arterial blood pressure and mean Doppler shift signals were used to calculate percentage changes in renal, mesenteric and hindquarters vascular conductances (Gardiner *et al.*, 1990c,d).

Data analysis

Changes relative to baseline and changes relative to pre-$MgSO_4$ were analysed by Friedman's test (Theodorsson-Norheim, 1987). A P value < 0.05 was taken as significant.

Drugs and peptides

$MgSO_4$ was dissolved in distilled water. L-Arginine hydrochloride and L-NAME hydrochloride (Sigma) were dissolved in isotonic saline (154 mmol l^{-1} NaCl). ET-1 (Peptide Institute) and AII (Bachem, UK) were dissolved in isotonic saline containing 1% bovine serum albumin (Sigma). ICI 118551 (erythro-(±)-1[7-methylindan-4-yloxy]-3-isopropyl-aminobutan-2-ol) hydrochloride (a gift from ICI Pharmaceuticals plc) was dissolved in sterile water by gentle warming.

Infusions were given at a rate of 0.3 ml h^{-1} for all substances except $MgSO_4$ which was infused at a rate of 0.15 ml min^{-1}.

Results

Resting values for cardiovascular variables on the different experimental days are shown in Table 1.

Effects of $MgSO_4$ alone

During the 20 min control period, prior to administration of $MgSO_4$, there were no significant changes in cardiovascular variables (Figures 1 and 2). At the end of the loading dose of $MgSO_4$ there was a slight hypotension accompanied by reductions in renal and mesenteric flow, but a marked increase in hindquarters flow (Figure 1). The latter was associated with a substantial increase in vascular conductance, but there were no changes in renal or mesenteric vascular conductance (Figure 2). By the end of the maintenance dose of $MgSO_4$ there was still a slight hypotension, accompanied by a modest bradycardia. There was no longer any significant reduction in

Table 1 Resting cardiovascular variables in the same conscious Long Evans rats ($n = 9$) prior to infusion of $MgSO_4$ N^G-nitro-L-arginine methyl ester (L-NAME), endothelin-1 (ET-1) or angiotensin II (AII) on different experimental days

	pre-$MgSO_4$	*pre-L-NAME*	*pre-ET-1*	*pre-AII*
Heart rate (beats min^{-1})	309 ± 6	299 ± 6	305 ± 7	293 ± 3
Mean arterial blood pressure (mmHg)	105 ± 2	103 ± 2	104 ± 3	100 ± 2
Renal Doppler shift (kHz)	7.8 ± 0.9	7.6 ± 0.9	7.3 ± 1.0	7.6 ± 1.0
Mesenteric Doppler shift (kHz)	5.0 ± 0.4	5.5 ± 0.6	5.1 ± 0.5	5.5 ± 0.5
Hindquarters Doppler shift (kHz)	4.6 ± 0.5	4.1 ± 0.4	4.3 ± 0.5	4.4 ± 0.5
Renal vascular conductance ([kHz $mmHg^{-1}$]10^3)	74 ± 7	73 ± 8	69 ± 7	75 ± 8
Mesenteric vascular conductance ([kHz $mmHg^{-1}$]10^3)	48 ± 4	54 ± 6	49 ± 5	56 ± 5
Hindquarters vascular conductance ([kHz $mmHg^{-1}$]10^3)	44 ± 5	40 ± 4	42 ± 5	44 ± 5

Values are means ± s.e.mean.

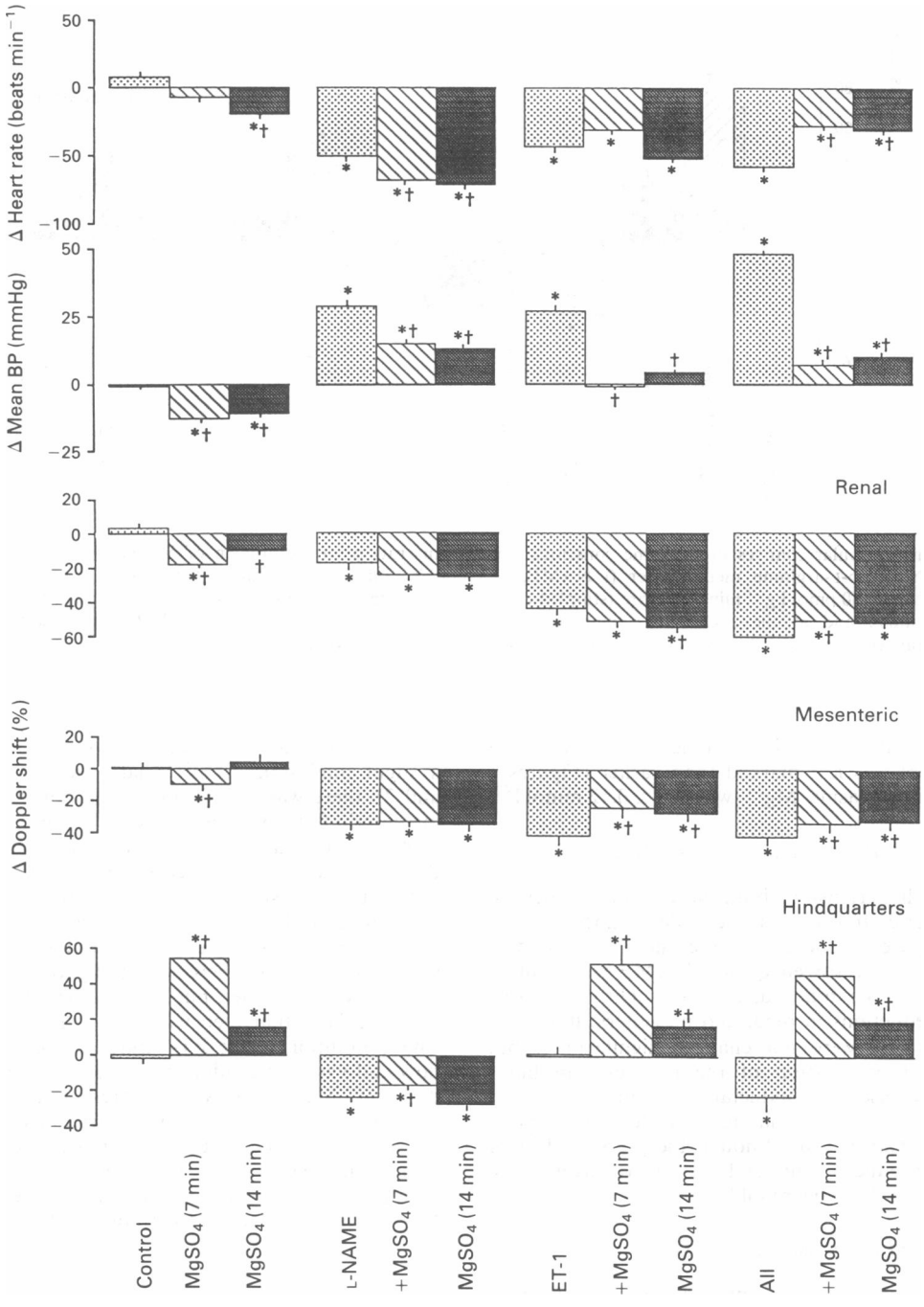

Figure 1 Cardiovascular changes in the same conscious Long Evans rats prior to infusion of $MgSO_4$ alone (control) or in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 183 nmol kg^{-1} min^{-1}), endothelin-1 (ET-1, 12.5 pmol kg^{-1} min^{-1}) or angiotensin II (AII, 50 pmol kg^{-1} min^{-1}) before (stippled columns) at the end of the loading dose of $MgSO_4$ (220 μmol kg^{-1} min^{-1}) (hatched columns), and at the end of the maintenance dose of $MgSO_4$ (56 μmol kg^{-1} min^{-1}) (solid columns). Values are mean ± s.e.mean ($n = 9$). *$P < 0.05$ versus baseline; †$P <$ versus pre-$MgSO_4$ value.

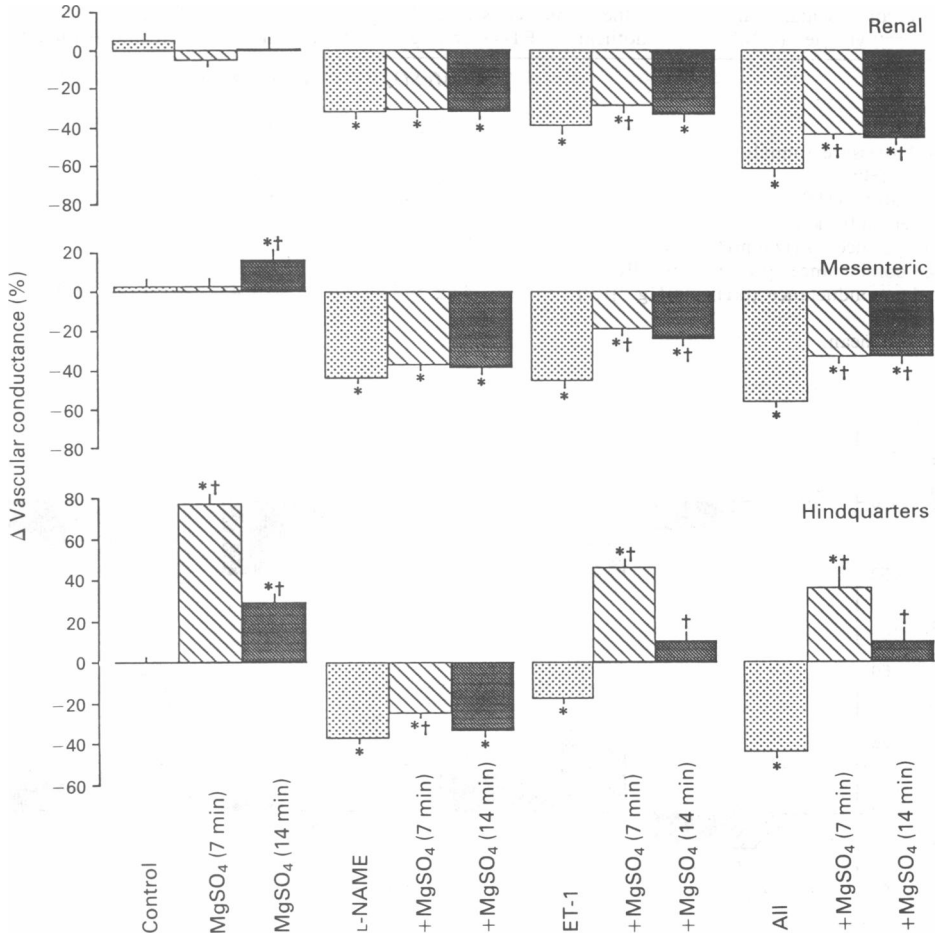

Figure 2 Cardiovascular changes in the same conscious Long Evans rats prior to infusion of $MgSO_4$ alone (control) or in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 183 nmol kg^{-1} min^{-1}), endothelin-1 (ET-1, 12.5 pmol kg^{-1} min^{-1}) or angiotensin II (AII, 50 pmol kg^{-1} min^{-1}) before (stippled columns) at the end of the loading dose of $MgSO_4$ (220 μmol kg^{-1} min^{-1}) (hatched columns), and at the end of the maintenance dose of $MgSO_4$ (56 μmol kg^{-1} min^{-1}) (solid columns). Values are mean ± s.e.mean ($n = 9$). *$P < 0.05$ versus baseline; †$P <$ versus pre-$MgSO_4$ value.

renal or mesenteric flow, and the hyperaemic vasodilatation in the hindquarters was less marked than earlier (Figures 1 and 2). At this time plasma Mg^{2+} was 3.9 ± 0.12 mmol l^{-1}.

Effect of $MgSO_4$ in the presence of L-NAME

L-NAME caused hypertension, bradycardia, and reductions in renal, mesenteric and hindquarters flows and vascular conductances (Figures 1 and 2). At the end of the loading dose of $MgSO_4$, the pressor effect of L-NAME was reduced by about 50%, but the bradycardia was slightly increased (Figure 1). The reductions in renal and mesenteric flows and vascular conductances were not changed significantly, but there was a slight attenuation of the reduction in hindquarters flow and vascular conductance (Figures 1 and 2). However, by the end of the maintenance dose of $MgSO_4$, these latter effects had waned, although the pressor effect of L-NAME was still reduced (Figures 1 and 2). At this juncture plasma Mg^{2+} was 3.9 ± 0.09 mmol l^{-1}.

Effect of $MgSO_4$ in the presence of ET-1

ET-1 caused hypertension and bradycardia, accompanied by constriction in the renal, mesenteric and hindquarters vascular beds, although only renal and mesenteric flows were reduced (Figures 1 and 2). The loading dose of $MgSO_4$ abolished the pressor effect of ET-1, but a bradycardia persisted (Figure 1). The ET-1-induced reduction in renal flow was not affected significantly by $MgSO_4$, although there was a slight inhibition of the renal vasoconstriction (Figures 1 and 2); there was significant attenuation of the reductions in mesenteric flow and vascular conductance (Figures 1 and 2). $MgSO_4$ caused a marked increase in hindquarters flow, and the ET-1-induced constriction in this vascular bed was reversed to a substantial dilatation (Figure 2).

At the end of the maintenance dose of $MgSO_4$, the pressor effect of ET-1 was still abolished, but a bradycardia was still present (Figure 1). There was a slightly greater reduction in renal flow than earlier, but the renal vasoconstriction was not different from that seen with ET-1 alone (Figures 1 and 2). The diminution in the ET-1-induced reduction in mesenteric flow and vascular conductance, seen at the end of the loading dose of $MgSO_4$, was still present at the end of the maintenance dose but the hindquarters hyperaemia had waned and the hindquarters vascular conductance was not significantly increased above baseline, although it was higher than in the presence of ET-1 alone (Figures 1 and 2). Plasma Mg^{2+} level was 4.4 ± 0.14 mmol l^{-1} at this time.

Effect of $MgSO_4$ in the presence of AII

AII caused hypertension, bradycardia, and reductions in renal, mesenteric and hindquarters flows and vascular conductances (Figures 1 and 2). Although the pressor effect of AII was greater than that of L-NAME or ET-1, the mesen-

teric and hindquarters vasoconstrictor effects of AII were not significantly different from those of L-NAME (Figure 2).

By the end of the loading dose of $MgSO_4$, the pressor and bradycardic effects of AII were significantly reduced (Figure 1), and there were slight inhibitions of the reductions in renal and mesenteric flows and vascular conductances (Figures 1 and 2). The AII-induced reductions in hindquarters flow and vascular conductance were reversed to significant increases (Figures 1 and 2).

At the end of the maintenance dose of $MgSO_4$ its inhibitory effects on the pressor, bradycardic, and renal and mesenteric vasoconstrictor actions of AII were still present (Figures 1 and 2). However, the hindquarters hyperaemia was less marked, and vascular conductance, although significantly higher than in the presence of AII alone, was not significantly above baseline (Figures 1 and 2). At this point plasma Mg^{2+} was 4.1 ± 0.11 mmol l^{-1}.

Influence of L-arginine on the effects of $MgSO_4$ in the presence of L-NAME

In the presence of L-arginine, L-NAME had no significant effect on renal vascular conductance ($-3 \pm 5\%$) or mesenteric vascular conductance ($3 \pm 22\%$), but there was still a significant reduction in hindquarters vascular conductance ($-31 \pm 6\%$). (Such a differential effect of L-arginine on the regional vasoconstrictor responses to L-NAME has been reported previously (Gardiner *et al.*, 1990c)). The hindquarters vasoconstrictor effect of L-NAME, in the presence of L-arginine, was abolished at the end of the loading dose of $MgSO_4$ (Δ vascular conductance $= 1 \pm 6\%$ (from $-31 \pm 6\%$)) and was still significantly reduced at the end of the maintenance dose ($-16 \pm 6\%$). Thus, in the presence of L-arginine and L-NAME, there was an underlying hindquarters vasodilator effect of $MgSO_4$ which was significantly greater than in the absence of L-arginine.

Effect of $MgSO_4$ in the presence of ICI 118551

In the presence of ICI 118551, the hindquarters vasodilator response to $MgSO_4$ (Δ vascular conductance at 7 min $= 15 \pm 6\%$, and at 14 min $= 5 \pm 5\%$) was significantly less than in the absence of ICI 118551 (at 7 min $= 67 \pm 9\%$ and at 14 min $= 29 \pm 5\%$).

Discussion

In the present work we measured renal, mesenteric and hindquarters responses to $MgSO_4$ infusion and found that the slight hypotensive effect of this intervention was accompanied by a selective hindquarters hyperaemia and vasodilatation. It is feasible that the apparently differential vasodilator influence of $MgSO_4$ was due to baroreflex-mediated sympathoadrenal stimulation, and activation of renin-angiotensin and vasopressin-mediated mechanisms, opposing any $MgSO_4$-induced vasodilatation in the renal and mesenteric vascular beds. If so, this situation might be analogous to that in which calcitonin gene-related peptide (CGRP), when infused *in vivo*, causes hindquarters vasodilatation and mesenteric vasoconstriction (Gardiner *et al.*, 1989a,b), in spite of the fact that, *in vitro*, CGRP is a potent mesenteric vasodilator (Marshall *et al.*, 1986; Kawasaki *et al.*, 1988). However, in that circumstance, the hypotensive response to CGRP was much greater than observed here with $MgSO_4$, and thus activation of vasoconstrictor mechanisms was probably more marked with CGRP. Moreover, since sympathoadrenal activity and vasopressin can exert hindquarters vasoconstrictor effects (Gardiner & Bennett, 1988; Gardiner *et al.*, 1988), it is probable (see later) that the early, sizeable increase in hindquarters flow and vascular conductance induced by $MgSO_4$ was due, at least partly, to more effective activation of vasodilator mechanism(s) in that vascular bed. We cannot dismiss the possibility that the waning vasodilator effect of $MgSO_4$ was due to the plasma Mg^{2+} level falling during infusion of the maintenance dose.

If the vasodilator effect of $MgSO_4$ *in vivo* was due to diminution in intracellular Ca^{2+} levels (Altura & Altura, 1985; D'Angelo *et al.*, 1992), it is not obvious why this influence should be confined to the hindquarters vascular bed. The finding that the hindquarters hyperaemic vasodilator action of $MgSO_4$ was clearly attenuated in the presence of L-NAME, and that this effect was largely prevented by pretreatment with L-arginine, indicates that a large component of the effect of $MgSO_4$ under normal conditions was due to activation of NO-mediated processes. However, as with a direct vasodilator action of $MgSO_4$ (above), it is, at first sight, puzzling that such an effect should be so prominent in the hindquarters vascular bed, particularly since, in other protocols, we have found the mesenteric vascular bed shows more marked vasodilatation than the hindquarters vascular bed in response to NO donors (Gardiner *et al.*, 1990d; 1991b; Phillips *et al.*, 1991). Thus, it would have to be argued that $MgSO_4$ more effectively activated NO-mediated processes in the hindquarters, than in the mesenteric, vascular bed. This is not without precedent, since CGRP has such an action (Gardiner *et al.*, 1991a). However, the influence of CGRP is a receptor-mediated event (Gardiner *et al.*, 1990e), whereas an effect of Mg^{2+} on NO release, for example, would be likely to be due to an influence on the disposition of Ca^{2+} (Altura & Altura, 1985). In this connection, *in vitro* findings indicate that elevated Mg^{2+} should inhibit NO release (Ku & Ann, 1991; Zhang *et al.*, 1992), thus our results are the opposite of what would be predicted from such *in vitro* studies. However, it is feasible that elevated Mg^{2+} levels suppress Na^+/Ca^{2+} exchange, thereby increasing intracellular Ca^{2+} levels and promoting NO release (Cocks *et al.*, 1988). In addition, there is evidence that Mg^{2+} is required for agonist-induced, endothelium-dependent vasorelaxation (Altura & Altura, 1987; Ku & Ann, 1991), and clearly our results could have been due to factors other than a direct stimulatory influence of Mg^{2+} on endothelial NO release, e.g., change in shear force on endothelial cells, or Mg^{2+}-induced release of other mediators (see below).

It is possible that the selective hindquarters vasodilator response to Mg^{2+} was due to increased prostacyclin production since there is evidence that Mg^{2+} has this action (Nadler *et al.*, 1987; Laurant *et al.*, 1992) and, in conscious rats, prostacyclin causes marked hindquarters hyperaemia (Steinberg *et al.*, 1988). However, such an effect should not be susceptible to L-NAME, unless NO mediates Mg^{2+}-induced prostacyclin release, but what evidence there is indicates that NO might suppress prostacyclin release (Mitchell *et al.*, 1993); thus L-NAME should augment any haemodynamic effects of Mg^{2+} that were due to prostacyclin release. It is feasible that the apparent inhibitory effect of L-NAME on the hindquarters response to Mg^{2+} was a non-specific influence, due to the change in baseline status caused by L-NAME, but this is not likely because, when animals were pretreated with a dose of AII that caused a reduction in hindquarters flow and vascular conductance matched to the effect of L-NAME, the hyperaemic vasodilator effect of $MgSO_4$ was not inhibited as it was in the presence of L-NAME. The hindquarters vasodilator effect of $MgSO_4$ was also seen in the presence of ET-1, although the latter did not have hindquarters haemodynamic effects that matched those of L-NAME, probably because ET-1, itself, activates hindquarters vasodilator mechanisms in conscious rats (Gardiner *et al.*, 1989c,d; 1990a).

Differences between the effects of $MgSO_4$ in the presence of L-NAME, compared to those seen in the presence of ET-1 and AII, were also apparent from the changes in mean arterial blood pressure and renal and mesenteric haemodynamics. Thus, $MgSO_4$ was more effective at reversing the pressor effects of ET-1 and AII than it was that of L-NAME. While this is consistent with the more marked hindquarters

vasodilator action of $MgSO_4$ in the former conditions, there was a clear dissociation between the slight vasodilator effect of $MgSO_4$ in the presence of L-NAME and its antihypertensive action. Thus, by the end of the maintenance dose of $MgSO_4$, in the presence of L-NAME, regional haemodynamic status was not different from that seen in the presence of L-NAME alone, but the pressor effect of L-NAME remained reduced. However, in this circumstance there was a significant bradycardia and hence a reduction in cardiac output might have been contributing to the reduction in mean arterial blood pressure.

Apart from the possibilities discussed above, we considered it feasible that the hindquarters hyperaemic vasodilator response to $MgSO_4$ might involve activation of β_2-adrenoceptors (consequent upon adrenal medullary adrenaline release), since the effect of adrenaline is also sensitive to L-NAME (Gardiner *et al.*, 1991c). We confirmed an involvement of β_2-adrenoceptors in the hindquarters haemodynamic effects of $MgSO_4$, inasmuch as pretreatment with ICI 118551 caused substantial inhibition of the responses to $MgSO_4$. It is likely that any effect of $MgSO_4$ on adrenaline release was indirect, since a direct effect of increased plasma Mg^{2+} would be expected to inhibit adrenaline secretion (Nakazoto *et al.*, 1986).

Although, as discussed above, the vasodilator effect of $MgSO_4$, and its susceptibility to L-NAME, was most obvious in the hindquarters vascular bed, $MgSO_4$ did exert significant, albeit modest, renal and mesenteric vasodilator effects in the presence of ET-1 and AII that were not apparent in the presence of L-NAME. Thus, it may be that $MgSO_4$ exerts NO-mediated vasodilator effects in all these vascular beds, but for the reasons given above this effect is most obvious in the hindquarters vascular bed. From our previous studies it appears that $MgSO_4$ also has a prominent, L-NAME-sensitive, vasodilator effect in the carotid vascular bed (Kemp *et al.*, 1993). It remains to be determined in which tissues in these vascular territories the vasodilator responses occur, but it is unlikely that β_2-adrenoceptor mechanisms are involved, since salbutamol does not increase carotid blood flow (Gardiner *et al.*, 1991b).

This work was supported by a grant (BHF 91/10) from the British Heart Foundation.

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(Received June 11, 1993
Revised September 20, 1993
Accepted September 29, 1993)

Br. J. Pharmacol. (1994), **111**, 332–338

GR113808: a novel, selective antagonist with high affinity at the 5-HT_4 receptor

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1 The 5-HT_4 receptor has only recently been identified but has yet to be cloned. This paper describes the pharmacology of a potent and selective 5-HT_4 receptor antagonist, GR113808, which will be useful in the further characterization of this receptor.

2 On the guinea-pig ascending colon, GR113808 (1 nM–0.1 μM) behaved as an antagonist of 5-hydroxytryptamine (5-HT)-induced contraction, producing rightward displacements of the concentration-effect curve to 5-HT and a concentration-related depression of the maximum effect. However, the compound had no effect on cholecystokinin (CCK-8)-induced contraction in concentrations up to 1 μM.

3 In the guinea-pig colon preparation, onset and offset of the antagonism by GR113808 of 5-HT-induced contraction was examined. Incubation of the tissues for either 15 min, 30 min or 60 min produced similar rightward displacements of the concentration-effect curves to 5-HT, with no increase in the degree of depression of the maxima with increasing time of incubation. Experiments examining offset of antagonism (0.01 μM) demonstrated that washout for 30 min was required to reverse fully the effects of the antagonist.

4 Potency estimates in the colon for GR113808 were made by determining approximate pA_2 values (30 min) using the Gaddum equation. The values obtained were 9.2, 9.7 and 9.2 when tested against the agonists 5-HT, 5-methoxytryptamine and R,S-zacopride respectively.

5 On the carbachol-contracted tunica muscularis mucosae preparation of the rat thoracic oesophagus, GR113808 behaved as an antagonist of 5-HT-induced relaxation, producing no reduction in maximum response. Analysis of these data yielded a pA_2 of 9.3. GR113808 also antagonised the relaxant effects of 5-methoxytryptamine ($pA_2 = 9.0$) and **R,S**-zacopride ($pA_2 = 9.4$). The compound had no effect on isoprenaline-induced relaxation of the carbachol-contracted oesophagus at a concentration of 1 μM.

6 In tests of selectivity, GR113808 had only low affinity for 5-HT_3 receptors ($pK_i = 6.0$) and had no functional activity at either 5-HT_2 or 5-HT_1-like receptors on vascular smooth muscle preparations. In a range of binding assays, GR113808 was shown to have no appreciable affinity for any other receptor type investigated.

7 In the anaesthetized piglet, GR113808 was a potent antagonist of 5-methoxytryptamine-induced tachycardia (mean $DR_{10} = 97.2\ \mu g\ kg^{-1}\ h^{-1}$). The compound was ineffective against isoprenaline-induced tachycardia.

8 The present results are discussed in comparison with those for existing antagonists at the 5-HT_4 receptor. The results of this study indicate that GR113808 will be a valuable antagonist for studying 5-HT_4 receptor mechanisms *in vitro* and *in vivo* and validate its use as a radioligand for determining 5-HT_4 receptor distribution.

Keywords: 5-HT; 5-HT_4 receptor; 5-HT_4-receptor antagonist; GR113808

Introduction

A new class of 5-hydroxytryptamine (5-HT) receptor termed 5-HT_4, which is positively coupled to adenylyl cyclase, has been identified in various isolated tissue preparations. The responses to 5-HT mediated by this receptor include, stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in neurones of mouse colliculi and guinea-pig hippocampus (Dumuis *et al.*, 1989; Bockaert *et al.*, 1990), neuronally-mediated contraction in guinea-pig ileum (Eglen *et al.*, 1990) and colon (Elswood *et al.*, 1991), relaxation of rat oesophagus (Baxter *et al.*, 1991; Reeves *et al.*, 1991) and a positive inotropic response in human and porcine atria (Kaumann, 1990; Kaumann *et al.*, 1990). In addition, the 5-HT_4 receptor has been identified *in vivo* mediating an increase in heart rate (Villalon *et al.*, 1990). In each of these preparations, high concentrations of tropisetron (ICS205-930), much larger than those required for 5-HT_3 receptor antagonism, inhibited the effect of selective agonists whereas other antagonists selective for 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors were without effect.

Until recently, tropisetron was the sole antagonist capable of blocking the effects of 5-HT at the 5-HT_4 receptor, albeit weakly with an affinity constant at the receptor of between 6.0 and 6.5 (Dumuis *et al.*, 1989; Baxter *et al.*, 1991; Elswood *et al.*, 1991). However, this compound has the disadvantages of high affinity for 5-HT_3 receptors and, at high concentrations, ion channel blocking activity (Richardson *et al.*, 1985; Scholtysik *et al.*, 1988). Two novel compounds SDZ205-557 and DAU 6285, have now been described for which increased 5-HT_4 receptor affinity is claimed with a concomitant reductin in affinity for the 5-HT_3 receptor (Buchheit *et al.*, 1992, Schiavone *et al.*, 1992). However, the affinities of these compounds for the 5-HT_4 receptor are relatively low, with pA_2 values of between 6.5 and 7.4. Furthermore, since their affinity for 5-HT_3 receptors has been assessed largely in guinea-pig tissues, a species in which 5-HT_3 receptor antagonists tend to have lower affinity than elsewhere, it is likely that their selectivities have been overestimated (Eglen *et al.*,

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1993). In this study we describe the pharmacology of GR 113808 (see chemical structure shown in Figure 1), a novel, selective antagonist with high-affinity for the 5-HT$_4$ receptor.

Methods

Guinea-pig ascending colon

Female Dunkin-Hartley guinea-pigs (300–400 g) were killed by cervical dislocation and the most proximal portion of the colon (a 10 cm segment, starting 1 cm from the caecum) was removed. Segments were then prepared as described by Elswood *et al.* (1991). Briefly, the colon was divided into 3 cm segments and opened longitudinally. Faecal matter was removed and the mucosa dissected away. The muscle strip was set up in the longitudinal plane in modified Krebs solution of the following composition (mM): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 1.3 and glucose 11.1 also containing methysergide (1 μM) and ondansetron (10 μM) to block 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptors. The modified Krebs solution was maintained at 32°C and bubbled with 95% O_2/5% CO_2. Tissues were allowed to equilibrate for 40 min prior to dosing with an agonist. Responses were measured isometrically.

Agonist concentration-effect curves were constructed by use of sequential dosing, leaving 10 min between doses. The agonist was left in contact with the tissue until the maximum effect was reached, or if there was no response for 2 min, then the tissues were washed. Thirty minutes were left between concentration-effect curves. Antagonists were incubated for 30 min before repeating the agonist concentration-effect curve.

Three agonist concentration-effect curves were constructed with each tissue. Preliminary experiments established that the second and third concentration-effect curves were superimposable. Therefore, in further experiments the second curve was used as a control and the third curve as the test curve in the presence of the antagonist.

As a control for specificity, the effect of the antagonist on concentration-effect curves to cholecystokinin (CCK-8) was investigated by use of a protocol similar to that outlined above. The responses to CCK-8, like those to 5-HT in this preparation, are cholinergically-mediated (see Elswood *et al.*, 1991).

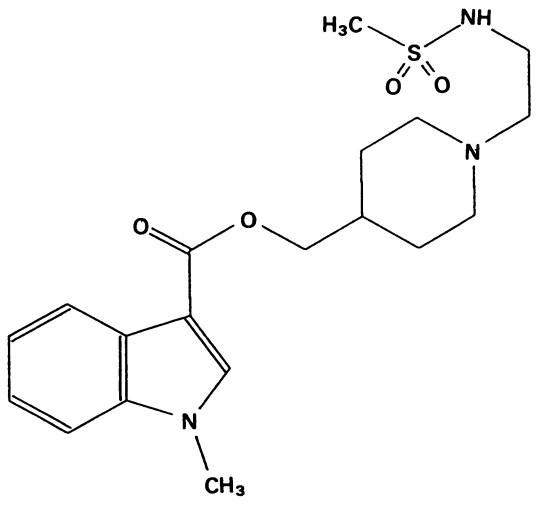

Figure 1 The structure of GR113808 ([1-[2-methylsulphonyl)amino] ethyl]-4-piperidinyl]methyl 1-methyl-IH-indole-3-carboxylate).

Investigation of onset and offset of antagonism due to GR113808

In the colon preparation, experiments were performed to determine the effect of increasing incubation time on the magnitude of antagonism produced with GR113808. Concentration-effect curves were constructed to 5-HT and then repeated following incubation with GR113808 (10.0 nM) for either 15, 30 or 60 min.

In addition, experiments were performed to determine the effect of increasing the washout time of GR113808 following tissue incubation. Concentration-effect curves were constructed to 5-HT and then tissues were incubated for 30 min with GR113808 (10.0 nM).Tissues were then washed every 5 min for either 15, 30 or 60 min and then a second concentration-effect curve to 5-HT was constructed.

Rat thoracic oesophageal muscularis mucosae

Female Wistar rats (120–180 g) were killed by cervical dislocation, the thorax was opened and a central 2 cm portion of the oesophagus removed. The oesophageal segments were prepared as described by Baxter *et al.* (1991). Briefly, the external muscularis propria, containing the outer longitudinal and circular muscle layers of the oesophagus, was carefully dissected in order to isolate the smooth muscle of the tunica muscularis mucosae. The preparations were suspended longitudinally under an initial tension of approximately 0.5 g in modified Krebs solution (composition as before) at 32°C and gassed with 95% O_2/5% CO_2. This solution routinely contained indomethacin (3 μM) and ketanserin (1 μM) to inhibit prostanoid formation and block 5-HT$_2$ receptors respectively. In addition, 3-isobuty-1-methyl-xanthine (IBMX) (3 μM) was included to prevent the breakdown of cyclic AMP and so maximize responses mediated through adenylyl cyclase (Reeves *et al.*, 1991). Responses were measured isometrically.

The oesophageal preparations were contracted by addition of a submaximal concentration of carbachol (1 μM) to the bathing solution. Upon establishment of a stable contraction, a cumulative concentration-effect curve for relaxations to an agonist was constructed. Following the construction of the control curve, the tissues were washed with fresh modified Krebs solution and allowed to recover for 1 h before recontracting with carbachol. Once the preparations had reattained their stable state of contraction to carbachol, the antagonist was added, incubated with the tissue for 30 min and the agonist re-added in a cumulative manner as before.

As a control for specificity, the effect of the antagonist on cumulative concentration-effect curves to isoprenaline was investigated using a protocol similar to the one outlined above on preparations precontracted with carbachol.

Anaesthetized piglet

Anaesthesia was induced in neonatal (10–14 days postpartum) Large White pigs of either sex (4.0–6.0 kg) with 5% isoflurane carried in a mixture of nitrous oxide (2.0 l min^{-1}) and oxygen (1.0 l min^{-1}). Anaesthesia was maintained with a bolus of sodium pentobarbitone (20 mg kg^{-1}, i.v.) followed by intravenous infusion (5 mg kg^{-1} h^{-1}). The animals were artificially respired with air via a tracheal cannula; the stroke volume of the respiration pump was adjusted to keep the arterial blood Po_2, Pco_2 and pH within normal limits (Po_2 = 70–110 mmHg, Pco_2 = 35–55 mmHg, pH = 7.35–7.45). A carotid artery was cannulated to allow measurement of arterial blood pressure and instantaneous heart rate was derived electronically from the signal. Both parameters were displayed continuously on a Devices MX8 pen recorder. Jugular veins were cannulated bilaterally for administration of drugs.

All experiments were carried out in the presence of ondansetron (0.3 mg kg^{-1}, i.v.; given every hour) and ketanserin (1.0 mg kg^{-1}, i.v.; single dose). Cumulative dose-effect curves to 5-methyoxytryptamine (5-MeOT, a 5-HT$_4$ receptor agon-

ist; Villalon et al., 1990) were constructed, leaving 30 min between successive curves.

In antagonist studies, two control dose-effect curves to 5-MeOT were constructed. This was followed by continuous intravenous infusion of the antagonist. After 20 min infusion, a further dose-effect curve to 5-MeOT was constructed.

As a control for specificity, the effect of intravenous infusion of the antagonist on cumulative dose-effect curves to isoprenaline, constructed as described above, was investigated.

Assessment of activity at 5-HT receptors

The activity of GR113808 at vascular 5-HT_2 receptors was assessed by the method of Apperley et al. (1976). Briefly, spiral strips were cut from thoracic aortae of New Zealand White rabbits of either sex (2–3 kg). The tissues were suspended in modified Krebs solution (composition as before), maintained at 37°C and bubbled with 95% O_2/5% CO_2. Cumulative concentration-effect curves were constructed to 5-HT (50 nM–50 μM). GR113808 was examined both as a potential agonist (10 nM–50 μM) and also as an antagonist (50 μM) against 5-HT concentration-effect curves.

The activity of GR113808 at the 5-HT_1-like receptor mediating contraction in the dog saphenous vein (Humphrey et al., 1988) and at the 5-HT_1-like receptor mediating relaxation in the porcine vena cava (Sumner et al., 1989) was assessed. Briefly, spiral strips were cut from beagle isolated saphenous vein and rings were cut from the vena cava of neonatal pigs. Preparations were suspended in modified Krebs solution (composition as before), maintained at 37°C and bubbled with 95% O_2/5% CO_2. Preparations of vena cava were precontracted with the thromboxane A_2-mimetic, U-46619 (10 nM). Cumulative concentration-effect curves were constructed to 5-HT (1 nM–10 μM). GR113808 was examined both as a potential agonist (10 nM–50 μM) and also as an antagonist (50 μM) against 5-HT concentration-effect curves.

Specificity data

Further analysis of the specificity and selectivity of GR 113808 was assessed in a range of binding assays performed by Battelle-Europe, Geneva. All tissues were from the rat unless otherwise stated. The following assays were used, the description of the assay being in the form of receptor, ligand, tissue: adenosine A_1, [^{3}H]-DPCPX, cerebral cortex; adenosine A_2, [^{3}H]-CGS-21680, striatum; α_1-adrenoceptor, [^{3}H]-prazosin, whole brain minus cerebellum; α_2-adrenoceptor, [^{3}H]-idazoxan, cerebral cortex; β_1-adrenoceptor, [^{3}H]-CGP-26505, cerebral cortex; β_2-adrenoceptor, [^{125}I]-iodocyanopindolol, guinea-pig lung; dopamine D_1, [^{3}H]-SCH-23390, striatum; dopamine D_2, [^{3}H]-YM-091512, striatum; $GABA_A$, [^{3}H]-muscimol, whole brain; $GABA_B$; [^{3}H]-baclofen, cerebellum; 5-HT_1-like, [^{3}H]-5-HT, cerebral cortex; 5-HT_{1C}, [^{3}H]-mesulergine, porcine choroid plexus; 5-HT_3, [^{3}H]-GR65630, cerebral cortex; muscarinic M_1 cholinoceptor, [^{3}H]-pirenzipine, cerebral cortex; muscarinic M_2 cholinoceptor, [^{3}H]-NMS, heart; muscarinic M_3 cholinoceptor, [^{3}H]-DAMP, pancreas; nicotinic cholinoceptor, [^{3}H]-NMCl, cerebral cortex; histamine H_1, [^{3}H]-mepyramine, cerebral cortex; histamine H_3, [^{3}H]-N-α-methylhistamine, cerebral cortex; NMDA [^{3}H]-CGS-19755, whole brain minus cerebellum; μ-opioid, [^{3}H]-CTOP, whole brain minus cerebellum; κ-opioid, [^{3}H]-U-69593, guinea-pig cerebral cortex; bradykinin, [^{3}H]-bradykinin, guinea-pig ileum; CCK_A, [^{3}H]-L 364718, pancreas; CCK_B, [^{3}H]-L365,260, guinea-pig whole brain minus cerebellum; neurokinin NK_1, [^{3}H]-substance P, whole brain minus cerebellum; neurokinin NK_2, [^{3}H]-neurokinin A, colon; neurokinin NK_3, [^{3}H]-senktide, cerebral cortex; CGRP, [^{125}I]-CGRP, hypothalamus; neuropeptide Y, [^{125}I]-neuropeptide Y, hippocampus; bombesin, [^{125}I]-bombesin, whole brain minus cerebellum; endothelin, [^{125}I]-endothelin, heart; somatostatin, [^{125}I]-somatostatin, cerebral cortex; vasopressin V_1, [^{3}H]-vasopressin, liver; vasopressin V_2, [^{3}H]-vasopressin, kidney; vasoactive intestinal polypeptide, [^{125}I]-VIP, cerebral cortex; galanin, [^{125}I]-galanin, cerebral cortex; benzodiazepine (central), [^{3}H]-flunitrazepam, cerebral cortex; glycine (strychnine insensitive), [^{3}H]-5,7-DCKA, cerebral cortex. Values for pK_i are expressed as the mean value from 3 observations (see Battelle Handbook, 7, Route de Drize, CH-1227 Carouge, Switzerland).

Analytical methods and statistical analysis

All results were expressed as either geometric means with 95% confidence limits or arithmetic means ± s.e.mean of n observations and were compared by Student's paired t test; a P value of <0.05 being considered significant. Concentration-ratios were determined by comparison of the EC_{50} values between control and test curves. EC_{50} values were calculated at the 50% level of control curves to each agonist. pA_2 estimates were determined for GR113808 against 5-HT and **R,S**-zacopride by comparison of the responses at the EC_{50} level for each agonist. In the colon preparation, an apparent pK_B for GR113808 was determined, against 5-methoxytryptamine, at a concentration of 10.0 nM, using the Gaddum equation (Gaddum, 1957). *In vivo*, DR_{10} values were calculated graphically from plots of agonist dose-ratio against dose of antagonist.

Compounds

The following compounds were obtained from commercial sources: 5-HT, 5-MeOT, indomethacin, IBMX and isoprenaline (Sigma Chemical Co), ketanserin (Janssen Pharmaceutica), methysergide (Sandoz) and cholecystokinin octapeptide (CCK-8, Research Plus Inc.). Compounds synthesized by Glaxo Group Research Ltd. were: **R,S**-zacopride, ondansetron, [^{3}H]-GR65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone), U-46619 (9,11-dideoxy methano-epoxy-9d,11α-prostaglandin $F_{2\alpha}$) and GR113808 (([1-[2-methylsulphonyl)amino]ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate).

All compounds were solubilized in distilled water except indomethacin (stock solution in 10% w/v $NaHCO_3$ with subsequent dilution in distilled water). Solutions of isoprenaline always contained ascorbic acid (10 μM) as an antioxidant.

Results

Guinea-pig ascending colon

5-HT (3 nM–1 μM), 5-MeOT (30 nM–10 μM) and **R,S**-zacopride (0.1 μM–10 μM) elicited concentration-dependent contractions of the preparations with EC_{50} values of 28.3 (20.5–39.8) nM ($n = 16$), 0.77 (0.35–1.72) μM ($n = 15$) and 0.37 (0.31–0.43) μM ($n = 15$) respectively. Compared to 5-HT, 5-MeOT and **R,S**-zacopride behaved as partial agonists, producing maximum contractions which were 80 ± 6% and 52 ± 4% (at 3 μM) respectively of that to 5-HT. Addition of GR113808 to the bathing medium had no effect on the colon preparation *per se*. In the presence of increasing concentrations of GR113808, the concentration-effect curves to 5-HT, 5-MeOT and **R,S**-zacopride were displaced to the right in a concentration-dependent manner (Figure 2). There was a concentration-related depression of the maximum response. At a concentration of 100 nM GR113808, the maximum effect of 5-HT was reduced to 71.0 ± 6.8% of control values, with 5-MeOT the maximum was reduced to 55.0 ± 10.0% and with **R,S**-zacopride to 62.0 ± 15.0%. By comparison of the responses at the original EC_{50} level for each agonist, Schild analysis yielded pA_2 estimates of: 9.2 ± 0.2 (Schild slope of 1.1 (0.8–1.3)) against 5-HT and 9.2 ± 0.2 (Schild slope of 0.9 (0.6–1.1)) against **R,S**-zacopride. Since the maximum response to 5-MeOT was most affected, the affinity

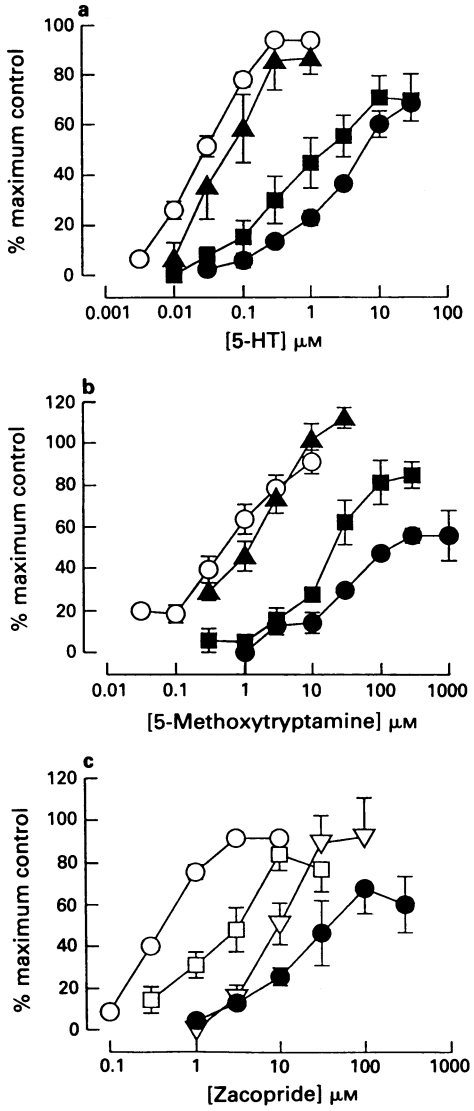

Figure 2 The antagonist effect of GR113808 on the contractile effects of 5-HT in the guinea-pig proximal colon. Curves represent concentration-effect curves to (a) 5-HT, (b) 5-methoxytryptamine and (c) **R,S**-zacopride in the absence (○) and presence of GR113808 1.0 nM (▲), 3.0 nM (□), 10.0 nM (■), 30.0 nM (▽) and 100.0 nM (●). Results are expressed as means ± s.e.mean, $n = 4-7$.

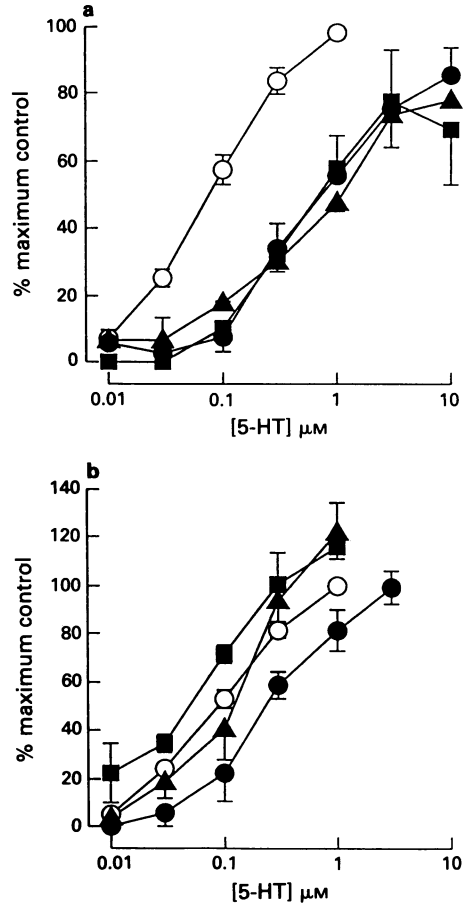

Figure 3 The effect of (a) increasing antagonist equilibration time and (b) increasing antagonist washout of GR113808 (10.0 nM) in the guinea-pig proximal colon. In (a), curves represent concentration-effect curves to 5-HT in the absence (○) and presence of GR113808 (10.0 nM) following tissue incubation for either 15 min (●), 30 min (▲) or 60 min (■). In (b), curves represent concentration-effect curves to 5-HT in the absence (○) of GR113808 and after pretreatment for 30 min with GR113808 (10.0 nM) followed by washout for either 15 min (●), 30 min (▲) or 60 min (■). Results are expressed as means ± s.e.mean, $n = 3-4$.

could only be estimated from a single antagonist concentration (10.0 nM). Use of the Gaddum equation yielded an apparent pK_B of 9.7 ± 0.2.

Cholecystokinin (CCK-8) (3 nM–0.3 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 5.5 (3.5–8.7) nM ($n = 6$). Incubation of the tissues with 1 μM GR113808 had no significant effect on the concentration-effect curves to CCK-8 (concentration-ratio = 1.9 (1.5–2.5)).

Investigation of onset and offset of antagonism due to GR113808

Onset 5-HT (10 nM–1 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 80.0 (60.0–110.0) nM ($n = 9$). Incubation with GR113808 (10.0 nM) for either 15, 30 or 60 min displaced 5-HT concentration-effect curves to the right, producing curves with EC_{50} values of 0.78 (0.46–1.33) μM ($n = 3$), 1.22 (0.79–1.88) μM ($n = 3$) and 0.88 (0.07–12.70) μM ($n = 3$) respectively (Figure 3a). The EC_{50} values calculated in the presence of antagonist were not signficantly different from one another ($P = 1.0$ comparing 15 min with 30 min, $P = 0.7$ comparing 15 min with 60 min). Furthermore, there was no increase in the depression of the maximum responses with increasing incubation time (see Figure 3).

Offset 5-HT (10 nM–1 μM) elicited concentration-dependent contractions of the colon preparation with an EC_{50} value of 90.0 (70.0–120.0) nM ($n = 16$). Incubation of the tissues for 30 min with GR113808 (10.0 nM) followed by repeated washout for either 15, 30 or 60 min and subsequent construction of concentration-effect curves to 5-HT yielded concentration-dependent contractile responses (Figure 3b). After washout for 15 min the EC_{50} value for the 5-HT concentration-effect curve was 0.21 (0.10–0.43) μM ($n = 4$). After washout for 30 min the EC_{50} value was 0.11 (0.04–0.29) μM ($n = 4$) and after washout for 60 min the EC_{50} was 0.11 (0.4–0.7) μM ($n = 4$). Only the EC_{50} value following washout for 15 min was significantly different from control values ($P < 0.01$).

Rat thoracic oesophageal muscularis mucosae

5-HT (1 nM–1 μM), 5-MeOT (1 nM–3 μM) and **R,S**-zacopride (1 nM–3 μM) elicited concentration-dependent relaxations of the precontracted rat thoracic oesophagus with EC_{50}

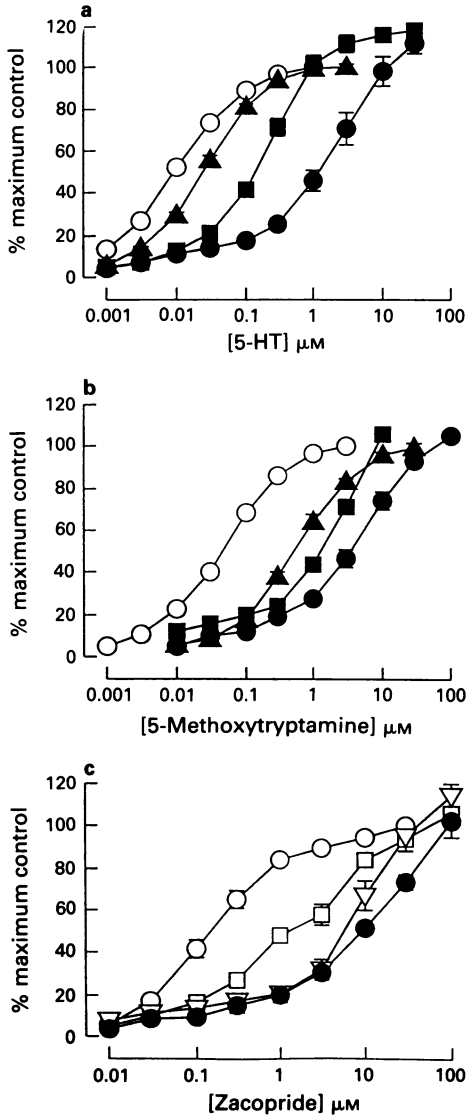

Figure 4 The antagonist effect of GR113808 on the relaxant response to (a) 5-HT, (b)-methoxytryptamine (5-MeOT) and (c) **R,S**-zacopride in the rat thoracic oesophagus. Curves represent concentration-effect curves to (a) 5-HT and (b) 5-MeOT in the absence (○) and presence of GR113808 1.0 nM (▲), 3.0 nM (□), 10.0 nM (■), 30.0 nM (▽) and 100.0 nM (●). Results are expressed as means ± s.e.mean, $n = 4-7$.

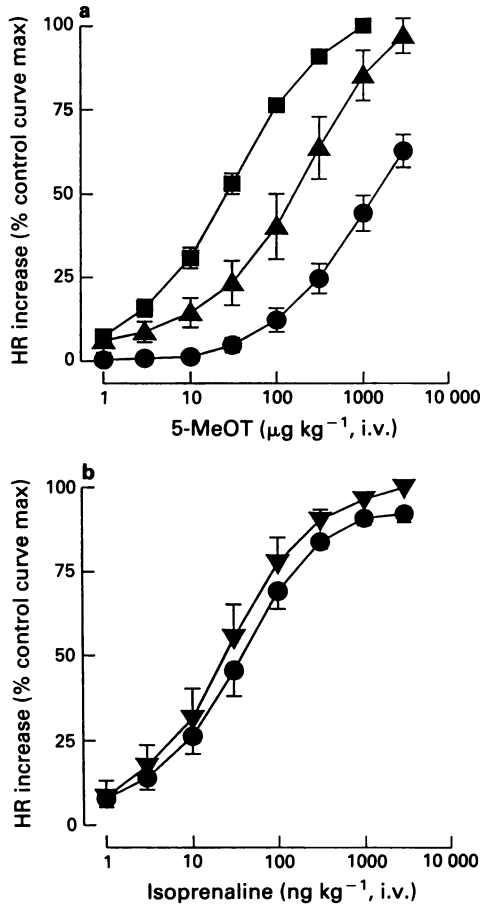

Figure 5 The antagonist effect of intravenous infusion of GR113808 on the increase in heart rate elicited by cumulative bolus intravenous administration of (a) 5-methoxytryptamine (5-MeOT) and (b) isoprenaline. Curves represent (a) 5-MeOT control (■) and dose-effect curves to 5-MeOT following 20 min infusion of GR113808 at 0.1 mg kg^{-1} h^{-1} (▲) and 0.5 mg kg^{-1} h^{-1} (●) or (b) isoprenaline dose-effect curves in the absence (▼) of GR113808 and following 20 min infusion of GR113808 at 0.5 mg kg^{-1} h^{-1} (●). Results are expressed as means ± s.e.mean, $n = 4$.

values of 9.0 (7.0–11.0) nM ($n = 12$), 47 (33–67) nM ($n = 10$) and 0.12 (0.07–0.20) μM ($n = 9$) respectively. Compared to 5-HT, 5-MeOT and **R,S**-zacopride behaved as full agonists. Addition of GR113808 to the bathing medium did not cause relaxation of the oesophagus preparation. In a number of tissues a small increase in the magnitude of the carbachol-induced contraction was observed following addition of the antagonist. In the presence of increasing concentrations of GR113808, the concentration-effect curves to 5-HT, 5-MeOT and **R,S**-zacopride were displaced to the right in a concentration-dependent manner (Figure 4). The maximum responses to all agonists was not affected by incubation with the antagonist. By comparing responses at the EC_{50} level for each agonist, Schild analysis yielded pA_2 estimates of 9.3 ± 0.1 (Schild slope of 0.9 (0.8–1.0)) against 5-HT, 9.0 ± 0.1 (Schild slope of 0.9 (0.8–1.1)) against 5-MeOT and 9.4 ± 0.2 (Schild slope of 0.8 (0.6–1.0)) against **R,S**-zacopride.

Isoprenaline (0.1 nM–0.3 μM) elicited concentration-dependent relaxations of the oesophageal preparations with an EC_{50} value of 1.27 (0.79–2.06) nM ($n = 6$). Incubation of the tissues with 1 μM GR113808 had no significant effect on the concentration-effect curves to isoprenaline (concentration ratio = 1.47 (0.96–2.27)).

Anaesthetized piglet

Intravenous bolus administration of 5-MeOT elicited dose-dependent increases in heart rate in the anaesthetized piglet, with an ED_{50} value of 25.3 (18.4–34.8) μg kg^{-1} ($n = 8$). In preliminary experiments, at least three dose-effect curves were shown to be reproducible (agonist dose-ratios between curves 1 and 2 and between 1 and 3 were 1.1 and 0.8 respectively). Following intravenous infusion of GR113808 at 0.1 mg kg^{-1} h^{-1} and 0.5 mg kg^{-1} h^{-1} for 20 min, the dose-effect curves to 5-MeOT were displaced to the right in a parallel fashion giving rise to agonist dose-ratios of 6.4 (1.9–21.6, $n = 4$) and 53.5 (15.9–180.0, $n = 4$) respectively (Figure 5a). The *in vivo* potency of the antagonist, expressed as a mean DR_{10} value calculated from both doses of antagonist, was 97.2 (53.4–177.0) μg kg^{-1} h^{-1}.

Intravenous bolus administration of isoprenaline elicited dose-dependent increases in heart rate in the piglet, with an ED_{50} value of 24.1 (3.8–153.8) ng kg^{-1} ($n = 3$). Successive dose-effect curves were shown to be repeatable (agonist dose-ratios between curves 1 and 2 and between curves 1 and 3 were 1.1. and 1.0 respectively). Following intravenous infu-

Table 1 The affinity of GR113808 for a range of receptor types assessed by binding assays (details in text)

Receptor	*Affinity* (pK_i)	*Receptor*	*Affinity* (pK_i)
Adenosine A_1	<5.0	Bombesin	<5.0
Adenosine A_2	<5.0	Endothelin	<5.0
α_1-Adrenoceptor	<5.0	Somatostatin	<5.0
α_2-Adrenoceptor	<5.0	Vasopressin V_1	<5.0
β_1-Adrenoceptor	5.1	Vasopressin V_2	<5.0
β-Adrenoceptor	<5.0	VIP	<5.0
Dopamine D_1	<5.0	Galanin	<5.0
Dopamine D_2	<5.0	Benzodiazepine	<5.0
$GABA_A$	<5.0	Glycine	<5.0
$GABA_B$	<5.0	μ-Opioid	<5.0
5-HT_1	<5.0	κ-Opioid	<5.0
5-HT_{1C}	<5.0	Bradykinin	<5.0
5-HT_3	6.0	CCK_A	<5.0
M_1 cholinoceptor	5.2	CCK_B	<5.0
M_2 cholinoceptor	<5.0	Neurokinin NK_1	<5.0
M_3 cholinoceptor	<5.0	Neurokinin NK_2	<5.0
Nicotinic cholinoceptor	<5.0	Neurokinin NK_3	<5.0
Histamine H_1	<5.0	CGRP	<5.0
Histamine H_3	<5.0	Neuropeptide Y	<5.0
NMDA	<5.0		

sion of GR113808 at 0.5 mg kg^{-1} h^{-1} for 20 min, the dose-effect curves to isoprenaline were unaffected (Figure 5b).

Selectivity at 5-HT receptors

GR113808 (10 nM–50 μM) did not exhibit any agonist activity at the 5-HT_2 receptor nor at the 5-HT_1-like receptors mediating contraction in the dog saphenous vein or 5-HT receptors mediating relaxation in the porcine vena cava. Furthermore, at 50 μM the compound did not affect contractile responses to 5-HT in either rabbit aorta or dog saphenous vein, nor the relaxant response to 5-HT in the porcine vena cava ($n = 2$). In addition, GR113808 was shown to have only low affinity at the 5-HT_3 receptor ($pK_i = 6.0$). Furthermore, GR113808 had no measurable affinity ($pK_i < 5.0$) at any of other 5-HT receptors examined in binding assays (Table 1).

Specificity

GR113808 had no significant binding affinity at the majority of receptor types examined. However, the compound was shown to have measurable affinity at the β_1-adrenoceptor ($pK_i = 5.0$) and the muscarinic M_1 cholinoceptor ($pK_i = 5.2$) (Table 1).

Discussion

In this study we have shown GR113808 to be a specific antagonist with high affinity for the 5-HT_4 receptor in guinea-pig isolated ascending colon and rat isolated oesophagus. It behaved as a surmountable antagonist in both preparations although some reduction in the maximum responses to agonists was observed in the colon. This effect on the maximum response to 5-HT_4 receptor agonists was apparently not attributable to a non-specific effect of GR113808 since responses to CCK-8 and isoprenaline were not affected in the colon and oesophagus preparations respectively.

A possible explanation for the depression of the maximum responses to agonists seen in the presence of GR113808 in the colon, but not in the oesophagus, may relate to a lack of steady-state conditions for the former preparation (for discussion of this topic, see Kenakin, 1987). Thus, the maxima of concentration-effect curves to agonists would be expected to be depressed if either (a) the antagonist dissociates slowly from the receptor or (b) the spare receptor population is low and/or the receptor-response coupling efficiency is poor, as would be so for a partial agonist (see Rang, 1966). Our data show that the offset rate for washout of GR113808 is slow in the colon preparation; after 15 min washing the effect of GR113808 was not fully reversed. However, this probably reflects the effects of diffusion of the drug from the tissue rather than slow dissociation of the antagonist from the receptor *per se*, since GR113808 has been shown to dissociate rapidly (<3 min) in radioligand binding studies using guinea-pig striatal membranes (Grossman *et al.*, 1993). Nevertheless, the fast, phasic response to agonists in the colon, in contrast to the slow response in the oesophagus, dictates that the agonist is only in contact for up to 2 min which might have contributed to the lack of steady-state conditions and apparent lack of competitive antagonism by GR113808 in colon. In addition, there seems to be a low reserve for 5-HT_4 receptors in the colon, as judged by the low potency of 5-HT and supported by the partial agonist activity of 5-MeOT and **R,S**-zacopride. This contrasts with the oesophagus where all the agonists tested produced similar maxima and were more active at lower concentrations than in the colon. The observed rise in tone in the precontracted oesophagus preparation, following application of the antagonist, is likely to be a result of antagonism of on-going relaxation produced by the release of endogenous 5-HT elicited by carbachol (Waikar *et al.*, 1993). These findings are entirely consistent with the concept that GR113808 is a competitive antagonist and that the reduction in agonist maxima that it produced in guinea-pig colon relates to a lack of steady-state conditions under the experimental conditions necessarily used for this preparation.

GR113808 is more potent than other 5-HT_4 receptor antagonists so far described, DAU 6285 (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride) and SDZ205-557 (2-methoxy-4-amino-5-chloro benzoic acid 2-(diethylamino) ethyl ester). These compounds have only modest affinity at the 5-HT_4 receptor. DAU 6285 has a pA_2 of between 6.64 and 7.16 in the guinea-pig ileum and human atrium respectively (Schiavone *et al.*, 1992), whereas SDZ205-557 has a slightly higher pA_2 of 7.4 in the guinea-pig ileum (Buchheit *et al.*, 1992) and a pA_2 of 7.3 in the rat oesophagus (Eglen *et al.*, 1993). In addition, *in vivo*, GR113808 was shown to be a very potent antagonist of the tachycardia response elicited by 5-MeOT ($DR_{10} = 97.2$ μg kg^{-1} h^{-1}); this response has previously been shown to be mediated by the 5-HT_4 receptor (Villalon *et al.*, 1990).

To date, tropisetron and DAU 6285 are the only 5-HT_4

receptor antagonists described in studies *in vivo* (Villalon *et al.*, 1990; Van Meel *et al.*, 1993). Tropisetron partially blocked the tachycardic effects of indole and benzamide agonists at the 5-HT_4 receptor on porcine heart. However, the compound exhibited only low potency, an intravenous bolus dose of 3 mg kg^{-1} being required to produce less than a 10 fold displacement to the right of the dose-effect curves (Villalon *et al.*, 1990). Similarly, between 1 and 3 mg kg^{-1}, i.v. DAU 6285 produced approximately a 10 fold displacement to the right of a 5-HT dose-effect curve in an anaesthetized pig model (Van Meel *et al.*, 1993). Although we are attempting to compare the effects of bolus doses of tropisetron and DAU 6285 with the effects of an intravenous infusion of GR113808, our data strongly suggests that GR 113808 is a considerably more potent 5-HT_4 receptor antagonist *in vivo*.

GR113808 exhibits a high degree of selectivity and specificity both *in vitro* and *in vivo*. The compound showed approximately 3000-fold selectivity between 5-HT_4 receptors and the other 5-HT receptors investigated. In particular, GR113808 exhibited a pK_i at the 5-HT_3 receptor of only 6.0 which makes the compound considerably more selective than the other 5-HT_4 receptor antagonists DAU 6285 and SDZ 205-557 (Buchheit *et al.*, 1992; Schiavone *et al.*, 1992; Eglen *et al.*, 1993). Furthermore, GR113808 demonstrated a high degree of specificity, showing at least a 10,000 fold specificity between 5-HT_4 receptor and the other non-5-HT receptor types examined.

Thus, GR113808 is a high affinity, selective and specific 5-HT_4 receptor antagonist which has potent activity *in vivo*. This compound therefore represents a useful pharmacological tool for investigating 5-HT_4 receptor-mediated events and for defining the role of this receptor in mammalian physiology and pathophysiology. Furthermore, the pharmacological profile validates its usefulness as a radioligand for the 5-HT_4 receptor (see Grossman *et al.*, 1993).

The authors would like to express their gratitude to Mrs Jill Darton and Mr Andy Green for expert technical assistance with the work presented in this paper.

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(Received June 29, 1993
Revised August 20, 1993
Accepted October 1, 1993)

Br. J. Pharmacol. (1994), **111**, 339–345

Contribution of thromboxane A_2 to the antigen-induced immediate asthmatic response mediated by IgG1 antibody by augmentation of bronchial responsiveness in guinea-pigs

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1 IgG1-mediated anaphylactic bronchoconstriction was elicited by intravenous administration of antigen to guinea-pig 2 days after passive sensitization with IgG1-rich serum, and this response was not affected by heating the serum (at 56°C, for 4 h). IgE-mediated bronchoconstriction, provoked 14 days after passive sensitization with IgE-rich serum, was completely abolished by the heating of the serum.

2 S-1452 (10 mg kg^{-1}, p.o.), a selective thromboxane (Tx) A_2 antagonist, significantly but incompletely suppressed the IgG1-mediated bronchoconstriction, but did not affect the IgE-mediated one, while diphenhydramine (5 mg kg^{-1}, i.v.), a histamine antagonist, almost completely inhibited both IgG1- and IgE-mediated bronchoconstriction.

3 Pretreatment with propranolol (1 mg kg^{-1}, i.v.), a β-adrenergic blocker, in addition to diphenhydramine, caused a long-lasting bronchoconstriction following antigen challenge in both animal models. This histamine-independent bronchoconstriction was markedly suppressed by S-1452 at a low dose of 0.1 mg kg^{-1}.

4 A significant increase in bronchial responsiveness to i.v. acetylcholine (ACh), compared to the prechallenge value, occurred as early as 3 min and persisted for 24 h after antigen challenge in the IgG1 model, but was not observed in the IgE model. S-1452 (10 mg kg^{-1}, p.o.) inhibited the IgG1-mediated bronchial hyperresponsiveness, as assessed 60 min after antigen challenge.

5 A marked elevation of TxB_2 levels was observed in bronchoalveolar lavage fluid (BALF) 3 min after antigen challenge in the IgG1 model, while levels were not changed in the IgE model. In contrast, the plasma TxB_2 level assessed 1 min after antigen challenge was increased in both the IgG1 and IgE models.

6 The results indicate that the inhibition of IgG1- but not IgE-mediated bronchoconstriction by higher doses of S-1452 may result from the suppression of increased bronchial responsiveness to allergic mediators such as histamine, which is probably due to TxA_2 generated in the airway lumen rather than in plasma. In both the IgG1 and IgE models, plasma TxA_2 appeared to contribute directly to the bronchoconstriction, its action being almost completely masked by histamine-mediated bronchoconstriction.

Keywords: S-1452; IgG1 antibody; IgE antibody; bronchoconstriction; bronchial hyperresponsivensss; thromboxane A_2

Introduction

It has been established that antibodies of the IgG and IgE classes can mediate allergic responses in man (Fagan *et al.*, 1982; Nakagawa & de Weck, 1983). Likewise, in guinea-pigs, both IgG1 and IgE antibodies have the capacity to sensitize pulmonary smooth muscle in antigen-induced contraction (Granziano *et al.*, 1984). However, in *in vitro* test systems, different mediators have been shown to be involved in the contraction of pulmonary tissues sensitized with the two homocytotropic antibodies (Regal, 1984; 1985). Thus it was demonstrated that a leukotriene antagonist inhibited only the IgG1-mediated contractile response in guinea-pig trachea and parenchyma, whereas a histamine antagonist suppressed both IgG1- and IgE-mediated responses. Furthermore, even at equivalent levels of contraction, the release of leukotrienes and histamine from pulmonary tissues taken from guinea-pigs sensitized with IgG1 antibody has been shown to be greater than such release in tissues from these animals in an IgE-sensitized state (Undem *et al.*, 1985; Ro *et al.*, 1991).

Thromboxane (Tx) A_2, an arachidonic acid metabolite, is an important mediator in the pathogenesis of bronchial asthma, as shown by the potent contractile response it induces in airway and vascular smooth muscle (Hamberg *et al.*, 1975; Svensson *et al.*, 1977), and by its release from lung tissues on immunological stimulation (Schulman *et al.*, 1981; Schleiner *et al.*, 1986). In addition, TxA_2 has been demonstrated to play an important role in the onset of bronchial hyperresponsiveness induced by inhalation of antigen and various stimuli such as leukotriene B_4 (LTB_4), platelet activating factor (PAF), ozone and lipopolysaccharide in animal models (O'Bryne *et al.*, 1985; Aizawa *et al.*, 1985; Chung *et al.*, 1986; Komatsu *et al.*, 1990; Arimura *et al.*, 1993). A potent and selective TxA_2 receptor antaonist, S-1452 (calcium (1**R**, 2**S**, 3**S**, 4**S**)-(5Z)-7-(((phenylsulphonyl)amino)bicyclo[2.2.1]hept-2-yl)hept-5-enoate dihydrate), has recently been identified (Ohtani *et al.*, 1991; Kishino *et al.*, 1991). This agent exerts an inhibitory effect on antigen-induced bronchoconstriction under conditions where the histamine-dependent contractile response was eliminated, on the action of various allergic spasmogens which are known to act directly or indirectly via stimulation of the TxA_2 receptor (Arimura *et al.*, 1992), and on bronchial hyperresponsiveness induced by lipopolysaccharide exposure in guinea-pigs (Arimura *et al.*, 1993).

In the present study, we examined the effects of S-1452 on IgG1- and IgE-mediated bronchoconstriction *in vivo*, using guinea-pigs passively sensitized with anti-ovalbumin (OA) IgG1-rich and anti-benzylpenicilloyl (BPO) IgE-rich sera, and we further investigated the role of TxA_2 in the immediately

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occuring anaphylactic bronchoconstriction. That is, we investiated whether TxA_2 acts as a bronchoconstrictor and/or as a potentiator of bronchial responsiveness to other spasmogenic chemical mediators.

Methods

Animals

Hartley albino guinea-pigs were obtained from Charles River Japan Inc. (Kanagawa, Japan). Males weighing 400–500 g were used throughout the study.

Antiserum

Guinea-pig anti-OA IgG1-rich serum was prepared as described in our previous papers (Arimura *et al.*, 1992). Briefly, guinea-pigs, immunized twice with i.p. injections of 0.2 ml of an emulsion containing 2 mg of OA and Freund's complete adjuvant (Difco, Detroit, Mich., U.S.A.) at 5-day intervals, were bled 16 days after the second injection. The antiserum was collected and stored at −20°C. Guinea-pig anti-BPO IgE-rich serum, which was kindly donated by Dr H. Nagai (Gifu Pharmaceutical University, Department of Pharmacology, Gifu, Japan), was prepared by the following procedure. Guinea-pigs were immunized 10 times at 3-week-intervals with an i.p. injection of 1.0 ml of 0.9% w/v NaCl solution (saline) containing 2 μg of bovine-γ-globulin conjugated with BPO haptens (Levine *et al.*, 1971) and 1 mg of aluminium hydroxide gel; they were bled 10 days after the last injection. The antiserum was collected and stored at −20°C.

To determine the IgG1 and IgE titers of each serum, 4 h and 14 day homologous passive cutaneous anaphylaxis (PCA), respectively, was performed in the guinea-pigs. A 0.05 ml portion of a two fold serial dilution of 100 fold diluted or undiluted antiserum was injected intradermally at several sites in the dorsal skin of guinea-pigs; this was followed 4 h or 14 days later by the intravenous injection of 1 mg of specific antigen, i.e., OA or bovine serum albumin conjugated with BPO haptens (BPO-BSA), together with 5 mg of Evans blue dye. Thirty minutes later, the animals were killed; the presence of blue stains with a diameter of more than 5 mm was taken as a positive reaction. The IgG1 antibody titer of the anti-OA serum was 1:1600; the IgE antibody was not detected. Both the IgE and IgG1 antibody titers of the anti-BPO serum were 1:2048.

Measurement of anaphylactic bronchoconstriction

After the animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.), the trachea, carotid artery, and left jugular vein were cannulated for the measurement of pulmonary parameters and systemic blood pressure and for the administration of antigen or drugs, respectively. Gallamine triethiodide (2 mg kg^{-1}, i.v.) was injected to suppress spontaneous breathing and the animals were then artificially ventilated with room air, using a small animal respirator (Model SN-480-7, Shinano, Tokyo, Japan) set to deliver a tidal volume of 3 ml $stroke^{-1}$ at a rate of 60 strokes min^{-1}. The body temperature was maintained at 37°C with a heating pad. A side arm to the trachea cannula was connected to a pressure transducer (TP-400T, Nihon Koden, Tokyo, Japan) to measure insufflation pressure continuously; this pressure was recorded on a polygraph according to the modified method of Konzett & Rössler (1940). An increase in insufflation pressure was taken to indicate bronchoconstriction.

IgG1- and IgE-mediated bronchoconstriction

To produce IgG1- and IgE-mediated bronchoconstriction, guinea-pigs were passively sensitized with anti-OA (0.2 ml per animal, i.p.) and 5 fold diluted anti-BPO (0.2 ml per animal, i.v.) sera, respectively. IgG1- and IgE-mediated bronchoconstriction were elicited by the i.v. administration of OA (70 μg kg^{-1}) 2 days and BPO-BSA (30 μg kg^{-1}) 14 days after the passive sensitization. To assess the involvement of chemical mediators other than histamine in IgG1- and IgE-mediated bronchoconstriction, we injected diphenhydramine (5 mg kg^{-1}, i.v.) and propranolol (1 mg kg^{-1}, i.v.) into the sensitized guinea-pigs 5 min before antigen challenge. This was done to suppress the bronchoconstrictor effect of the histamine released by antigen challenge and to enhance the bronchoconstriction induced by other mediators. The challenge doses of OA and BPO-BSA were reduced to 30 μg kg^{-1} and 20 μg kg^{-1}, respectively, to prevent animal death.

Measurement of bronchial responsiveness to acetylcholine (ACh)

Bronchial responsiveness was measured 3, 60 and 120 min and 24 h after the i.v. injection of antigen (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}) or saline in the IgG1 and IgE models. Guinea-pigs were anaesthetized with urethane (2 g kg^{-1}, i.p.) instead of sodium pentobarbitone and gallamine triethiodide, since gallamine is known to block ACh receptors. For the measurement of bronchial responsiveness at 3, 60 and 120 min, the animals were given i.v. specific antigen or saline after the surgical operation, following the pretreatment with diphenhydramine (5 mg kg^{-1}, i.v.) to avoid the influence of antigen-induced bronchoconstriction. Additional urethane was administered as required to maintain adequate anaesthesia. For the assessment of bronchial responsiveness at 24 h, specific antigen or saline was administered i.v. to the conscious guinea-pigs pretreated with diphenydramine (5 mg kg^{-1}, i.v.), and 23 h later, the animals were anaesthetized with urethane (2 g kg^{-1}, i.p.) and surgical operation was performed. Bronchial responsiveness was assessed by measuring the changes in insufflation pressure induced by increasing doses of i.v. ACh (6.25–100 μg kg^{-1}) at 5 min intervals.

Antigen-induced prostaglandin production

Antigen-induced mediator production was assessed in plasma 1 min and in BALF 3 min after antigen challenge, as previously described by Nambu *et al.* (1990). Blood samples (5 ml) were obtained 1 min after the i.v. administration of saline or antigen (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}), via the catheters inserted into the carotid arteries of the sensitized animals. The samples were withdrawn into polyethylene syringes containing EDTA (final concentration: 7.7×10^{-3} M) and indomethacin (final concentration: 10^{-5} M) to prevent the artificial generation of cyclo-oxygenase products. In addition, bronchoalveolar lavage (BAL) was performed before and 3 min after the antigen challenge in sensitized animals pretreated with diphenhydramine (5 mg kg^{-1}) to prevent bronchoconstriction. The BAL procedure was as follows: the animals were killed with an overdose of urethane; the trachea was then cannulated and 10 ml of 0.9% sterile saline, including 10 μg ml^{-1} indomethacin, was slowly injected into the lungs, withdrawn, and reinjected twice more. The blood samples and BAL fluid were centrifuged at 300 *g* for 10 min at 4°C. Aliquots of the supernatants were collected and stored at −20°C until required for anlaysis of TxB_2 and 6-keto-$PGF_{1\alpha}$.

Radioimmunoassay of TxB_2 and 6-keto-$PGF_{1\alpha}$

The blood samples and BAL fluid were acidified to pH 3 with 1 M HCl and loaded on Waters SEP-PAK C_{18} cartridges (Millipore Co., Milford, Ma., U.S.A.) that were washed with 99.5% ethanol and water before use. The cartridges were then washed with 4% acetic acid. Prostanoids were finally eluted with 2 ml of methanol. The eluant was evaporated under a stream of nitrogen gas at room temperature, and the

residue was dissolved in assay buffer solution. TxB_2 and 6-keto-$PGF_{1\alpha}$ in the extract were assayed by radioimmunoassay, with kits from New England Nuclear (Boston, Ma, U.S.A.).

Materials

Egg ovalbumin (OA; Seikagaku Kougyo, Tokyo, Japan), acetylcholine chloride, diphenhydramine hydrochloride and gallamine triethiodide (Sigma, St Louis, Mo., U.S.A.) were dissolved in saline. S-1452 (calcium (1**R**, 2S, 3S, 4S)-(5Z)-7-(((phenylsulphonyl)amino)bicyclo[2.2.1]hept-2-yl)hept-5-enoate dihydrate) was synthesized in our laboratories. For oral administration, S-1452 was suspended in 0.1% gum arabic solution.

Statistical analysis

All data are expressed as the mean ± s.e. Statistical significance was assessed by Dunnett's test or Student's *t* test and a *P* value of less than 0.05 was considered significant.

Results

IgG1- and IgE-mediated bronchoconstriction

IgG1-mediated bronchoconstriction was elicited by the i.v. administration of OA (70 μg kg^{-1}) 2 days after the passive sensitization, with a maximal increase in insufflation pressure of 68 ± 13 cmH_2O by 3–4 min. To verify whether IgE class antibodies were present in the IgG1 model, guinea-pigs were sensitized with anti-OA serum which had been previously heated at 56°C for 4 h to inactivate IgE (Parish, 1970). The OA-induced bronchoconstriction in animals sensitized with the heated serum was not different from that in animals sensitized with unheated serum, indicating that this response was not due to contaminating IgE class antibodies (Figure 1a). IgE-mediated bronchoconstriction was elicited by the i.v. administration of BPO-BSA (30 μg kg^{-1}) 14 days after the passive sensitization, with a peak increase in insufflation pressure of 64 ± 11 cmH_2O by 3–4 min. This bronchoconstriction was almost completely abolished in animals sensitized with serum heated at 56°C for 4 h (Figure 1b), indicating that this response was not mediated by IgG1 class antibodies.

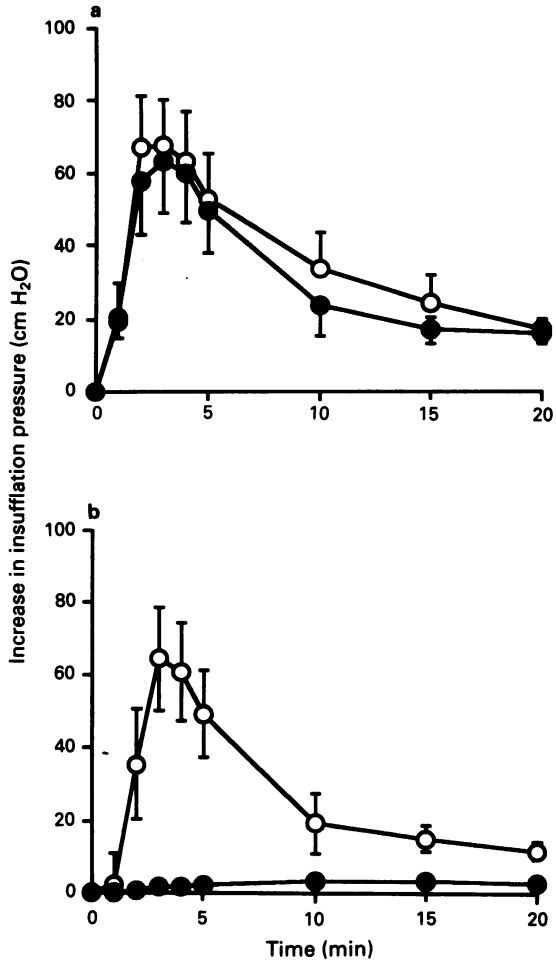

Figure 1 Antigen-induced bronchoconstriction in guinea-pigs passively sensitized with either heated (56°C, 4 h) (●) or unheated (○) anti-OA IgG1-rich serum (a: 0.2 ml, i.p.) and 5 fold diluted anti-BPO-IgE-rich serum (b: 0.2 ml, i.v.). Each specific antigen (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}) was administered to the anaesthetized animals after 2 day and 14 day sensitization periods, respectively. Results are expressed as the mean ± s.e. of 4–7 animals.

Effects of S-1452 and diphenhydramine on IgG1- and IgE-mediated bronchoconstriction

When S-1452 was administered orally 2 h before antigen challenge, IgG1-mediated bronchoconstriction was inhibited in a dose-dependent manner at doses of 3 to 10 mg kg^{-1}, the ED_{50} value (dose required for 50% inhibition of the peak increase in insufflation pressure of control group) being 6.3 mg kg^{-1}; however, there was no inhibition of IgE-mediated bronchoconstriction, even at the higher dose of 10 mg kg^{-1} (Figure 2). In contrast, i.v. administration of diphenhydramine (5 mg kg^{-1}) 5 min prior to antigen challenge almost completely suppressed both IgG1- and IgE-mediated bronchoconstriction (Figure 3).

Effect of S-1452 on histamine-independent IgG1- and IgE-mediated bronchoconstriction

To investigate the involvement of chemical mediators other than histamine in IgG1- and IgE-mediated bronchoconstriction, we pretreated guinea-pigs i.v. with diphenhydramine (5 mg kg^{-1}) and propranolol (1 mg kg^{-1}) before antigen challenge, thus allowing detection of histamine-independent bronchoconstriction. As illustrated in Figure 4, IgG1-mediated, histamine-independent bronchoconstriction developed slowly, reaching a maximum 5 to 10 min after antigen challenge, with the peak increase in insufflation pressure being 52 ± 8 cmH_2O. The IgE-mediated bronchoconstriction peaked at 10 to 20 min, with a maximal increase in insufflation pressure of 66 ± 6 cmH_2O. When administered orally 2 h before the antigen challenge, S-1452, at doses ranging from 0.01 to 0.1 mg kg^{-1}, markedly suppressed both IgG1- and IgE-mediated histamine-independent bronchoconstrictions, with ED_{50} values of 0.026 and 0.021 mg kg^{-1}, respectively.

Bronchial responsiveness after antigen challenge

Bronchial responsiveness to the i.v. administration of ACh was assessed 3, 60 and 120 min and 24 h after the i.v. administration of antigen or saline in sensitized guinea-pigs which had been pretreated with diphenhydramine (5 mg kg^{-1}, i.v.) to prevent bronchoconstriction. In guinea-pigs passively sensitized with anti-OA serum, bronchial responsiveness was markedly increased as early as 3 and persisted for at least 24 h after i.v. injection of OA (70 μg kg^{-1}), compared with the response at the same times after saline injection (Figure 5a–d). In contrast there was no or only a slight difference in bronchial responsiveness between BPO-BSA- and saline-injected animals passively sensitized with anti-BPO serum (results assessed at 3 and 60 min postchallenge are described in Figure 5e and f).

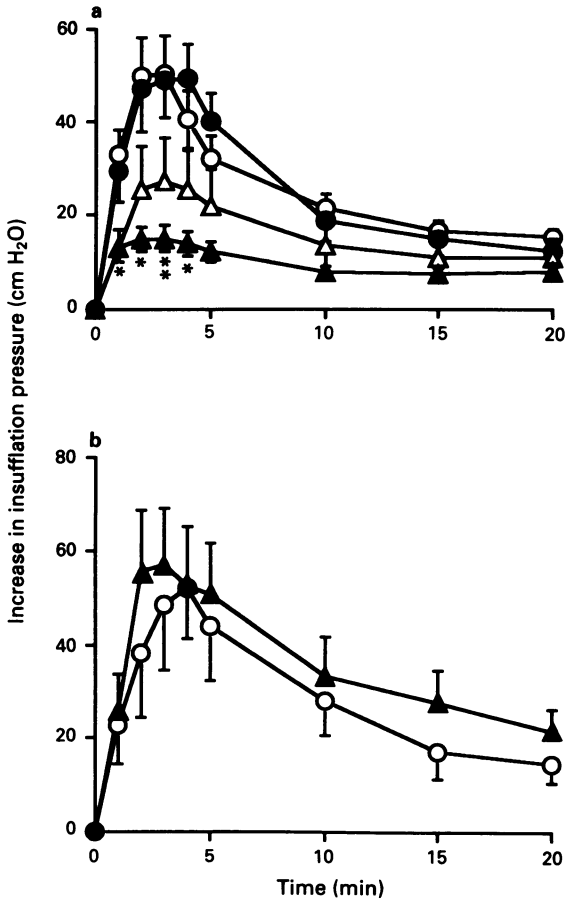

Figure 2 Effect of S-1452 on antigen-induced bronchoconstriction mediated by IgG1 (a) and IgE (b) antibodies in anaesthetized guinea-pigs. IgG1- and IgE-mediated bronchoconstrictions were elicited by i.v. administration of specific antigen (OA: 70 $\mu g\,kg^{-1}$; BPO-BSA: 30 $\mu g\,kg^{-1}$) 2 days and 14 days after passive sensitization with anti-OA IgG1-rich serum (0.2 ml, i.p.) and 5 fold diluted anti-BPO IgE-rich serum (0.2 ml, i.v.), respectively. Vehicle (○) or S-1452, at doses of 3 (●), 5.5 (△) and 10 mg kg^{-1} (▲), was administered orally 2 h before antigen challenge. Results are expressed as the means ± s.e. of 8 to 10 animals. Statistical significance: $^*P<0.05$; $^{**}P<0.01$, compared to vehicle control.

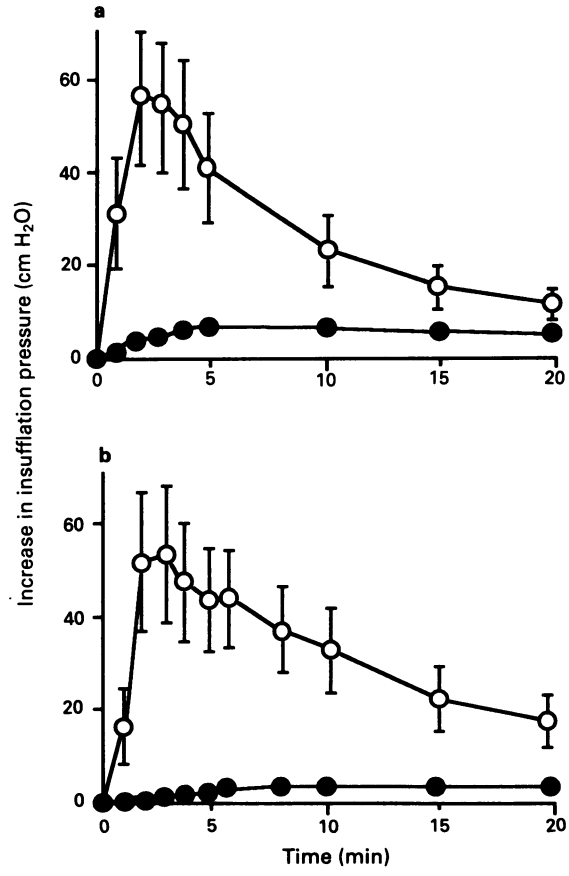

Figure 3 Effect of diphenhydramine on antigen-induced bronchoconstriction mediated by the IgG1 (a) and IgE (b) antibodies in anaesthetized guinea-pigs. Vehicle (○) or diphenydramine, at a dose of 5 mg kg^{-1} (●), was administered i.v. 5 min before antigen challenge. Results are expressed as the means ± s.e. of 6 to 8 animals. For other details, see the legend to Figure 2.

Effect of S-1452 on IgG1-mediated bronchial hyperresponsiveness

When administered orally 1 h before the i.v. injection of OA, S-1452, at doses of 1 to 10 mg kg^{-1}, suppressed the increase in bronchial responsiveness to i.v. ACh, as assessed at 60 min postchallenge, in a dose-dependent manner (Figure 6), although there was no significant change in the dose-response curve for i.v. ACh in normal guinea-pigs (data not shown).

Prostaglandin production after antigen challenge

Plasma levels of TxB_2 measured 1 min after antigen challenge were increased significantly in both the IgG1- and IgE-models, compared to the control TxB_2 levels following saline injection (Table 1). However, there were no significant differences in 6-keto-$PGF_{1\alpha}$ levels between antigen-treated and saline-injected groups in either the IgG1- or the IgE-model (Table 1). In contrast, there was a significant increase in TxB_2 levels in BALF obtained 3 min after antigen challenge in the IgG1 model, but not in the IgE model, although there was no significant increase in 6-keto-$PGF_{1\alpha}$ levels after antigen challenge in either the IgG1- or the IgE-model (Table 2).

Discussion

In the present study, to investigate whether the contribution of TxA_2 to antigen-induced anaphylactic bronchoconstriction in an *in vivo* system differed with sensitizing antibody class, i.e. with IgG1 and IgE antibodies, we passively sensitized guinea-pigs with anti-OA IgG1-rich and anti-BPO IgE-rich sera. A PCA examination of the antibody classes involved with each type of serum showed that the anti-OA serum, which was obtained from guinea-pigs sensitized with large amounts of antigen, contained only IgG1 type antibody, and the anti-BPO serum, which was obtained from animals immunized with small amounts of antigen together with alum, contained not only IgE but also IgG1 antibodies (Anderson, 1980). As expected, there was no contamination of the IgE-mediated response in the bronchoconstriction caused by the i.v. injection of OA 2 days after sensitization with anti-OA serum, as shown by our finding that OA induced bronchoconstriction was not changed in guinea-pigs sensitized with anti-OA serum that had been heated at 56°C for 4 h (Ovary *et al.*, 1963). To exclude IgG1-mediated responses from antigen-induced bronchoconstriction in guinea-pigs sensitized with anti-BPO serum, we performed antigen challenge after a long latent period (14 days) following the i.v. administration of 5 fold diluted antiserum, based on the assumption that IgE antibody persists in the tissue for relatively longer periods than the IgG1 antibody (Ovary *et al.*, 1963; 1976). This bronchoconstriction was almost completely

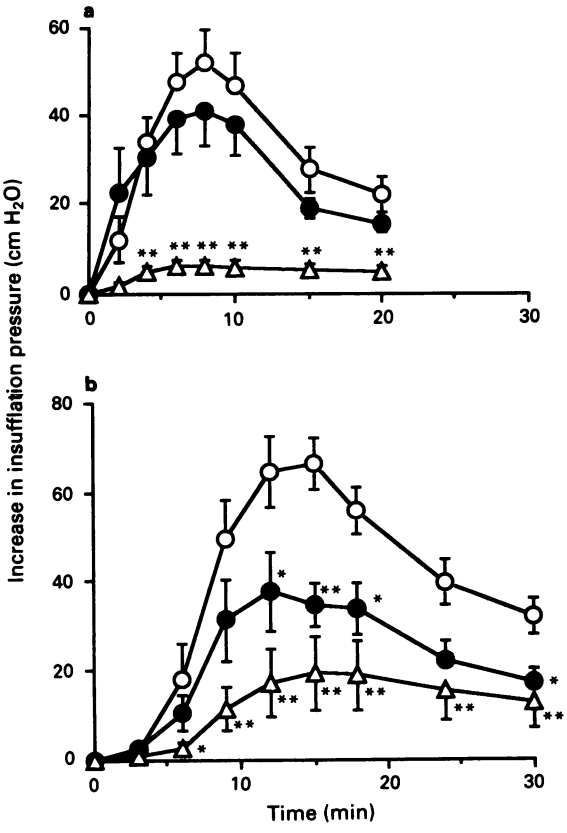

Figure 4 Effect of S-1452 on antigen-induced histamine-independent bronchoconstriction mediated by the IgG1 (a) and IgE (b) antibodies in anaesthetized guinea-pigs. Animals were treated with diphenhydramine (5 mg kg^{-1}, i.v.) and propranolol (1 mg kg^{-1}, i.v.) 5 min before antigen challenge. The procedures of passive sensitization and antigen challenge are described in the legend to Figure 2, but the challenge doses of OA and BPO-BSA were reduced to 30 and 20 μg kg^{-1} to prevent animal death by severe anaphylactic bronchoconstriction. Vehicle (○) or S-1452, at doses of 0.01 (●) and 0.1 mg kg^{-1} (△), was administered orally 2 h before antigen challenge. Results are expressed as the means ± s.e. of 5 to 10 animals. Statistical significance: $*P<0.05$; $**P<0.01$, compared to vehicle control.

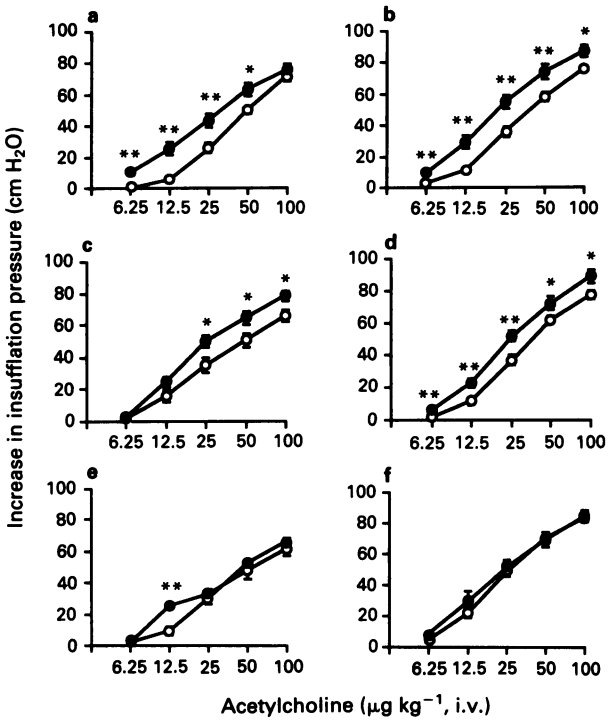

Figure 5 Time-dependent changes in increase in bronchial responsiveness to i.v. acetylcholine after antigen challenge in the IgG1 and IgE models as described in legend to Figure 3. Bronchial responsiveness in the IgG1 model was assessed 3 (a), 60 (b) and 120 min (c) and 24 h (d) after, and that in the IgE model assessed 3 (e) and 60 min (f) after, the i.v. injection of saline (○) or each antigen (●) (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}). To abolish the influence of antigen-induced bronchoconstriction on the measurement of bronchial responsiveness, animals were routinely treated with diphenhydramine (5 mg kg^{-1}, i.v.) 5 min before antigen challenge. Results are expressed as the means ± s.e. of 7 to 8 animals. Statistical significance: $*P<0.05$; $**P<0.01$, compared to saline-injected control.

reduced in guinea-pigs sensitized with 5 fold diluted anti-BPO serum that had been heated at 56°C for 4 h, indicating that there was no contamination of the IgG1-mediated response in this model.

Initially, we examined the effects of a selective TxA_2 antagonist, S-1452 (Ohtani *et al.*, 1991; Kishino *et al.*, 1991), and those of a histamine antagonist, diphenydramine, on IgG1- and IgE-mediated bronchoconstriction. Although diphenhydramine (5 mg kg^{-1}, i.v.) almost completely suppressed both IgG1- and IgE-mediated bronchoconstriction, oral administration of S-1452 at 10 mg kg^{-1} significantly, but incompletely, inhibited IgG1-mediated bronchoconstriction and did not affect IgE-mediated bronchoconstriction. The dose of S-1452 required to inhibit IgG1-mediated bronchoconstriction was more than 100 times greater than the dose required to suppress bronchoconstriction induced by a TxA_2-mimetic, U-46619 (Arimura *et al.*, 1992), while the dose required to inhibit the onset of bronchial hyperresponsiveness to i.v. ACh induced by inhalation of bacterial lipopolysaccharide in guinea-pigs (Arimura *et al.*, 1993) is similar to that required for the inhibition of IgG1-mediated bronchoconstriction. Morooka *et al.* (1991) have demonstrated that the doses of a platelet-activating factor (PAF) antagonist required to suppressed antigen-induced airway hyperreactivity were more than 100 times higher than the dose required to inhibit PAF-induced responses. These findings lead to the

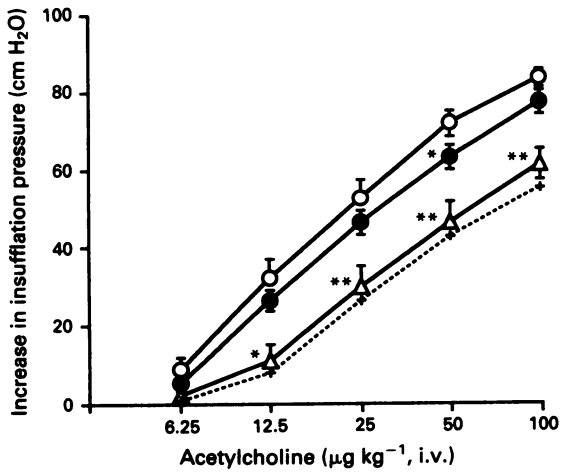

Figure 6 Effect of S-1452 on bronchial hyperresponsiveness to i.v. acetylcholine occurring after antigen challenge in the IgG1 model. Bronchial responsiveness was assessed 60 min after the i.v. injection of OA (70 μg kg^{-1}) in guinea-pigs sensitized with anti-OA IgG-rich serum and pretreated with diphenhydramine (5 mg kg^{-1}, i.v.). Vehicle (○) or S-1452, at doses of 1 (●), and 10 mg kg^{-1} (△), was administered orally 1 h before antigen challenge. Dashed line indicates bronchial responsiveness of unchallenged sensitized animals. Results are expressed as the means ± s.e. of 7 to 10 animals. Statistical significance: $*P<0.05$; $**P<0.01$, compared to vehicle control.

Table 1 Plasma levels of thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) after antigen challenge in the IgG1 and IgE models

	Challenge	*TxB_2* (pg ml^{-1})	*6-keto-$PGF_{1\alpha}$* (pg ml^{-1})
IgG1 model	Saline	67.0 ± 3.1	120.0 ± 5.2
	OA	340.0 ± 57.6**	193.3 ± 31.9
IgE model	Saline	103.2 ± 13.8	210.0 ± 21.0
	BPO-BSA	315.0 ± 32.4**	215.0 ± 6.7

Blood samples were obtained 1 min after the i.v. injection of saline or specific antigen (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}) in anaesthetized guinea-pigs which were sensitized with anti-OA IgG1-rich and anti-BPO IgE-rich sera as described in the legend to Figure 2. Results are expressed as the means ± s.e. of 6 animals. Statistical significance. $^{**}P<0.01$, compared to saline-injected control.

Table 2 Levels of thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in bronchoalveolar lavage fluid (BALF) after antigen challenge in the IgG1 and IgE models

	Time (min)	*TxB_2* (pg ml^{-1})	*6-keto-$PGF_{1\alpha}$* (pg ml^{-1})
IgG1 model	Pre	57.5 ± 7.8	25.8 ± 5.7
	3	107.2 ± 15.0*	43.4 ± 6.2
IgE model	Pre	49.0 ± 12.0	20.5 ± 2.8
	3	51.2 ± 5.0	23.6 ± 2.3

BAL was performed before (Pre) and 3 min after the i.v. injection of specific antigen (OA: 70 μg kg^{-1}; BPO-BSA: 30 μg kg^{-1}) in anaesthetized guinea-pigs which were sensitized with anti-OA IgG1-rich and anti-BPO IgE-rich sera as described in the legend to Figure 2. To prevent bronchoconstriction, animals were treated with diphenhydramine (5 mg kg^{-1}, i.v.) 5 min before antigen challenge. Results are expressed as the means ± s.e. of 5–6 animals. Statistical significance: $^{*}P<0.05$, compared to prechallenge value.

possibility that the inhibition of IgG1-mediated bronchoconstriction by higher doses of S-1452 is dependent upon the suppression of bronchial responsiveness to allergic spasmogens such as histamine, rather than upon the suppression of bronchoconstriction directly caused by TxA_2.

To test this hypothesis, we assessed bronchial responsiveness after the i.v. administration of specific antigen challenge in the IgG1 and IgE models. Since it was not possible to measure bronchial responsiveness under conditions where bronchoconstriction was occurring after antigen challenge, we pretreated guinea-pigs with diphenhydramine. After that, the bronchoconstriction following antigen challenge was almost completely abolished, and bronchial responsiveness to i.v. ACh could be assessed. In the IgG1 model, a significant increase in bronchial responsiveness to i.v. ACh was noted as early as 3 min after antigen challenge, compared to saline challenge, and this increase lasted for at least 24 h. However, in the IgE model, little or no increase in bronchial responsiveness was observed after antigen challenge. Furthermore, oral administration of S-1452, at 10 mg kg^{-1}, inhibited the onset of bronchial hyperresponsiveness in the IgG1 model. This dose was close to that required for the inhibition of IgG1-mediated histamine-dependent bronchoconstriction. These findings suggest that TxA_2 acts as a potentiator of bronchoconstriction induced by allergic mediators such as histamine rather than as a bronchoconstrictor in the IgG1- but not IgE-mediated anaphylactic bronchoconstriction, and may support the possibility that S-1452 suppresses the IgG1-mediated bronchoconstriction through the prevention of non-specific bronchial hyperresponsiveness which occurred as early as the onset of IgG-mediated bronchoconstriction.

Interestingly, levels of TxB_2, a stable metabolite of TxA_2, were significantly elevated in BALF, compared to the prechallenge value, 3 min after antigen challenge in the IgG1 model, but not in the IgE model. In contrast, plasma TxB_2 levels were significantly increased in both models, as assessed 1 min after antigen challenge. These results indicated that TxA_2 generated in the lung lumen may contribute to the onset of bronchial hyperresponsivess in the IgG1 model.

Although the precise reasons for the differences in TxA_2 production in the IgG1 and IgE antibody models remains unclear, it is likely that differences in the cell types activated by the two antibodies are responsible. It is possible that alveolar macrophages, which account for more than 80% of resident cells in lung lumen of normal guinea-pigs and can produce TxA_2 in response to antigen (Bachelet *et al.*, 1990), may be activated more easily by an IgG-mediated pathway than by an IgE-mediated one, since these macrophages possess a large number of Fc_γ receptors (Reynolds *et al.*, 1975). In addition, the release of leukotrienes and histamine from pulmonary tissues taken from guinea-pigs sensitized with IgG1 antibody has been shown to be much greater than that from tissues sensitized with IgE antibody, even though equivalent levels of contractions were obtained (Undem *et al.*, 1985; Ro *et al.*, 1991). TxA_2 production is known to be stimulated by leukotrienes in lung tissues of guinea-pigs (Folco *et al.*, 1981). It is therefore possible that the differences in TxB_2 generation in the IgG and IgE models may be the results of the differences in leukotriene production.

We also investigated whether circulating TxA_2 contributed to antigen-induced bronchoconstriction in the IgG1 and IgE models. As noted above, histamine is the major mediator in both IgG1- and IgE-mediated bronchoconstriction. We therefore eliminated the participation of histamine in antigen-induced bronchoconstriction by carrying out pretreatment with a histamine antagonist, diphenhydramine, and produced histamine-independent bronchoconstriction with a β-adrenoceptor blocker, propranolol (Ney, 1983). Thereafter, slowly developing and long-lasting bronchoconstriction was observed following antigen challenge in both the IgG1 and the IgE models, and these responses were markedly suppressed by the oral administration of S-1452 at low doses, ranging from 0.01 to 0.1 mg kg^{-1}, which are close to those required to inhibit U-46619-induced bronchoconstriction (Arimura *et al.*, 1992). These results indicate that plasma TxA_2 may contribute to bronchoconstriction in both the IgG1 and the IgE models, in guinea-pigs, but that this bronchoconstriction is completely masked by histamine-mediated bronchoconstriction.

Taken together, our results in the guinea-pig model indicate that TxA_2, probably generated in the lung lumen, plays an important role in IgG1-mediated bronchoconstriction by augmenting bronchial responsiveness to histamine. Our results also indicate that the suppression of IgG1-mediated bronchoconstriction by S-1452 (given orally) may have been due to the inhibition of the increased bronchial responsiveness that occurred immediately after the i.v. administration of antigen rather than to the inhibition of the direct bronchoconstrictor action of TxA_2. In contrast, IgE-mediated bronchoconstriction, which is caused primarily by histamine, is not likely to be potentiated by TxA_2. However, it is possible that systemically produced TxA_2 in both the IgG1 and the IgE models may exert bronchoconstrictor effects in certain conditions in which the participation of histamine in antigen-induced bronchoconstriction is excluded.

We thank Prof. Dr Nagai (Gifu Pharmaceutical University, Department of Pharmacology, Gifu, Japan) for supplying the anti-BPO IgE-rich serum used in this study.

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(Received May 17, 1993
Revised September 29, 1993
Accepted October 4, 1993)

Br. J. Pharmacol. (1994), **111**, 346–350

5-HT_3 receptors do not mediate vagally-induced relaxation or contraction of the isolated stomach of the guinea-pig

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1 We have tested whether 5-HT_3 receptors mediate vagally-induced relaxation or contraction of the isolated stomach of the guinea-pig.

2 The antagonists of 5-HT_3 receptors, ondansetron (1–10 μM) or metoclopramide (1–30 μM) did not inhibit vagally-induced relaxations at low concentrations but partially inhibited them at 30 μM or 60 μM, respectively. These higher concentrations of ondansetron and metoclopramide also inhibited relaxations induced by 1,1-dimethyl-4-phenylpiperazinium iodide (30 μM) but did not affect those induced by glyceryl trinitrate (0.7–1.1 μM).

3 Desensitization to 5-HT (100 μM) or 2-Me-5-HT (100 μM) did not affect relaxations or contractions induced by vagal stimulation.

4 Ondansetron (30 μM) or metoclopramide (60 μM) did not inhibit vagally-induced gastric contraction.

5 Thus, 5-HT_3 receptors do not mediate vagally-induced relaxation or contraction in the guinea-pig stomach.

Keywords: Guinea-pig stomach; 5-HT_3 receptors; vagal relaxation; vagal contraction

Introduction

We have shown that nitric oxide (NO) mediates the relaxation of the guinea-pig stomach induced by increasing the luminal pressure or vagal stimulation and have proposed that it is the final common mediator of inhibitory gastric reflexes (Desai *et al.*, 1991a,b). 5-Hydroxytryptamine (5-HT) is also a widely distributed neurotransmitter in the enteric nervous system (Wood, 1989; Mawe *et al.*, 1989). Within the gastrointestinal (GI) tract it stimulates both excitatory (Yamaguchi, 1972; Moen *et al.*, 1983) and inhibitory neurones (Gershon, 1967; Meulemans & Schuurkes, 1991) and releases NO from nonadrenergic, noncholinergic (NANC) neurones (Bogers *et al.*, 1991; Briejer *et al.*, 1992). It has also been proposed as a neurotransmitter of vagal inhibitory innervation in the guinea-pig stomach (Bülbring & Gershon, 1967; Bugge *et al.*, 1989) and the lower oesophageal sphincter in the opossum (Rattan & Goyal, 1978). These effects may be mediated by 5-HT_3 receptors, stimulation of which causes depolarization in guinea-pig and rat isolated vagus nerves, rat and rabbit superior cervical ganglia, and the rabbit nodose ganglion (Burridge *et al.*, 1989; Ireland *et al.*, 1982; Round & Wallis, 1986; Ireland & Tyers, 1987). Stimulation of 5-HT_3 receptors leads to acetylcholine (ACh) release from cholinergic nerve terminals in the guinea-pig myenteric plexus (Eglen *et al.*, 1990; Fox & Morton, 1990), noradrenaline (NA) release from sympathetic nerve terminals (Fozard *et al.*, 1979) and NO release from NANC neurones (Bogers *et al.*, 1991). In addition, stimulation of 5-HT_3 receptors on neurones of the myenteric plexus of the guinea-pig stomach antrum induces fast depolarization (Tack *et al.*, 1992). However, the physiological role of 5-HT in GI motility remains to be defined clearly, as do the receptor subtypes that mediate these effects.

Thus, our aim was to examine whether 5-HT_3 receptors mediate vagally-induced stimulation of inhibitory nitrergic neurones or excitatory cholinergic neurones in the isolated stomach of the guinea-pig. In addition, as ondansetron (GR38032F), a selective 5-HT_3 receptor antagonist (Butler *et al.*, 1988), is a potent and successful antiemetic (see Milne & Heel, 1991), we also wanted to find whether it acted in part by inhibiting vagally-mediated gastric receptive relaxation. This phenomenon is of interest, for it precedes vomiting (Cannon, 1898; Cannon & Lieb, 1911; Lumsden & Holden, 1969) and allows for a reversal of the gastroduodenal pressure gradient and reflux of the duodenal contents into the proximal stomach before expulsion (Davenport, 1971).

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Methods

Male Hartley guinea-pigs (300–350 g) were killed by cervical dislocation and exsanguination. The stomach along with 2 cm of oesophagus and adherent vagus nerves was immediately removed after cutting the duodenum just beyond the pylorus. It was immersed in oxygenated (95% O_2:5% CO_2) Krebs solution (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.17, $MgSO_4$ 2.5, $CaCl_2$ 2.5, glucose 5.6, pH 7.4) and a flexible cannula (i.d. 5 mm) placed in the pyloric end. The oesophagus was ligated at its proximal end without damaging the vagi. The contents of the stomach were gently flushed out with Krebs solution and the stomach was placed in an isolated organ bath containing 100 ml of gassed (95% O_2:5% CO_2) Krebs solution at 37°C. The pyloric cannula was connected through a warming coil to a reservoir filled with 1 litre of Krebs solution. The reservoir was placed on an adjustable stand so that it could be moved up or down to increase or decrease the pressure of fluid filling the stomach (Paton & Vane, 1963). Intragastric pressure was measured by a sidearm cannula connected to a Statham pressure transducer. The reservoir was sealed and connected to a float recorder to measure intragastric volume changes by means of a Harvard isotonic transducer. The volume and pressure changes were recorded on a Graphtec WR 3310 recorder. The oesophagus and the vagus were passed through a pair of ring electrodes connected to a Harvard Grass S 88 stimulator for electrical stimulation of the vagus, and kept immersed in Krebs solution to prevent drying. The experiments were started after a 45–60 min equilibration period.

To study the NANC inhibitory effects of vagal stimulation, some experiments were performed in the presence of atropine (3 μM) and guanethidine (5 μM) to inhibit cholinergic muscarinic and adrenergic effects, respectively. In other experiments, where only the excitatory (contractile) effect of

vagal stimulation was studied, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 100 μM) was used to inhibit the NANC inhibitory fibres (Desai *et al.*, 1991b), in the absence of atropine and guanethidine.

To determine the threshold pressure for an adaptive response, the reservoir was positioned such that Krebs solution in it was at the same level as the stomach. The pressure was thus zero and no fluid entered the stomach. The reservoir was then elevated in 1 cm increments, leading to a gradual filling of the stomach up to a threshold pressure (about 7 cmH_2O; 1 cmH_2O = 98.18 Pa) at which the fundus relaxed abruptly. This reproducible adaptive response, which is independent of extrinsic innervation and is NANC in nature (Paton & Vane, 1963; Desai *et al.*, 1991a), resulted in a substantial increase in intragastric volume (approx. 50% of the total volume), with no further increase in the pressure. The stomach was emptied after this relaxation via a side cannula and the next response was elicited after 10 min. The pressure was then elevated to 1–2 cmH_2O below the threshold for an adaptive response and the vagus nerve stimulated with square wave pulses (supramaximal voltage, 10–16 Hz, 1 ms, 50–60 s). The responses induced by vagal stimulation were reproducible over a period of 5–6 h at 10 min intervals in control experiments. The compounds were preincubated, using the same concentration both intra- and extraluminally simultaneously, to determine their effects on the responses to vagal or drug stimulation.

Statistical analysis

The volume changes due to vagal or drug stimulation are expressed as a percentage of the total gastric volume after stimulation, for relaxations; or as a percentage reduction of the volume before stimulation, for contractions. All results are expressed as the mean ± s.e.mean of n observations. The data were analysed for statistical significance by analysis of variance and post-hoc Bonferroni test or Student's unpaired two-tailed t test, with a P value <0.05 taken as significant.

Drugs used

5-Hydroxytryptamine creatinine sulphate, noradrenaline bitartrate, atropine sulphate, guanethidine sulphate, metoclopramide monohydrochloride, 1,1-dimethyl-4-phenyl piperazinium iodide and N$^{\omega}$-nitro-L- arginine methyl ester were purchased from Sigma Chemical Co., U.K. Glyceryl trinitrate (Nitronal) was purchased from Lipha Pharmaceuticals Ltd., U.K. Ondansetron hydrochloride (GR38032F) and 2-methyl-5-hydroxytryptamine hydrochloride (AH 21009 AC) were kindly provided by Glaxo Group Research Ltd., U.K. All other reagents used were of the highest commercially available purity and were purchased from either BDH or Sigma. The drug solutions were made in deionised water.

Results

Effect of ondansetron or metoclopramide on gastric relaxation induced by vagal stimulation

In the presence of atropine and guanethidine, vagal stimulation induced a reproducible relaxation, seen as an increase in intragastric volume (Figure 1). Low concentrations of ondansetron (1–10 μM for 20 min) or metoclopramide (1–30 μM for 30 min) did not inhibit this relaxation, nor did they affect the baseline tone or volume. At higher concentrations, which did not affect baseline tone, ondansetron (30 μM, Figure 1) or metoclopramide (60 μM) partially inhibited the relaxations induced by vagal stimulation or by 1,1-dimethyl-4-phenyl piperazinium (DMPP, 30 μM, 40–50 s) (see Table 1). These inhibitory effects were reversed on washing out the antagonist by repeated changing of the bathing Krebs solution (5–6 times over 45–60 min).

Ondansetron (30 μM) or metoclopramide (60 μM) did not inhibit the relaxation induced by glyceryl trinitrate (GTN, 0.7–1.1 μM, 60–70 s: control, 49 ± 8%; + ondansetron 45 ± 1%, $n = 3$, $P > 0.05$: control, 36 ± 6%; + metoclopramide, 29 ± 7%, $n = 5$, $P > 0.05$).

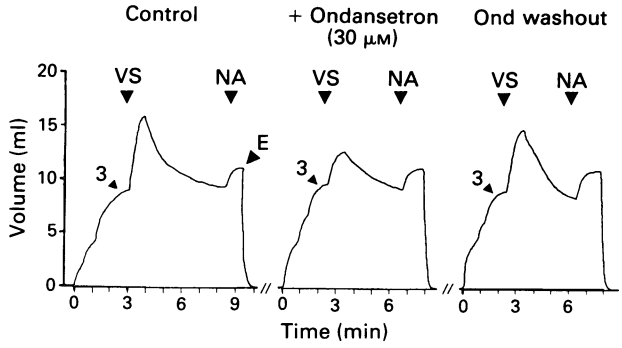

Figure 1 High concentrations of ondansetron reduce gastric relaxation in response to vagal stimulation. After determining the reproducible threshold pressure for a pressure-induced adaptive relaxation, the intragastric pressure was raised to 2 cmH_2O below this threshold pressure and vagal stimulation (VS) or exogenous noradrenaline (NA, 2 μM, 1 min) applied to induce a relaxation at this same pressure (3 cmH_2O). Incubation with ondansetron (Ond, 30 μM) reduced the relaxation induced by vagal stimulation but not by NA. Repeated washout of ondansetron for 45–60 min reversed the inhibitory effect on relaxation to VS. E denotes emptying of the stomach after a response and arrowheads indicate vagal stimulation. Small arrowheads show a baseline volume at a pressure of 3 cmH_2O. Experiments were performed in the presence of atropine (3 μM) and guanethidine (5 μM). This trace is typical of at least four other experiments.

Table 1 Ondansetron (30 μM) or metoclopramide (60 μM) inhibit gastric relaxations induced by vagal stimulation or 1,1-dimethyl-4-phenylpiperazinium (DMPP) in the guinea-pig

	Control	*Ondansetron*			
		1 μM	3 μM	10 μM	30 μM
Vagally-induced gastric relaxation	49 ± 2%	55 ± 2%	53 ± 3%	49 ± 6%	33 ± 4%**

	Control	*Metoclopramide*				
		1 μM	3 μM	10 μM	30 μM	60 μM
Vagally-induced gastric relaxation	40 ± 2%	44 ± 2%	46 ± 4%	45 ± 4%	33 ± 5%	22 ± 3%**

	Control	*Ondansetron* (30 μM)	*Control*	*Metoclopramide* (60 μM)
DMPP-induced relaxation	22 ± 2%	6 ± 2%**	17 ± 4%	3 ± 1%*

Responses are expressed as % of total volume. *$P<0.05$, **$P<0.01$, $n = 5$–6 for each group.

Effect of desensitization to 5-HT or 2-Me-5-HT, on relaxation induced by vagal stimulation

In the presence of atropine and guanethidine, exogenous 5-HT (1–50 μM, 60–75 s), given after elevation of the intragastric pressure, had no effect on the stomach. However, 5-HT in a concentration of 100 μM (60–75 s), induced a relaxation (26.7 ± 3.1%, $n = 5$, Figure 2), which was maximal and a higher concentration of 200 μM did not induce a greater relaxation. This relaxation was not always reproducible even after washout for 20–30 min, due to desensitization to its relaxant effect. Repeated (3–4 times) and prolonged (20–30 min total) exposure to 5-HT (100 μM) induced complete desensitization to its relaxant effect. This desensitization did not affect relaxations induced by vagal stimulation (control, 45 ± 3%; after desensitization, 46 ± 2%, $n = 4$, $P > 0.05$) or noradrenaline (2 μM, 1 min: control, 37 ± 5.0%; after desensitization, 32 ± 5%, $n = 4$, $P > 0.05$).

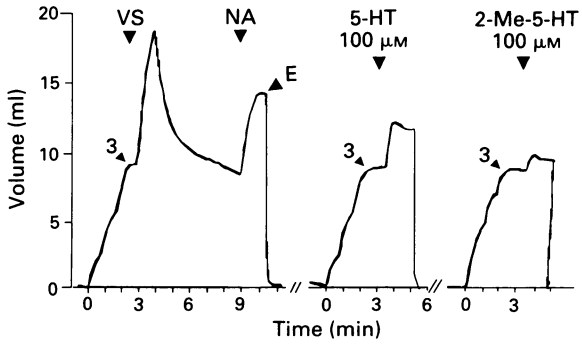

Figure 2 The relaxation of the guinea-pig stomach, induced by 5-hydroxytryptamine (5-HT) or 2-Me-5-HT does not match that induced by vagal stimulation. After elevating the intragastric pressure to 3 cmH_2O, stimulation of the vagus nerve (VS) or administration of noradrenaline (NA, 2 μM), 5-HT or 2-Me-5-HT induced a relaxation. The relaxations induced by 5-HT or 2-Me-5-HT did not match the magnitude of that induced by vagal stimulation. The experiments were done in the presence of atropine (3 μM) and guanethidine (5 μM). Small arrowheads show a baseline volume at a pressure of 3 cmH_2O. E denotes emptying of the stomach. This figure shows responses from three different stomachs and is representative of at least four experiments.

The selective 5-HT_3 receptor agonist, 2-Me-5-HT, had no effect in lower concentrations from 1–50 μM but in a concentration of 100 μM it induced a small relaxation (11.3 ± 1.6%, $n = 4$, Figure 2) on first application. This relaxation was maximal, for 200 μM did not induce a greater relaxation. Moreover, it was not always reproducible upon repeated application even after 20–30 min washout, due to desensitization. Repeated (3–4 times) and prolonged (20–30 min total) exposure to 2-Me-5-HT (100 μM) induced complete desensitization to its relaxant effect. This desensitization did not affect relaxations induced by vagal stimulation (control, 39 ± 1%; after desensitization, 37 ± 3%, $n = 4$, $P > 0.05$), or noradrenaline (2 μM, 1 min: control, 25 ± 2%; after desensitization, 22 ± 3%, $n = 4$, $P > 0.05$).

Effect of ondansetron or metoclopramide on gastric contraction induced by vagal stimulation

Preincubation with ondansetron (30 μM, 20 min) or metoclopramide (60 μM, 30 min, Figure 3) did not inhibit gastric contractions induced by vagal stimulation (Table 2). Ondansetron (30 μM) also did not affect contractions induced by acetylcholine (2 μM, 1 min).

Effect of desensitization to 5-HT or 2-Me-5-HT, on gastric contraction induced by vagal stimulation

Application of 5-HT in lower concentrations (1–50 μM, 60–90 s) had no effect on the stomach but in a higher concentration of 100 μM (60 s) it induced a contraction in the absence of atropine and guanethidine. This contraction was maximal and 200 μM 5-HT did not induce a greater contraction. Repeated (4–5 times) and prolonged exposure (20–30 min total) to 5-HT (100 μM) resulted in desensitization to its contractile effect but not to contractions induced by vagal stimulation or ACh (Table 3).

2-Me-5-HT in low concentrations (1–50 μM, 60–90 s) had no effect on the stomach. However, a concentration of 100 μM (60 s) induced a small maximal contraction (200 μM did not have a greater effect) in some experiments, followed by a small relaxation, while in others it induced a small relaxation but no contraction with the same concentration. In experiments in which 2-Me-5-HT (100 μM) induced a small contraction, desensitization to this effect could be induced by exposure to 2-Me-5-HT (100 μM, 3–4 times) for 20–30 min total. This desensitization did not affect contractions induced by vagal stimulation (Table 3).

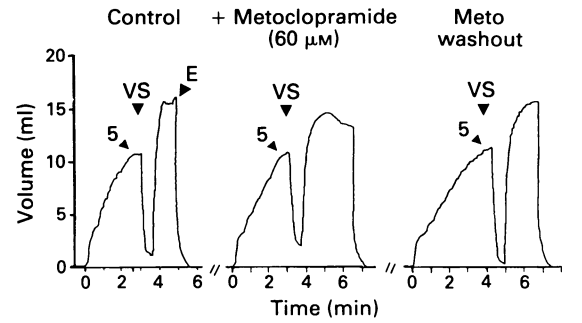

Figure 3 Metoclopramide (Meto) does not inhibit contraction induced by vagal stimulation. Stimulation of the vagus nerve (VS) in the presence of N^{ω}-nitro-L-arginine methyl ester (100 μM), after raising the intragastric pressure to 5 cmH_2O, induced a contraction. Incubation with metoclopramide (60 μM) did not inhibit contraction induced by vagal stimulation. Small arrowheads show a baseline volume at a pressure of 5 cmH_2O. E denotes emptying of the stomach after a response and arrowheads indicate vagal stimulation. This figure is typical of at least four experiments.

Table 2 Ondansetron (Ondan, 30 μM) or metoclopramide (Metoclo, 60 μM) do not inhibit gastric contraction induced by vagal stimulation

	Control	*Ondansetron* (30 μM)	*After Ondan. washout*
Vagal contraction	85 ± 5%	83 ± 7%	76 ± 6%
ACh contraction	37 ± 1%	45 ± 6%	35 ± 2%
	Control	*Metoclopramide* (60 μM)	*After Metoclo. washout*
Vagal contraction	92 ± 4%	84 ± 6%	93 ± 4%

Responses are expressed as the % reduction in volume. $n = 3$–4.

Table 3 Desensitization to 5-hydroxytryptamine (5-HT) or 2-Me-5-HT does not affect vagally-induced gastric contraction in the guinea-pig

	Control	*After desensitization to 5-HT*
Vagal contraction	92 ± 4%	92 ± 5%
ACh contraction	38 ± 4%	42 ± 8%
	Control	*After desensitization to 2-Me-5-HT*
Vagal contraction	76 ± 7%	71 ± 10%

Responses are calculated as % reduction in gastric volume. $n = 3-4$.

Discussion

NO is a major transmitter of gastric relaxation induced by an increase in luminal pressure or by vagal stimulation, in the guinea-pig, most probably secondary to the release of acetylcholine from vagal preganglionic fibres (Desai *et al.*, 1991a, b). However, other neurotransmitters could be involved in the transduction pathway within the myenteric plexus. 5-HT is a prime candidate as such a transmitter for desensitization to 5-HT has been reported to reduce the gastric relaxations resulting from vagal stimulation (Bülbring & Gershon, 1967). Moreover, 5-HT has also been shown to release nitric oxide from the NANC neurones in the GI tract through neuronal 5-HT$_3$ (Bogers *et al.*, 1991) or 5-HT$_1$ receptors (Briejer *et al.*, 1992). Hence, we investigated the possible role of 5-HT$_3$ receptors in the vagally-stimulated pathways leading to NO production and relaxation, or acetylcholine release and contraction of the stomach muscle.

Ondansetron or metoclopramide at low concentrations did not inhibit relaxations following vagal stimulation. Thus, it is unlikely that these are mediated by 5-HT$_3$ receptors, for ondansetron is a potent and selective antagonist of this receptor (Butler *et al.*, 1988). This may appear surprising as stimulation of 5-HT$_3$ receptors by 5-HT, induces fast depolarization in the myenteric plexus neurones of the guinea-pig stomach antrum. However, as the 5-hydroxytryptaminergic nerve fibres are outnumbered a thousand fold by cholinergic nerve fibres, their contribution to the stimulus-evoked fast e.p.s.p. response may be masked (Tack *et al.*, 1992). Conversely, high concentrations of ondansetron or metoclopramide did partially inhibit relaxation induced by vagal stimulation. This may be due to a non-specific effect, such as antagonism at histamine H_2 or ganglionic nicotinic receptors (Ireland *et al.*, 1982; Fortune & Ireland, 1984; Butler *et al.*, 1988). However, neither ondansetron nor metoclopramide inhibited the gastric contraction induced by vagal stimulation, which is mediated by ganglionic nicotinic receptors.

Experiments with either 5-HT or the selective 5-HT$_3$ agonist 2-Me-5-HT (Richardson *et al.*, 1985) also argue against the involvement of 5-HT$_3$ receptors. Desensitization to 5-HT, or 2-Me-5-HT, in marked contrast to a previously published report (Bülbring & Gershon, 1967), was not accompanied by any changes in vagally-induced relaxation. In addition, 2-Me-5-HT induced a maximal relaxation very much smaller than that induced by vagal stimulation (Figure 2) or by DMPP, whereas it would have been of equal size if the vagally-induced relaxation depended on 5-HT$_3$ receptors.

Ondansetron or metoclopramide in high concentrations that partially inhibited vagally-induced relaxations, did not affect vagally-induced contractions. Nor was desensitization to the contractile effect of 5-HT accompanied by any changes in the contractile responses to vagal stimulation. Hence, 5-HT or 5-HT$_3$ receptors do not appear to mediate stimulation of excitatory cholinergic neurones by the vagus nerves in the stomach. This is despite the fact that the release of ACh induced by exogenous 5-HT in the myenteric plexus of the guinea-pig ileum, is mediated by 5-HT$_3$ receptors (Eglen *et al.*, 1990; Fox & Morton, 1990). On the other hand, the contraction induced by 2-Me-5-HT or 5-HT was not seen in the presence of atropine. This was, therefore, most likely due to the release of ACh (Moen *et al.*, 1983; Eglen *et al.*, 1990).

Thus, NO is the major NANC inhibitory neurotransmitter (Desai *et al.*, 1991a,b), and ACh the main excitatory neurotransmitter, as well as the preganglionic neurotransmitter of the vagal motor innervation in the guinea-pig stomach. We did not find any strong evidence supporting a role for 5-HT in either contraction or relaxation induced by vagal stimulation, although there is a possibility that 5-HT could be involved in other circuits controlling gastric motility, such as pressure-induced relaxation, contraction or peristalsis. Our data also strengthen the idea that the potent anti-emetic effect of ondansetron is most probably by an effect on vagal afferent nerves, the chemoreceptor trigger zone and the nucleus tractus solitarius (see Milne & Heel, 1991), without any influence at the level of vagally-mediated gastric relaxation.

This work was supported by Glaxo Group Research Ltd, U.K.

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(Received June 7, 1993
Revised September 1, 1993
Accepted October 4, 1993)

Br. J. Pharmacol. (1994), **111**, 351–357

Modulation by the endothelium of sympathetic vasoconstriction in an *in vitro* preparation of the rat tail artery

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1 The influence of the endothelium on transmural electrical stimulation was investigated in isolated and perfused segments of the rat tail artery. Noradrenaline release (NA, quantified by h.p.l.c. – electrochemical detection) and changes in perfusion pressure (PP, measured at constant flow rate) were simultaneously recorded in unstimulated and stimulated arterial segments, in the absence and in the presence of drugs. The ratio PP/NA release (mmHg pg^{-1}) was taken as an index of the noradrenergic effectiveness.

2 Removal of the endothelium produced an increase in NA release and PP, in unstimulated and stimulated arteries. This can be taken as evidence of an endothelium-derived inhibitory factor (EDIF) acting at the prejunctional level, inhibiting NA release. Furthermore, in unstimulated arteries, the ratio PP/NA release decreased suggesting the existence of an endothelium-derived contracting factor (EDCF).

3 Perfusion of arteries with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10 μM) or methylene blue (MeB, 0.5 μM) had no effect on PP or NA release in unstimulated arteries. In stimulated arteries, both drugs potentiated the increase in PP without changing NA release and therefore, led to an increase in noradrenergic effectiveness. After removal of the endothelium, neither L-NAME nor MeB affected the increases in PP and NA release following electrical stimulation.

4 Carbachol (1 μM) attenuated both NA release and the increase in PP during electrical stimulation, and increased the ratio PP/NA release. L-NAME and MeB did not modify the inhibitory effect of carbachol on NA release, or the facilitatory effect of carbachol on the noradrenergic effectiveness.

5 Angiotensin II (AII, 0.1 μM) potentiated the increase in PP, without modifying NA release following electrical stimulation, and facilitated the vasoconstriction induced by perfusion of NA. In the absence of endothelium, AII potentiated both the increase in PP and NA release in arteries stimulated electrically but had no effect on the vasoconstriction induced by perfusion of NA. This suggests an endothelium-dependent activity of AII in this preparation.

6 These findings suggest that, in the rat tail artery, sympathetic vasoconstriction is modulated by three endothelial factors: (1) nitric oxide (NO), the release of which seems NA-dependent; (2) EDCF, predominant in the unstimulated state, the release of which can be stimulated by AII; and (3) EDIF, unmasked by removal of the endothelial layer, the release of which can be stimulated by AII.

Keywords: Rat tail artery *in vitro*; noradrenaline; endothelium; angiotensin II; carbachol

Introduction

The vascular endothelium modulates responses to stimulation of adrenergic nerves by releasing an endothelium-derived relaxing factor (EDRF) now identified as being nitric oxide (NO) (Palmer *et al.*, 1987), which acts directly on the vascular smooth muscle cells and produces vasodilatation (Hynes *et al.*, 1988; Reid *et al.*, 1991). In addition, the endothelium releases an endothelium-derived inhibitory factor (EDIF) which antagonizes the release of noradrenaline (NA) at the prejunctional level by an unknown mechanism (Cohen & Weisbrod, 1988; Greenberg *et al.*, 1989). Although it is generally accepted that angiotensin II (AII) amplifies sympathetic vasoconstriction by facilitating NA release (Ellis & Burnstock, 1989), a postjunctional endothelium-derived contracting factor (EDCF) mechanism may also be involved (Yen *et al.*, 1990). All these three factors could potentially interact in the modulation of vascular tone.

To test this hypothesis, we used the richly innervated rat tail artery which allows the simultaneous measurement of NA release and change in perfusion pressure (PP) (Fouda *et al.*, 1987). With this experimental model, we first tested the effect of removal of the endothelium on sympathetic vasoconstriction. Then, we used three different classes of pharmacological tools in order to find out if the endothelium-derived factors cited above, play an important role in the regulation of the vasomotor tone. N^{ω}-nitro-L-arginine methyl ester (L-NAME) and methylene blue (MeB) were used as blockers of EDRF/NO activity (Martin *et al.*, 1985; Palmer *et al.*, 1987). Carbachol, a muscarinic agonist, was used to antagonize sympathetic nerve-dependent vasoconstrictor, presumably by acting both pre- and postjunctionally (Langer, 1981; Toda *et al.*, 1990). Finally, AII was used to unmask an endothelium-dependent vasoconstrictor influence. Some of these results have been presented to the British Pharmacological Society (Thorin *et al.*, 1991).

Methods

Twelve month old male Wistar rats (400–450 g, Iffa-Credo SA, L'Arbresle, France) were used in all experiments. Animals received food and water *ad libitum* and were housed under standard conditions for at least 10 days prior to experimentation. Rats were exsanguinated under sodium pentobarbitone (50 mg kg^{-1} intraperitoneally) anaesthesia and two segments (1 cm length) of the proximal tail artery dissected out. The arterial segments were kept in Petri dishes containing a physiological salt solution at room temperature. The endothelium was removed from one segment by inserting a metal wire into the lumen.

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The presence or absence of the endothelium was confirmed by direct histological examination of the endothelium with silver staining at the conclusion of each experiment (Caplan *et al.*, 1974).

Perfusion and superfusion experiments

The proximal end of the arterial segment was cannulated with a polypropylene catheter (Portex, LSA, Fontenay-sous-bois, France). The distal end was cannulated with a curved catheter, thus allowing sampling of the perfusate for NA determination (see below). The segment was suspended vertically with the distal end uppermost under a resting tension of 0.4 g to restore its *in vivo* length, and was then perfused at a constant flow rate of 2 ml min^{-1}. Superfusion of modified Krebs bicarbonate was achieved by using a second pump at the same constant flow rate. The modified Krebs bicarbonate was of the following composition (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, NaH_2PO_4 1, $CaCl_2$ 2.6, $NaHCO_3$ 25 and glucose 11.1. Cocaine (4 μM) was added to inhibit the neuronal uptake of NA ($uptake_1$). In preliminary experiments, addition of propranolol (a β-adrenoceptor antagonist) to the modified Krebs bicarbonate had no effect on either change in perfusion pressure or NA release during an electrical stimulation (unpublished results). We have previously shown that β-adrenoceptors can affect noradrenergic effectiveness following electrical stimulation or hyperpotassium depolarization but that this is an important element at stimulation intensities above the ones used in the present paper (Fouda *et al.*, 1991). The perfusion pH and temperature were 7.4 ± 0.2 and 37.0 ± 0.2°C, respectively ($n > 50$). The modified Krebs bicarbonate was oxygenated with 95% O_2/5% CO_2.

Electrical stimulation

Segments were perfused and superfused for an equilibration period of 30 min before an initial series of electrical stimulation (1 Hz for 5 s, five stimulations, at 5 min intervals for 25 min; pulse generator Alvar SA, Montreuil, France).

At the end of this equilibration period, once vasoconstrictor responses to electrical stimulation were stable, basal perfusion pressure (PP, mmHg) was recorded with a low volume, strain gauge pressure transducer (Statham P23Db, Statham Ins., Puerto Rico). The pressure created by the tubing of the perfusion system was measured in the absence of the arterial segment, and all pressure values given were corrected by the subtraction of this value. The perfusate was collected for 2 min for the measurement of basal NA release (see below).

Experimental protocols

Following measurement of basal parameters, tail artery segments were subjected to electrical field stimulation (30 s, square wave pulses, 0.2 ms duration, supramaximal voltage, frequencies 0.3 Hz and 1 Hz at 5 min intervals). Vasoconstriction, estimated from the increase in PP above baseline was measured. The perfusate was collected during each 30 s electrical stimulation period for the determination of NA release (see below).

Segments were then perfused and superfused with the octapeptide AII (0.1 μM, in the presence of bovine serum albumin 0.1%) for 30 s and electrically stimulated (0.3 Hz). After a further 3 min perfusion of AII, segments were stimulated at 1 Hz.

Tail artery segments were next perfused and superfused for 10 min with the muscarinic agonist carbachol (1 μM) after which electrical stimulation (1 Hz) was performed.

A similar protocol was repeated in the presence of the nitric oxide synthase inhibitor, L-NAME (10 μM), the soluble guanylate cyclase inhibitor, MeB (0.5 μM), or the cyclo-oxygenase inhibitor, indomethacin (2.5 and 10 μM).

Low frequencies of stimulation and pulse duration were used in order to eliminate the release of co-transmitters such as ATP or neuropeptide Y (NPY) (Duckles & Dubaï, 1990). Moreover, phentolamine and reserpine pretreatment, completely blocked electrical stimulation-induced vasoconstriction (see below) suggesting a pure α-adrenergic component involved in the vasoconstrictor response of the isolated rat tail artery. Thus, it is unlikely that ATP or NPY play a major role in our experimental conditions.

Measurement of noradrenaline release

In all experiments, the perfusate was collected for 2 min before (spontaneous NA release) and for 30 s during electrical stimulation of the arterial segment (stimulated NA release). Samples were collected in ice cold tubes containing 15 mg of aluminium oxide, 20 μl of 0.3 N perchloric acid solution containing 36 nM of 3-4 dihydroxybenzylamine as internal standard and 100 μl of a solution containing 0.13 M Na_2EDTA and 53 mM $Na_2S_2O_5$ (sodium metabisulphite).

The pH of the solution was adjusted to 8.6 with 0.25 ml of 2 M Tris (hydroxymethyl-aminomethane)-HCl buffer (pH 8.8). Samples were placed on a rotator at 4°C and shaken for 15 min to allow adsorption of catecholamines onto the alumina. The supernatant was discarded, and the alumina washed three times with 2 ml triple distilled water at 4°C. NA was eluted with 50 μl of 0.3 N perchloric acid, filtered (0.45 μm) and 25 μl of eluate were injected in the chromatographic system. The high performance liquid chromatographic (h.p.l.c.) system (Waters, Bedford, U.S.A.) consisted of a pump (model 510), an injector (model U6K), a 5 μm silica column (Resolve C18) and an electrochemical detector (model 460) equipped with a glass carbon electrode. Samples were eluted at a flow rate of 0.8 ml min^{-1} with 0.1 M NaH_2PO_4, 0.05 mM Na_2EDTA, 0.52 mM octyl sodium sulphate and 8% (v/v) methanol. The electrochemical detector was set at + 0.6 V. The limit of detection was 0.4 pg of NA injected and the recovery of NA was 62–65%. The amount of NA present in each sample was calculated by using the peak height relative to that of the internal standard. NA release is expressed as pg $30\ s^{-1}$.

Samples of tail artery (1 cm of the proximal segment) were stored at − 80°C for further quantification of tissue NA content after reserpine pretreatment. Tissue content in NA was not affected by storage conditions for at least 2 months (unpublished results). Tissue samples were blotted dry, weighed and homogenized in perchloric acid (510 μl, 0.3 N) containing 3-4 dihydroxybenzylamine (7.2 μM) as internal standard. After centrifugation at 4°C and 8,000 *g* for 30 min, the supernatant was filtered (0.45 μm) and injected (25 μl) into the chromatographic column after dilution (100 times in perchloric acid 0.3 N). The protein content of the supernatant was determined (Lowry *et al.*, 1951). Tissue NA content is expressed as ng μg^{-1} of protein.

Preliminary experimental protocols

Optimal concentration of cocaine Cocaine (4 μM) was added to inhibit $uptake_1$. In order to determine the optimal concentration of cocaine to be used, electrical stimulation was performed in the presence of stepwise increases in the concentration of cocaine. Following equilibration, electrical stimulation (1 Hz, 30 s) was applied ($n = 6$ segments), the modified Krebs bicarbonate was replaced by modified Krebs bicarbonate plus cocaine (0.04 μM), and 15 min later the stimulation was repeated. The concentration of cocaine was increased in a stepwise manner (0.4 to 400 μM) and stimulation repeated at each step. Control arteries ($n = 6$) were subjected to the same protocol, in the absence of cocaine.

The maximum effect of cocaine on both vasoconstriction and NA release was observed at 4 and 40 μM (61 ± 15 mmHg and 4.9 ± 0.4 pg $30\ s^{-1}$, and 66 ± 15 mmHg and 4.7 ± 0.3 pg $30\ s^{-1}$, respectively).

At a lower concentration (0.4 μM: 31 ± 13 mmHg and 1.3 ± 0.2 pg $30\ s^{-1}$), and a higher concentration of cocaine

(400 μM: 31 ± 13 mmHg and 2.8 ± 0.4 pg 30 s^{-1}), stimulation-induced vasoconstriction and NA release were lower. Electrical stimulation (1 Hz) of control arteries in the absence of cocaine produced vasoconstrictor responses of between 27 and 35 mmHg and NA release was at the limit of detection of our h.p.l.c. method.

In summary, a concentration of cocaine of 4 μM appeared to be optimal for blocking NA $uptake_1$ in this caudal artery preparation.

Reproducibility of responses Several tissue controls were carried out. In order to evaluate the stability of the responses to repeated stimulation of the same segment, following equilibration, caudal artery segments were electrically stimulated (1 Hz, 30 s) at 15 min intervals for 90 min, in the absence of cocaine. Vasoconstrictor responses after the 1st and 6th electrical stimulation were 27 ± 5 and 28 ± 8 mmHg, respectively ($n = 6$). When this protocol was repeated in the presence of cocaine (4 μM, $n = 6$), vasoconstrictor responses and NA release after the 1st and 6th stimulations were 57 ± 6 mmHg and 6.6 ± 0.5 pg 30 s^{-1}, and 63 ± 8 mmHg and 7.6 ± 0.7 pg 30 s^{-1}, respectively. There were no time-related differences between either the vasoconstrictor responses or the stimulation-induced NA release.

In order to evaluate the reproducibility of the protocol, electrical stimulations (0.3 and 1 Hz) were performed, following equilibration, in the presence of cocaine (4 μM) in four separate groups (i to iv) of caudal artery segments ($n = 6$ per group). Responses at 0.3 Hz were (i) 30 ± 4 and 3.6 ± 0.5, (ii) 27 ± 5 and 3.2 ± 0.7, (iii) 35 ± 9 and 3.6 ± 0.5, and (iv) 43 ± 5 mmHg and 3.5 ± 0.5 pg 30 s^{-1}, and at 1 Hz: (i) 59 ± 5 and 8.0 ± 0.6, (ii) 60 ± 6 and 7.4 ± 0.8, (iii) 55 ± 7 and 6.2 ± 0.5, and (iv) 74 ± 8 mmHg and 6.6 ± 0.5 pg 30 s^{-1}. There were no significant differences amongst the four groups for either the vasoconstrictor responses or the stimulation-induced NA release.

In summary, this caudal artery preparation gives consistent, reproducible responses over at least a 90 min period.

Depletion of NA with reserpine In order to evaluate the role of NA release in the vasoconstrictor response to electrical stimulation, six rats were injected with reserpine (2.5 mg kg^{-1}, i.p.), 18 h prior to the experiment. One segment of the caudal artery was used for the determination of tissue NA content, the other was subjected to electrical stimulation (1 Hz, 30 s) in the presence of cocaine (4 μM). Tissue NA content of the caudal arteries of rats pretreated with reserpine was very low (0.03 ± 0.01 ng μg^{-1} protein) compared to control arteries (0.9 ± 0.1 ng μg^{-1} protein).

In reserpine pretreated rats, basal and stimulation-induced NA release was below the limit of detection of our h.p.l.c. system. Basal PP decreased in caudal arteries from pretreated rats (8.6 ± 0.6 versus 13.5 ± 0.8 mmHg in control group, $P < 0.05$) and electrical stimulation did not induce vasoconstriction.

In summary, reserpine pretreatment which depleted the caudal artery NA content, decreased basal PP and abolished NA release as well as vasoconstriction following electrical stimulation. This suggests that part of the basal PP, as well as the vasoconstrictor response to electrical stimulation, is of noradrenergic origin.

Effect of phentolamine on responses to electrical stimulation The α-adrenoceptor antagonist, phentolamine (1 μM) in the presence of cocaine (4 μM), abolished 1 Hz electrical stimulation-induced vasoconstriction, presumably via antagonism at the postjunctional α_1-adrenoceptor. In caudal arteries from male normotensive Wistar rats, the $-\log K_B$ for the competitive antagonism of NA-induced vasoconstriction is 8.1 (Fouda *et al.*, 1991). Phentolamine greatly potentiated NA release following electrical stimulation (20.1 ± 3.0 versus 6.6 ± 1.0 pg 30 s^{-1} in the absence of phentolamine, $n = 6$, $P < 0.05$), presumably via antagonism at the level of the prejunctional α_2-adrenoceptor involved in the negative feedback control of NA release (Langer, 1981).

In summary, in this caudal artery preparation, a high concentration of the nonspecific α-adrenoceptor antagonist, phentolamine, blocked presynaptic α_2-adrenoceptors and so increased NA release, and blocked postsynaptic α_1-adrenoceptor and so abolished vasoconstriction.

Solutions and drugs

Carbachol chloride, N^{ω}-nitro-L-arginine methyl ester (L-NAME), methylene blue, noradrenaline bitartrate, cocaine hydrochloride, 3-4dihydroxybenzylamine hydrobromide, bovine serum albumin and sodium octyl sulphate were purchased from Sigma, St Louis, MO, U.S.A. Phentolamine (Regitine), angiotensin II (Hypertensin Ciba) and reserpine (Serpasil) were gifts from Ciba Geigy, Basel, Switzerland. Sodium disulphite, disodium EDTA (ethylene diamine tetra-acetic acid), perchloric acid, Tris (hydroxymethyl-aminomethane) and all chemicals for the preparation of the modified Krebs bicarbonate solution were h.p.l.c. grade and purchased from Merck, Darmstadt, Germany. Aluminium oxide was purchased from Millipore-Waters, Bedford, U.S.A. Methanol (h.p.l.c. grade) was purchased from Carlo Erba, Milan, Italy. Indomethacin was purchased from Merck Sharp and Dohme-Chibret, Riom, France.

Statistical analysis

Values are presented as the mean ± s.e.mean. Part of the results are expressed as the ratio of PP/NA released (mmHg pg^{-1}). Statistical differences between means were evaluated by Student's unpaired or paired t test as appropriate. A probability level of $P < 0.05$ was accepted as being statistically significant. The number of arterial segments per protocol was $\geqslant 6$.

Results

Histological assessment of the state of the endothelium

After each experiment, arterial segments were stained with silver nitrate. The physical integrity of the endothelium was not altered by the protocol used in this study. In all cases, after rubbing, only a few spots of remaining endothelial cells were visible.

Effect of removal of endothelium on unstimulated and stimulated vasoreactivity

Removal of endothelium increased the spontaneous NA release from 0.6 ± 0.2 to 1.8 ± 0.4 pg 30 s^{-1} ($n = 12$, $P < 0.05$) and basal PP from 7.8 ± 0.8 to 11.3 ± 0.9 mmHg ($n = 12$, $P < 0.05$). The ratio PP/NA release, however, decreased by 46%, from 11.7 ± 0.4 to 6.3 ± 0.4 mmHg pg^{-1} ($P < 0.05$).

Removing the endothelium potentiated electrical stimulation-induced NA release by 122 and 95%, and the increase in PP by 150 and 57%, at 0.3 and 1 Hz, respectively (Figure 1, a and c). The ratio PP/NA release did not change in the absence of endothelium (9.4 ± 0.7 and 7.9 ± 0.5 versus 8.3 ± 1.1 and 8.6 ± 0.7 mmHg pg^{-1}, at 0.3 and 1 Hz, respectively).

Effect of L-NAME and MeB on vasoreactivity

In unstimulated, intact arterial segments, L-NAME (10 μM) had no effect on either PP or spontaneous NA release (data not shown). Moreover, L-NAME had no effect on either PP or NA release in either unstimulated (data not shown) or stimulated arterial segments without endothelium (Figure 2).

In the presence of endothelium, vasoconstrictions were potentiated for both electrical stimulation frequencies tested in the presence of L-NAME; there was no change in NA

release (Figure 2). Therefore, the ratio PP/NA release increased (14.7 ± 1.4 and 12.6 ± 1.6 versus 8.3 ± 1.1 and 8.6 ± 0.7 mmHg pg^{-1} in the absence of L-NAME, at 0.3 and 1 Hz, respectively, $n = 4$, $P < 0.05$).

MeB (0.5 μM), had no effect on unstimulated PP and NA release (data not shown). MeB potentiated the vasoconstriction induced either by electrical stimulation at 0.3 Hz (103 ± 5 versus 47 ± 3 mmHg, $n = 6$, $P < 0.05$) or 1 Hz (140 ± 13 versus 74 ± 8 mmHg, $n = 6$, $P < 0.05$). Therefore, the ratio PP/NA release increased (32.8 ± 7.0 and 16.2 ± 1.2 versus 12.3 ± 1.1 and 11.2 ± 1.1 mmHg pg^{-1} at 0.3 and 1 Hz, respectively, $P < 0.05$). MeB had no effect in the absence of endothelium (data not shown). The results obtained with MeB confirm those with L-NAME.

Effect of carbachol on vasoreactivity

Carbachol (1 μM) had no effect on basal PP and NA release (results not shown). Electrically stimulated NA release and increase in PP were antagonized by carbachol by 79% and 61%, respectively (Table 1). The ratio PP/NA release was increased by over 240%. Removal of endothelium significant-

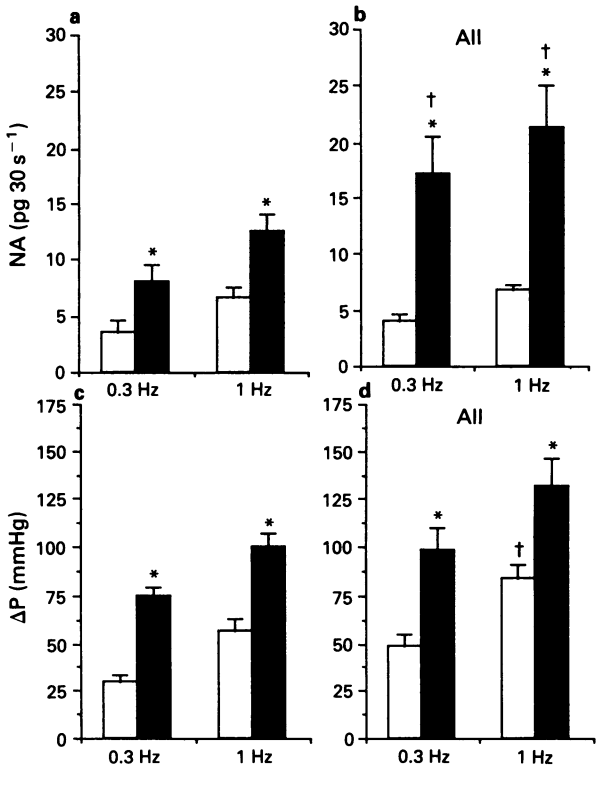

Figure 1 Electrical stimulation-induced noradrenaline release (NA, a,b) and contraction (ΔP, c,d) of the rat tail artery with (open column) or without (solid column) endothelium, in the absence (a,c) or in the presence (b,d) of angiotensin (AII, 0.1 μM). All values shown are means ± s.e.mean of 12 experiments. *$P < 0.05$ versus with endothelium (t test); †$P < 0.05$ versus without angiotensin II (t test).

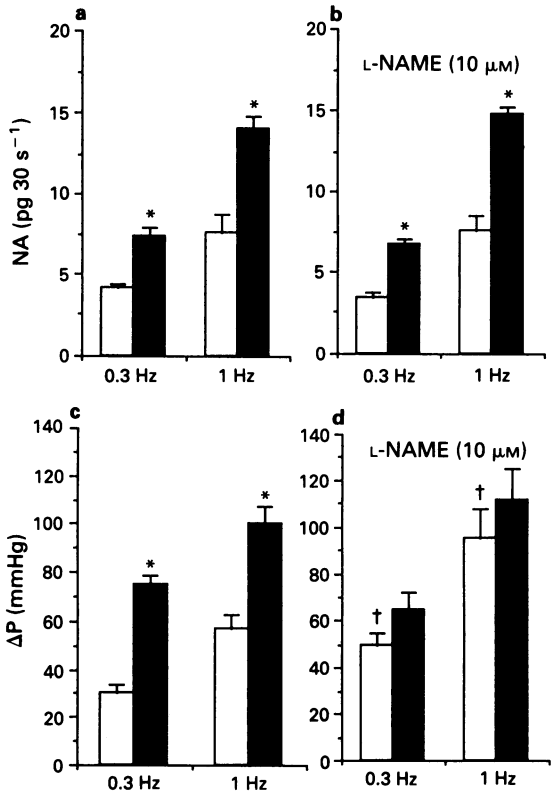

Figure 2 Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (b,d) on noradrenaline release (NA, a,b) and contraction (ΔP, c,d) of the electrically stimulated (0.3 and 1 Hz) rat tail artery, with (open columns) or without (solid columns) endothelium. All values shown are means ± s.e.mean of 4 experiments. *$P < 0.05$ versus with endothelium (t test); †$P < 0.05$ versus without L-NAME (t test).

Table 1 Effect of carbachol in the absence or in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on electrical stimulation-induced (1 Hz) noradrenaline release (NA, pg 30 s^{-1}), increase in perfusion pressure (PP, mmHg) and change in the ratio PP/NA release (mmHg pg^{-1}) of rat tail arterial segments with or without endothelium

Groups	*PP*	*NA*	*Ratio*
	Endothelium present		
Control	57 ± 6	6.6 ± 1.0	8.6 ± 0.7
Carbachol (1 μM)	25 ± 5*	1.2 ± 0.4*	21.0 ± 4.0*
L-NAME (10 μM)	95 ± 13*	7.6 ± 0.9	12.6 ± 1.6*
Carbachol + L-NAME	74 ± 12[c]	2.4 ± 0.2*[d]	31.0 ± 6.0*[d]
	Endothelium absent		
Control	100 ± 7†	12.3 ± 1.4†	8.1 ± 0.7
Carbachol (1 μM)	67 ± 8†*	5.8 ± 1.4†*	11.6 ± 2.8†
L-NAME (10 μM)	112 ± 13	14.8 ± 0.4†	7.6 ± 0.5†
Carbachol + L-NAME	87 ± 10	7.2 ± 1.8*†[d]	12.0 ± 2.0†

All values shown are means ± s.e.mean of 4 to 6 experiments.
*$P < 0.05$ versus control (t test).
†$P < 0.05$ versus with endothelium (t test).
[c]$P < 0.05$ versus carbachol (t test).
[d]$P < 0.05$ versus L-NAME (t test).

ly altered the effect of carbachol: NA release was decreased by 35% during a 1 Hz stimulation and the vasoconstriction by 42% only (Table 1). The ratio PP/NA release was not significantly modified by carbachol in denuded segments.

L-NAME (10 μM) had no effect on carbachol-induced inhibition of NA release, but suppressed the vasorelaxant property of the muscarinic agonist. The ratio PP/NA release was similar to that determined in the presence of carbachol alone (Table 1).

When arterial segments with endothelium were stimulated in the presence of both carbachol (1 μM) and MeB (0.5 μM), NA release decreased by 38% as opposed to 79% in the absence of MeB (5.4 ± 1.2 versus 1.2 ± 0.4 pg 30 s^{-1}, $n = 6$, $P < 0.05$), and vasoconstriction decreased by 27% as opposed to 61% (103 ± 12 versus 25 ± 5 mmHg, $n = 6$, $P < 0.05$). The ratio PP/NA release did not change in the presence of MeB (19 ± 3 versus 21 ± 4 mmHg pg^{-1}).

The results obtained with MeB confirm those obtained with L-NAME.

Effect of angiotensin II on vasoreactivity

At the concentration used (0.1 μM), AII affected neither the basal PP (results not shown) nor electrical stimulation-induced NA release (Figure 1). However, the octapeptide facilitated the vasoconstrictor response by 71% and 34% at 0.3 and 1 Hz, respectively (Figure 1). The ratio PP/NA release increased in the presence of AII (12.2 ± 1.7 and 12.3 ± 0.7 versus 8.3 ± 1.1 and 8.6 ± 0.7 mmHg pg^{-1} at 0.3 and 1 Hz, respectively, $P < 0.05$).

After removal of the endothelium, AII potentiated both NA release (by 115% and 65%), and the increase in PP (by 30% and 32% at 0.3 and 1 Hz, respectively, Figure 1), but had no effect on the ratio PP/NA release (5.7 ± 1.9 and 6.2 ± 0.9 versus 9.4 ± 0.7 and 7.9 ± 0.5 mmHg pg^{-1} at 0.3 and 1 Hz, respectively).

In order to investigate whether AII potentiated vasoconstriction by inhibition of EDRF/NO formation, experiments ($n = 6$) were carried out in the presence of MeB (0.5 μM). Vasoconstriction was still potentiated by AII, significantly so at the higher stimulation frequency (130 ± 15 and 190 ± 13 versus 105 ± 16 and 140 ± 13 mmHg at 0.3 and 1 Hz, respectively). MeB did not modify the effect of AII on the ratio PP/NA release (22.0 ± 2.3 and 20.0 ± 2.3 versus 32.8 ± 7.0 and 16.1 ± 1.2 mmHg pg^{-1} at 0.3 and 1 Hz, respectively).

In order to investigate whether eicosanoids were involved in the endothelium-dependent effect of AII on vasoreactivity, indomethacin (2.5 and 10 μM) was used to block synthesis of prostanoid-related substances. Indomethacin did not modify electrically stimulated NA release, vasoconstriction (Figure 3) or the effects of AII (Figure 3).

Effect of angiotensin II on noradrenaline-induced contraction

Intraluminally perfused NA produced an increase in perfusion pressure of 29 ± 11 and 100 ± 11 mmHg at 1 and 3 μM, respectively. Removal of endothelium potentiated the vasoconstrictor response to NA for the lower (1 μM, 61 ± 10 mmHg, $P < 0.05$) but not for the higher concentration (3 μM, 119 ± 9 mmHg).

AII (0.1 μM) potentiated NA-induced vasoconstriction of arterial segments in the presence of endothelium (76 ± 19 and 147 ± 12 mmHg, at 1 and 3 μM, respectively, $P < 0.05$), but not in the absence of endothelium (76 ± 12 and 128 ± 10 mmHg, at 1 and 3 μM, respectively).

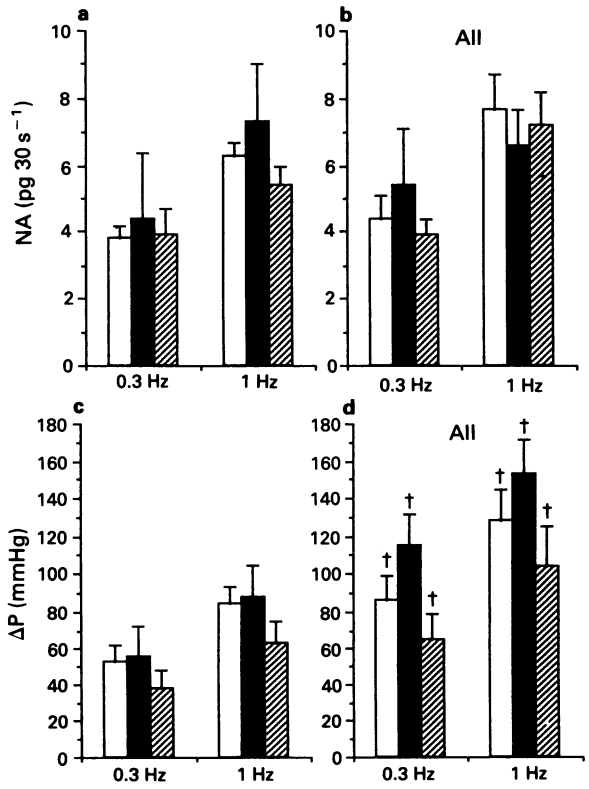

Figure 3 Electrical stimulation-induced noradrenaline release (NA, a,b) and vasoconstriction (ΔP, c,d) of the rat tail artery in the absence (open columns) or presence of indomethacin (2.5 μM, solid columns, or 10 μM, hatched columns) in the absence (a,c) or in the presence (b,d) of angiotensin II (AII, 0.1 μM). All values shown are means ± s.e.mean of 4 experiments. †$P < 0.05$ versus without angiotensin II (t test).

Discussion

This work provides evidence that the vascular endothelium can modulate sympathetic vasoconstriction by liberating at least three factors: (i) EDRF/NO, as demonstrated by potentiation of vasoconstriction following pharmacological blockade of this pathway, (ii) an EDCF, as demonstrated by the increase in noradrenergic effectiveness in the presence of AII, and (iii) an EDIF, inhibiting NA release, as demonstrated by the increase in NA release following removal of endothelium. This working hypothesis depends on several premises. Firstly, the amount of NA released into the perfusate can be taken as a reliable index of the amount of NA released into the synaptic cleft. This is likely to be the case, as (i) we used cocaine in order to block the neuronal uptake of NA, and (ii) the endothelium-dependent uptake of the neurotransmitter is insignificant in this preparation (Palaty, 1988). The second premise is that the ratio PP/NA release can be used as an index of noradrenergic effectiveness. This implies that, within the range of the vasoconstrictor responses obtained, increases in PP are linearly related to NA release. The frequencies of electrical stimulation chosen were between threshold and half maximum – the maximal response to electrical stimulation in the caudal artery from normotensive male adult Wistar rats is between 200 and 240 mmHg (Fouda *et al.*, 1991). The value of this ratio was similar at frequencies of 0.3 and 1 Hz. In denuded segments, the values of this ratio were the same in both the unstimulated and stimulated state and drugs were without effect. Therefore, changes in the ratio in intact segments can be attributed to modulation by the endothelium. An increase in the ratio PP/NA release can be taken as evidence of the suppression of EDRF activity or an increase in EDCF activity, and *vice versa*. Firstly, we used relatively low stimulation frequencies, as there exists an inverse relationship between stimulus intensity and the efficiency of the different presynaptic receptors – muscarinic (receptors stimulated by carbachol) and α_2-adrenoceptors

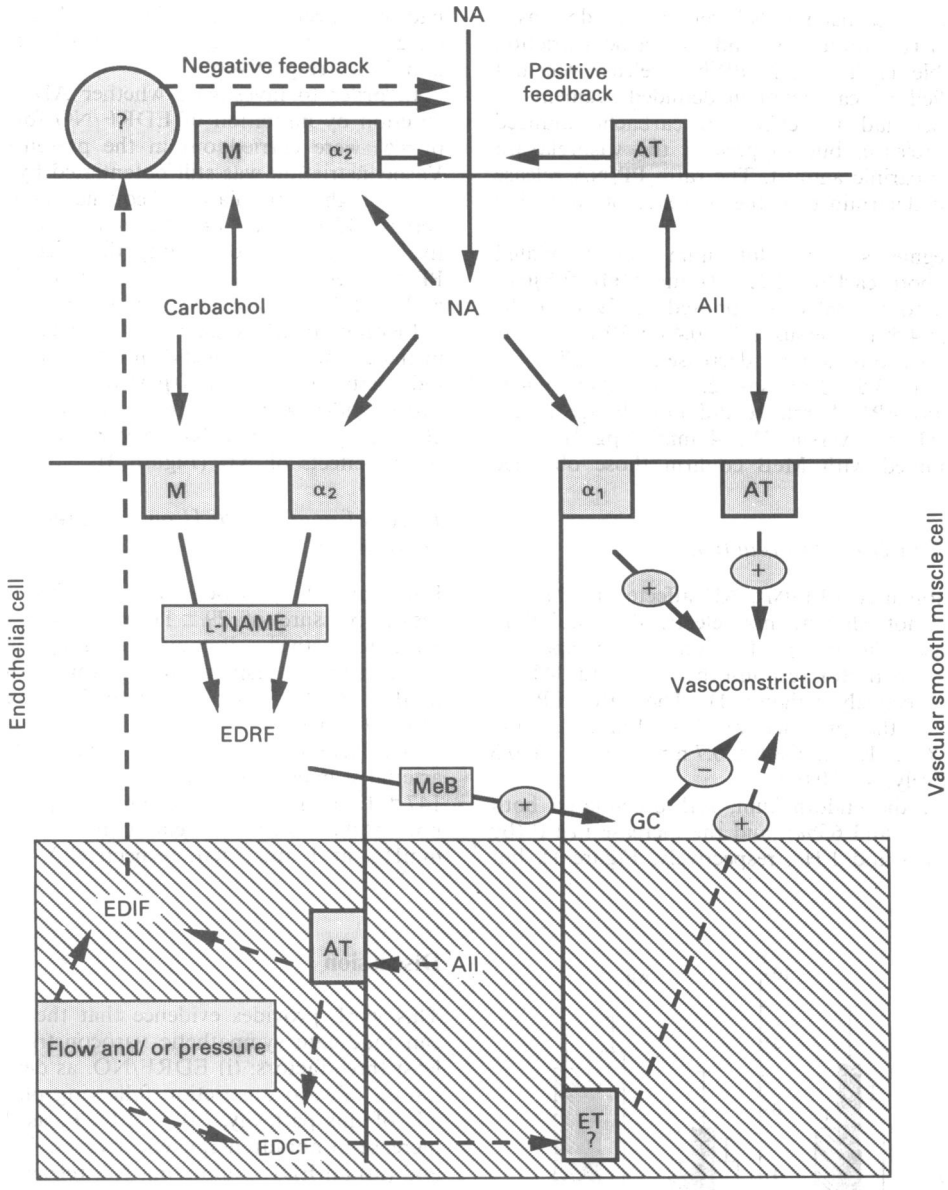

Figure 4 A working hypothesis of the possible interactions at the endothelial level between noradrenaline (NA), carbachol and angiotensin II (AII) involved in the modulation of sympathetic transmission in the rat caudal artery. Receptors are shown as rectangles and designated as follows: M, muscarinic; α_1, α_1-adrenoceptor; α_2, α_2-adrenoceptor; AT, angiotensin receptor. ET represents a putative receptor for endothelin. EDRF, endothelium-derived relaxing factor; EDCF, endothelium-derived contracting factor; EDIF, endothelium-derived inhibitory factor; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MeB, Methylene Blue. The lower part of the figure (hatched area) represents our working hypothesis for the postjunctional effects of AII described in this paper.

(blocked by phentolamine) – that inhibit transmitter release (Duckles & Dubaï, 1990).

L-NAME and MeB potentiated the stimulation-induced increase in PP but did not modify NA release. This suggests that EDRF/NO is released during the activation of sympathetic nerve endings. Further evidence of this is given by the observation that the muscarinic agonist, carbachol, halves stimulation-induced increase in PP which can be restored to its original value by the nitric oxide synthase inhibitor, L-NAME. Many experiments show that carbachol can relax vascular preparations by activating endothelial muscarinic receptors leading to the release of EDRF/NO (Palmer *et al.*, 1987). Carbachol also produced a decrease in stimulation-induced NA release. This effect was observed in intact and denuded preparations and its mechanism is presumably via a prejunctional muscarinic receptor, independent of endothelium. A decrease in NA release would lead to a decrease in vasoconstriction independently of any release of EDRF/NO.

In order to decide which of these two factors is the more important – decrease in NA release or increase in EDRF/NO release – one has to examine the PP/NA release ratio. Were EDRF/NO release to be of prime importance, this ratio should be lower in the presence of carbachol. In fact the opposite occurred, suggesting that it is the decrease in NA release which is mainly involved in the vasorelaxant response to carbachol during an electrical stimulation in this preparation. This is confirmed by the fact that coperfusion of L-NAME does not modify the ratio. Taken together, these observations suggest that EDRF/NO is not of major importance in the vasorelaxant properties of carbachol during an electrical stimulation, as recently reported by Toda *et al.* (1990).

L-NAME and MeB had no effect in unstimulated arteries. This suggests that NA, reaching the lumen of the vessel, is the stimulus required for EDRF/NO formation in this preparation, thus explaining the lack of effect of blockade of the

EDRF/NO pathway in unstimulated arterial segments, i.e., under conditions of low sympathetic activity. Furthermore, NA can relax precontracted vascular preparations (Cocks & Angus, 1983), and fluid shear stress produced by a change in perfusate velocity can stimulate the release of EDRF/NO from endothelial cells (Buga *et al.*, 1991). In our perfused preparation, an increase in endothelial shear stress following noradrenergic vasoconstriction may provide the stimulus for EDRF/NO release, as recently hypothesised by Vo *et al.* (1992). This remains a possibility although, in this study, we have no direct evidence of such an interaction. Although we did not carry out specific experiments to examine the effect of the flow rate on EDIF activity or noradrenergic effectiveness, certain preliminary data suggest that the latter, at least, is relatively independent of flow rate. Using the same protocol as the one described in the present paper (but without measurement of NA overflow) we have previously shown that the ED_{50} and slope values for the response curve for electrical stimulation were very similar at flow rates of 2, 4 or 6 ml min^{-1} (Atkinson *et al.*, 1986).

AII did not produce vasoconstriction in this preparation but increased the vasoconstrictor responses to electrical stimulation, without increasing NA release. The increase in noradrenergic effectiveness suggests either the release of an EDCF, as proposed by Yen *et al.* (1991), or the suppression of EDRF activity. This latter possibility is unlikely because of the lack of influence of MeB on AII-induced potentiation of vasoconstriction in electrically stimulated arterial segments. Furthermore, AII potentiated the vasoconstriction induced by perfusion with NA, an effect which was abolished by removal of the endothelium, suggesting once again the existence of an EDCF whose release is stimulated by AII (see Figure 4).

EDCF may also be involved in the maintenance of tone in unstimulated artery as removal of endothelium produced a fall in PP/NA release ratio. Reid *et al.* (1991) have proposed that endothelin-1 could be the EDCF involved.

NA release increased following removal of endothelium. Several hypotheses can be put forward to explain this increase. Firstly, the endothelium can take up and metabolize NA but this would appear unlikely in this preparation (Palaty, 1988). Secondly, the endothelium could act as a barrier for diffusion of NA into the lumen. This is unlikely, as Cohen & Weisbrod (1988) showed that removal of the endothelium increased NA overflow on the adventitial side of the arterial segment as well as into the lumen. The third hypothesis concerns the existence of an EDIF which is produced by the endothelium and which inhibits NA release via an as yet undefined prejunctional mechanism (Tesfamarian *et al.*, 1989). The facilitatory effect of AII on NA release following removal of the endothelium suggests that AII can also stimulate the release of EDIF. It is unlikely that the absence of the endothelium 'unmasks' presynaptic AII receptors. AII-induced release of EDIF presumably counterbalances the endothelium-dependent, prejunctional facilitatory effect of AII (Story & Ziogas, 1986) or the possible inhibition of NA reuptake by AII (Hilgers *et al.*, 1993).

In conclusion, this study provides evidence for a functional interaction between three endothelium-derived factors. All these factors, EDRF/NO, EDCF and EDIF, could be involved in the regulation of local vascular tone.

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(Received May 20, 1993
Revised August 26, 1993
Accepted September 2, 1993)

Br. J. Pharmacol. (1994), **111**, 358–363

A dose-response analysis of the beneficial effects of the ACTH-(4-9) analogue, Org 2766, on behavioural recovery following unilateral labyrinthectomy in guinea-pig

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1 After removal of the peripheral vestibular receptors in one inner ear (unilateral labyrinthectomy, UL), oculo-motor and postural symptoms occur but disappear over time in a process of recovery known as vestibular compensation.

2 ACTH-(4-10), a fragment of the adrenocorticotrophic hormone (ACTH) molecule, which is devoid of corticotrophic activity, has been shown to enhance vestibular compensation. The present study investigated the effect of the ACTH-(4-9) analogue, Org 2766, on vestibular compensation in guinea-pig. Org 2766 is reported to be more potent behaviourally than ACTH-(4-10).

3 After UL, Org 2766 was delivered via an osmotic minipump implanted s.c. to 30 animals randomly assigned to one of five conditions: 1, 5, 10, 20 or 40 nmol kg^{-1} Org 2766, every 4 h for 52 h post-UL. Although infusion was continuous, in the present study the doses are expressed as nmol per 4 h in order to compare the results to a previous study in which animals received a discrete dose of ACTH-(4-10) at the end of each 4 h period. All animals were compared to saline controls ($n = 6$).

4 Three symptoms of UL, spontaneous ocular nystagmus, roll head tilt and yaw head tilt, were measured every 4 h for 52 h, beginning at 10 h post-UL.

5 Rates of infusion of 1, 5 and 10 nmol kg^{-1} accelerated spontaneous nystagmus compensation; 20 nmol kg^{-1} produced a significant decrease in the frequency of spontaneous nystagmus, as well as accelerating its compensation; 40 nmol kg^{-1} had no significant effect on spontaneous nystagmus compensation.

6 In comparison to the effects of Org 2766 on spontaneous nystagmus compensation, Org 2766 had little effect on the compensation of the postural symptoms, yaw head tilt and roll head tilt. Only 5 and 40 nmol kg^{-1} produced a significant change in postural compensation, and this was a reduction in the rate of roll head tilt compensation.

7 At the optimal infusion rate of 20 nmol kg^{-1} every 4 h, Org 2766 produced a similar effect on spontaneous nystagmus compensation to that of ACTH-(4-10). However, Org 2766 was effective in accelerating spontaneous nystagmus compensation at much smaller doses per 4 h period than ACTH-(4-10). Org 2766 did not have the same effect on postural compensation as it had on the compensation of spontaneous nystagmus.

Keywords: Org 2766; ACTH-like peptides; vestibular compensation; enhanced recovery; unilateral labyrinthectomy

Introduction

It has been well documented that neuropeptide fragments of the adrenocorticotrophic hormone (ACTH), which are devoid of corticotrophic activity (Greven & de Wied, 1973), facilitate lesion-induced plasticity in both the peripheral and central nervous systems (CNS) (see Strand *et al.*, 1989 for a review). In the CNS, short ACTH fragments enhance functional recovery following hippocampal lesions (Hannigan & Isaacson, 1985), parafascicular lesions (Nyakas *et al.*, 1985), septal lesions (Isaacson & Poplawski, 1983), lesions of the dopaminergic system in nucleus accumbens (Wolterink *et al.*, 1990), and lesions of the fimbria fornix (Spruijt *et al.*, 1990; Pitsikas *et al.*, 1991). It is possible that some of the ACTH fragments may have clinical potential to promote lesion-induced plasticity in humans. In a recent clinical study of 55 women with ovarian cancer, the ACTH-(4-9) analogue Org 2766 was shown to have a protective action against cisplatin neurotoxicity, a dose-limiting side effect of the cisplatin treatment (Gerritsen van der Hoop *et al.*, 1990).

Short ACTH fragments also enhance functional recovery after removal of the peripheral vestibular receptors in one inner ear (unilateral labyrinthectomy, UL) (Flohr & Lüneburg, 1982; Igarashi *et al.*, 1985; Gilchrist *et al.*, 1990). UL removes normal vestibular input to the brainstem vestibular nuclei on the ipsilateral side, producing a disturbance of both oculo-motor and postural reflexes (see Smith & Curthoys, 1989 for a review). Over time, these symptoms disappear in a recovery process known as vestibular compensation. Although recent evidence suggests that hair cells within the inner ear are capable of regeneration following damage due to aminoglycoside toxicity (Forge *et al.*, 1993; Warchol *et al.*, 1993), there is no evidence to suggest that such regeneration can occur even up to a year following surgical removal of the vestibular receptor cells (Fermin & Igarashi, 1984; Cass *et al.*, 1989). Furthermore, electrophysiological studies have shown that no significant functional recovery occurs in the vestibular nerve following surgical UL (see Smith & Curthoys, 1989 for a review). The behavioural recovery following UL correlates with a return of resting activity to medial vestibular nucleus type I neurones ipsilateral to the lesion (Precht *et al.*, 1966; Smith & Curthoys, 1988; Newlands & Perachio, 1990; see also Smith & Curthoys, 1989 for a review). Vestibular compensation is therefore attributed to CNS plasticity. In guinea-pig, frog and squirrel monkey, ACTH-(4-10) has been shown to accelerate vestibular compensation (Flohr & Lüneburg, 1982; Igarashi *et al.*, 1985; Gilchrist *et al.*, 1990).

In most animal models of lesion-induced plasticity, the

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enhanced recovery observed after treatment with ACTH-like peptides appears to be produced by biological activity located within the 4–10 sequence of the fragment (Greven & de Wied, 1967; Bijlsma *et al.*, 1981; Flohr & Lüneburg, 1989). However, modifications to the ACTH-(4-9) fragment have produced a peptide with a marked increase in biological activity (Witter *et al.*, 1975; Verhoef & Witter, 1976; Fekete & de Wied, 1982). Org 2766 (H-Met(0_2)-Glu-His-Phe-D-Lys-Phe-OH) is 1000 times more potent than ACTH-(4-10) in some behavioural tests (Greven & de Wied, 1973), an effect attributed to the peptide's resistance to enzymatic degradation (Witter *et al.*, 1975). Although ACTH-(4-10) has been shown to enhance vestibular compensation in three different species, the effect of Org 2766 has been investigated only in frog. Flohr *et al.* (1985) have reported that Org 2766 was the most effective ACTH-like peptide, enhancing vestibular compensation at very small doses. However, in frog the symptoms produced by UL may take as long as 60 days to compensate (Flohr & Lüneburg, 1982). In guinea-pig, vestibular compensation of the static symptoms (those which occur in the absence of head movement) occurs within 2–3 days post-UL. In most species, including man, it is during this early period post-UL that the most severe symptoms occur. At present no study has investigated the effect of Org 2766 on vestibular compensation in a mammalian species. Given that Gerritsen van der Hoop *et al.* (1990) have already demonstrated that Org 2766 has clinical potential, it is possible that drug therapy in man during the first 2–3 days post-UL may also be clinically useful. The present dose-response study investigates the effect of Org 2766 on vestibular compensation in guinea-pig.

Methods

Subjects

Thirty-six guinea-pigs (340–360 g) were used in the present study. Post-surgery all animals were housed individually in a room with a 24 h light cycle. The containers in which the animals were housed had a perspex front and for each animal all video measurements (see apparatus and measurement section) were made in their home container; this procedure prevented possible loss of compensation (decompensation) due to lifting the animal from one box to another (e.g. Jensen, 1979). Water and food were available *ad lib.*

Surgery

Animals were anaesthetized with xylazine (12 mg kg^{-1}, i.m.) and ketamine hydrochloride (100 mg kg^{-1}, i.m.). Procaine hydrochloride (2% in 2 ml) was locally infused into all wound margins and pressure points and electrocardiograph electrodes were inserted into the forelimb muscles in order to monitor heart rate. Under microscopic control, a right surgical UL was performed by making an incision from the nose to the occipital bone along the midsaggital suture and retracting the skin to expose the temporal bone. A portion of the temporal bone was removed and the anterior and horizontal semicircular canal ampullae were opened with a dental drill using a fine burr. Once both of these structures were removed, the opening in the utricular duct was widened and the utricle was destroyed by drilling and aspiration; the saccule and the posterior canal ampulla were probed and aspirated. On completion of the inner ear surgery, Furacin antiseptic cream (Norwich Eaton Pharmaceuticals) was inserted into the vestibule to prevent infection and the temporal bone was sealed with dental cement. The initial midline incision to expose the temporal bone was extended caudally and an osmotic minipump (Alzet 1003D) was implanted subcutaneously (s.c.) in the dorsal neck region. Once the pump was in place the wound was sutured.

Drug administration

Org 2766 (Organon, Netherlands) was dissolved in saline and delivered via the osmotic minipump. McDaniel *et al.* (1989) have previously used an Alzet osmotic minipump to deliver Org 2766 s.c. to rats. The Alzet 1003D used in the present study delivered 1.0 µl h^{-1} for 3 days. To ensure that the pump began to deliver its contents immediately, the filled pumps were incubated in saline at 37°C for at least 4 h prior to implantation. The desired concentration for each condition was attained by adding the appropriate volume of saline to 1 mg of Org 2766. All solutions were prepared on the day of surgery. Each animal was randomly assigned to one of six conditions: 1 nmol kg^{-1} every 4 h for 52 h ($n = 6$); 5 nmol kg^{-1} every 4 h for 52 h ($n = 6$); 10 nmol kg^{-1} every 4 h for 52 h ($n = 6$); 20 nmol kg^{-1} every 4 h for 52 h ($n = 6$); 40 nmol kg^{-1} every 4 h for 52 h ($n = 6$); saline controls ($n = 6$). The osmotic minipump continously delivered the solution for the entire 52 h of the experiment; however, in the present study the doses are expressed as nmol per 4 h in order to compare the results with a previous study in which animals received a discrete dose of ACTH-(4-10) at the end of each 4 h period (Gilchrist *et al.*, 1990). Following the experiment, animals were killed and the osmotic minipump was removed and cut in half, crosswise. According to manufacturer's instructions this provides a qualitative assessment of pump functionality. In all cases the pump reservoir had collapsed to indicate that the pump had been functioning.

Apparatus and measurements

Measurements were made of three static symptoms of UL: spontaneous ocular nystagmus, yaw head tilt and roll head tilt. For all three symptoms measurements were made every 4 h for 52 h post-UL, beginning at 10 h post-UL. At each measurement time all three symptoms were videotaped on a video camera (Panasonic NV-M7) with a zoom lens and a videorecorder (Mitsubishi E7 Black Diamond). Spontaneous nystagmus frequency was measured by gently retracting the skin behind the animal's left eye and visually counting the number of quick phase beats in a 15 s interval, as indicated by an electronic timer which emitted an audio signal at the end of the specified interval. This procedure was repeated five times at each measurement time for each animal and the average of the five measurements was obtained. For all animals at all times, the spontaneous nystagmus measurements were videotaped. The quick phase of spontaneous nystagmus is easy to detect visually as a rapid, large amplitude eye movement, contralateral to the UL, which produces a large reduction in the visible area of the sclera (Jensen, 1979; Smith *et al.*, 1986; Curthoys *et al.*, 1988; Gilchrist *et al.*, 1992). To avoid contamination of spontaneous nystagmus with vestibulo-ocular reflex nystagmus evoked by head movement, and high spontaneous nystagmus frequencies produced by stress (Jensen, 1979; Smith *et al.*, 1986), animals were allowed to adopt a natural and stationary position for all measurements. Upon completion of the study, a trained independent observer measured a representative sample of spontaneous nystagmus from all animals and conditions for each time (see data analysis). The independent observer was naive with respect to the experimental conditions. The independent observations were compared to those of the original experimenter as a measure of reliability. A colour monitor (Sony Trinitron) and the videorecorder were used to replay the eye movements. Yaw head tilt was defined as the difference (in degrees) between a line through the midscapular point and sacrum and a line through the midscapular point and the animal's snout. Roll head tilt was defined as the difference (in degrees) between gravitational vertical and a line through the vertical axis of the animal's head (see Curthoys *et al.*, 1988 for a review). Roll head tilt was videotaped by positioning the home container so the perspex panel at the front of the container faced a video-

camera. A second videocamera positioned directly above the animal videotaped yaw head tilt. A digital production mixer (Panasonic, WJ-mx 10) was used in order to display both yaw head tilt and roll head tilt on the colour monitor simultaneously. The videotapes were replayed on the videorecorder and the freeze frame facility was used to pause the tape at the appropriate moment. A large, specially constructed protractor was fitted over the screen of the colour monitor in order to measure roll head tilt and yaw head tilt (Flohr & Lüneburg, 1982). All measurements began at 10 h post-UL in order to allow all animals to recover sufficiently from the effects of anaesthetic and display typical UL symptoms.

Data analysis

For all conditions, the mean spontaneous nystagmus, yaw head tilt and roll head tilt at each measurement time was calculated for each group of animals. For all three symptoms, drug-treated animals were compared to saline controls by use of 2-factor analysis of variance (ANOVA) with repeated measures on time (Statview 512$^+$). Factor A represented the effect of drug administration (Org 2766 or saline) on spontaneous nystagmus frequency, or the magnitude of yaw head tilt or roll head tilt; factor B represented the repeated measure, time; and the interaction (AB) represented the change in the rate of compensation due to treatment (Winer, 1971). During compensation the severity of the UL symptoms decreases over time; as a result factor B was always significant and will not be discussed further. For all comparisons, the significance rate (α) was set at 0.05.

To measure reliability the independent observer rated 72 measurements of spontaneous nystagmus. In total there were only 16 disagreements, 13 of which were within $\pm$ 1 beat of each other. A kappa reliability statistic was calculated and yielded a value of 0.765 (Bartko & Carpenter, 1976). Although agreement was significantly better than chance ($P <$ 0.00001; Z test, two tailed), reliability may in fact be much greater than this as differences of 1 beat or less are probably

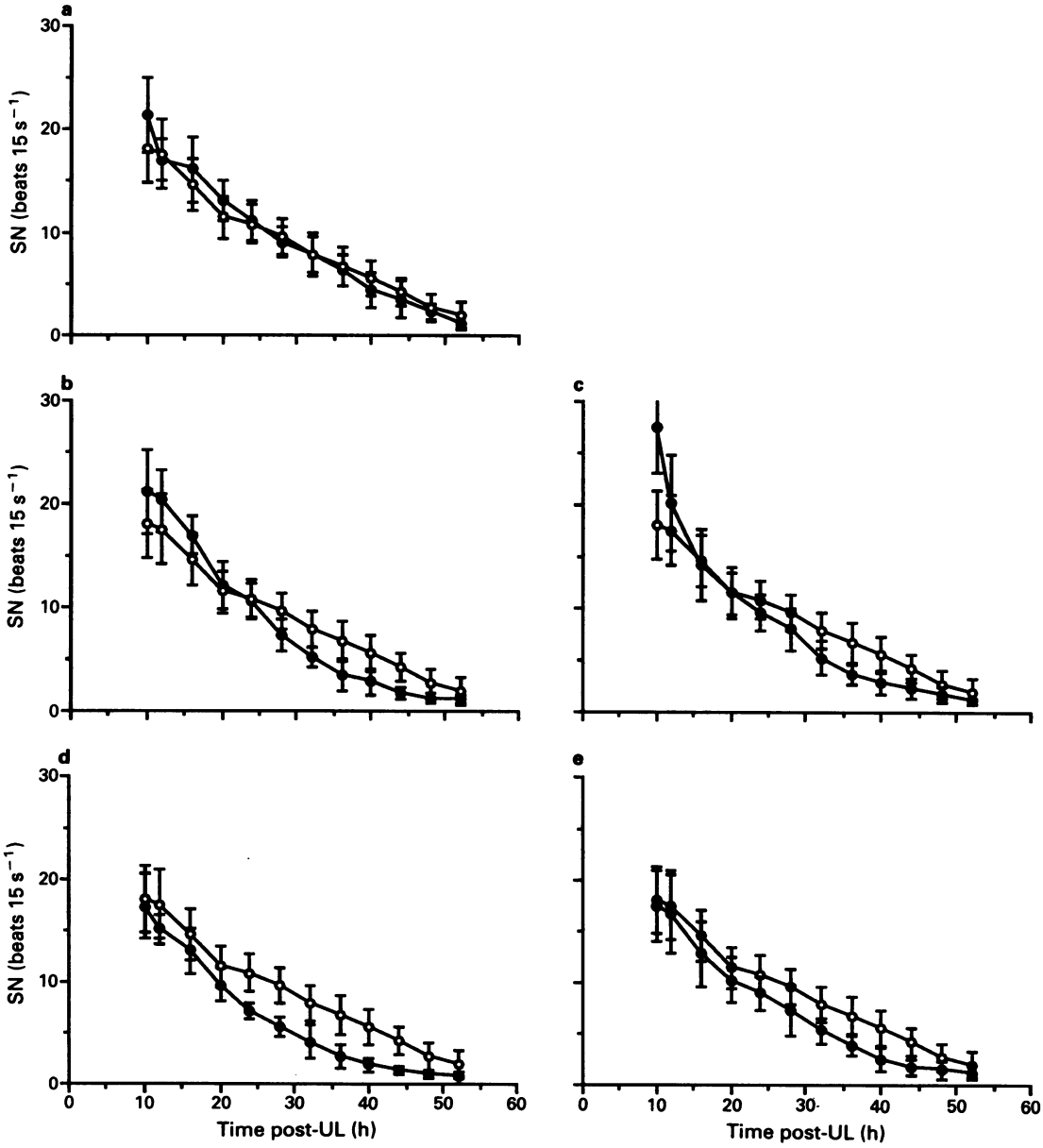

Figure 1 Comparison of vestibular compensation of spontaneous nystagmus (SN, in beats 15 s^{-1}) for guinea-pigs receiving either Org 2766 (●) or saline (○) s.c. via an osmotic minipump (Alzet 1003D). All data points represent a mean of six animals $\pm$ 1 s.d. Saline controls are compared to animals receiving: (a) 1 nmol kg^{-1} every 4 h to 52 h; (b) 5 nmol kg^{-1} every 4 h to 52 h; (c) 10 nmol kg^{-1} every 4 h to 52 h; (d) 20 nmol kg^{-1} every 4 h to 52 h or (e) 40 nmol kg^{-1} every 4 h to 52 h.

within the measurement error of visual analysis of spontaneous nystagmus.

Results

Effect of Org 2766 on compensation of spontaneous nystagmus

Administration of 1, 5 and 10 nmol kg^{-1} every 4 h for 52 h post-UL produced no significant change in the frequency of spontaneous nystagmus; however, for all three doses it did produce a significant increase in the rate of compensation (F (11, 110) = 2.32, $P \leqslant 0.01$, 1 nmol kg^{-1} condition; F (11, 110) = 8.31, $P \leqslant 0.0001$, 5 nmol kg^{-1} condition; F (11, 110) = 5.73, $P \leqslant 0.0001$, 10 nmol kg^{-1} condition) (see Figure 1). It should be noted that despite reaching statistical significance, the effects of 1 nmol kg^{-1} Org 2766 on the rate of spontaneous nystagmus compensation were very small and probably within the measurement error of the method used to analyse spontaneous nystagmus frequency (see Figure 1a). Administration of 20 nmol kg^{-1} every 4 h for 52 h post-UL produced a significant decrease in the spontaneous nystagmus frequency (F (1, 110) = 11.06, $P \leqslant 0.008$) and a significant increase in the rate of spontaneous nystagmus compensation (F (11, 110) = 2.68, $P \leqslant 0.004$) (see Figure 1d). Administration of 40 nmol kg^{-1} every 4 h for 52 h post-UL did not have a significant effect on either spontaneous nystagmus frequency or the rate of spontaneous nystagmus compensation (see Figure 1e) (see also Table 1 for summary).

Effect of Org 2766 on compensation of yaw head tilt and roll head tilt

Except for decreases in the rate of roll head tilt compensation for the 5 and 40 nmol kg^{-1} conditions (F (11, 110) = 3.79, $P \leqslant 0.0001$ and (F (11, 110) = 2.88, $P \leqslant 0.002$ respectively), administration of Org 2766 had no other significant effects on either yaw head tilt or roll head tilt (see Figure 2 and Table 1).

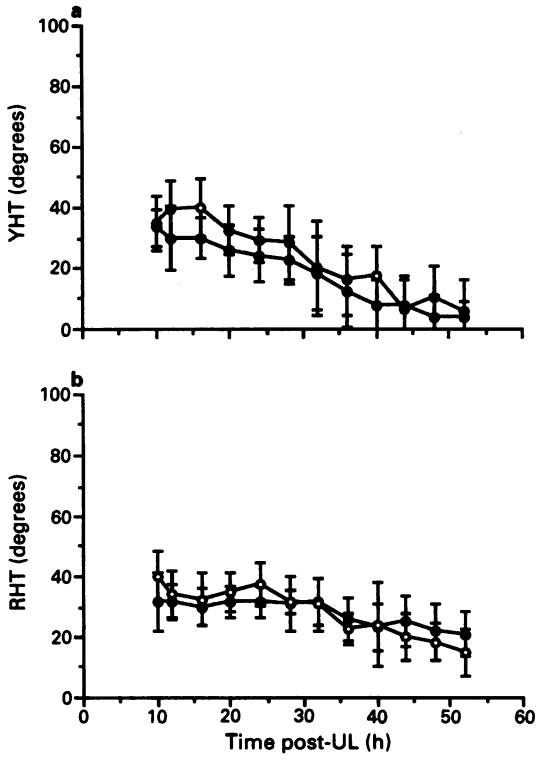

Figure 2 Compensation of yaw head tilt (YHT) (a) and roll head tilt (RHT) (b) for guinea-pigs receiving either 20 nmol kg^{-1} Org 2766 every 4 h for 52 h ($n = 6$) (●) or saline ($n = 6$) (○) s.c. via an osmotic minipump (Alzet 1003D). All data points represent means ± 1 s.d.

Table 1 The effect of Org 2766 on spontaneous nystagmus (SN), yaw head tilt (YHT) and roll head tilt (RHT)

	Dose (nmol kg^{-1}) 1	5	10	20	40
SN					
Effect of drug treatment on level of symptom	−	−	−	+	−
Effect of drug treatment on rate of compensation	+	+	+	+	−
YHT					
Effect of drug treatment on level of symptom	−	−	−	−	−
Effect of drug treatment on rate of compensation	−	−	−	−	−
RHT					
Effect of drug treatment on level of symptom	−	−	−	−	−
Effect of drug treatment on rate of compensation	−	+	−	−	+

(+) indicates the drug-treated animal was significantly different from saline control, ($P \leqslant 0.05$). (−) indicates a non-significant effect.

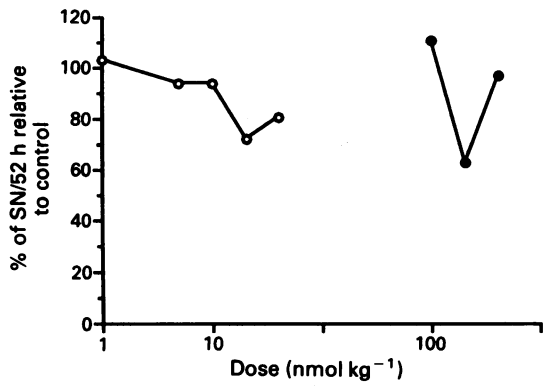

Figure 3 Dose response comparison of the effect of different doses of Org 2766 (○) and ACTH-(4-10) (●) (Gilchrist *et al.*, 1990) on the compensation of spontaneous nystagmus (SN) in guinea-pig. Each data point represents the total average SN over 52 h for a particular dose/4 h period as a % of the total average SN for the control group.

Discussion

At an optimal s.c. infusion rate of 20 nmol kg^{-1} every 4 h for 52 h, Org 2766 reduced spontaneous nystagmus frequency and accelerated its compensation in guinea-pig. The pattern of the acceleration of spontaneous nystagmus compensation by Org 2766 was very similar to that produced by 200 μg kg^{-1} ACTH-(4-10) in a previous study (Gilchrist *et al.*, 1990) (see Figure 3). Doses above and below the optimal dose of the two drugs (200 μg kg^{-1} ACTH-(4-10) and 20 nmol kg^{-1} Org 2766) had less or no effect on spontaneous nystagmus compensation. According to Strand *et al.* (1989), dose-response studies of the effects of ACTH peptides on peripheral nerve regeneration have often produced inverted U-shaped

dose-response functions. Acceleration of spontaneous nystagmus compensation in guinea-pig parallels the effect of Org 2766 on vestibular compensation in frog (Flohr *et al.*, 1985) in that in both species, Org 2766 enhances compensation at much smaller doses than ACTH-(4-10).

For 1 nmol kg^{-1} Org 2766, the statistically significant interaction between drug effect and time may not reflect true acceleration of spontaneous nystagmus compensation, but rather the fact that animals in the Org 2766 condition had a higher average spontaneous nystagmus frequency at 10 and 20 h post-UL; this may also account for the significant interactions for the 5 and 10 nmol kg^{-1} Org 2766 conditions, compared to the lack of effect in the case of 40 nmol kg^{-1} (see Figure 1, b,c and e). However, when the data are expressed as a percentage of spontaneous nystagmus frequency over 52 h post-UL relative to controls (see Figure 3), the overall differences in spontaneous nystagmus frequency for each dose condition become apparent.

In contrast to the effects of Org 2766 on spontaneous nystagmus compensation, Org 2766 had little effect on the compensation of the postural symptoms, roll head tilt and yaw head tilt. At a rate of infusion of 5 and 40 nmol kg^{-1}, Org 2766 decreased the rate of roll head tilt compensation. We have previously reported that ACTH-(4-10) also had inconsistent effects on the compensation of posture; for yaw head tilt some doses of ACTH-(4-10) increased symptoms, some decreased symptoms, and others had no effect; for roll head tilt, ACTH-(4-10) produced no significant effects relative to controls (Gilchrist *et al.*, 1993). The different effects that ACTH-like peptides have on spontaneous nystagmus compared to posture suggest that different mechanisms may underlie the compensation of the oculo-motor and postural symptoms following UL (Curthoys *et al.*, 1988). At present the mechanisms responsible for the generation and compensation of static postural symptoms are less well understood (e.g. Fukushima *et al.*, 1988) than those responsible for the generation and compensation of spontaneous nystagmus (e.g. Nakao *et al.*, 1982).

The exact mechanism by which ACTH-like peptides accelerate vestibular compensation or other forms of lesion-induced plasticity is not clear. Recently, a family of genes has been characterized that encodes receptors for the proopiomelanocortin peptides (Mountjoy *et al.*, 1992). It is of particular interest that one of the receptors characterized is specific for peptides with melanotrophic (ACTH/melanocyte stimulating hormone (MSH)) activity. This raises the possibility that ACTH-like peptides may exert their effects on CNS function by acting at a specific melanotrophic ACTH site. Florijn *et al.* (1993) have provided evidence suggesting that in some brain regions, melanotrophic peptides bind to an adenylate cyclase-coupled ACTH/α-MSH receptor, activating an adenosine 3':5'-cyclic monophosphate (cyclic-AMP)-dependent second messenger pathway. Although Tatro (1990) has reported that there is a wide distribution of melanotrophic receptors in rat brain, at present there is no evidence for receptors for ACTH-like peptides, or ACTH-containing cell fibres, within the vestibular nucleus (Schwartzberg & Nakane, 1983; Romagnano & Joseph, 1983; see also Palkovits, 1984 for a review). In the majority of binding studies to date it is only the longer ACTH fragments like ACTH-(1-24) that bind to specific receptors, and ACTH-(4-10) does not bind to the ACTH-(1-24) binding sites (Hnatowich *et al.*, 1989). However, a lack of suitable radioligand probes had previously made it difficult to determine the distribution of brain melanotrophic receptors (Tatro, 1990). ACTH-(1-24) and some of the shorter fragments inhibit binding of ligands to opiate receptors (Terenius *et al.*, 1975; Akil *et al.*, 1980), muscarinic acetylcholine receptors (Tonnaer *et al.*, 1986), dopamine receptors (Florijn *et al.*, 1991) and the glutamatergic N-methyl-D-aspartate (NMDA) receptor (Ito *et al.*, 1988; Trifiletti & Pranzatelli, 1992).

It is possible that the effects produced by ACTH-like peptides on vestibular compensation may be due to direct action on specific subnuclei of the vestibular nucleus complex (Darlington *et al.*, 1990). Guinea-pig medial vestibular nucleus neurones *in vitro* respond to ACTH-(4-10) at picomolar concentrations (Darlington *et al.*, 1990; 1992; 1993). The effect of ACTH-(4-10) on medial vestibular nucleus neurones is dose-dependent and also follows an inverted U-shaped dose-response function. Darlington *et al.* (1990) have suggested that because ACTH-(4-10) produces its effects on medial vestibular nucleus neurones at such low concentrations, the neuropeptide may be functioning as a neurotransmitter within the nucleus (Darlington *et al.*, 1990). However, Darlington *et al.* (1992) argue that the acceleration of vestibular compensation by ACTH-(4-10) is unlikely to be associated with changes in the medial vestibular nucleus ACTH-(4-10) binding sites: the effect of ACTH-(4-10) on medial vestibular nucleus neurones in brainstem slices removed from normal guinea-pigs was similar to that for medial vestibular nucleus neurones in slices from compensated guinea-pigs, indicating no change in the sensitivity of medial vestibular nucleus neurones to ACTH-(4-10) before and after compensation (Darlington *et al.*, 1992; 1993). The challenge for future studies will be to determine exactly how ACTH-like peptides facilitate vestibular compensation.

This research was supported by project grants from the New Zealand Neurological Foundation and the Health Research Council of New Zealand. D.G. is a W & B Miller Neurological Foundation Scholar. We are grateful to Organon, Netherlands for generously providing the Org 2766 used in these experiments. The authors wish to thank Mr Barry Dingwall and his staff for their excellent technical assistance and Miss J. Greenstock for her assistance in reliability analysis.

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(Received July 20, 1993
Revised September 27, 1993
Accepted October 5, 1993)

Br. J. Pharmacol. (1994), **111**, 364–369

Modulation of cyclic AMP formation by putative metabotropic receptor agonists

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1 The effects of various metabotropic glutamate receptor agonists on [^{3}H]-cyclic AMP accumulation and phosphoinositide hydrolysis were investigated in guinea-pig cerebral cortical slices prelabelled with [^{3}H]-adenine or [^{3}H]-inositol.

2 1-Aminocyclopentane-1S,3R-dicarboxylate (1S,3R-ACPD), L-2-amino-4-phosphonobutanoate (L-AP4) and (2S,3S,4S)-α-(carboxycyclopropyl)glycine (L-CCG-I), elicited concentration-dependent inhibitions of forskolin-stimulated [^{3}H]-cyclic AMP accumulation, with IC_{50} values of 2.1 ± 0.3, 71 ± 17 and 0.2 ± 0.1 μM respectively.

3 1S,3R-ACPD and L-CCG-I increased the cyclic AMP responses to histamine H_2 receptor stimulation with EC_{50} values of 7 ± 2 μM and 19 ± 2 μM respectively. 1S,3R-ACPD (EC_{50} value 17 ± 2 μM) and L-CCG-I (EC_{50} value 15 ± 3 μM) potentiated the cyclic AMP responses to the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA, 10 μM). This potentiating effect of L-CCG-I was reduced in the presence of a protein kinase C inhibitor, and also in the absence of extracellular calcium. In contrast, L-AP4 inhibited the NECA response in a concentration-dependent manner, with an IC_{50} value of 120 ± 20 μM.

4 L-AP4 (at concentrations up to 1 mM) failed to stimulate phosphoinositide hydrolysis in guinea-pig cerebral cortical slices, but both 1S,3R-ACPD (EC_{50} value 35 ± 6 μM) and L-CCG-I (approximately 160 μM) elicited concentration-dependent stimulations of phosphoinositide turnover.

5 These results confirm the existence of at least two distinct subtypes of metabotropic receptor in guinea-pig cortex. The data also substantiate previous studies indicating the importance of the agonist L-CCG-I as a pharmacological tool for distinguishing metabotropic receptor subtypes.

Keywords: Metabotropic receptors; excitatory amino acid receptors; ACPD; L-AP4; L-CCG-I

Introduction

Excitatory amino acid receptors are broadly classified into two main families. The first major group consists of receptors thought to contain integral cation-specific channels and these are termed 'inotropic receptors'. The second group are responsible for slower signalling via second messenger transduction mechanisms and are known as 'metabotropic receptors' (mGluRs) (Monaghan *et al.*, 1989; Mayer & Miller, 1990). Glutamate, the most abundant excitatory amino acid in the vertebrate central nervous system, activates both groups of receptors, and in order to investigate distinct properties of metabotropic receptors, the use of agonists more selective than glutamate is necessary. The introduction of *trans*-1-aminocyclopentane-1,3-dicarboxylate (*t*-ACPD, a racemic mixture of the 1S,3R- and 1R,3S-isomers of ACPD), a rigid glutamate analogue selective for metabotropic receptors (Palmer *et al.*, 1989), enabled the metabotropic receptor-mediated effects on second messenger transduction systems to be elucidated. The subsequent purification of *trans*-ACPD and *cis*-ACPD lead to the isolation of the four isomers of ACPD, 1S,3R-, 1S,3S-, 1R,3S- and 1R3R- (Curry *et al.*, 1988). The 1S,3R-isomer of ACPD is more selective for metabotropic receptors than the racemic mixture *t*-ACPD (Cartmell *et al.*, 1992), but 1S,3R-ACPD does not distinguish subtypes of the metabotropic receptor recently cloned by Nakanishi and coworkers ($mGluR_1$-$mGluR_6$; Nakanishi, 1992), although we have recently found that 1S,3R-ACPD is significantly more potent an agonist of metabotropic receptors negatively coupled to adenylyl cyclase than phospholipase C-linked metabotropic receptors in guinea-pig cerebral cortex (Cartmell *et al.*, 1993). The metabotropic receptor subtypes are grouped according to distinct transduction mechanisms; $mGluR_1$ and $mGluR_5$ are coupled to phospholipase C, and $mGluR_2$, $mGluR_3$, $mGluR_4$ and $mGluR_6$ appear to be negatively-coupled to adenylyl cyclase (Masu *et al.*, 1991; Houamed *et al.*, 1991; Tanabe *et al.*, 1992; Abe *et al.*, 1992).

It has recently been shown that isomers of a second rigid glutamate analogue α-(carboxycyclopropyl) glycine (CCG) have agonist properties at metabotropic receptors. Nakagawa *et al.* (1990) reported that the (2S,3S,4S) isomer of α-(carboxycyclopropyl) glycine (L-CCG-I) elicited significant chloride currents (induced through phosphoinositide breakdown) in *Xenopus* oocytes injected with rat brain poly $(A)^+$ RNA. L-CCG-I is also an effective stimulant of phosphoinositide metabolism in both hippocampal synaptoneurosomes (Nakagawa *et al.*, 1990) and $mGluR_1$-expressing Chinese hamster ovary (CHO) cells (Hayashi *et al.*, 1992). Forskolin-stimulated cyclic AMP accumulation was inhibited by L-CCG-I in $mGluR_2$-expressing CHO cells with a potency about 100 fold greater than that at the $mGluR_1$ and $mGluR_4$ receptors. This evidence strongly suggests that L-CCG-I is a potent agonist at metabotropic receptors and may serve as an important tool in distinguishing putative receptor subtypes.

It has been reported that L-2-amino-4-phosphonobutyrate (L-AP4), an unconstrained glutamate analogue, inhibits neurotransmitter release via a G-protein-coupled glutamate receptor (Trombley & Westbrook, 1992) and, recently L-AP4 has been shown to have a potency in the high nanomolar range for inhibition of cyclic AMP formation in CHO cells trans-

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fected with the $mGluR_4$ receptor, but is without effect on second messenger formation in any of the other mGluR-expressing cells (Tanabe *et al.*, 1993).

On the basis of their effects on transfected cells, L-CCG-I and L-AP4 would appear to be potentially useful agents for distinguishing between mGluR-mediated events in the central nervous system. Therefore, in this study we have compared the effects of L-CCG-I and L-AP4 with those of 1S,3R-ACPD on second messenger generation in slices of guinea-pig cerebral cortex.

Methods

The accumulation of [^{3}H]-adenosine 3':5'-cyclic monophosphate ([^{3}H]-cyclic AMP) was assessed in guinea-pig cerebral cortical slices prelabelled with [^{3}H]-adenine as previously described (Donaldson *et al.*, 1990). Briefly, after washing the slices, antagonists were added for 15 min and excitatory amino acid (EAA) agonists for 5 min, before the addition of forskolin (30 μM), NECA (10 μM, in the presence of 1.2 u ml^{-1} adenosine deaminase) or histamine (1 mM, in the presence of 3 μM mepyramine and 10 μM rolipram). The incubation was terminated after 10 min by the addition of 1 M HCl. [^{3}H]-cyclic AMP was isolated by sequential Dowex 50/alumina column chromatography.

Agonist-stimulated phosphoinositide turnover was measured in slices labelled with [^{3}H]-inositol, for 45 min in the presence of 5 mM LiCl (Alexander *et al.*, 1990). The incubation was terminated with 7.5% perchloric acid and [^{3}H]-inositol phosphates were isolated by Dowex 1 (chloride form) column chromatography.

Curves were fitted to data using a logistic equation from the computer programme GraphPad (GraphPad, California, U.S.A.). Statistical significance was determined by Student's *t* test.

[^{3}H]-adenine (888 GBq $mmol^{-1}$) and [^{14}C]-cyclic AMP (1.6 GBq $mmol^{-1}$) were purchased from Amersham International, Bucks. [^{3}H]-inositol (640 GBq $mmol^{-1}$) was obtained from NEN Du Pont, Herts. Forskolin, histamine, mepyramine, [ethylene*bis* (oxyethelenenitrilo)] tetraacetic acid, 2-amino-3-phosphonopropionate, L-2-amino-4-phosphonobutyrate and L-2-amino-5-phosphonopentanoate were all purchased from Sigma Chemical Company Ltd., Poole, Dorset. 6,7-Dinitroquinoxaline-2,3-dione and 1-aminocyclopentane-1S,3R-dicarboxylate were obtained from Tocris Neuramin, Bristol. 5-Methyl-10,11-dihydro-5H-dibenzo[*ad*]cyclohepten-5,10-imine maleate was manufactured at Merck, Sharp and Dohme Neuroscience Centre, Terling's Park, Harlow and 5'-N-ethylcarboxamidoadenosine was obtained from Research Biochemicals Incorporated, St. Albans, Herts. Adenosine deaminase was purchased from Boehringer Mannheim, Sussex. Rolipram and Ro 31-8220 (3-{1-[3-(2-isothionreido)propyl]-indol-3-yl}-4-(1-methyl indol-3-yl)-3-pyrrolin-2,5-dione) were gifts from Schering, Germany and Hoffmann La Roche, U.K., respectively.

Results

Inhibition of forskolin-stimulated cyclic AMP accumulation

L-CCG-I had no direct effects on basal levels of [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, but inhibited forskolin-stimulated [^{3}H]-cyclic AMP accumulation in a concentration-dependent manner with an IC_{50} value of 0.24 ± 0.06 μM ($n = 5$) (Figure 1b). L-CCG-I inhibited the forskolin response by 91 ± 4% ($n = 5$), a value similar to the inhibition of the forskolin cyclic AMP response by 1S,3R-ACPD (85 ± 2%, Cartmell *et al.*, 1993). The inhibition of forskolin-stimulated cyclic AMP accumulation by L-CCG-I was not reversed by the EAA receptor antagonists 6,7-

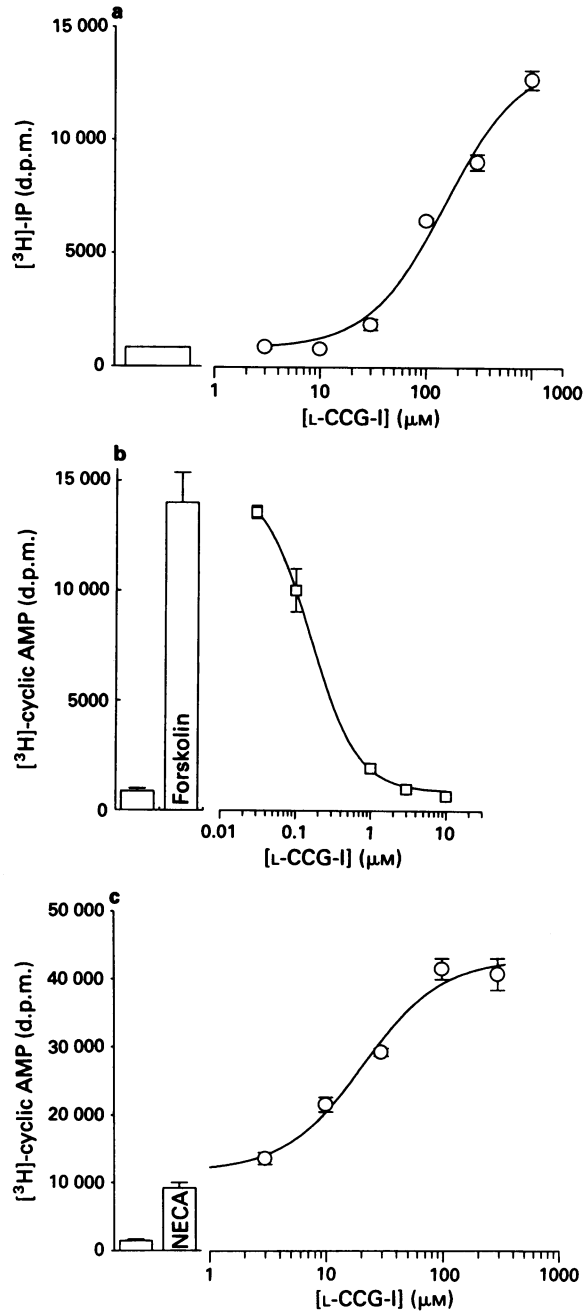

Figure 1 (a) The effect of L-CCG-I on phosphoinositide turnover in guinea-pig cerebral cortical slices in the presence of 5 mM LiCl. Slices were incubated with increasing concentrations of L-CCG-I for 45 min. Unlabelled column represents control values. Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean ± s.e.mean values. (b) The inhibitory effects of L-CCG-I on forskolin-stimulated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, in the presence of adenosine deaminase (1.2 u ml^{-1}). Slices were incubated with 30 μM forskolin in the absence or presence of increasing concentrations of L-CCG-I added 5 min previously. Unlabelled column represents control values, Forskolin indicates forskolin alone. Data are d.p.m. from a single experiment conducted in quadruplicate. The mean ± s.e.mean IC_{50} value for five such experiments was 0.24 ± 0.06 μM. Data are represented as mean ± s.e.mean values. (c) The effects of L-CCG-I on NECA-stimulated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, in the presence of adenosine deaminase (1.2 u ml^{-1}). Slices were incubated with 10 μM NECA in the absence or presence of increasing concentrations of L-CCG-I added 5 min previously. Unlabelled column represents control values, NECA indicates NECA alone. Data are d.p.m. from a single experiment conducted in quadruplicate. The mean ± s.e.mean IC_{50} value for three such experiments was 15 ± 3 μM. Data are represented as mean ± s.e.mean values.

dinitroquinoxaline-2,3-dione (DNQX, 100 μM), L-2-amino-5-phosphonopentanoate (L-AP5, 100 μM) and 2-amino-3-phosphonopropionate (DL-AP3, 1 mM) (data not shown). However, the effects of the inotropic receptor antagonists are complicated by the finding that DNQX and L-AP5 alone also inhibited the forskolin response by 39 ± 3 and 30 ± 3% respectively. L-CCG-I appears to be approximately 10 fold more potent than 1S,3R-ACPD (IC_{50} 2.14 ± 0.29 μM ($n = 4$); Cartmell *et al.*, 1993).

L-AP4 also inhibited forskolin-stimulated cyclic AMP accumulation in a concentration-dependent manner but with an IC_{50} value of 71 ± 17 μM ($n = 5$) (Figure 2), indicating a lower potency of L-AP4 in comparison with 1S,3R-ACPD and L-CCG-I. The inhibition of the forskolin cyclic AMP response by L-AP4 was also not reversed by the inotropic receptor antagonists DNQX (100 μM), L-AP5 (100 μM) and DL-AP3 (1 mM).

Effects on receptor-mediated cyclic AMP formation

In order to investigate the effects of putative metabotropic receptor agonists on receptor-mediated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, adenosine A_{2b} receptors were stimulated with 5'-N-ethylcarboxamidoadenosine (NECA, 10 μM), in the presence of 1.2 u ml^{-1} adenosine deaminase; and histamine H_2 receptors stimulated with 1 mM histamine, in the presence of 3 μM mepyramine and 10 μM rolipram. L-AP4 elicited inhibitory effects on both adenosine A_{2b} receptor- and histamine H_2 receptor-stimulated [^{3}H]-cyclic AMP accumulation (Figure 2). IC_{50} values obtained for these inhibitory effects were 120 ± 20 μM ($n = 3$) and 71 ± 14 μM ($n = 3$) respectively. At an L-AP4 concentration of 1 mM the NECA [^{3}H]-cyclic AMP response was inhibited by 86 ± 4% ($n = 3$) and the [^{3}H]-cyclic AMP response to histamine was inhibited by 80 ± 2% ($n = 3$).

In contrast to these inhibitory effects, L-CCG-I enhanced the [^{3}H]-cyclic AMP responses to both adenosine A_{2b} receptor- (Figure 1c) and histamine H_2 receptor- stimulation (Table 1). The response to NECA was enhanced by L-CCG-I with an EC_{50} value of 15 ± 3 μM ($n = 3$); a maximal potentiation (297 ± 14% of the NECA response) was achieved at 100 μM. The EC_{50} value obtained for L-CCG-I-elicited potentiation is similar to that previously obtained for the enhancement of the NECA response by 1S,3R-ACPD (17 ± 2 μM ($n = 5$); Cartmell *et al.*, 1993). L-CCG-I also had a potentiatory effect on the [^{3}H]-cyclic AMP response to histamine with an EC_{50} value of 19 ± 2 μM (438 ± 42% increase in the histamine response at 100 μM) compared to a value of 7 ± 2 μM ($n = 3$) for the potentiation of the histamine [^{3}H]-cyclic AMP response by 1S,3R-ACPD.

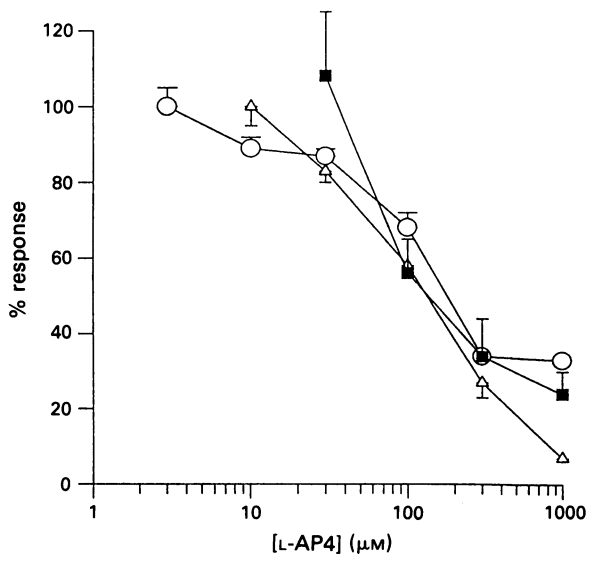

Figure 2 The inhibitory effects of L-AP4 upon forskolin (30 μM, ○), NECA-(10 μM, △), and histamine- (1 mM, ■) stimulated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices. To normalize responses from different experiments data are expressed as a percentage of the response to the stimulus. Data are expressed as mean ± s.e.mean. For abbreviations, see text.

Phosphoinositide hydrolysis

In addition to potentiating the [^{3}H]-cyclic AMP responses to NECA and histamine, L-CCG-I stimulated phosphoinositide hydrolysis in a concentration-dependent manner. Due to constraints imposed by the availability of the material, concentrations of L-CCG-I greater than 1 mM (at which a maximum effect had not been achieved) could not be examined. The EC_{50} value is therefore in excess of 160 μM (the best fit estimate for the curve in Figure 1a). L-CCG-I is therefore a less potent agonist at the phospholipase C-coupled metabotropic receptor(s) than 1S,3R-ACPD (EC_{50} value 35 ± 6 μM, $n = 8$; Table 1). L-CCG-I-stimulated phosphoinositide hydrolysis was not inhibited by the inotropic receptor antagonists 5-methyl-10, 11-dihydro-5*H*-dibenzo [*a,d*] cyclohepten-5, 10-imine maleate (MK-801, 100 μM), L-AP5 (1 μM) or DNQX (100 μM). L-AP4 failed to stimulate significantly phosphoinositide hydrolysis at 1 mM (78 ± 26 d.p.m. over basal, $n = 3$).

The mechanism of L-CCG-I enhancement of cyclic AMP accumulation

In brain slice preparations, stimulation of many receptor systems linked to phosphoinositide hydrolysis e.g. histamine H_1, α_1-adrenoceptors, 5-HT_2 can indirectly potentiate cyclic AMP formation mediated by other receptor systems (Hill & Kendall, 1989). This augmentation is thought to be due to products of phosphoinositide turnover, increasing protein kinase C activity and intracellular Ca^{2+} concentration (Donaldson *et al.*, 1990). In order to investigate the possible role of protein kinase C in the augmentation of the NECA [^{3}H]-cyclic AMP response by L-CCG-I, the effects of the protein kinase C inhibitor Ro 31-8220 were studied. Slices were incubated for 5 min with L-CCG-I (100 μM) prior to a 10 min stimulation with either NECA (10 μM) or forskolin (30 μM), in the presence or absence of 10 μM Ro 31-8220 (Davis *et al.*, 1989). Ro 31-8220 had no effect on basal levels of [^{3}H]-cyclic AMP but reduced the potentiation of the NECA [^{3}H]-cyclic AMP response by both L-CCG-I (100 μM) and β-phorbol-12,13-dibutyrate (PDBu, 1 μM) (Figure 3a). Potentiation of the NECA response by L-CCG-I was 547 ± 58% ($n = 3$) of the [^{3}H]-cyclic AMP response to NECA in the absence and 356 ± 50% ($n = 3$) in the presence of the PKC inhibitor. Similarly, the PDBu-elicited increase in the NECA [^{3}H]-cyclic AMP response was reduced from 628 ± 83% in the absence to 172 ± 23% in the presence of Ro 31-8220 (10 μM). These data suggest that protein kinase C plays a role in the potentiation of the [^{3}H]-cyclic AMP response to NECA by L-CCG-I.

The effects of removing Ca^{2+} from the extracellular medium were investigated by incubating slices in Ca^{2+}-free Krebs solution containing 250 μM EGTA. The presence of EGTA had no effect on basal levels of cyclic AMP, nor on the inhibition of forskolin-stimulated cyclic AMP accumulation by 100 μM L-CCG-I (although the forskolin response was increased in the absence of extracellular Ca^{2+} presumably due to the deactivation of Ca^{2+}-dependent phosphodiesterases, Figure 3b). Conversely, the L-CCG-I-elicited potentiation of the NECA [^{3}H]-cyclic AMP response was reduced by omission of Ca^{2+} from the extracellular medium. The L-CCG-I-elicited increase in the NECA [^{3}H]-cyclic AMP response was reduced from 634 ± 32% ($n = 3$) in the presence to 193 ± 25% ($n = 3$) in the absence of extracellular Ca^{2+}. Interestingly, the potentiating effects of PDBu on the NECA

Table 1 Values of agonist potencies (μM) of 1S,3R-ACPD, L-CCG-I and L-AP4

	Forskolin-stimulated cyclic AMP	*NECA-stimulated cyclic AMP*	*Histamine-stimulated cyclic AMP*	*Phosphoinositide hydrolysis*
1S,3R-ACPD	2.14 ± 0.29(7)↓	17 ± 2 (5)↑	7 ± 2 (3)↑	35 ± 6(8)↑
L-CCG-I	0.24 ± 0.06(5)↓	15 ± 3 (3)↑	19 ± 2 (3)↑	~160(3)↑
L-AP4	71 ± 17(5)↓	120 ± 20(3)↓	71 ± 14(3)↓	No effect

Values are mean ± s.e.mean of 3–8 experiments (*n* given in parentheses).
↑ represents increased response
↓ represents inhibition of response.
For abbreviations, see text.

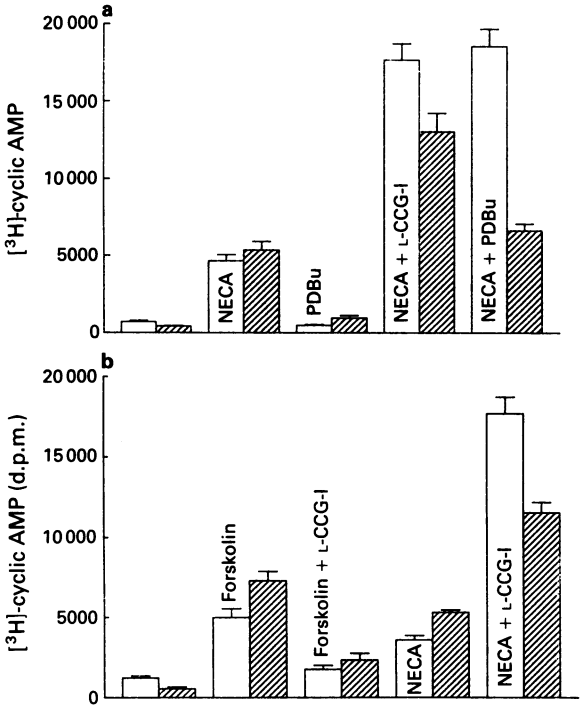

Figure 3 (a) The effect of the protein kinase C inhibitor Ro-31–8220 (10 μM) on potentiation of the NECA-stimulated [^{3}H]-cyclic AMP response. NECA indicates incubation with 10 μM NECA only and PDBu indicates incubation with 1 μM PDBu only. Hatched columns represent data from slices incubated in the presence of Ro-31–8220 (10 μM). Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean ± s.e.mean values. Two similar experiments gave essentially identical results. (b) The effects of EGTA (250 μM) on the inhibition of forskolin-stimulated [^{3}H]-cyclic AMP accumulation and potentiation of the NECA-stimulated [^{3}H]-cyclic AMP response by L-CCG-I (100 μM). Forskolin indicates incubation with 30 μM forskolin, and NECA represents incubation with 10 μM NECA. Unlabelled columns represent control values, hatched columns represent data from slices incubated in the presence of EGTA (250 μM). Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean ± s.e. mean values. Two similar experiments gave essentially identical results.

response were also reduced (~400% in the presence and ~200% in the absence of extracellular Ca^{2+}), confirming that the action of PKC is Ca^{2+}-dependent and suggesting that the concentration of EGTA used (250 μM) was sufficient to reduce cytoplasmic Ca^{2+}. These results implicate an important role for the phosphoinositide-linked metabotropic receptor in 1S,3R-ACPD- and L-CCG-I-elicited potentiations of adenosine A_{2b}- and histamine H_2-receptor-mediated responses.

Discussion

The aim of this study was to determine the selectivity, in a brain-derived preparation, of two agonists, L-CCG-I and L-AP4 which have been shown to have different potencies for metabotropic receptor subtypes expressed in model cell systems.

L-CCG-I stimulated phosphoinositide hydrolysis in a concentration-dependent manner, but due to a limited availability of the compound the maximum concentration that could be achieved was 1 mM at which inositol phosphates accumulation was still increasing. The EC_{50} of L-CCG-I-stimulated inositol phosphates was therefore in excess of 160 μM, somewhat less potent than the EC_{50} of 5 μM reported for the compound in studies on rat hippocampal synaptoneurosomes (Nakagawa *et al.*, 1990) or the 50 μM determined for $mGluR_1$ receptors expressed in CHO cells (Hayashi *et al.*, 1992). The differences could be due to the existence of different receptor reserves in the various preparations or to the mediation of the responses in the brain preparations by metabotropic receptors other than $mGluR_1$ (e.g. $mGluR_5$) for which the potency of L-CCG-I is not yet known. L-CCG-I is also a less potent agonist of the $mGluR_1$ receptor than quisqualate, glutamate, ibotenate or *trans*-ACPD (Tanabe *et al.*, 1993).

L-AP4 was without effect on basal phosphoinositide hydrolysis in guinea-pig cerebral cortex although it has previously been suggested that it is an antagonist in rat cerebral cortex (Schoepp *et al.*, 1990) and it is conceivable therefore that some of the effects of L-AP4 on neurotransmitter release could be due to blockade of Ca^{2+}-mobilizing metabotropic receptors, for instance those involved in the metabotropic receptor-mediated enhancement of glutamate release (Herrero *et al.*, 1992).

In contrast to their effects on forskolin-stimulated cyclic AMP formation, glutamate (Donaldson *et al.*, 1990) and 1S,3R-ACPD (Cartmell *et al.*, 1993, Table 1) enhanced adenosine A_{2b} receptor-mediated responses. In the present study, L-CCG-I was observed to potentiate both NECA- and histamine-stimulated cyclic AMP formation with EC_{50} of 15 μM and 19 μM respectively. PDBu also enhanced the cyclic AMP response to NECA, and this enhancement was completely reversed in the presence of the protein kinase C inhibitor, Ro 31–8220. The augmentation elicited by L-CCG-I was only partially reversed by Ro 31–8220 suggesting that protein kinase C (presumably activated by diacylglycerol generated by stimulation of phosphoinositide turnover) mediates only part of the potentiated response (Figure 3). Calcium influx also appears to be involved in the augmented response to L-CCG-I since a reduced extracellular Ca^{2+} concentration resulted in a reduction of the enhanced response. Similarly, the ACPD-evoked phosphoinositide response in guinea-pig cerebral cortex requires the presence of extracellular calcium (Alexander *et al.*, 1990). These findings suggest that L-CCG-I-evoked potentiations of receptor-stimulated cyclic AMP formation are mediated by products of phosphoinositide hydrolysis. The potency of 1S,3R-ACPD in potentiating receptor-stimulated cyclic AMP formation was

in accordance with that for stimulating phosphoinositide hydrolysis (Table 1) suggesting a similar mechanism, but L-CCG-I was at least 10 fold more potent in potentiating the cyclic AMP responses to histamine and NECA compared with its effect on phosphoinositide hydrolysis. It is therefore possible that the L-CCG-I-mediated potentiations involve a considerable amplification step although it is puzzling that a similar amplification does not appear to be involved in the enhanced cyclic AMP response to 1S,3R-ACPD–this mechanism clearly requires further investigation.

Since L-AP4 failed to stimulate phosphoinositide hydrolysis, it is not surprising that L-AP4 did not increase the [^{3}H]-cyclic AMP responses to either NECA or histamine. In fact L-AP4 inhibited these responses in a concentration-dependent manner (IC_{50} values $120 \pm 20\,\mu M$ and $71 \pm 14\,\mu M$ respectively), and these reductions might also be involved in L-AP4-mediated inhibition of synaptic neurotransmission. The inhibitory actions of L-AP4 are not due to non-specific toxic effects since in preliminary experiments on cultured rat astrocytes, L-AP4 (up to a concentration of 1 mM) had no effect on isoprenaline-stimulated cyclic AMP formation (Cartmell *et al.*, unpublished observations).

When adenylyl cyclase was stimulated by forskolin (30 μM), L-CCG-I elicited a concentration-dependent inhibition of [^{3}H]-cyclic AMP accumulation. L-CCG-I was approximately 10 fold more potent (IC_{50} 0.24 μM) in this regard than the selective metabotropic receptor agonist 1S,3R-ACPD (IC_{50} value $2.14 \pm 0.29\,\mu M$, $n = 7$; Cartmell *et al.*, 1993, Table 1). Somewhat surprisingly, the extent of this inhibition at 1 μM was $91 \pm 4\%$ ($n = 5$) of the forskolin response (1S,3R-ACPD also caused an $85 \pm 2\%$ inhibition of the forskolin [^{3}H]-cyclic AMP response at 10 μM, Cartmell *et al.*, 1993). This contrasts with the much smaller inhibitions of the forskolin response mediated by some other receptors negatively coupled to adenylyl cyclase (e.g. α_2-adrenoceptor, Duman & Enna, 1986) in brain slices. The simplest explanation for this difference is that most of the cells responding to forskolin contain metabotropic but not α_2-adrenoceptors. The EC_{50} for L-CCG-I mediated inhibition of the forskolin response (240 nM) is very close to the 300 nM quoted by Hayashi *et al.* (1992) for L-CCG-I at the $mGluR_2$ but much more potent than at the $mGluR_4$ receptor (IC_{50} 50 μM) expressed in CHO cells. Whether the steepness of the inhibition curve (Figure 2b) indicates the presence of multiple co-operating receptors or an additional, non-receptor-mediated effect of L-CCG-I remains to be determined.

L-AP4 also inhibited forskolin-stimulated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices (IC_{50} value $71 \pm 17\,\mu M$, $n = 5$), but was much less potent than either 1S,3R-ACPD or L-CCG-I. The shallow concentration-response curve may be due to L-AP4 acting at a number of receptor subtypes with different potencies. Indeed, on the basis of the data from transfected receptor studies (Nakanishi, 1992; Thomsen *et al.*, 1992; Tanabe *et al.*, 1993), L-AP4 would be expected to have a potency at the $mGluR_4$ receptor in the high nanomolar range and it is likely that the guinea-pig cortex contains only a minor proportion of $mGluR_4$s with the majority of the inhibition of the forskolin response due to L-AP4 being mediated by other cyclase-linked mGluRs.

We may therefore conclude that there are at least two different subtypes of metabotropic glutamate receptors in guinea-pig cerebral cortex. The first group of receptors are coupled to phosphoinositide turnover via phospholipase C, and the second group are negatively linked to adenylyl cyclase. Furthermore, interaction between phospholipase C-linked and adenylyl cyclase-coupled receptors appears to cause an indirect enhancement of cyclic AMP accumulation. It is apparent from these results (Table 1) that 1S,3R-ACPD, L-AP4 and L-CCG-I are all more potent inhibitors of cyclic AMP accumulation than stimulators of phosphoinositide hydrolysis, but it is evident that L-CCG-I is by far the most selective for metabotropic receptors negatively-coupled to adenylyl cyclase in this tissue preparation. These data confirm previous studies indicating that L-CCG-I is an important pharmacological tool for the differentiation of metabotropic glutamate receptor subtypes.

We are grateful for support from the Wellcome Trust and Medical Research Council.

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(Received June 29, 1993
Revised October 1, 1993
Accepted October 6, 1993)

Br. J. Pharmacol. (1994), **111**, 370–376

Attenuated 5-hydroxytryptamine receptor-mediated responses in aortae from streptozotocin-induced diabetic rats

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1 This study was designed to examine further the attenuated contractile responses to 5-hydroxytryptamine (5-HT) previously observed in aortae from diabetic rats.

2 Cumulative concentration-response curves to 5-HT, and the 5-HT receptor agonists, α-methyl 5-HT (α-Me-5-HT, 5-$HT_{2/1C}$ agonist), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 5-$HT_{2/1C}$ agonist) and 5-carboxamidotryptamine (5-CT, 5-$HT_{1A/1B/1D}$ agonist), were examined in endothelium-intact and -denuded aortae from 2-week streptozotocin (STZ)-diabetic and control rats.

3 In endothelium-intact and -denuded aortae from diabetic rats, maximum responses to 5-HT and α-Me-5-HT were significantly reduced compared to those of aortae from control rats. Responses to these agonists were inhibited by the 5-$HT_{2/1C}$ receptor antagonist, ketanserin (0.1 μM).

4 The attenuated responses to 5-HT of aortae from diabetic rats were normalized by chronic insulin treatment of the rats (5 units day^{-1}, s.c.), but not by altering the glucose concentration of the bathing fluid.

5 The nitric oxide synthase inhibitor N-nitro-L-arginine (NOLA, 0.1 mM) significantly potentiated responses to both 5-HT and α-Me-5-HT in endothelium-intact aortae. However, the difference between maximum responses of aortae from diabetic and control rats was still evident in the presence of NOLA.

6 Endothelium-intact rings, in the presence of ketanserin (0.1 μM) and preconstricted with the thromboxane A_2-mimetic, U46619 (0.1–0.3 μM), from control and diabetic rats, did not relax to cumulative additions of 5-HT (1 nM–30 μM).

7 Contractile responses to DOI were obtained only in endothelium-denuded aortae, and in endothelium-intact aortae in the presence of NOLA, from control rats.

8 Contractile responses to 5-CT were obtained only in endothelium-denuded aortae from both control and diabetic rats, and in endothelium-intact aortae in the presence of NOLA, from control rats.

9 [^{3}H]-ketanserin binding studies showed that there was no significant change in the affinity or density of [^{3}H]-ketanserin for binding sites in membrane preparations of aortae from control and diabetic rats.

10 These results suggest that 5-HT contracts aortae from rats via 5-$HT_{2/1C}$ receptor activation. However, the simultaneous release of EDRF from endothelial cells in response to 5-HT does not appear to be receptor-mediated. The attenuated contractile responses observed to 5-HT in aortae from 2-week diabetic rats do not appear to be mediated by changes in either endothelial cell function or an alteration in 5-HT receptor affinity or density.

Keywords: Diabetes mellitus; rat aortae; endothelium; 5-hydroxytryptamine; N-nitro-L-arginine

Introduction

Previous work from our laboratory has shown that contractile responses to the 5-hydroxytryptamine (5-HT) receptor agonists (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and 5-HT are attenuated in aortic rings from streptozotocin (STZ)-diabetic rats (Sikorski *et al.*, 1993). This latter finding supported the earlier work of Hagen *et al.* (1985) and Head *et al.* (1987). However, the mechanism(s) behind this abnormality has yet to be elucidated. We have postulated that functional and/or structural changes of the endothelium of vessels from diabetic rats are unlikely to be responsible for these changes but that a downregulation of the 5-HT receptor population in aortae from diabetic rats may contribute (Sikorski *et al.*, 1993). The aim of the present study was to identify further the mechanisms underlying the attenuated response to 5-HT receptor agonists of isolated aortae from 2-week STZ-diabetic rats. This included examining the effects of altering the glucose level of the physiological solution, chronic insulin administration and radioligand binding studies.

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Methods

Male Wistar rats (300–420 g) were treated with STZ (60 mg kg^{-1}, i.v.) or vehicle (50 mM citrate buffer) under 4% halothane anaesthesia (2:1 O_2/N_2O). The animals were then housed in treatment pairs, being allowed free access to food and water at all times. Rat body weights and blood glucose levels were measured on the day of STZ or citrate buffer administration and again after 2 weeks. Only rats displaying elevated blood glucose levels (>16 mM, Ames Minilab 1) after 2 weeks were considered to be diabetic. Vehicle control rats had blood glucose levels of between 3–8 mM when tested at the same time. After 2 weeks, a STZ-induced diabetic and a control animal were killed, and 5 mm rings cut from each descending thoracic aorta. Where indicated, endothelial cells were removed from aortae by rubbing the intimal surface with thin wire. Rings were placed in 15 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2 and glucose 11.1 Where indicated, high-glucose Krebs consisted of Krebs solution with 30 mM glucose. Physiological solutions were maintained at 37°C, and bubbled with 5% CO_2 in O_2. Rings were placed under 10 g resting tension throughout the experiment, as this approximates the physiological wall tension (Fulton *et al.*, 1991; Sikorski *et al.*, 1993). After 1 h equilibration, a submaximal concentration of phenylephrine (0.3 μM) was added to the

bath. At the plateau of contraction, acetylcholine (10 μM) was added. The presence of functional endothelial cells was indicated by subsequent relaxation. Following this procedure, a single cumulative concentration-response curve was constructed, on each tissue, either in the presence or absence of antagonists/inhibitors. NOLA was allowed to equilibrate for 15 min before the addition of any agonist. Ketanserin and indomethacin were allowed 30 min equilibration.

Insulin treatment

Where indicated, STZ-treated rats were injected with a single daily dose of Lente MC insulin zinc suspension (5 units day^{-1}, s.c.) commencing on the second day after STZ administration (Hodgson & King, 1992).

Binding studies

Tissue preparation The remaining segments of thoracic aortae, obtained from control and diabetic rats at the time of killing, were denuded of endothelial cells (as above) and the tissues stored at − 80°C until required.

Radioligand binding

Aortae from 8–10 diabetic or control rats were weighed and suspended in 10 ml ice-cold Krebs phosphate buffer (KPB) of composition, (mM): NaCl 119, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.7, NaH_2PO_4 1.3 and Na_2HPO_4 8.7. The tissues were crudely chopped before being homogenized (12 s) in a polytron (Kinematica PT 10-35). The chopped tissues were then homogenized in a glass homogenizer (4–6 strokes) prior to centrifugation (30 *g*, 7 min, 4°C; Sorvall model RC5C), passed through a double layer of gauze, centrifuged again (45,000 *g*, 15 min, 4°C), and the supernatant removed. The membrane pellet was resuspended (10 ml KPB) and allowed to equilibrate (10 min, 37°C, to promote the breakdown of endogenous catecholamines). The suspension was cooled in an ice-bath and spun again (45,000 *g*, 15 min, 4°C). The membrane pellets were resuspended in KPB (8–10 ml). Membrane binding to [^{3}H]-ketanserin (specific activity 60.0 Ci $mmol^{-1}$, NEN Research Products) was carried out by incubating 100 μl of tissue homogenate (30–63 μg protein) with [^{3}H]-ketanserin for 10 min (37°C) in a final volume of 200 μl. [^{3}H]-ketanserin reaches binding equilibrium with 5-HT_2-receptors within 5 min and remains stable for at least a further 15 min (Leysen *et al.*, 1982). Methysergide (30 μM) was used to estimate non-specific binding (as previously described by Leysen *et al.* (1982)). The level of specific binding was approximately 30%. The reaction was stopped with 5 ml ice-cold KPB, the reaction mixture was then drawn, under vacuum, through Gelman A/E filter papers using a Brandel M-24 cell harvester. Filter papers were washed five times with 4 ml ice-cold KPB and then partially dried under vacuum. The filter papers were placed in scintillation vials, 4 ml PCS (Amersham) was added and c.p.m. were recorded over a 5.5 min period. Protein determinations were made by the modified Lowry method (Schacterle & Pollack, 1973).

Saturation studies

For saturation studies eight concentrations of [^{3}H]-ketanserin (0.05–28 nM) were run in triplicate.

Competition studies

For competition studies eight concentrations of methysergide, WB-4101 and 5-HT (0.1 nM–0.1 mM) were used to displace [^{3}H]-ketanserin (2.4 nM) from binding sites. Competition study points were run in triplicate.

Binding analysis

Data from both saturation and competition studies were analysed initially using the computer programme EBDA (McPherson, 1983), to obtain estimates of B_{max}, K_D and K_i and then LIGAND (Munson & Rodbard, 1980), to obtain final parameter estimates (± s.e.mean) of B_{max}, K_D and K_i.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), 5-carboxamidotryptamine maleate (Glaxo), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (Research Biochemicals Inc.), 5-hydroxytryptamine creatine sulphate (Sigma), iproniazid PO_4 (Sigma), indomethacin (Sigma), insulin (Lente MC zinc suspension, CSL-Novo), ketanserin tartrate (Janssen), α-methyl-5-hydroxytryptamine maleate (Research Biochemicals Inc.), methysergide maleate (Sandoz), N-nitro-L-arginine (Sigma), phenylephrine HCl (ICN Pharmaceutical), streptozotocin (Sigma), U-0521 (3′,4′-dimethyl-2-methylpropiophenone; Upjohn), U46619 ((15S)-hydroxy-11α,9α-(epoxymethano) prosta-5Z, 13E-dienoic acid; Upjohn), 2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1, 4-benzodioxane hydrochloride (WB-4101 HCl, Research Biochemicals Inc.).

Phenylephrine was dissolved in catecholamine diluent (0.312 g NaH_2PO_4 and 0.08 g ascorbic acid per litre of 0.9% (w/v) saline). Ketanserin was dissolved in dimethyl sulphoxide. Indomethacin was dissolved in 1% $NaCO_3$. U46619 was prepared in ethanol, which was evaporated under a stream of N_2, and redissolved in distilled water. Further dilutions of these drugs, and stock solutions of all other drugs (except for phenylephrine), were prepared in distilled water.

For binding experiments WB-4101, methysergide and 5-HT were diluted in KPB containing 0.4% ascorbic acid, 40 μM iproniazid and 120 μM of the catechol-*O*-methyl transferase inhibitor, U-0521 (Henseling, 1983).

Statistics

Comparisons between responses in aortae from STZ-treated and control rats were made by two-way analysis of variance (ANOVA). Multiple comparisons were analysed by Tukey's test. Values shown are means ± s.e.mean. Shifts between linear portions of concentration-response curves were calculated by the Apple IIe COMPAR programme. EC_{50} values were determined from the E_{max} of each individual curve (Apple IIe LINUS) and the geometric mean (i.e. mean of the log values) determined. In all cases, statistical significance is indicated by $P<0.05$.

Results

As shown in Table 1, body weights of 2-week diabetic rats were significantly less than those of age-matched controls. In addition, although control rats gained weight in the 2 weeks following vehicle injection, weights of diabetic rats decreased significantly. The reduction in body weight observed in diabetic rats was not evident in insulin-treated diabetic rats.

Blood glucose levels of diabetic rats 2 weeks after STZ-treatment were elevated significantly compared to those of control rats and compared to their pre-injection levels. However, blood glucose levels of insulin-treated diabetic rats were no different from those of control rats (Table 1).

The dry weights of aortae from diabetic rats were significantly less than those of control rats and insulin-treated diabetic rats (Table 1).

As shown in Figure 1, 5-HT (0.1 μM–0.1 mM) produced concentration-dependent contractile responses in aortae from both control and diabetic rats. Removal of the endothelium significantly potentiated maximum responses to 5-HT in aortae from both control and diabetic rats (Table 2). Maximum responses to 5-HT of endothelium-intact and -denuded aor-

Table 1 Body weights, blood glucose levels and aortic ring dry weights of control and streptozotocin (STZ)-treated rats

		Body weights (g)		*Blood glucose levels* (mM)		*Aortic ring dry weights* (mg)	
	(n)	Initial	Final	Initial	Final	With E	Without E
Control	59	353 ± 3	399 ± 4†	5.5 ± 0.2	6.3 ± 0.1	1.98 ± 0.02	1.97 ± 0.03
STZ	62	343 ± 3	308 ± 4*†	5.7 ± 0.2	22.0 ± 0.6*†	1.78 ± 0.03*	1.84 ± 0.02*
STZ/insulin	6	328 ± 8	366 ± 4*†§	6.2 ± 0.4	5.0 ± 0.3§	2.02 ± 0.02§	2.01 ± 0.04§

Initial measurements were made at the time of STZ or vehicle injection, and final measurements made 2 weeks later.
E = endothelial cells.
*Significantly different from corresponding value in control group, $P<0.05$ ANOVA.
†Significantly different from initial value in same treatment group, $P<0.05$ ANOVA.
§Significantly different from corresponding value in diabetic group, $P<0.05$ ANOVA.

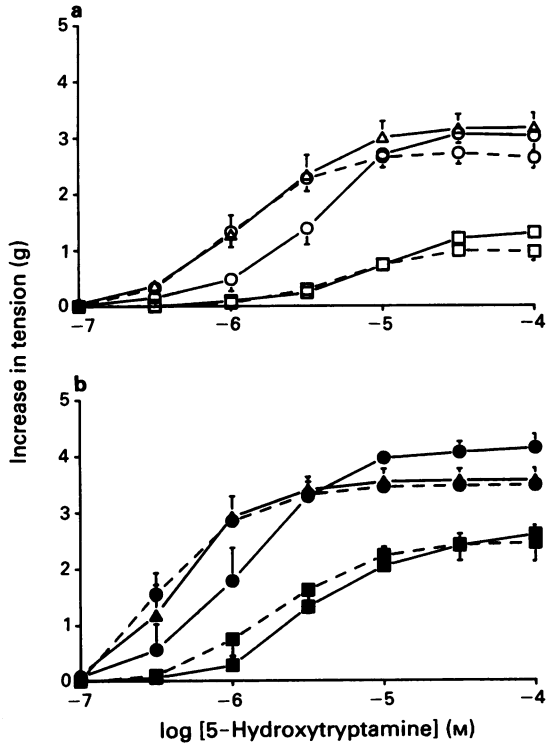

Figure 1 Responses of rat aortic rings to 5-hydroxytryptamine ($n = 5-6$). (a) Diabetic (□—□; □---□ 30 mM glucose), insulin-treated diabetic (△) and control (○—○; ○---○ 30 mM glucose) with endothelium. (b) Diabetic (■—■; ■---■ 30 mM glucose), insulin-treated diabetic (▲) and control (●—●; ●---● 30 mM glucose) without endothelium. Values indicate mean ± s.e.mean.

tae from diabetic rats were significantly attenuated compared to those of control rats (Table 2). Maximum responses to 5-HT of aortae from insulin-treated diabetic rats were not significantly different from those of aortae from control rats (Table 2). When experiments were performed on aortae bathed in 30 mM glucose Krebs solution, maximum responses of endothelium-denuded aortae from control rats, but not diabetic rats, were significantly reduced compared to those of aortae bathed in normal Krebs solution. However, the difference between responses of aortae from control and diabetic rats was still significant (Table 2).

In the presence of indomethacin (10 μM), maximum responses to 5-HT of endothelium-intact or -denuded aortae from diabetic rats were still significantly reduced compared to controls (Figure 2). However, a comparison of Figures 1b and 2b shows that maximum responses of endothelium-denuded aortae from diabetic rats were significantly lower than those obtained to 5-HT in the absence of indomethacin (2.6 ± 0.2 g without indomethacin; 1.9 ± 0.1 g with indomethacin, $P<0.05$).

In the presence of ketanserin (0.1 μM), 5-HT concentration-response curves were shifted to the right in endothelium-denuded aortae from both control and diabetic rats by factors of 42 (26, 71, 95% confidence limits (c.l.); 30 degrees of freedom (d.f.)) and 29 (15, 54, 95% c.l.; 30 d.f.), respectively (Figure 2b). In the presence of ketanserin (0.1 μM), endothelium-intact aortae, from both groups of rats, did not respond to increasing concentrations of 5-HT ($n = 6$, data not shown). However, responses to 5-HT (10 μM–0.3 mM) were obtained in endothelium-intact aortae, from both control and diabetic rats, in the combined presence of ketanserin (0.1 μM) and NOLA (0.1 mM). In the presence of these two agents, maximum responses of aortae from diabetic rats were still significantly reduced compared to those of aortae from control rats ($P<0.05$, ANOVA; Figure 2a).

DOI (10 nM–10 μM) failed to produce contractile responses in aortae (with or without endothelial cells) from diabetic rats (Figure 3), and produced only small responses in endothelium-intact aortae from control rats (Figure 3a). However, marked contractile responses were obtained in endothelium-denuded aortae (Figure 3b), and in endothelium-intact aortae in the presence of NOLA, from control rats (Figure 3a).

α-Me-5-HT (0.1 μM–0.1 mM) produced concentration-dependent constriction in aortae from both control and diabetic rats (Figure 4). Maximum responses to α-Me-5-HT were significantly potentiated by the addition of NOLA to endothelium-intact aortae from both control and diabetic rats ($P<0.05$, ANOVA, Figure 4a). Maximum responses were significantly attenuated in aortae from diabetic rats, in the absence or presence of NOLA, compared to those obtained in aortae from control rats ($P<0.05$, ANOVA, Figure 4a). In the presence of ketanserin (0.1 μM), α-Me-5-HT concentration-response curves were shifted to the right in endothelium-denuded aortae from both control and diabetic rats by factors of 422 (210, 1046, 95% c.l.; 36 d.f.) and 257 (92, 1017, 95% c.l.; 36 d.f.), respectively (Figure 4b). However, in the presence of ketanserin (0.1 μM) endothelium-intact aortae, from both groups of rats, did not respond to increasing concentrations of α-Me-5-HT ($n = 4$, data not shown).

5-CT (0.1 μM–0.3 mM) produced concentration-dependent contractile responses in endothelium-denuded aortae from different control and diabetic rats (Figure 5b) and in endothelium-intact aortae, in the presence of NOLA (0.1 mM), from control rats (Figure 5a). NOLA had no significant effect on responses to 5-CT in endothelium-intact aortae from diabetic rats (Figure 5a). Maximum responses to 5-CT in endothelium-denuded aortae from diabetic rats were significantly attenuated compared to those obtained in aortae from control rats (Table 2). Ketanserin (0.1 μM) completely abolished responses to 5-CT (0.1 μM–0.3 mM) in endothelium-denuded aortae from both control and diabetic rats ($n = 3$, data not shown).

When the above results were expressed as tension developed per mg of tissue weight, contractile responses to 5-HT,

Table 2 Maximum responses and EC_{50} values for agonists on aortae from streptozotocin (STZ)-treated and control (C) rats with (+) and without (−) endothelial cells (E)

		C + E	*STZ + E*	*C − E*	*STZ − E*
5-HT	Maximum (g)	3.06 ± 0.17	1.30 ± 0.07*	4.14 ± 0.24†	2.60 ± 0.16*†
	EC_{50} (− log M)	5.54 ± 0.12	5.07 ± 0.19*	6.04 ± 0.25†	5.36 ± 0.21
5-HT/	Maximum (g)	2.72 ± 0.20	0.98 ± 0.15*	3.48 ± 0.09*†	2.45 ± 0.33*†
30 mM glucose	EC_{50} (− log M)	5.97 ± 0.06	5.22 ± 0.15*	6.74 ± 0.28†	5.67 ± 0.15*†
5-HT/insulin	Maximum (g)	NA	3.17 ± 0.26§	NA	3.56 ± 0.22§
	EC_{50} (− log M)		5.85 ± 0.12§		6.53 ± 0.30†§
DOI	Maximum (g)	0.43 ± 0.09	0.04 ± 0.02*	2.14 ± 0.12†	0.08 ± 0.03*
	EC_{50} (− log M)	5.59 ± 0.36	NA	7.06 ± 0.16	NA
α-Me-5-HT	Maximum (g)	2.80 ± 0.21	0.64 ± 0.14*	3.32 ± 0.36	1.40 ± 0.37*
	EC_{50} (− log M)	6.30 ± 0.23	5.73 ± 0.09	7.49 ± 0.17†	6.04 ± 0.06*†
5-CT	Maximum (g)	0.40 ± 0.16[1]	0.30 ± 0.23[1]	3.25 ± 0.07†	1.57 ± 0.23[1]*†
	EC_{50} (− log M)	NA	NA	4.64 ± 0.22	NA

Values are mean ± s.e.mean; $n = 5-6$.
*Significantly different from corresponding value in control group, $P<0.05$ unpaired t test.
†Significantly different from corresponding value in same treatment group with endothelial cells, $P<0.05$ unpaired t test.
§Significantly different from corresponding value in diabetic group, $P<0.05$, ANOVA.
NA = not applicable. For other abbreviations, see text.
[1]As these responses were not maximum, EC_{50} values have not been calculated.

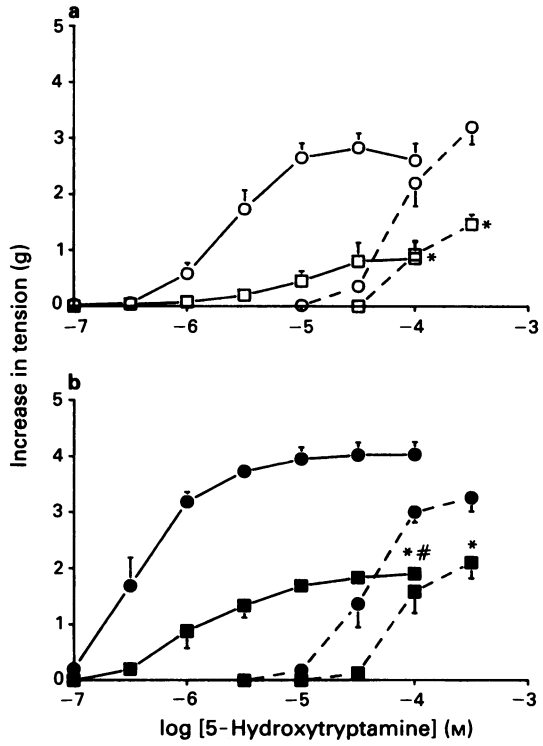

Figure 2 Responses of rat aortic rings to 5-hydroxytryptamine in the presence of indomethacin, ketanserin, or ketanserin and N-nitro-L-arginine (NOLA) combined ($n = 5$). (a) Diabetic (□—□ indomethacin; □---□ ketanserin and NOLA) and control (○—○ indomethacin; ○---○ ketanserin and NOLA) with endothelium. (b) Diabetic (■—■ indomethacin; ■---■ ketanserin) and control (●—● indomethacin; ●---● ketanserin) without endothelium. Values indicate mean ± s.e.mean. *$P<0.05$, significantly different from maximal value in corresponding control group, ANOVA. #$P<0.05$, significantly different from without indomethacin (Figure 1b), ANOVA.

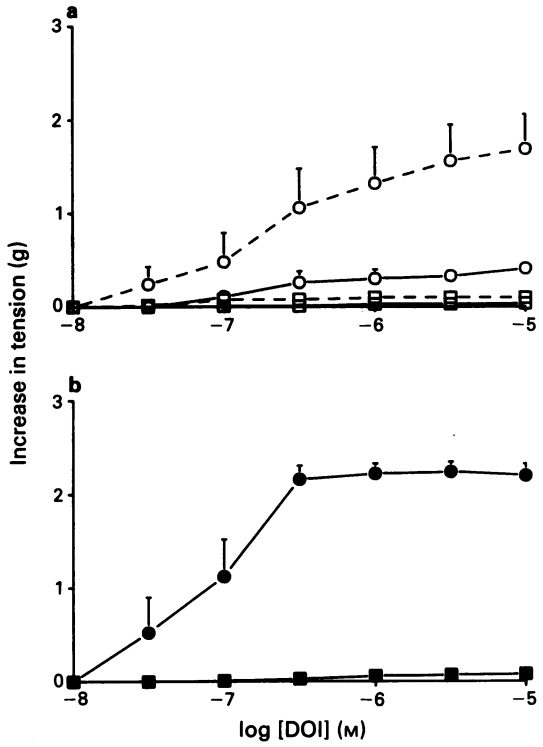

Figure 3 Responses of rat aortic rings to (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) alone, in the presence of N-nitro-L-arginine (NOLA), indomethacin or indomethacin and NOLA combined ($n = 5$). (a) Diabetic (□—□; □---□ with NOLA) and control (○—○; ○---○ with NOLA) with endothelium. (b) Diabetic (■) and control (●) without endothelium. Values indicate mean ± s.e.mean.

α-Me-5-HT, DOI and 5-CT of aortae from diabetic rats were significantly attenuated compared to those obtained in aortae from controls ($n = 5-6$ all groups, data not shown).

NOLA (0.1 mM) had no significant effect on basal tone in unconstricted endothelium-intact aortic rings from control or diabetic rats.

The relaxant effects of 5-HT were examined in endothelium-intact rings, in the presence of ketanserin (0.1 μM), preconstricted with a submaximal concentration of the thromboxane $(Tx)A_2$-mimetic, U46619 (0.1–0.3 μM). 5-HT (1 nM–30 μM) did not induce significant relaxation ($n = 3$, data not shown) in aortae from control or diabetic rats. In fact at higher concentrations of 5-HT (3–30 μM) some additional contraction was observed.

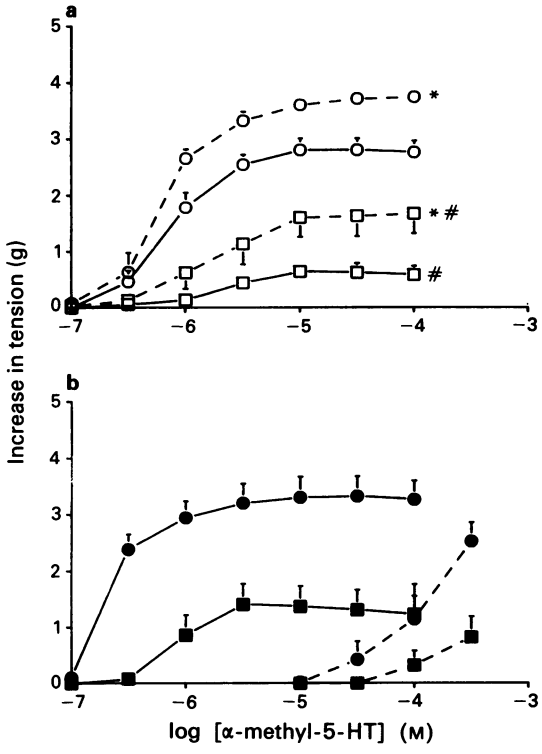

Figure 4 Responses of rat aortic rings to α-methyl-5-HT alone and in the presence of N-nitro-L-arginine (NOLA) or ketanserin (n = 5–6). (a) Diabetic (□—□; □---□ with NOLA) and control (○—○; ○---○ with NOLA) with endothelium. (b) Diabetic (■—■; ■---■ with ketanserin) and control (●—●; ●---● with ketanserin) without endothelium. *$P<0.05$, significantly different from corresponding group without NOLA, ANOVA. #$P<0.05$, significantly different from corresponding control, ANOVA. Values indicate mean ± s.e.mean.

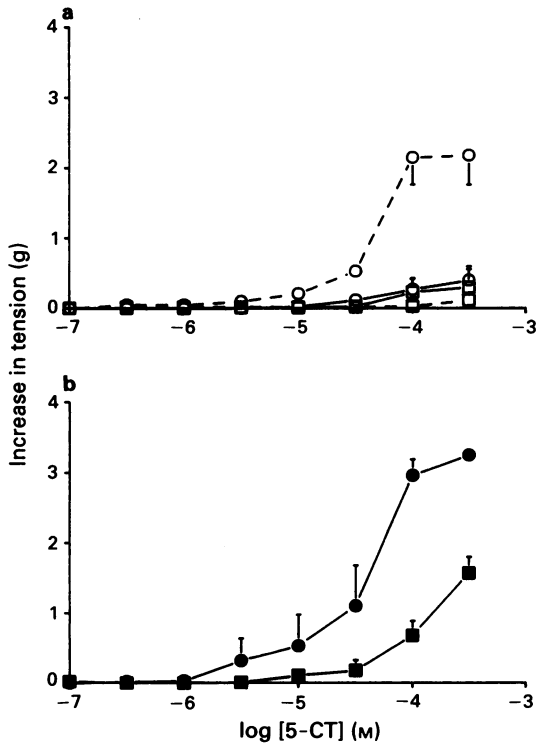

Figure 5 Responses of rat aortic rings to 5-carboxamidotryptamine (5-CT) alone or in the presence of N-nitro-L-arginine (NOLA) (n = 4–6). (a) Diabetic (□—□; □---□ with NOLA) and control (○—○; ○---○ with NOLA) with endothelium. (b) Diabetic (■) and control (●) without endothelium. Values indicate mean ± s.e. mean.

Radioligand binding saturation studies

In membrane preparations of aortae from control and diabetic rats, [^{3}H]-ketanserin bound to single saturable binding sites (K_D 1.33 (0.76,2.34; 95% c.l.) nM, B_{max} 17.4 ± 3.6 (fmol mg^{-1} protein), n = 3 and (K_D 2.31 (1.02,5.23; 95% c.l.)nM, B_{max} 16.3 ± 5.2, n = 3; respectively).

Radioligand binding competition studies

In membrane preparations of aortae from control and diabetic rats, both WB-4101 and methysergide competed for two [^{3}H]-ketanserin binding sites (Table 3). In both treatment groups 5-HT competed for one [^{3}H]-ketanserin binding site (Table 3).

Discussion

The present study confirms earlier reports that contractile responses to 5-HT in aortae from diabetic rats are attenuated (Owen & Carrier, 1979; Hagen *et al.*, 1985; Head *et al.*, 1987; Sikorski *et al.*, 1993). This decrease in responsiveness was no longer apparent in aortae from insulin-treated diabetic rats suggesting that the changes observed were not due to the diabetogen *per se* but linked to the metabolic imbalance produced by insulin deficiency. However, responses to 5-HT obtained in aortae bathed in high glucose Krebs, from diabetic rats, were still attenuated compared to those obtained in aortae from controls. This was despite the fact that maximum responses to 5-HT in endothelium-denuded aortae from control rats were significantly reduced by altering the glucose concentration in the bathing medium. These results suggest that transient changes in blood glucose levels may contribute to the altered responsiveness to 5-HT but that other factors are more important. We have previously shown that contractile responses to endothelin-1, in aortae from control rats, are also attenuated in high glucose Krebs solution (Hodgson & King, 1992).

It has been postulated that the majority of the contractile response to 5-HT in rat aortae is mediated by 5-HT$_2$ receptors (Cohen *et al.*, 1981). Results of the present study, using the 5-HT$_2$ receptor antagonist, ketanserin, appear to confirm this hypothesis for aortae from both control and diabetic rats. However, the fact that indomethacin significantly inhibited responses to 5-HT in endothelium-intact aortae from diabetic rats suggests that a contractile metabolite of arachidonic acid, possibly TxA$_2$, may be contributing to the response in this tissue. This finding supports the earlier work of Hagen *et al.* (1985) who used the cyclo-oxygenase inhibitor, meclofenamic acid. However, they found that meclofenamic acid also produced a significant reduction in 5-HT responses in aortae from control rats.

Contractile responses to the 5-HT receptor agonists, α-Me-5-HT, DOI and 5-CT were also diminished in aortae from diabetic rats. Responses to these agonists were still significantly reduced in endothelium-denuded aortae and when EDRF production was inhibited by NOLA. This suggests that altered EDRF release, as has been previously observed in aortae from diabetic rats (Oyama *et al.*, 1986; Kamata *et al.*, 1989), was not responsible for the attenuated 5-HT receptor-mediated constriction. However, it does appear from the present study that the magnitude of the contractile response to 5-HT, in aortae from both diabetic and control rats, is attenuated by the simultaneous release of EDRF as responses were potentiated by the removal of the

Table 3 Mean molar inhibition constants, K_i for competition by WB-4101, methysergide and 5-hydroxytryptamine at [^{3}H]-ketanserin binding sites in membrane preparations of rat aortae

		Control	*Diabetic*
WB-4101	K_{iH}	0.16 (0.09, 0.28) nM (41%)	0.21 (0.01, 4.00) nM (38%)*
	K_{iL}	4.68 (2.39, 9.15) μM (59%)	0.55 (0.14, 2.18) μM (62%)*
Methysergide	K_{iH}	1.40 (0.15, 13.20) nM (43%)	10.36 (2.32, 46.24) nM (46%)
	K_{iL}	7.85 (0.41, 150.0) μM (57%)	54.5 (12.26, 243.3) μM (54%)
5-HT	K_i	268 (89, 804) nM	150 (34, 662) nM

$n = 3$ (except *, $n = 2$).
Values shown are from three competition study replicates with 95% confidence limits and percentage of high and low affinity binding sites.
WB-4101 and methysergide competed for two [^{3}H]-ketanserin binding sites, 5-HT competed for one site only.

endothelium and by the presence of NOLA. The exact mechanism responsible for this release of EDRF is unclear. However, there appears to be three likely options. These are; (1) basal release, (2) 5-HT receptor-mediated release and (3) stimulated release due to smooth muscle contraction. As NOLA had no effect on resting tone in aortae from either group of rats it does not appear that there was any significant basal EDRF release. 5-HT receptor-mediated release of EDRF has been demonstrated in canine and pig isolated coronary arteries via 5-HT$_1$-like receptor activation (Cocks & Angus, 1983; Angus, 1989). However, in the present study, relaxant responses to 5-HT could not be obtained in rat aortae preconstricted with U46619, when the 5-HT receptors mediating contraction were blocked with ketanserin. This finding is in agreement with the previous work of Martin *et al.* (1986) and indicates that in rat aortae 5-HT is probably causing the release of EDRF by producing an increase in smooth muscle tone (Vargas *et al.*, 1990; Vo *et al.*, 1991). However, this alternative does not adequately explain why contractile responses to 5-HT in endothelium-intact aortae, in the presence of ketanserin, could be obtained only in the additional presence of NOLA because in these experiments there was no induced tone. Also, responses to the more specific agonists DOI and 5-CT were obtained only in endothelium-intact rings, from control rats, when NOLA was present. Therefore, the exact mechanism (or stimulus) for the release of the EDRF remains to be elucidated.

The reason/s for the absence of responses to DOI and 5-CT in endothelium-intact rings, even in the presence of NOLA, from diabetic rats is still unclear. However, if, as indicated by our binding studies, there is no significant change in receptor affinity/density between aortae from control and diabetic rats, it is possible that the alteration is due to post-receptor changes.

Interestingly, in endothelium-denuded aortae from both control and diabetic rats, the 5-HT$_2$ receptor antagonist, ketanserin, produces an approximately 10 fold greater shift in concentration-response curves to the 5-HT$_2$ receptor agonist, α-Me-5-HT than to 5-HT. This suggests that in rat aortae, 5-HT may be acting at more than one 5-HT receptor subtype.

It would appear also that in the present study the 5-HT$_{1A/1B/1D}$ receptor agonist 5-CT (Fozard, 1987) produces vasoconstriction via the activation of 5-HT$_{2/1C}$ receptors. This is likely as responses to 5-CT were abolished by ketanserin. Indeed, it has been shown previously that high concentrations of 5-CT can interact with 5-HT$_2$ receptors in rabbit aorta (Feniuk *et al.*, 1985).

Previous work in our laboratory has shown that contractile responses to DOI can be obtained only in aortae from control rats, and only when the endothelial cells have been removed or a nitric oxide synthase inhibitor present (Sikorski *et al.*, 1993). This finding was confirmed in the present study. In contrast, contractile responses to another 5-HT$_{2/1C}$ agonist, α-Me-5-HT, were obtained in aortae, with and without endothelial cells, from both control and diabetic rats. The reason for the differing results between these two agonists is unknown.

We have previously suggested (Sikorski *et al.*, 1993) that in aortae from diabetic rats, peripheral 5-HT$_2$ receptors may be downregulated, as activation of these receptors promotes hyperglycaemia (Chaouloff *et al.*, 1990). However, results of the present study suggest that there is no significant change in the density or affinity of [^{3}H]-ketanserin for binding sites in membrane preparations of aortae from control and diabetic rats, nor is there a significant change in the affinity of 5-HT for these sites. However, the binding studies indicate that both methysergide and WB-4101 compete with [^{3}H]-ketanserin at two binding sites. [^{3}H]-ketanserin has been shown to bind significantly with α_1-adrenoceptors in human and pig frontal cortex membrane preparations (Hoyer *et al.*, 1987). In the present study, it is likely that [^{3}H]-ketanserin is also binding to α_1-adrenoceptors present in rat aortae (Wenham & Marshall, 1992). The high and low affinity binding sites observed for methysergide may then represent 5-HT$_2$ and α_1-adrenoceptor binding, respectively. Conversely, WB-4101 may be competing with low affinity at 5-HT$_2$ and high affinity at α_1-adrenoceptors.

Aortic ring dry weights from diabetic rats were significantly decreased compared to rings from control rats. However, this difference in tissue weight is unlikely to be responsible for the altered responsiveness observed in aortae from diabetic rats as when results were expressed as tension developed per mg of tissue, responses to all the contractile agents used were still attenuated compared to responses obtained in aortae from control rats.

It has been shown that 5-HT-induced contraction in rat aorta can be analysed into two distinct components (i.e. phasic and tonic). The phasic component appears to be mediated by calcium influx via voltage-dependent channels and the tonic component by phosphoinositide hydrolysis products (Nakaki *et al.*, 1985). We have previously shown that responses to KCl are diminished in the aortae of 2-week diabetic rats (Fulton *et al.*, 1991), indicating that there is likely to be an impairment in the influx of calcium through voltage-operated channels. However, although this impairment probably contributes to the changes observed in the present study, the changes observed in 5-HT receptor-mediated responses are markedly greater than those observed to KCl. Therefore, it is likely that there is also an alteration in the second messenger system (IP$_3$/DAG). This hypothesis is supported by previous work from our laboratory which has shown that responses to endothelin-1 (Fulton *et al.*, 1991; Hodgson & King, 1992), an agonist which utilizes the same second messenger system are also attenuated during diabetes.

In conclusion, in aortae of rats with diabetes of 2-weeks duration, 5-HT receptor-mediated contractile responses are attenuated compared to controls. The decreased responses do not appear to be due to altered EDRF release since this difference was still apparent in the presence of NOLA but may be due to alterations in the second messenger system.

We thank Glaxo Group Research (U.K.) and Upjohn (U.S.A.) for the generous gifts of 5-CT and U46619, respectively.

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(Received July 26, 1993
Revised September 28, 1993
Accepted October 8, 1993)

Br. J. Pharmacol. (1994), **111**, 377

Erratum

Br. J. Pharmacol. (1993), **110**, 1126–1132

J.R.A. Wooltorton & A. Mathie. Block of potassium currents in rat isolated sympathetic neurones by tricyclic antidepressants and structurally related compounds.

In the above article, one of the final paragraphs of the Discussion appeared incorrectly due to the repetition of three lines and omission of three others. The paragraph is shown in its correct form below.

> For example, chlorpromazine has been known for some time to block the muscle-type of nicotinic acetylcholine receptors. The structure of these receptor-channels is well documented and the binding sites for chlorpromazine in the M2 (pore-forming) region of the receptor-channel have been identified. These binding sites are made up of seven amino-acids which lie at three distinct positions on the five subunits that form the functional nicotinic receptor-channel (Revah *et al.*, 1990). Comparison of the sequence of the amino-acids that make up the chlorpromazine binding site and the surrounding structure of nicotinic receptors with known sequences of *Shaker*-like K^+ channels (Pongs, 1992a) shows that there is no comparable sequence in the H5 (pore-forming) segment of *Shaker*-like K^+ channels. It would be of interest then to determine whether chlorpromazine binds to a completely different sequence of amino acids either in the H5 segment or elsewhere or whether it binds to an homologous sequence in a region of the K^+ channel that does not form the pore. There is, for example, a sequence in the S4/S5 segment of these channels which shows reasonable homology with the chlorpromazine binding site on nicotinic receptors. This region is thought to form an amphipathic helix toward the intracellular face of the middle pore of the channel and regulate channel opening and closing (Pongs, 1992b). As the tricyclic compounds are fairly hydrophobic (Jack, 1992) they could easily reach such a site.

British Journal of Pharmacology

VOLUME 111 (1) JANUARY 1994

The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

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Authors should consult the Instructions to Authors and the Nomenclature Guidelines for Authors in Vol. **111,** 378–387. These Instructions and Guidelines also appear with the journal Index for Volumes **108–110,** 1993. A checklist of the essential requirements is summarised in each issue of the journal, or as the last page of the issue.

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Br. J. Pharmacol. (1994) **111**, 378–384

INSTRUCTIONS TO AUTHORS

With effect from 1 January 1994

The *British Journal of Pharmacology* welcomes contributions in all fields of pharmacology for publication as full papers or as high priority Special Reports.

Papers should normally be based on new results obtained experimentally and should constitute a significant contribution to pharmacological knowledge. Papers which reassess pharmacological concepts based on earlier results will also be considered as will purely theoretical papers. Papers dealing only with descriptions of methods are acceptable if new principles are involved.

Contributions that have already been published, or accepted or are under consideration for publication, with essentially the same content will not be considered. This restriction does not apply to results published as abstracts of communications, letters to editors, or as contributions to symposia, provided that the submission adds significantly to the information available in the previously published contribution.

Papers are only accepted if accompanied by a Declaration which must be signed by all Authors. This Declaration concerns the originality of the submitted paper and assigns the copyright of all papers accepted for publication to Macmillan Press Ltd. on behalf of the British Pharmacological Society. See pages 381 and 382 for details.

The Journal will not consider papers which describe experiments on animals which do not fall clearly within the current laws governing animal experimentation in the United Kingdom. Authors must make it clear that the procedures they use were as humane as possible and the doses (initial and subsequent) of anaesthetics and analgesics should be clearly stated; the method of assessing anaesthesia, particularly after the administration of skeletal muscle relaxants (neuromuscular blocking drugs), must be well defined. The Society has an Ethics Committee which can be consulted by authors through the Secretaries to the Editorial Board.

When investigations on normal human subjects are reported, evidence of approval by a local Ethics Committee must be given. Papers concerned with clinical trials or investigations of the effects of drugs on patients are not appropriate for this Journal.

Authors are strongly urged to keep their manuscripts as short as they reasonably can. An effective way is to reduce the Discussion and the number of figures to a minimum and to avoid repetition of information that has already been published. Authors should remember that a reader may be influenced by literary style and will appreciate simple but accurate prose.

It is important to note that failure to comply with 'Instructions to Authors' may lead to considerable editorial delays.

FULL PAPERS

Manuscripts must be typed on one side of A4 paper. Words at the end of lines should not be divided because they may become incorrectly hyphenated. Handwritten characters or symbols (e.g. Greek letters) should be spelled out in full in the margin. Papers in recent issues of the *British Journal of Pharmacology* should be consulted for the general layout of the paper and also for details. The following subsections are used:

1. Title page
2. Summary
3. Introduction
4. Methods
5. Results
6. Discussion and conclusions
7. Acknowledgements
8. List of references
9. Tables
10. Figures and captions

The type must not be smaller than 12 pitch or 10 point. Each section must be typed in **double spacing** with margins of not less than 2.5 cm all round and each page should be numbered. **The original and one copy of the typescript should be supplied.**

Title page

The **title** should normally contain **no more than 150 characters** and should not consist of a sentence (statement or conclusion) or be interrogative. A **short running title** containing not more than 50 characters and spaces **is also required**. The title page should include the names of authors and their appropriate addresses. It should be made clear which address relates to which author. Authors' present addresses differing from those at which the work was carried out should be given as footnotes on the title page and references at the appropriate place in the author list by superscript numbers. A footnote may also be used to indicate the author to whom correspondence should be sent. The use of footnotes for any other reason is not allowed. If the address to which proofs should be sent is not that of the first mentioned author, clear instructions should be given in a covering note and not on the title page. The title page should be paginated as page 1 of the paper.

Summary

The summary will be printed at the beginning of the paper. It should not exceed 5% of the length of the paper and should contain a brief account of the problem, the methods, results and the conclusions. It should be arranged **in numbered and concise paragraphs**. Up to ten **keywords** or phrases of two to three words (including names and terms used in the title) should be displayed at the end of the summary. Keywords will be used to compile the annual index. The quality of the index will thus be determined by the appropriateness of the keywords. These may be selected by reference to the most recent Index of the Journal. Avoid unhelpful or unqualified terms such as 'rat', 'drug' etc.

Introduction

The introduction should give a short and clear account of the background of the problem and the rationale of the investigation. Only previous work that has a direct bearing on the present problem should be cited.

Methods

The methods must be described in sufficient detail to allow the experiment to be interpreted and repeated by the reader. However, detailed repetition of methods which have been adequately described previously should be avoided and references given, although a brief outline is often helpful.

Drugs should be listed in a separate paragraph. Their names should be 'approved names' as published previously in British Approved Names, 1990 (HMSO). If a drug has no 'approved name' its chemical name must be used and the rules set out in the current *Handbook for Chemical Society*

Authors (London, Chemical Society) observed, or its structural formula given. Cumbersome chemical names should be suitably abbreviated for later reference in the paper.

The doses of drugs should be given as unit weight per body weight, e.g. mmol kg^{-1} or mg kg^{-1}; concentrations should be given in terms of molarity, e.g. nM or μM.

Reference should be made to any statistical analyses that have been performed on the results in order, for example, to determine the significance of differences between results obtained under different conditions.

Results

The description of the experimental results should be succinct but, nevertheless, in sufficient detail to allow the experiments to be repeated by others. Typical single experiments may be presented with a clear statement that *n* number of similar experiments had similar results. Where appropriate, however, the mean results with confidence limits or with standard errors of the means and the number of observations should be given. Statistical tests of significance should be performed where appropriate. The results of such tests should be stated as the numerical value of the probability (*P*) that is calculated, with any necessary clarification (e.g. one-tail or two-tail test).

Every effort should be made to avoid unnecessary repetition of data in the text, tables and figures. Conclusions and theoretical considerations should not be elaborated in this section.

Discussion

The purpose of the discussion is to present a brief and pertinent interpretation of the results against the background of existing knowledge. Any assumptions on which conclusions are based must be stated clearly. A mere recapitulation of the results is not acceptable. A review-like treatment, which reduces the impact on the reader, should also be avoided. The main conclusion should be conveyed in a final paragraph.

Acknowledgements

Acknowledgements should be brief but should include reference to sources of support. Sources of drugs not widely available commercially should be acknowledged.

References

In the text, references to other work should take the form: (Bolton & Kitamura, 1983) or, 'Bolton & Kitamura (1983) showed that . . .'. If there are more than two authors, the first author's name should be given followed by *et al.* (Bülbring *et al.*, 1981).

References to 'unpublished observations' or 'personal communications' should be mentioned in the text only, and not included in the list of references. Papers which have been **submitted and accepted** for publication, should be included in the list of references with the names of the periodicals and 'in press'. **A photocopy should normally be submitted with the manuscript**. If this is not possible, authors should indicate whether the work cited is an abstract or a full paper. **Papers in preparation or which have been submitted but not yet finally accepted for publication must not be included in the list of references.**

The reference list at the end of the manuscript must be arranged alphabetically according to the surname of the first author. When the surnames of authors are identical, the alphabetical order of their initials takes precedence over the year of publication. The AUTHORS' names are followed by the year of publication in brackets. If more than one paper by the same authors in one year are cited, a, b, c, etc. are placed after the year of publication, both in the text and in the list of references. The title of the article is given in full, followed by the *abbreviated title of the periodical*, volume **number** and first and last page numbers. The abbreviations used for periodicals are those of the most recent edition of the International List of Periodical Title Word Abbreviations. References to articles in books should consist of names of authors, year of publication, title of article followed by the *title of the book*, the editors, volume numbers, if any, and page numbers, the place of publication and the names of the publishers. For example:

BOLTON, T.B. & KITAMURA, K. (1983). Evidence that ionic channels associated with the muscarinic receptor of smooth muscle may admit calcium. *Br. J. Pharmacol.*, **78**, 405–416.

BRADING, A.F. (1981). Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In *Smooth Muscle: An Assessment of Current Knowledge*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 65–92. London: Edward Arnold.

Tables

Each table should be given on a separate page, paginated as part of the paper. Tables should be numbered consecutively with arabic numerals and the number should be followed by a brief descriptive caption, occupying not more than two lines, at the head of the table. The proportions of the text area should be borne in mind when designing the layout of tables. For the sake of clarity, tables should not have more than 120 characters to a line, with spaces between columns counted as four characters. The absolute maximum is 180 characters to a line. Each column should have a heading and the units of measurement should be given in parentheses in the heading. Except in special circumstances, tables should be self-explanatory; the necessary descriptions should be at the bottom of the table.

Figures

To avoid unnecessary Figures, particularly those requiring half-tone reproduction, only critical points of the text should be illustrated. If coloured Figures are desired, the Authors should discuss their requirements with the Secretaries, preferably before submission.

Please note that unsatisfactory Figures will be returned to the Author for revision. The Journal reserves the right to reject a manuscript if the Figures are unacceptable.

Submission Requirements

(a) The Authors' names and the Figure number must be indicated lightly *in pencil* on the back of each Figure; if necessary, use an adhesive label to avoid damage to the Figure.

(b) *Each* copy of the manuscript must be accompanied by one set of labelled Figures (i.e. complete with lettering and numbering, arrows, etc.). An original set and one high quality photocopy will suffice.

(c) **Another original set of Figures identical in size but without any letters or numbers must also be supplied for the use of the Publisher.** Arrows and event marks on experimental records may be retained, provided they are larger than 3 mm wide. The Publisher will choose the correct style of typeface of an appropriate size to suit the final size of the Figure on the printed page.

(d) No submitted Figure should exceed 210 × 297 mm (A4).

(e) Each Figure must be accompanied by a legend; each legend should be typed on a *separate* sheet of paper and paginated as part of the manuscript. Legends should explain the Figures in sufficient detail that, whenever possible, they can be understood without reference to the text.

Line width (axes)	Line width (graphs)	Symbol size	Figure will reduce to this percentage of the original size
		△□○	100 (No reduction)
		△□○	80
		△□○	70
		△□○	60
		△□○	50
		△□○	40

Line Figures

It is best to submit an original drawing (black ink on heavy white paper or faint blue graph paper) which has been prepared to conform with the style and convention of the Journal, because redrawing is expensive. The original drawing should be lettered in pencil and should be larger (up to two times as large) than the intended size in the Journal.

It is important that the printed symbols and lines should retain their clarity. To achieve this the symbols and lines in original drawings should be sharply defined and of an even density and breadth. When graphs are generated by computer, lines must not show noticeable stepping. Heavier (broader) lines should be used for curves than for the axes of graphs. The table above illustrates line widths and symbol sizes to be used on a figure and the appropriate reductions in the final printed form.

Symbols should be chosen from the following set

○ ● □ ■ △ ▲ ▽ ▼ ◇ ◆ + ×

The preferred order to shading of histogram columns is: open (clear), closed (solid), cross-hatched, heavily stippled and other (if required).

The explanation of the symbols and column headings should be given in the Figure legend and not as a key in the Figure itself.

Line Figures should normally have only left and bottom axes; box-style Figures and those using 3-dimensions are not acceptable.

Where the Figure is a composite of more than one graph, experimental record, etc., particular care is needed to minimise the spaces between each part, without overcrowding the entire Figure.

Figure 1 illustrates a simple properly-drawn graph in its original form (a) and in its reduced form (b) as it would appear in the Journal.

Photographs and photomicrographs

These should be submitted, twice as large as their intended published size, as good quality prints of high contrast especially where traces and records are illustrated. The originals must not contain arrows, lettering or numbering; these must be accurately located on a duplicate print (or photocopy). When submitting half-tone illustrations for publication authors should remember that it is not possible to reproduce Figures to a finer quality than the original photographs/photomicrographs provided. Critical areas should be marked on a second copy or on an overlay, so that the Printer can choose the correct exposure. Maximum trim areas should be marked on a second copy of the photograph/photomicrograph or on a tracing overlay, i.e. authors should show any parts of the photographs that could be excluded from the finished half-tone illustration. **A calibration bar must be provided on the photomicrograph** to ensure that, if the Printer reduces the plate, the scale is reduced in the correct proportion.

Proofs

Two sets of page proofs will be supplied, one of which should be retained by the authors. The other should be corrected immediately and returned to the Publisher. Corrections should be kept to a mimimum.

SPECIAL REPORTS

The purpose of *Special Reports*, which have superseded 'Short Communications', is to provide rapid publication for new and important results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Publisher within 48 hours of receipt**. Authors should ensure that their submitted material conforms exactly to the following requirements.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page, a Summary consisting of a single short paragraph, followed by keywords (maximum of 10), Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers.

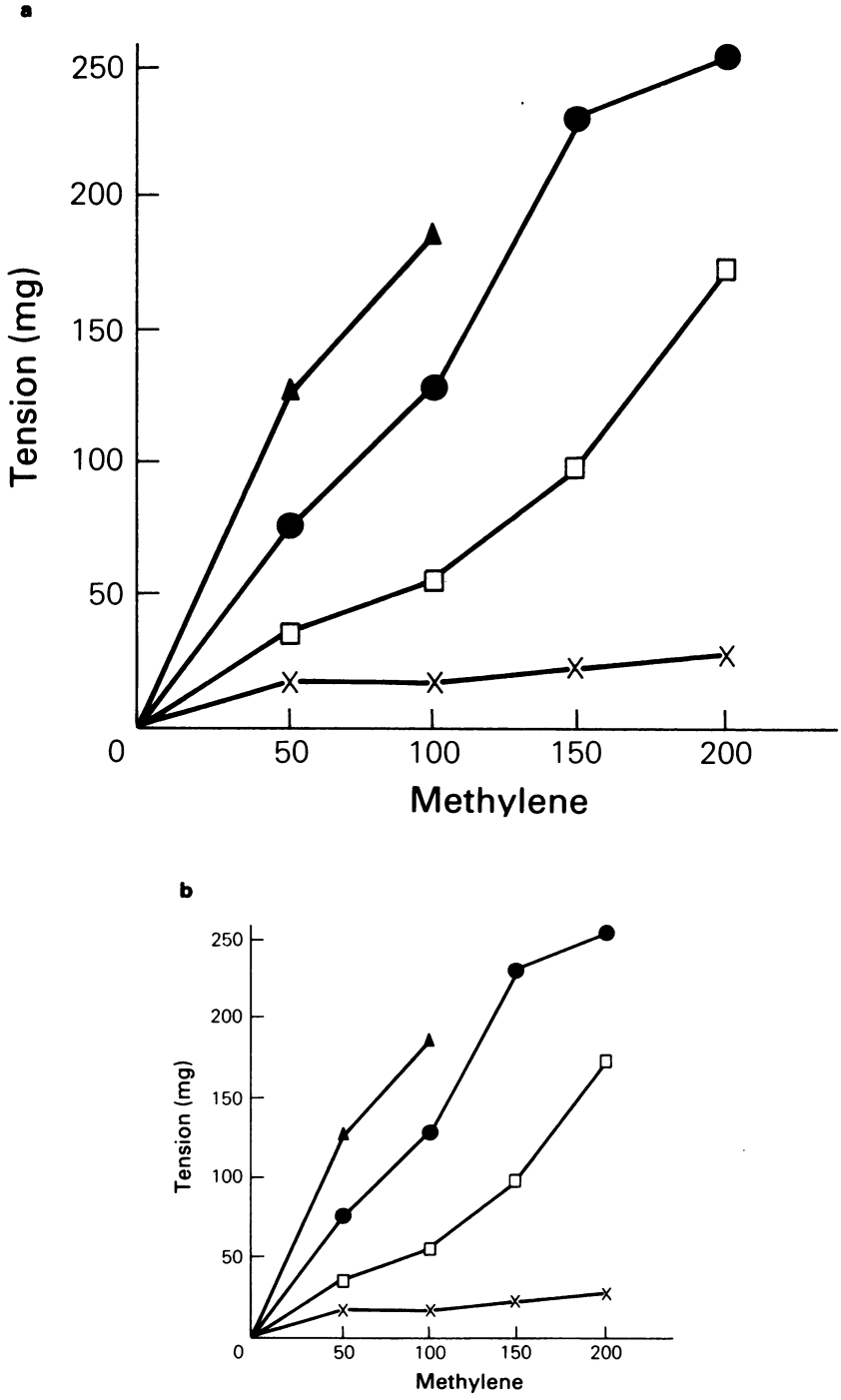

Figure 1 (a) Artwork as drawn. (b) Artwork reduced to 60 per cent of its original size for publication in the Journal.

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1. Submission of a manuscript will be taken to imply that the Authors have obtained permission to publish from (a) their employers or institution, if they have a contractual or moral obligation to do so, and (b) those whose unpublished work, including papers accepted for publication (i.e. in press), has been cited or those whom the Authors wish to acknowledge as having improved the content or presentation of the manuscript.
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When submitting a manuscript for editorial consideration, Authors should confirm their acceptance of these terms by signing a Declaration to that effect. The recommended wording is given in the example. No paper will be accepted for publication without such a Declaration being signed by each Author (see paragraph 6 above). If the manuscript is not accepted for publication, the assignment will be null and void.

ABBREVIATIONS AND SYMBOLS

Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixed for multiples of units should be used:

Multiplier	*Prefix*	*Symbol*
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Multiplier	*Prefix*	*Symbol*
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G
10^{12}	tera	T

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus m μg should be ng. The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g. mg kg^{-1} rather than mg/kg; pmol mm^{-2} min^{-1} rather than pmol/mm^2/min.

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. $P_{A,\,CO_2}$ denotes partial pressure of CO_2 alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

Authors should also consult *Nomenclature Guidelines for Authors* contained in this issue of the Journal. The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

Use abbreviations sparingly as extensive use can make the text hard to follow.

Physico-chemical quantities

Quantity	*Preferred unit*	*Symbol*
Amount (of substance)	mole	mol
Capacitance	farad	F
Concentration	moles per litre	M or mol l^{-1}
Current	ampere	A
Electrical conductance	siemens	S
Electromotive force	volt	V
Flow (blood or other liquid)	litres per second (or min)	1 s^{-1} or 1 min^{-1}
Flow (air or other gas)	litres per second (or min)	1 s^{-1} or 1 min^{-1}
Force	newton	N
Frequency of regular event	hertz	Hz
Length	metre	m
Mass	gram	g
Power	watt	W
Pressure (or partial pressure)	pascal*	Pa
Radioactivity	becquerel or curie	Bq (60 d.p.m.) or Ci (3.7×10^{10} Bq)
Resistance (electrical)	ohm	Ω
Temperature	degree celsius	°C
Time	second (preferred)	s
	minute	min
	hour	h
Volume (blood or other liquid)	litre	l
Volume (air or other gas)	litre	l
Work	joule	J

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

Chemical and biological abbreviations

acetylcholine	ACh
acetylcholinesterase	AChE
adenosine 3′:5′-cyclic monophosphate	cyclic AMP
adenosine 5′-phosphate	AMP
adenosine triphosphatase	ATPase
γ-aminobutyric acid	GABA
analysis of variants	F
adrenaline	Ad
analytical standard of reagent purity	A.R.
anhydrous	anhyd.
approximate(ly)	approx.
approximately equals	≈
aqueous	aq.
arg-vasopressin	AVP
boiling point	b.p.
bovine serum albumin	BSA
cardiovascular system	CVS
catechol-*O*-methyl transferase	COMT
central nervous system	CNS
cerebrospinal fluid	CSF
chi-squared (statistics)	χ^2
clearance	*c*
coenzyme A	CoA
concentrated	conc.
correlation coefficient	*r*
cubic	cu.
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D-
dextro-(optical rotation)	(+)-
diameter	diam.
diameter, inside	i.d.
diameter, outside	o.d.
diffusion coefficient	*D*
3,4-dihydroxyphenylalanine	DOPA
3,4-dihydroxyphenylethylamine	dopamine
direct current	d.c.
disintegration per minute	d.p.m.
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
distilled	dist.
dry ice	solid CO_2
edition	edn
editor(s)	ed.
effective concentration	EC_{50}
effective dose, median	ED_{50}
electrocardiogram	ECG
electrocorticogram	ECoG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	e.s.r.
endothelial-derived relaxing factor	EDRF
epithelial-derived relaxing factor	EpDRF
equilibrium constant	*K*
equivalent (general use)	equiv.
erythrocyte	r.b.c.
erythrocyte sedimentation rate	ESR
ethylenediaminetetracetic acid	EDTA
excitatory postsynaptic potential	e.p.s.p.
experiment	expt
experimental	exptl

fatty acids, nonesterified	NEFA
figure(s) (with reference number)	Figure(s)
figure (diagram)	figure
gas-liquid chromatography	g.l.c.
glomerular filtration rate	GFR
haemoglobin	Hb
half-life	$t_{\frac{1}{2}}$
high-frequency	h.f.
high performance liquid chromatography	h.p.l.c.
human serum albumin	HSA
hydrogen-ion concentration	$[H^+]$
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH
6-hydroxydopamine	6-OHDA
N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]	HEPES
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptamine	5-HT
immunoglobulins	IgA, IgD, IgE, IgG, IgM
inhibitor constant	K_i
inhibitory concentration	IC_{50}
inhibitory postsynaptic potential	i.p.s.p.
insoluble	insol.
international unit	iu
intra-arterial	i.a.
intracellular fluid	ICF
intradermal	i.d.
intramuscular	i.m.
intraperitoneal	i.p.
intracerebroventricular	i.c.v.
intravenous	i.v.
isotope (atomic mass) e.g. iodine-131	^{131}I
isotopically substituted compounds e.g.	[^{14}C]-ethanol
laevo-(absolute configuration)	L-
laevo-(optical rotation)	(−)-
lethal dose, median	LD_{50}
leukotriene	LT
logarithm to base e	$\log_e$ or ln
logarithm to base 10	$\log_{10}$
maximum	max.
mean arterial pressure	MAP
mean value of (statistics)	$\bar{x}$
melting point	m.p.
meta	*m*-
Michaelis constant	K_M
minimum	min.
mobility (electrophoresis)	*m*
monoamine oxidase	MAO
noradrenaline	NA
nuclear magnetic resonance	n.m.r.
number	no. or No.
number of observations (statistics)	*n*
ortho	*o*-
packed cell volume	PCV
page/pages	p./pp.
para-	*p*-
paragraph	para. or ¶
parts per millon	p.p.m.
per cent	%
platelet activating factor	PAF
posterior	post.
probability (significance level in a statistical test)	*P*
radioimmunoassay	RIA
rectus (configuration by the sequence rule)	**R**
red blood corpuscle	RBC
relative band speed to front (chromatography)	R_F
relative molecular mass	M_r
relative retention time (gas chromatography)	t_r
renal plasma flow	RPF
resistance (respiratory)	*R*
respiratory conductance	Sgaw
revolutions per minute	r.p.m.
ribonucleic acid	RNA
section	§
sedimentation coefficient (ultracentrifugation)	*s*
sinister (configuration by the sequence rule)	**S**
soluble	sol.
solution	soln.
Spearman rank coefficient	r_s
standard deviation (of observed sample)	s.d.
standard error (of estimate mean value)	s.e.mean
standard error (of sampling)	s.e.
standard temperature and pressure	STP
subcutaneous	s.c.
sum (statistical): of hypothetical population	**Σ**
of observed sample	S or **Σ**
temperature	temp.
thin layer chromatography	t.l.c.
time, clock – 24 h clock used e.g. 18 h 30 min	*t*
time constant	τ
2-amino-2-hydroxymethyl-propan-1,3-diol	Tris
ultraviolet	u.v.
unit	u
vacuum	vac.
valency	e.g. Fe^{2+}; Fe(II) protoporphyrin
volume by volume	v/v
wavelength	λ
weight	wt.
weight by volume	w/v

Br. J. Pharmacol. (1994), **111**, 385–387

NOMENCLATURE GUIDELINES FOR AUTHORS

With effect from 1 January 1994

The Nomenclature Working Party (NWP) of the Editorial Board of the *British Journal of Pharmacology* has consulted many acknowledged experts in an effort to clarify and standardize receptor and other nomenclature systems for use by Editors until the recommendations of the IUPHAR Commission on Receptor Nomenclature and Classification are made known.

NWP is unanimous in its view that, with rare exceptions, the Journal should use spellings, names and abbreviations that have been chosen by international bodies or specialist groups specially convened for the purpose.

For receptor nomenclature, with few exceptions, the Journal generally follows the guidelines laid down in the current Trends in Pharmacological Sciences (TiPS) Receptor Nomenclature Supplement.

1 *Definition of receptors and subtypes*

Receptors and their subtypes are defined in relation to structural information where this is available and on the basis of functional studies. With the latter, they are defined in terms of the relative potencies of agonists and selectivities of antagonists and by the binding of such ligands, without reference to second (or other) messenger systems.

2 *Format of receptor names*

It was agreed that, until the IUPHAR Commission on Receptor Nomenclature and Classification make their recommendations:

(a) Editors will permit with reluctance new nomenclature systems in papers accepted for publication if and only if there are compelling reasons to introduce a new terminology (or modify an accepted one). The criteria upon which the new receptor type or subtype are defined must be given, together with adequate explanations of the relationship between the previous nomenclature (fully referenced) and the proposed one.

N.B. The new nomenclature should not appear in the Title, Short Title or Keywords, unless qualified by the adjective putative (e.g. . . . mediated by the putative α_{2A}-adrenoceptor).

(b) Only well-established and universally accepted subtype names (e.g. muscarinic and nicotinic acetylcholine receptors; α-and β-adrenoceptors) will be acceptable without any reference to the originator of these terms. In cases of controversy concerning further subdivision of the subtype, full referencing must be given.

(c) Receptor subtypes should be designated by means of a subscript numeral or capital letter. Some double subscripts (i.e. numerical plus letter) are acceptable.

3 *Types of receptor*

The NWP accepts that there are additional receptors to those described below which can be considered as well established. In many cases, however, their existence has been confirmed only in cloning studies and it is as yet unclear how they relate to similar subdivisions proposed on the grounds of differences in agonist and antagonist potencies in various tissues.

(a) *Acetylcholine receptors* The two principal subfamilies are muscarinic and nicotinic acetylcholine receptors (the abbreviations muscarinic AChRs and nicotinic AChRs are acceptable, but not mAChR or nAChR, which may be confused with muscle and neuronal subtypes).

Muscarinic acetylcholine receptors The principle subtypes are M_1, M_2, M_3 and M_4.

Nicotinic acetylcholine receptors The principal subgroups are muscle and neuronal nicotinic acetylcholine receptors.

(b) *Adenosine receptors* Known also as P_1 purinoceptors (see purinoceptors, 3t).

(c) *Adrenoceptors* The principal subtypes are α_1-, α_2-, β_1-, β_2- and β_3-adrenoceptors. Additional subtypes must be fully referenced.

(d) *Angiotensin receptors* At present only the AT_1 receptor is recognised. The AT_2 binding site should be fully referenced.

(e) *Bombesin receptors* Proposed subtypes such as BB_1, BB_2 may be used but must be fully referenced.

(f) *Bradykinin receptors* The principle subtypes are B_1 and B_2 receptors. Additional subtypes must be fully referenced.

(g) *Calcitonin gene-related peptide (CGRP) receptors* Proposed CGRP receptor subtypes must be fully referenced.

(h) *Cholecystokinin (CCK) receptors* The principal subtypes are CCK_A and CCK_B receptors.

(i) *Dopamine receptors* D_1 and D_2 dopamine receptors are recognised. Other subtypes must be fully referenced.

(j) *Endothelin receptors* The principle subtypes are ET_A and ET_B receptors.

(k) *Excitatory amino acid receptors* Three ionotropic subtypes are recognised and named: (1) NMDA (N-methyl-D-aspartate) receptors; (2) AMPA receptors, and (3) kainate receptors. Subtypes of metabotropic receptors must be fully referenced.

(l) *γ-Aminobutyric acid (GABA) receptors* The principal subtypes are $GABA_A$ and $GABA_B$ receptors. Regulatory sites on the $GABA_A$ receptor should be referenced.

(m) *Histamine receptors* The principle subtypes are H_1, H_2 and H_3 receptors.

(n) *5-Hydroxytryptamine (5-HT) receptors* The principle subtypes are 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4. Further subdivision, e.g. 5-$HT_{1D\alpha}$, 5-HT_{2C}, require full referencing.

(o) *Leukotriene receptors* When first mentioned, the style leukotriene (LT) receptor should be used, thereafter LT receptor. Receptors should be designated according to the leukotriene that selectively or preferentially binds to them. All leukotriene receptor subtypes should be fully referenced.

(p) *Neuropeptide Y (NPY) receptors* Proposed subtypes should be fully referenced.

(q) *Opioid receptors* The principal subtypes are μ-, δ- and κ-opioid receptors. Other proposed subtypes should be fully referenced.

(r) *Oxytocin receptors* (see Vasopressin and oxytocin receptors).

(s) *Prostanoid receptors* The principal types are DP, EP, FP, IP and TP receptors. When first mentioned, the style prostanoid (XP) receptor should be used, thereafter XP receptor (where X denotes the type). Proposed subtypes should be referred to as XP_n, (e.g. EP_1, EP_2) and referenced.

(t) *Purinoceptors* The principal subtypes are P_1 and P_2 receptors. Subdivision of P_1 into A_1 and A_2 types and of P_2 into P_{2X} and P_{2Y} are permitted. Other subtypes e.g. P_{2T}, P_{2Z} should be fully referenced.

(u) *Somatostatin (SS) receptors* Proposed subtypes should be fully referenced.

(v) *Tachykinin receptors* The term tachykinin is preferred to neurokinin. The principle subtypes are NK_1, NK_2 and NK_3 receptors.

(w) *Vasoactive intestinal peptide (VIP) receptors* Proposed subtypes should be fully referenced.

(x) *Vasopressin and oxytocin receptors* The principle subtypes are V_{1A}, V_{1B}, V_2 and OT receptors; V_{1A} was formerly V_1, and V_{1B} formerly V_3.

4 *Naming of nerve fibres*

Many nerve fibres are now known to release more than one transmitter, and future work may show that this is in fact the general rule. In that case, the concept of the same transmitter being released either at different developmental stages or under various experimental conditions would no longer hold, and single adjectives that imply this (e.g. cholinergic, noradrenergic) would become inappropriate when applied to nerve fibres, as distinct from transmitter functions. For the present, those nerve fibres that are known to function by releasing more than one identified transmitter may be described accordingly; for example, noradrenergic-purinergic, cholinergic-peptidergic (in alphabetical order, the order implying no priority of function). N.B. The suffix 'ergic' should continue to be applied only to nerve fibres and to the transmission event, in accordance with Dale's intentions. For example, 'cholinergic' indicates that the nerve fibre, or the transmission, functions under particular conditions through the release of a choline-like substance. The suffix should not be used loosely to mean 'pertaining to'. Hence, for example, the expression 'cholinergic receptor' (rather than acetylcholine receptor) is an inappropriate use of the term. Transmission events involving nitric oxide may be referred to as *nitrergic*. However, *nitrergic* may be used to describe axons only when there is sufficient evidence that nitric oxide is released from them as a neurotransmitter.

(a) *Catecholamine releasing nerve fibres* The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic).

Nerve fibres that are known to function by releasing noradrenaline are to be described as noradrenergic. The term adrenergic should be reserved for either a nerve fibre that functions by releasing a catecholamine, the identity of which is unknown, or one known to release adrenaline.

(b) *Some other adjectives describing nerve fibre function* NANC is an acceptable abbreviation of non-adrenergic, non-cholinergic for peripheral efferent nerve fibres when the identity of the transmitter(s) is unknown other than the fact that neither (nor)-adrenaline nor acetylcholine is involved. It should be defined when introduced. NANCergic, e-NANC (or NANC-e) and i-NANC (or NANC-i) are not acceptable terms.

Glutamatergic, not glutaminergic, should be used to describe nerve fibres releasing glutamate. In referring to peptide-releasing nerve fibres (e.g. those that may release substance P or vasoactive intestinal peptide) the nomenclature to be used is peptidergic (X), e.g. peptidergic (SP), peptidergic (VIP), not SPergic, VIPergic.

The terms 5-hydroxytryptamine (5-HT) and 5-hydroxytryptaminergic (i.e. nerves releasing 5-hydroxytryptamine) are preferred to those of serotonin and serotoninergic. The term 5-HTergic is not acceptable, except to avoid frequent repetition of 5-hydroxytryptaminergic.

Likewise, the terms purinergic (ATP) and purinergic (adenosine) are preferred.

5 *Terms used to describe agonist and antagonist action*

The following terms can be used without full definition. Where appropriate, other terms may be used but must be accompanied by a full definition.

Terms used to describe affinity and potency

(a) EC_{50} The concentration of an agonist that produces 50% of the maximal response for that agonist *in vitro*. The agonist may be stimulatory or inhibitory. When EC_{50} values are determined in the presence of other agonists or antagonists the concentration of the latter should be stated. Related terms, e.g. EC_{25}, are acceptable if accompanied by a full definition.

(b) IC_{50} This term may be used in the following ways: (i) The concentration of antagonist that reduces the response to a sub-maximal concentration of agonist by 50%; the concentration of agonist should be stated. (ii) The concentration of competing agonist or antagonist that inhibits the binding of a radioligand by 50%; the concentration of radioligand should be stated.

(c) ED_{50} This term may be used in the following ways: (i) The dose of an agonist or antagonist that produces 50% of the maximal possible effect of that agonist or antagonist *in vitro*. (ii) The dose of drug that produces the effect under investigation in 50% of the population.

(d) K_A The equilibrium dissociation constant ($mol\,l^{-1}$) for an agonist determined in a functional study, e.g. by Furchgott analysis. The reciprocal is called the affinity constant or association constant.

(e) K_B The equilibrium dissociation constant ($mol\,l^{-1}$) for an antagonist determined in a functional study using the Gaddum equation or a Schild plot in which the slope has been constrained to unity when not significantly different from this value. The reciprocal is called the affinity constant or association constant.

(f) K_D The equilibrium dissociation constant ($mol\,l^{-1}$) for a radiolabelled agonist or antagonist determined in a radioligand binding study by saturation analysis. The reciprocal is called the affinity constant or association constant.

(g) K_I The equilibrium dissociation constant ($mol\,l^{-1}$) for a competing agonist or antagonist determined in a radioligand binding assay. It can be calculated from the IC_{50} value using the Cheng-Prusoff equation when the Hill coefficients of the radioligand and competing ligand are not significantly different from unity. If the

Hill coefficient of the radioligand or competing ligand is significantly different from unity, IC_{50} values and the concentration of radioligand should be given.

(h) n_H The Hill coefficient.

(i) pA_2 The negative logarithm to base 10 of the concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit a given submaximal response. Note that the definition is empirical and does not pre-suppose the mechanism of antagonism. The pA_2 value can be determined from a Schild plot with unconstrained slopes, but only provides an estimate of the pK_B if the antagonism has been shown to meet all of the criteria of competition.

(j) pD_2 The negative logarithm to base 10 of the EC_{50}.

(k) pIC_{50} The negative logarithm to base 10 of the IC_{50}.

(l) pK_A The negative logarithm to base 10 of K_A.

(m) pK_B The negative logarithm to base 10 of K_B.

(n) pK_D The negative logarithm to base 10 of K_D.

(o) pK_I The negative logarithm to base 10 of K_I.

Terms used to describe the mode of antagonism

(a) *Competitive antagonism* Used to describe antagonists that bind reversibly to the agonist binding site.

(b) *Competitive irreversible antagonism* Used to describe antagonists that bind irreversibly to the agonist binding site.

(c) *Non-competitive* Used to describe antagonists that bind reversibly to a distinct (allotopic) site.

(d) *Non-competitive irreversible antagonism* Used to describe antagonists that bind irreversibly to a distinct (allotopic) site.

6 *Enzymes*

The IUB Enzyme Commission (EC) number and full name (Enzyme Nomenclature 1984, Academic Press, New York and London) must be quoted when first mentioned in text. Subsequently the accepted trivial name is used. Trivial names may be used in the title.

7 *Other nomenclature requirements*

(a) *Racemates* Authors must state unambiguously in the Methods section of papers which isomers were used, e.g. (+)- or (−)-propranolol, and must bring to the attention of the reader the composite character of drugs that are mixtures of stereoisomers. Furthermore, the implications of the composite nature of such drugs studied for the interpretation of the data measured and the conclusions drawn must be made explicit. Note that the terms d- or l- for dextro- and laevo-rotatory are now obsolete, and the prefixes (+)- or (−)- respectively should be used. Capital D and L refer to the absolute configurations and of course remain acceptable when appropriate.

(b) *Platelet activating factor (acetyl-glyceryl-ether-phosphorylcholine)* The acronym to be used is PAF (not AGEPC, Paf, Paf-acether or other variant). The alkyl chain should be specified for synthetic PAF e.g. C_{16}-PAF.

(c) *Ligands for NMDA receptors* N-methyl-D-aspartate (NMDA) and N-methyl-DL-aspartate (NMDLA) are to be given in full when introduced in the text.

(d) *Purines* This term should not be used as a synonym for purine nucleotides or nucleosides.

(e) *Eicosanoids* The system of nomenclature to be used for eicosanoids is that published in *Methods in Enzymology*, (1990), **187**, 1–9. This scheme incorporates recent changes in the style of abbreviation of hydroperoxy-, epoxy- and oxo-unsaturated fatty acids e.g. 12(S)-hydroperoxyeicosatetraenoic acid which was formerly abbreviated as 12(S)-HPETE now becomes 12(S)-HpETE. In manuscripts, the first use of the full chemical name of any eicosanoid should indicate double bond geometry when this is known.

(f) *Peptide nomenclature* The preferred style is capital letters to designate the first letter of the word. Otherwise, upper and lower case letters should be used (e.g. Enk-IR, enkephalin-like immunoreactivity). When numbers are used these should be placed after a hyphen on the same line as the abbreviation, e.g. ET-1.

(g) *Cell lines* Cell type, sources and originating species need to be defined.

(h) *Molecular biology* Abbreviations pertaining to molecular biological techniques need to be defined or presented in such a way that they can be recognised by the non-specialist e.g. the oligonucleotide sequence, TAGC.

(i) *Tension* Tension is force and should be calibrated in newtons (1 newton = 1kg ms^{-1}) or in kg weight, g weight, or mg weight etc. It should not be calibrated in units of mass (e.g. kg). (See Miller D.J. in *Trends Pharmacol. Sci.*, 1988, **9**, 124–5).

(j) *Ions* When referring to ions, the charge should be indicated. e.g. Na^+, Ca^{2+}, $2Na^+/Ca^{2+}$ exchange, etc.

(k) *Inhibitors of nitric oxide synthase* The most commonly used and currently accepted abbreviation for N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester are L-NOARG and L-NAME. Unless alternative international agreement is reached, these will be the abbreviations accepted by the journal.

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1993, **108**, 275–284) before submitting a manuscript for publication in the *British Journal of Pharmacology*. The manuscript and cover letter should be checked against the following list before mailing.

The original and one copy of the manuscript must be supplied. Manuscripts must be typed in double-line spacing on one side of A4 paper, in type not smaller than 12 characters per inch or 10 point. Both copies to include Tables and a set of labelled Figures. One set of Figures without numbers or letters is also to be included. The text to be arranged in the following subsections:

1. **Title**—To have no more than 150 characters on a separate page, which should also include a Short Title (50 characters maximum) and the name and address of the author for correspondence.
2. **Summary**—To be arranged in numbered paragraphs (Full Papers) or a single paragraph (Special Reports).
   —to include aims, principal results and conclusions.
   —to include Key words (10 maximum) at end of summary.
3. **Introduction**—To contain concise statements of the problem and the aims of the investigation.
4. **Methods**—To have brief but adequate account of the procedures; *full names of drugs* (*including those referred to by manufacturer's code*), sources of drugs and statistical tests to be stated.
5. **Results**—To have no repetition of data in Figures, Tables and text.
6. **Discussion**—Findings and conclusions to be placed in context of other relevant work.
   NB Simple repetition of results and unwarranted speculation are not acceptable.
7. **Acknowledgments**—Sources of support. Sources of drugs not widely available commercially.
8. **References**—All references in the text to be included in the Reference List and *vice versa*. References in alphabetical order with complete citations; Journals publishing 'in press' papers identified.
   References to manuscripts submitted to other journals but not yet accepted are not allowed.
9. **Tables**—Each on a separate page and prepared in accordance with current requirements of the Journal.
10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors*, 1993, **108**, 275–281) and provided with Figure Number and Authors' names on back (*in pencil*).
    —each legend to be typed on a separate page and carrying keys to symbols.
    —keys to symbols and histograms must not appear on the figures themselves, but in the respective legends.
    —'box style' figures are not in keeping with the Journal style; line drawings etc must have only left-hand and bottom axes.
11. **Manuscripts**—To be accompanied by a declaration signed by each author that
    (a) results are original
    (b) approval of all persons concerned has been given to submit manuscripts for consideration (see also 12b)
    (c) the same material is neither 'in press' (i.e. is in proof or has definitely been accepted for publication) nor under consideration elsewhere. Furthermore it will not be submitted or published elsewhere before a decision has been reached by the Editorial Board of the *British Journal of Pharmacology* and will not be submitted elsewhere if accepted by the *British Journal of Pharmacology*.
    (d) Copyright assignment is included.
12. **Cover letter**—To state clearly
    (a) Corresponding author's full postal address, telephone, telex or Fax number
    (b) where appropriate, that *either* ethical approval has been given for investigation *or* Company or Institutional permission to publish work has been received.
13. **Reminder**—Packaging to be sufficiently robust to protect Figures and to withstand mailing.

Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors*, as published in the *British Journal of Pharmacology*, *Br. J. Pharmacol.*, 1994, **111**, 385–387.

SPECIAL REPORTS

The purpose of *Special Reports* is to provide rapid publication for **new** and **important** results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Production Office within 48 hours of receipt.** Authors should ensure that their submitted material conforms exactly to the following requirements.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page with key words (maximum of 10), a Summary consisting of a single short paragraph, followed by Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers (see current 'Instructions to Authors').

BRITISH JOURNAL OF PHARMACOLOGY VOLUME 111 (1) 1994

SPECIAL REPORTS

PAPERS

Contents continue inside back cover

Typeset by Elite Typesetting Techniques, Southampton, Hampshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey