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SPECIAL REPORT

Evidence for the involvement of a 5-HT_4 receptor in the secretory response of human small intestine to 5-HT

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5-Hydroxytryptamine increases transmucosal short-circuit current across human isolated small intestinal mucosa. The competitive 5-HT_4 antagonist, DAU 6285 evoked a concentration-dependent, dextral and parallel shift of the concentration-response curve to 5-HT, with no alteration of the maximum response. Schild analysis of this antagonism produced a Schild regression with a slope of 1.00 and an apparent pA_2 estimate of 6.17. It appears that a 5-HT_4 receptor may mediate the short-circuit current response of human small intestinal mucosa to 5-HT.

Keywords: Human ileum; intestinal mucosa; 5-HT_4 receptor; short-circuit current

Introduction *In vitro*, 5-hydroxytryptamine (5-HT) has been shown to increase transmucosal short-circuit current (SCC) across isolated sheets of intestinal mucosa in a number of species (Brown & Miller, 1991). In the rat colon and guinea-pig ileum, 5-HT elicits a SCC response which is partly due to stimulation of a 5-HT_4 receptor (Bunce *et al.*, 1991; Scott *et al.*, 1992).

The present investigation seeks to determine whether such receptors are involved in mediating the SCC response of human terminal ileum to 5-HT. The compound DAU 6285 has been used as a competitive antagonist at 5-HT_4 receptors (Schiavone *et al.*, 1992).

Methods Sheets of mucosa complete with submucosa were prepared by sharp dissection from specimens of human terminal ileum (within 20 cm of ileocaecal junction) resected at right-hemicolectomy operations for carcinoma of caecum or ascending colon ($n = 5$) or for Crohn's disease ($n = 2$). Tissue removed from specimens with Crohn's disease was judged to be macroscopically normal by a Consultant histopathologist. Tissue was transported in Dulbecco's Modified Eagle's Medium plus Ham's F12 Medium (1:1) with 10% foetal bovine serum added and placed in gassed Krebs solution within 60 min of removal from the patient.

Mucosal sheets were mounted in Ussing chambers in which mannitol (11.5 mM) replaced glucose in Krebs fluid bathing the mucosal side of the tissue. Electrical stimulation of mucosal nerves and measurement of SCC were performed as described previously (Burleigh, 1991), except that agar bridges were made up in modified Krebs fluid (minus glucose and calcium). Following electrical stimulation, two cumulative concentration-response curves to 5-HT were obtained, with a 3 min contact time for each concentration and at least 60 min between the construction of the two consecutive curves. During this time SCC returned to a stable level and the tissue was exposed to either DAU 6285 (0.3 to 10 μM) or control vehicle, for a contact period of 30 min. Control experiments were performed in order to allow for measurement of changes in preparation sensitivity. Maximum responses to forskolin (49 μM) were obtained at the end of each experiment in all preparations. 5-HT was added to the serosal side of the tissue, forskolin and DAU 6285 were applied to both sides.

Responses to 5-HT were converted to a % of the maximum response to forskolin and EC_{50} values were determined graphically from individual concentration-response curves. Concentration-ratios were calculated from these values and were expressed as geometric mean with 95% confidence limits; all other data are given as arithmetic mean ± s.e.mean. Statistical comparisons used the Mann Whitney U-test, with $P < 0.05$ being taken to represent a significant difference. 5-Hydroxytryptamine creatinine sulphate and forskolin were obtained from Sigma, DAU 6285 (1H-benzimidazole-1-carboxylic acid,2,3-dihydro-6-methoxy-2-oxo-8-methyl-8-azabicyclo(3,2,1)oct-3-yl ester chloridate) was kindly donated by Boehringer Ingelheim Italia.

Results After 60 min equilibration, basal SCC was 81.9 ± 7.3 μA cm^{-2} and tissue conductance was 16.9 ± 1.1 millisiemens cm^{-2}. Conductance did not alter significantly throughout the experiment. Electrical field stimulation of mucosal nerves produced an increase in basal SCC of 61.8 ± 5.8 μA cm^{-2} ($n = 36$).

Cumulative serosal application of 5-HT (0.3 to 300 μM) gave monophasic, concentration-dependent increases in SCC. In control experiments the first concentration-response curve to 5-HT gave a maximal increase in SCC of 66.3 ± 4.3 μA cm^{-2}. This value did not alter significantly for a second control response curve (56.7 ± 4.2 μA cm^{-2}, $P > 0.05$), however a decrease in preparation sensitivity caused a dextral shift of the concentration-response curve with a concentration-ratio between the two curves of 3.35 (Figure 1). This was taken into consideration when assessing antagonist effects. Addition of forskolin (49 μM) to the preparation gave a mean increase in SCC of 105.0 ± 8.0 μA cm^{-2}.

Application of DAU 6285 (1 to 10 μM) to the tissue after the first 5-HT response curve evoked concentration-dependent dextral shifts of the second response curve, with no depression of the maximum response (Figure 1). DAU 6285 at a concentration of 0.3 μM had no significant effect on the second response to 5-HT. DAU 6285 was also shown to cause a brief but significant fall in basal SCC, which was found to be concentration-dependent and was maximal 15 min after addition of antagonist (Table 1).

Schild analysis of the antagonism produced by DAU 6285 yielded a Schild plot with a slope of 1.00 (95% c.l. 0.67–1.33) and an apparent pA_2 estimate of 6.17 ± 0.06 (Figure 1).

Discussion In human terminal ileum, the 5-HT_4 receptor antagonist, DAU 6285, evoked parallel, dextral shifts of the concentration-response curve to 5-HT. There was no alteration of the maximum response to 5-HT, and Schild analysis of the antagonism produced by DAU 6285 yielded a Schild regression with unit slope. According to Arunlakshana & Schild (1959), this is in accordance with competitive anta-

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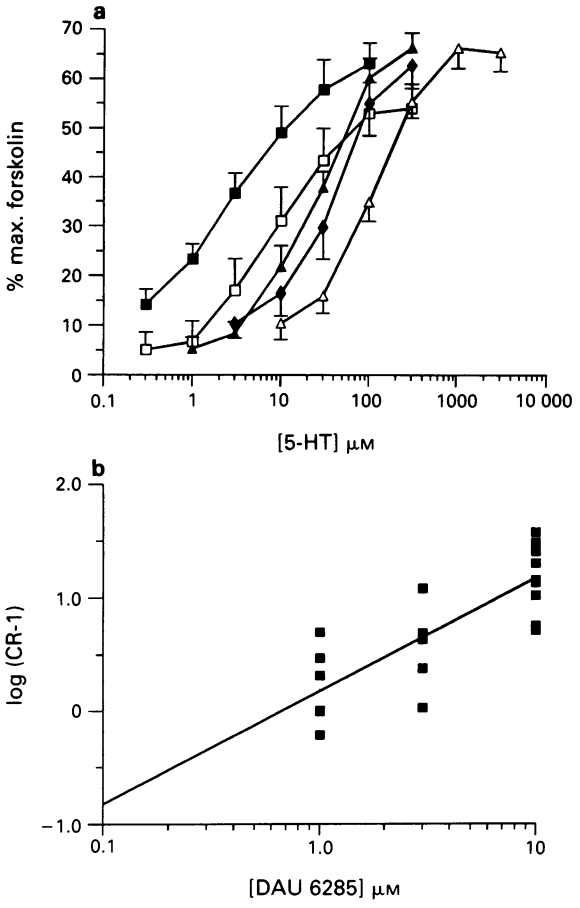

Figure 1 Effect of DAU 6285 on the SCC response of human small intestinal mucosa to cumulative concentrations of 5-HT. (a) First control response curve (■), and second curve in absence (□) and presence of DAU 6285 at 1 μM (▲), 3 μM (◆) and 10 μM (△). (b) Schild plot for DAU 6285 against SCC responses of human small intestinal mucosa to 5-HT.

gonism and this allowed a pA_2 value of 6.17 to be estimated. Although this value is somewhat lower than has been reported at the 5-HT_4 receptor in some tissues, for example 6.8 in mouse collicular neurones (Bockaert *et al.*, 1992), it is approaching the pA_2 value achieved at the 5-HT_4 receptor in guinea-pig ileum and human atrium (6.5–7.1) and is significantly higher than has been reported for interaction at the 5-HT_3 receptor (pA_2 for DAU 6285 of 5.0; Schiavone *et al.*, 1992). It can thus be stated that the pA_2 value for DAU 6285 most closely resembles competitive antagonism at the 5-HT_4 receptor.

Table 1 Effect of DAU 6285 on basal SCC and concentration-ratio between two consecutive response curves to 5-HT

[DAU 6285] (μM)	*Basal SCC* (μA cm^{-2}) *15 min after addition*[a]	*5-HT concentration ratio*[b]	n
Control	−3.3 ± 1.6	3.35 (1.44–7.83)	7
0.3	−9.5 ± 1.8*	3.91 (1.77–8.64)	6
1	−16.1 ± 1.9**	9.31* (5.53–15.68)	6
3	−22.5 ± 3.0***	17.20** (10.19–29.03)	7
10	−26.4 ± 2.2***	56.38*** (36.94–88.43)	10

[a]Change in basal SCC 15 min after addition of antagonist or control vehicle. Data expressed as mean ± s.e.mean. [b]5-HT concentration-ratio given as geometric mean with 95% confidence limits.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to control values.

Application of DAU 6285 to the tissue was shown to cause a concentration-dependent fall in basal SCC levels, which was maximal 15 min after addition to the tissue, and showed recovery to control levels over the subsequent 15 min. It is not known if this is due to non-specific effects of DAU 6285. However, as there are no reports of such effects at these concentrations, it seems more likely that this fall may result from antagonism of endogenous 5-HT by DAU 6285. A similar effect has been reported in guinea-pig ileum and rat oesophagus (Waikar *et al.*, 1993), and in this case could indicate a possible 5-hydroxytryptaminergic component of the resting SCC of human ileal mucosa. If this were to be the case, however, we would expect the fall in SCC to be sustained; the reason why it is relatively transient is unknown and so further work would be required to substantiate or refute this claim.

In conclusion, this investigation has provided evidence that the SCC response of human isolated small intestinal mucosa to 5-HT is mediated by a receptor of the 5-HT_4 sub-type. It is as yet unclear whether the variability in pA_2 values at the 5-HT_4 receptor is a real difference, that is, a possible indication of receptor variation. In addition, DAU 6285 appears to have uncovered a possible 5-HT-mediated component of the resting secretory tone of human small intestine.

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Evidence for a functional β_3-adrenoceptor in man

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1 The existence of a functional β_3-adrenoceptor in man was investigated by studying the lipolytic action of selective β-adrenoceptor agents in isolated white omental and subcutaneous fat cells.

2 The non-selective β_1/β_2-adrenoceptor antagonist, CGP 12177 was lipolytic in both omental and subcutaneous fat cells. The intrinsic activity relative to isoprenaline was greater in omental than in subcutaneous cells.

3 Addition of the β_2-adrenoceptor antagonist, ICI 118,551 and the β_1-adrenoceptor antagonist CGP20712A in combination or the non-selective β-adrenoceptor antagonist propranolol alone (all 10^{-7} M), induced a rightward shift of the dose-response curves for isoprenaline- and BRL37344-stimulated lipolysis of about 4 and 2 log-units, respectively. However, the antagonists did not alter lipolysis induced by CGP12177.

4 Several concentrations of β-adrenoceptor antagonists were used to determine the pA_2 values by Schild analysis. The values for CGP 20712A and ICI 118,551 (6.63 ± 0.20 and 6.25 ± 0.12) as antagonists of the lipolytic effects of CGP 12177 were over 2 units lower than the pA_2 value for CGP 20712A against the response to the selective β_1-agonist dobutamine (8.58 ± 0.23) and the pA_2 value for ICI 118,551 against the response to the selective β_2-agonist terbutaline (9.15 ± 0.26).

5 β_3-Adrenoceptor mRNA expression, investigated with a polymerase chain reaction assay, was demonstrated in both types of adipocytes in the same cell preparations that had a lipolytic response to CGP 12177.

6 In conclusion, human white fat cells express an atypical β-adrenoceptor in addition to β_1- and β_2-adrenoceptors. This receptor is stimulated more selectively by the β_1-/β_2-antagonist CGP 12177 than by BRL 37344 and is poorly sensitive to blockade by selective β_1- and β_2-antagonists. On the basis of the pharmacological properties and the mRNA analyses, we suggest that this atypical receptor corresponds to the β_3-adrenoceptor subtype.

Keywords: β_3-Adrenoceptors; lipolysis; adipocytes; mRNA expression; CGP 12177; BRL 37344

Introduction

Catecholamines mediate a wide variety of tissue-specific responses by interaction with different β-adrenoceptor subtypes, including stimulation of lipolysis in fat cells. Originally, the stimulatory effects of catecholamines were thought to be mediated by only two β-adrenoceptors, called β_1 and β_2 (Lands *et al.*, 1967). However this early classification is insufficient to account for the thermogenic and lipolytic responses of rat brown and white adipose tissues (Harms *et al.*, 1974; Arch, 1989; Zaagsma & Nahorski, 1990). The availability of an increasing number of selective β-adrenoceptor ligands suggested that an additional, or 'atypical' β-adrenoceptor is present in adipose tissue (Arch, 1989; Zaagsma & Nahorski, 1990).

Following the molecular characterization of a third β-adrenoceptor in man (Emorine *et al.*, 1989) and rodents (Nahmias *et al.*, 1991; Granneman *et al.*, 1991; Muzzin *et al.*, 1991), the importance of this receptor in catecholamine action has to be considered. Structurally, the β_3-AR resembles the β_1- and β_2-adrenoceptor (Emorine *et al.*, 1989) but exhibits pharmacological properties very different from those of the β_1- and β_2-subtypes. First, classical β_1- and β_2-adrenoceptor antagonists have weak effects on the β_3-adrenoceptor and some, such as CGP 12177, are even agonists on this receptor (Feve *et al.*, 1991). Secondly, new selective agonists, which have a weak effect on β_1- and β_2-adrenoceptors are potent on β_3-adrenoceptors in animal fat cells (Arch *et al.*, 1984). The involvement of the β_3-adrenoceptor in rodent adipocyte lipolysis and thermogenesis is well documented (Arch, 1989; Zaagsma & Nahorski, 1990; Feve *et al.*, 1991), but its presence and biological function in man remain controversial (Hollenga *et al.*, 1991; Langin *et al.*, 1991).

We have recently demonstrated that β_3-adrenoceptor mRNA is expressed in human fat cells (Krief *et al.*, 1993). The aim of this study was therefore to investigate the presence of a functional β_3-adrenoceptor in man by measuring lipolysis in isolated fat cells, with a highly sensitive assay (Wahrenberg *et al.*, 1992). The pharmacological data were correlated to β_3-adrenoceptor mRNA expression in fat cells from the same individuals. Since the number of binding sites for β_1- and β_2-adrenoceptors is twice as high in omental as in subcutaneous human fat cells (Hellmér *et al.*, 1992), we also compared β_3-adrenoceptors in adipocytes from these two regions.

Methods

Patients

The study comprised 28 patients (11 men and 17 women) undergoing elective cholecystectomy at Huddinge University Hospital. In all other respects they were healthy and not on any medication. The ages of the subjects ranged from 20 to 66 years (mean ± s.e.: 42 ± 3 years) and the body mass index ($kg\ m^{-2}$) from 21.9 to 28.0 (mean ± s.e.: 24.4 ± 0.6). General anaesthesia was induced by a short-acting barbiturate and maintained by fentanyl and a mixture of oxygen and nitrous oxide. The patients had fasted overnight and intravenous saline was administered prior to the biopsies, which were taken from abdominal subcutaneous and omental fat tissues.

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The study was approved by the Ethics Committee of the Karolinska Institute.

Isolation of fat cells

The amount of tissue obtained from each depot ranged from 2–5 g. The adipose tissue was immediately transported to the laboratory and the preparation of isolated fat cells, by Rodbell's method (Rodbell, 1964), was started within 10 min, after collection. Samples were cut into 5 to 10 mg fragments. Adipocytes were isolated from the stroma cells by incubation with 0.5 g l^{-1} of collagenase for 60 min in 5 ml of Krebs-Ringer phosphate buffer (pH 7.4) with 40 g l^{-1} of dialysed bovine serum albumin at 37°C in a shaking bath. Adipocytes were washed through a silk cloth 3 times with a collagenase-free buffer. A portion of about 1 ml of each cell type was frozen in liquid nitrogen and stored at − 80°C for subsequent RNA analysis. The remaining cells were used for lipolysis experiments.

Lipolysis

The lipolysis assay has previously been described in detail (Arner *et al.*, 1986). Briefly, dilute suspensions of isolated fat cells (5000–10000 cells ml^{-1}) were incubated in duplicate for 2 h at 37°C in Krebs-Henseleit phosphate buffer (pH 7.4), supplemented with glucose (1 g l^{-1}), bovine serum albumin (20 g l^{-1}) and ascorbic acid (0.1 g l^{-1}) and with or without β-adrenoceptor ligands. All ligands were added simultaneously at the start of the incubation. Isoprenaline (non-selective β-adrenoceptor agonist), terbutaline (selective β_2-agonist), dobutamine (selective β_1-agonist), BRL 37344 (selective β_3-agonist in animal studies), CGP12177 (β_1- and β_2-antagonist, partial β_3-agonist in animals) (Langin *et al.*, 1991), ICI 118,551 (selective β_2-antagonist), CGP20712A (selective β_1-antagonist) and propranolol (non-selective β_1- and β_2-antagonist) were added to the incubation medium. Twelve different concentrations, ranging from 10^{-14} to 10^{-3} mol l^{-1}, were used for isoprenaline, terbutaline, dobutamine, BRL 37344 and CGP 12177, while the concentration range for ICI 118,551 and CGP20712A ranged from 10^{-10} to 10^{-3} mol l^{-1}. Propranolol was added at 10^{-7} mol l^{-1}. The same batches of collagenase or albumin and the same stock solutions of β-adrenoceptor agents were employed throughout the study.

The release of glycerol was used as an index of lipolysis. The glycerol concentration after a 2-h incubation was determined in a cell-free aliquot with a bioluminescence method (Hellmér *et al.*, 1989), and was expressed per g lipid. Methodological experiments revealed that the rate of glycerol release from fat cells was linear for at least 4 h in the basal state and in the presence of maximal effective concentrations of lipolytic drugs.

Dose-response curves for glycerol release were used to determine the concentrations of agonists giving half of their own maximum stimulation (EC_{50}) and of antagonists yielding half of their own maximum inhibition (IC_{50}). The values for these concentrations represent pD_2 ($-\log$ mol l^{-1} for EC_{50}) and pIC_{50} ($-\log$ mol l^{-1} for IC_{50}). Intrinsic activities of the lipolytic agents were determined as the percentage of the maximal isoprenaline response for each experiment separately.

Antagonist potencies were evaluated by calculating their pA_2 values. Concentration-response curves for agonists were made in the presence of 3–5 different concentrations of antagonists. Schild plots were constructed from the individual experiments (Arunlakshana & Schild, 1959) and the pA_2 values ($-\log$ mol l^{-1}) were calculated.

RNA analysis

The method has previously been described in detail (Krief *et al.*, 1993). Briefly, total RNA was prepared from human isolated fat cells with a single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction (Chomczynski & Sacchi, 1987). For polymerase chain reaction (PCR) analysis, RNA was treated for 1 h at 37°C with 6 u of RNase-free DNase I per μg of RNA in Tris-HCl 100 mM, pH 7.5 and $MgCl_2$ 50 mM, in the presence of 2 u μl^{-1} of placenta RNase inhibitor. Following phenol extraction and ethanol precipitation, 1 μg of RNA was treated with 400 u of Maloney murine leukaemia virus reverse transcriptase in 80 μl of PCR buffer (Tris-HCl 67 mM, pH 8.4; $MgCl_2$ 6.7 μM; EDTA 6.7 μM; β-2-mercaptoethanol 10 mM; $(NH_4)2SO_4$ 16 mM; gelatine 0.1 mg ml^{-1}) containing 0.4 mM of each dNTP; 10 μM random hexanucleotides, 2 u μl^{-1} RNase inhibitor. A negative control without reverse transcriptase was performed to ensure that amplification did not proceed from the residual genomic DNA. β_1- and β_2-adrenoceptor cDNA were then amplified by 29 temperature cycles (92°C, 1 min; 57°C, 1.5 min; 72°C, 1.5 min) followed by 7 min of extension at 72°C in a temperature cycler (LEPPREM) in 100 μl of PCR buffer containing 2.5 u of *Thermophylus aquaticus* (Taq) polymerase, 125 nM of each sense and antisense oligonucleotide primers, 125 μM of each dNTP, 10% (v/v) dimethylsulphoxide. Formamide 5% (v/v) was also added for β_1-adrenoceptor amplification. Amplification of cDNA was linear up to 500 ng of initial RNA for β_1- and β_3-adrenoceptors and up to 250 ng for β_2-adrenoceptors. The use of 250 ng of RNA for β_1- and β_3-adrenoceptor amplifications and 125 ng for β_2-adrenoceptor allowed a rough approximation of the relative levels of expression of each mRNA.

The sequences of the sense and antisense oligonucleotides corresponded to amino acid 178 to 265 for β_1, aminoacid 143 to 252 for β_2 and aminoacid 2 to 106 for β_3 (Krief *et al.*, 1993). Using these primers, the lengths of the fragments, calculated from the structure of the corresponding genes, were 265, 329 and 314 base pairs (bp) for β_1-, β_2- and β_3-receptors, respectively.

Statistical analysis

The values presented are the means ± standard error of the means (s.e.). Regression analysis and the Student's two-tailed paired or unpaired *t* tests were used for statistical comparisons of the results.

Drugs and chemicals

Bovine serum albumin (fraction V) (lot 63F-0748), *Clostridium histiolyticum* collagenase type I, glycerol kinase from *E. coli* (G4509) and (±)-propranolol were obtained from Sigma (St. Louis, MO. U.S.A.). (−)-Isoprenaline hydrochloride came from Hässle (Mölndal, Sweden), terbutaline sulphate from Draco (Lund, Sweden), dobutamine hydrochloride from Lilly (Indianapolis, IN, U.S.A.) and ICI 118,551 (erythro- (±) -1- (7-methyllindane-4-yloxy) -3-isopropylaminobutane-2-olhydrochloride) from Cambridge Research Biochemicals Limited (Cheshire, U.K.). BRL 37344 (4- [2- [(2-hydroxy-2 (3-chlorophenyl) ethyl) amino] propyl] phenoxyacetic acid) was kindly supplied by SmithKline Beecham (Epsom, U.K.) and CGP (±)-12177 ((−)-4-(3-*t*-butylamino-2-hydroxy-propoxy) benzimidazole-2-one) and CGP 20712A ((±)-(2-(3-carbamoyl-4-trifluormethyl-2-imidazolyl)-phenoxy)-2-propanol methane sulphonate) were kindly supplied by Ciba Geigy (Basel, Switzerland). ATP monitoring reagent containing firefly luciferase was from LKB Wallac (Turku, Finland). Maloney murine leukemia virus reverse transcriptase from Gibco (Bethesda, MD, U.S.A.), *Thermophylus aquaticus* (Taq) polymerase from Perkin Elmer-Cetus (Emeryville, CA, U.S.A.) and DNase I and placenta RNase inhibitor were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of the highest grade of purity commercially available.

Results

Lipolysis

The lipolytic effects of increasing concentrations of various adrenoceptor agonists were measured in omental and subcutaneous fat cells from 16 subjects. The mean dose-response curves for omental cells are depicted in Figure 1. Similar curves were obtained with subcutaneous cells (graph not shown). The intrinsic activities of selective agonists in relation to isoprenaline are shown in Table 1. In both types of adipocytes, dobutamine and terbutaline were full agonists relative to isoprenaline, a finding in accordance with our previous data (Lönnqvist *et al.*, 1992). However, the β_3-adrenoceptor agents, BRL 37344 and CGP 12177, both acted as partial agonists. The intrinsic activities of BRL 37344 and CGP 12177 in relation to the isoprenaline response were significantly higher in omental than in subcutaneous adipocytes. In omental fat cells, the intrinsic activity of BRL 37344 was about 70% and that of CGP 12177 about 40%.

The experiments in Figure 2 were performed in order to investigate the effects of β-antagonists on lipolysis induced by isoprenaline as compared to lipolysis induced by β_3-agonists. Since the lipolytic response was much more pronounced in omental fat cells, experiments were performed mainly on this cell type. Isoprenaline, BRL 37344 or CGP 12177 was added in increasing concentrations in the presence or the absence of 10^{-7} M of either the selective β_1-antagonist CGP20712A plus the selective β_2-antagonist ICI 118,551, or the non-selective β_1/β_2-antagonist, propranolol. In a previously published study the competition binding experiments on human isolated omental fat cells with [^{125}I]-cyanopindolol, ICI 118,551 and CGP 20712A were re-calculated to find out whether 10^{-7} M of the selective antagonists was sufficient to block the β_1- and β_2-responses (Hellmér *et al.*, 1992). The displacement curves were analysed by non-linear regression to determine receptor occupancy. At 10^{-7} M, ICI 118,551 or CGP 20712A blocked >95% of its corresponding β-subtype in all experiments ($n = 8-9$).

In the present study, maximum lipolytic responses were not affected by the antagonists. The isoprenaline dose-response curves were markedly shifted (≈10,000 times decreased agonist sensitivity, $P<0.001$) to the right when either propranolol or the combination of β_1- and β_2-antagonists had been added. There was a less marked shift to the right (≈100 times decreased agonist sensitivity) for the BRL 37344 dose-response curve in the presence of β_1/β_2-antagonists or propranolol. This difference in shift, as compared to the isoprenaline-propranolol shift, was significant ($P<0.01$). The CGP 12177-stimulated lipolysis was not influenced by either of the antagonists. The individual pD_2

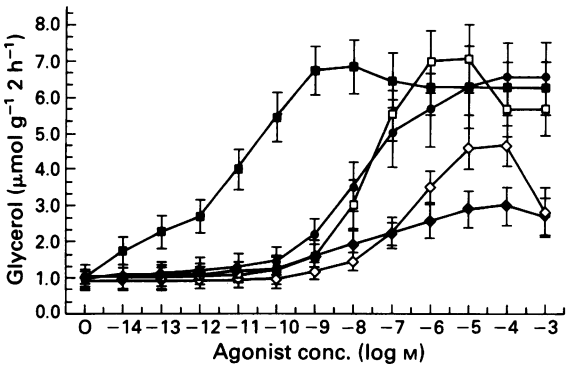

Figure 1 β-Adrenoceptor agonist-induced glycerol release in isolated omental adipocytes from 16 individuals. The fat cells were incubated with increasing concentrations of isoprenaline (■), dobutamine (□), terbutaline (●), BRL 37344 (◇) and CGP 12177 (◆). The results (means ± s.e.) are expressed as μmol glycerol/g lipid/2 h.

Table 1 Intrinsic activity of β-adrenoceptor agonists in human fat cells

	Omental fat cells	*Subcutaneous fat cells*
Terbutaline	100 ± 2	92 ± 5*
Dobutamine	102 ± 4	98 ± 5
BRL 37344	66 ± 7	48 ± 6*
CTP 12177	37 ± 6	*24 ± 4**

Intrinsic activities are expressed as % of the maximal isoprenaline response ($n = 16$). Values obtained with omental and subcutaneous cells were compared by Student's paired t test.
*$P<0.05$; **$P<0.005$.

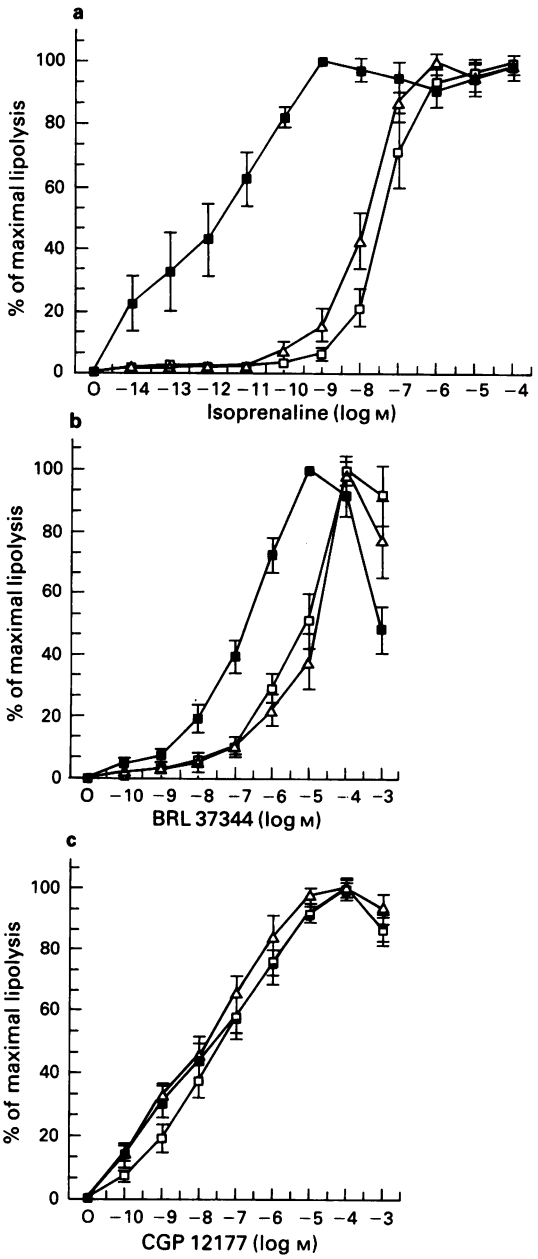

Figure 2 Resistence of omental fat cell lipolysis to blockade by β_1- and β_2-adrenoceptor antagonists. Adipocytes were incubated with increasing concentrations of isoprenaline (a), BRL 37344 (b) and CGP 12177 (c) in the absence (■) or presence of the selective β_1-antagonist CGP 20712A plus the selective β_2-antagonist ICI 118,551 (□) or the non-selective β-antagonist propranolol (△). The antagonists were added at a concentration of 0.1 μM. The results of six experiments (means ± s.e.) are expressed as the percentage of the maximal lipolytic response obtained with each agonist.

Table 2 pD_2 values for β-adrenoceptor agonists in the presence or absence of selective or non-selective β-antagonists

Agonist	*No β-receptor antagonist*	*+ ICI 118,551 + CGP 20712A* 10^{-7} M	*+ Propranolol* 10^{-7} M
Isoprenaline	11.19 ± 0.33	7.02 ± 0.32***	7.78 ± 0.19***
BRL	6.49 ± 0.10	4.72 ± 0.26**	4.93 ± 0.29**
CGP	7.73 ± 0.28	7.39 ± 0.31	7.80 ± 0.21

Student's paired t test was used for statistical comparisons of results with and without antagonists in the incubation system ($n = 6$). **$P < 0.005$, ***$P < 0.001$.

values of the experiments in Figure 2 are shown in Table 2. These results confirm the high, intermediate and low sensitivities of isoprenaline, BRL 37344 and CGP 12177, respectively, to blockade by propranolol or by selective β_1- and β_2-antagonists. The results in Table 2 were also used to calculate the dose-ratio, and hence the apparent pA_2 value for propranolol and the β_1-/β_2-antagonist combination, using MacKay's method (MacKay, 1978): $pA_2 = -\log([\text{antagonist}]/DR - 1)$, where DR is the dose-ratio between the EC_{50} value for an agonist in the presence of a certain antagonist concentration and the EC_{50} value in the absence of the same antagonist. This gives pA_2 values for propranolol of 10.4 and 8.5 for isoprenaline and BRL 37344, respectively, and values of 11.2 and 8.8 for the combination of selective antagonists. The interactions between CGP 12177 and the antagonists were too small to allow calculations of pA_2. However, it is of interest that the pD_2 values for isoprenaline and CGP 12177 differed by about 3.5 units in the absence of antagonists, but were almost the same (about 7.5) in the presence of antagonists.

To determine whether the antagonistic effects of CGP 20712A(β_1) or ICI 118,551(β_2) on lipolysis stimulated with CGP 12177 differed from their effects on β_1- and β_2-agonist-induced lipolysis, increasing concentrations of the antagonists were added to a fixed, submaximal dose of the agonist. The mean dose-response curves are shown in Figure 3. Both antagonists could maximally inhibit lipolysis induced by agonists. However, the sensitivity to the antilipolytic effect of CGP 20712A was much more pronounced for the lipolysis induced by dobutamine than for the lipolysis induced by CGP 12177. Furthermore, the sensitivity to the antilipolytic effect of ICI 118,551 was much more pronounced for the terbutaline-induced lipolysis than for the CGP 12177-induced lipolysis. The mean pIC_{50} values are given in Table 3. The pIC_{50}-value for the β_1-antagonist was about 2 units lower for CGP 12177-stimulated than for dobutamine-stimulated lipolysis i.e., 5.86 ± 0.27 versus 8.26 ± 0.69, $P < 0.05$. In a corresponding way the pIC_{50}-value for the β_2-antagonist was also about 2 units lower for CGP 12177-stimulated than for terbutaline-stimulated lipolysis i.e., 4.81 ± 0.20 versus 6.93 ± 0.27, $P < 0.001$.

An additional, indirect, way of investigating the existence of a functional β_3-adrenoceptor is to determine the pA_2 values for selective β_1- and β_2-blockers in agonist dose-response experiments performed at several concentrations of antagonist in each individual experiment. The pA_2 values are obtained by the construction of Schild plots (Arunlakshana & Schild, 1959). The mean values are given in Table 3. One of four experiments is shown in Figures 4 and 5. The pA_2 values of ICI 118,551 and CGP 20712A versus CGP 12177 were 2–3 units lower ($P < 0.005$ and $P < 0.05$, respectively) than those of ICI 118,551 versus terbutaline and of CGP 20712A versus dobutamine.

It should be stressed that the pA_2 values and the pIC_{50} values in Table 3 are not directly comparable. First, the calculation of the pA_2 value for an antagonist (i.e., the K_i) from the pIC_{50} value for the same substance requires a compensation for the agonist concentration and the affinity of the agonist to the β_3-adrenoceptor (which is unknown) in the IC_{50} experiment, according to Cheng & Prusoff (1973).

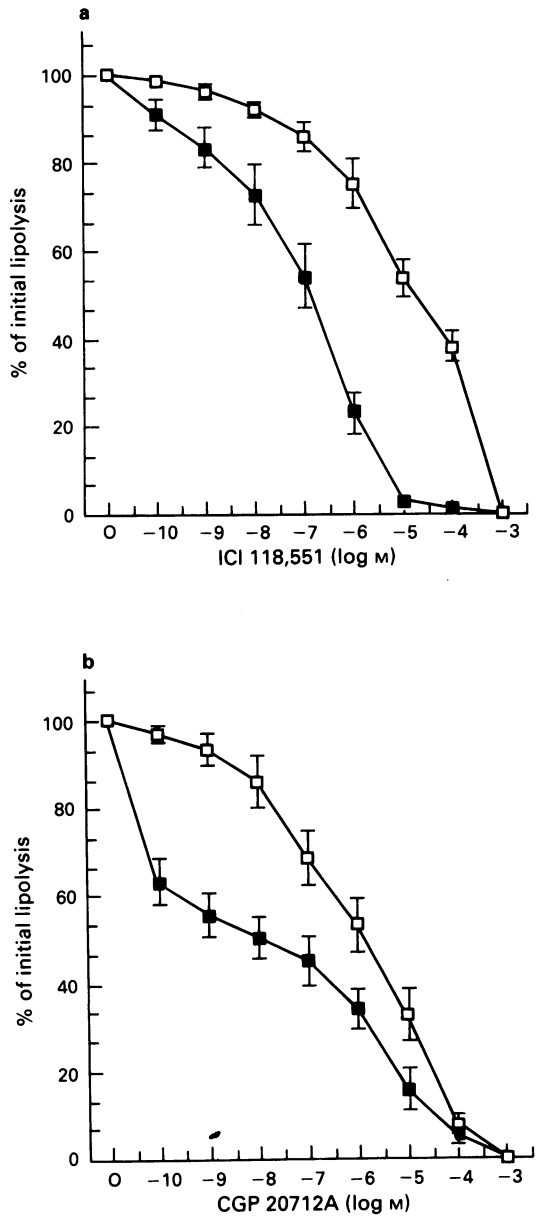

Figure 3 Antilipolytic effects of the β_2-adrenoceptor antagonist ICI 118,551 (a) and the β_1-antagonist CGP 20712A (b) on β-adrenoceptor agonist-stimulated lipolysis in omental adipocytes. In (a), lipolysis was induced by terbutaline 10 μM (■) or CGP 12177 0.1 μM (□) whereas in (b), lipolysis was stimulated with either dobutamine 0.1 μM (■) or CGP 12177 0.1 μM (□). Values for basal lipolysis were subtracted from the agonist-induced lipolysis. The antagonist effect is expressed as a percentage; 100% is the lipolysis in the absence of antagonists. At the maximum effective concentration, all antagonists reduced agonist-induced lipolysis to zero in all individual experiments. Values are means ± s.e. of duplicate experiments performed on fat cells from 6 patients.

Table 3 pIC_{50}-values and pA_2-values for the interactions between selective β-adrenoceptor agonists and antagonists

	Antagonist					
	CGP 20712A			*ICI 118,551*		
Agonist	*pIC_{50}*	*pA_2*	*Slope*	*pIC_{50}*	*pA_2*	*Slope*
Terbutaline	–	–	–	6.93 ± 0.27 (n = 6)	9.15 ± 0.26 (n = 4)	1.05 ± 0.13
Dobutamine	8.26 ± 0.69 (n = 6)	8.58 ± 0.23 (n = 4)	0.94 ± 0.26	–	–	–
CGP 12177	5.86 ± 0.27 (n = 6)	6.63 ± 0.20 (n = 4)	0.97 ± 0.17	4.81 ± 0.20 (n = 6)	6.25 ± 0.12 (n = 4)	0.84 ± 0.17

Fat cells were incubated *in vitro* with various combinations of agonists and antagonists, as described in the legends to Figures 4 and 5, for the determinations of pIC_{50} and pA_2. In the latter experiments the slopes of the Schild plots are also given, n = number of experiments.

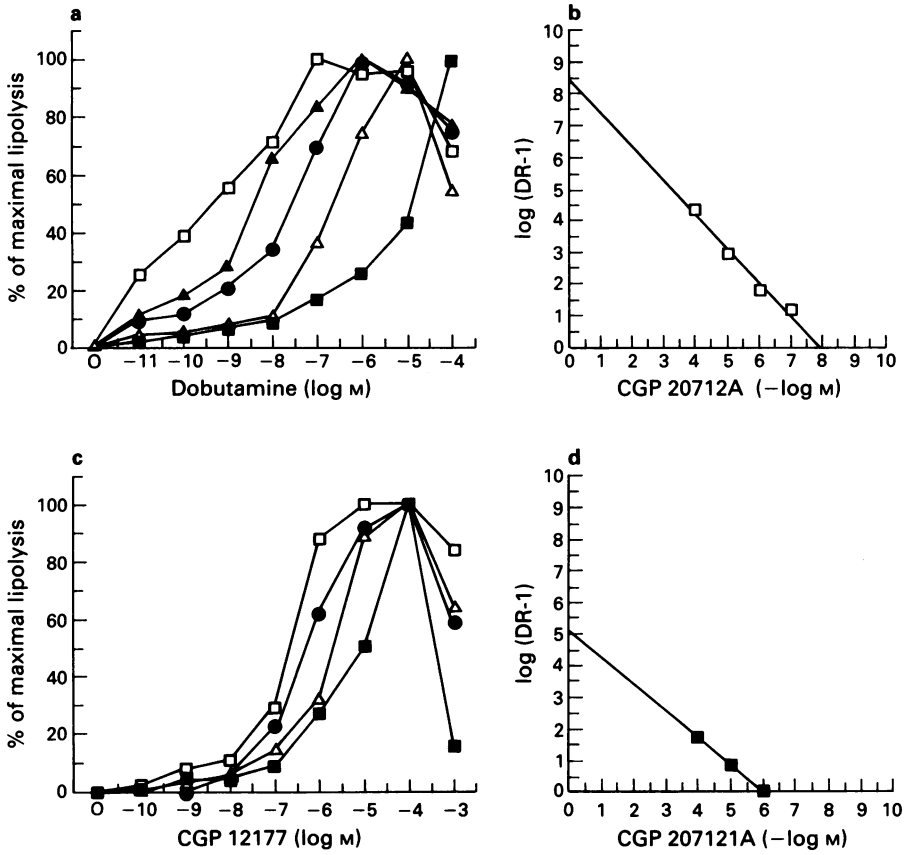

Figure 4 Determination of the pA_2 values for CGP 20712A (β_1-adrenoceptor antagonist). Dose-response curves for the antagonistic effect of increasing concentrations of CGP 20712A on dobutamine- (a) and CGP 12177-stimulated lipolysis (c) and the corresponding Schild plots used for the calculation of the pA_2 values for CGP 20712A versus dobutamine (b) and CGP 12177 (d) are shown. The concentrations of CGP 20712A used to counteract the lipolytic effects of dobutamine (□) and CGP 12177 (■) were 0 (□) 100 nM (▲), 1 μM (●) 10 μM (△) and 100 μM (■). The concentrations of dobutamine and CGP 12177 ranged from 10^{-11} to 10^{-3} M and 10^{-10} to 10^{-3} M, respectively. The pA_2 values were calculated for each antagonist by plotting: log (dose ratio − 1) vs. − log (antagonist) concentration as described in Methods. One typical experiment out of four is shown. The mean values of these experiments are given in Table 3.

Secondly, the relative stimulation of the lipolysis rate induced by an agonist should be the same in all experiments (ideally half-maximum stimulation). The latter is difficult to achieve in human experiments, since β-agonist sensitivity varies between adipocytes from different subjects (Lönnqvist *et al.*, 1992). However, Table 3 shows that, for CGP 20712A versus dobutamine, the pA_2 and pIC_{50} values agree and that the discrepancies for CGP 12177 versus the two antagonists are small (between 0.8 and 1.5 units). However, the ICI 118,551 versus terbutaline pA_2 was clearly different from pIC_{50}. This might suggest that the concentration of terbutaline (10^{-5} M) was more than the submaximal. In order to test this possibility, four additional experiments were performed, in which increasing concentrations of ICI 118,551 were added to 10^{-7} M of terbutaline. The antagonist caused a complete dose-dependent inhibition of terbutaline-induced lipolysis. In all these experiments pIC_{50} for ICI 118,551 was 9.58 ± 1.17, which is similar to the pA_2 value in Table 3 (9.15 ± 0.26).

β-Adrenoceptor mRNA expression in human fat cells

In order to obtain a biological correlation with the pharmacological evidence for a functional β_3-adrenoceptor in man, the mRNA expression of this gene was studied in isolated fat cells from the same preparations as in the lipolysis investigation, using a PCR assay. mRNA for β_1-, β_2- and β_3-adrenoceptor was expressed in both omental and subcutaneous adipocytes. A typical experiment is shown in

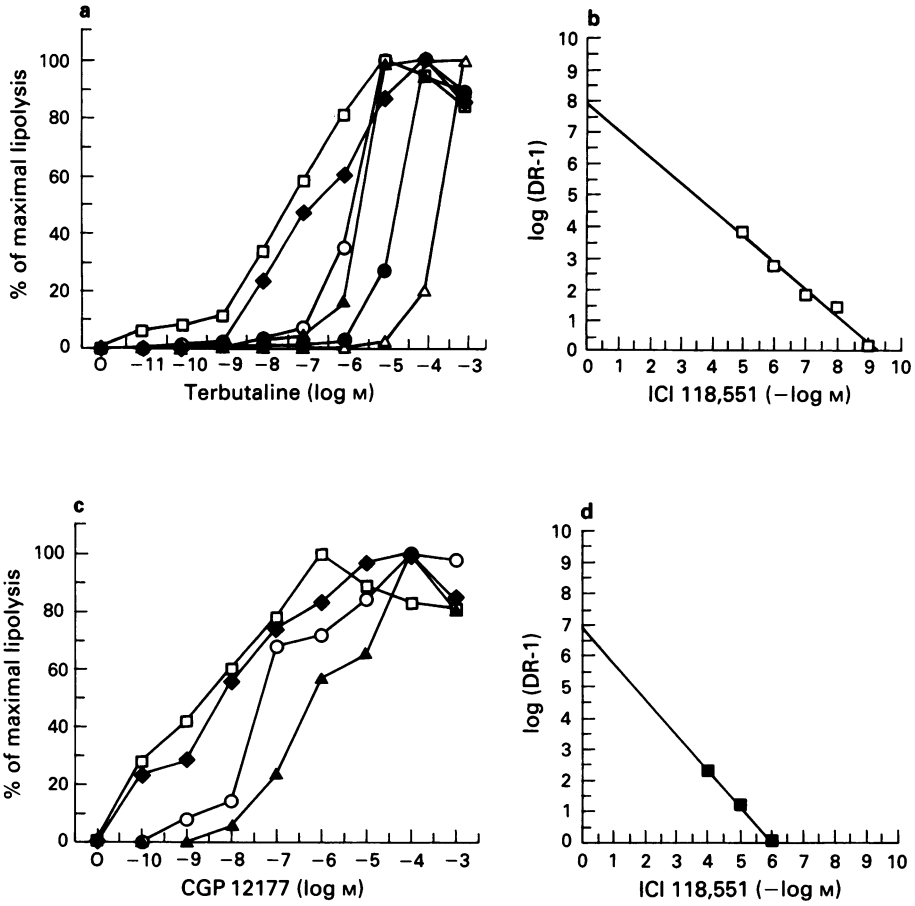

Figure 5 Determination of the pA_2 values for ICI 118,551 (β_2-adrenoceptor antagonist). Dose-response curves for the antagonistic effect of increasing concentrations of ICI 118,551 on terbutaline- (a) and CGP 12177-stimulated lipolysis (c) and the corresponding Schild plots used for the calculation of the pA_2 values for ICI 118,551 versus terbutaline (b) and CGP 12177 (d) are shown. The concentrations of ICI 118,551 used to counteract the lipolytic effects of terbutaline (□) and CGP 12177 (■) were 0 (□) 1 nM (◆), 10 nM (○), 100 nM (▲), 1 μM (●), 10 μM (△) and 100 μM (■). The concentrations of terbutaline and CGP 12177 ranged from 10^{-11} to 10^{-3} M and 10^{-10} to 10^{-3} M, respectively. The pA_2 values were calculated for each antagonist by plotting: log (dose ratio − 1) vs. − log (antagonist) concentration as described in Methods. One typical experiment out of four is shown. The mean values of these experiments are given in Table 3.

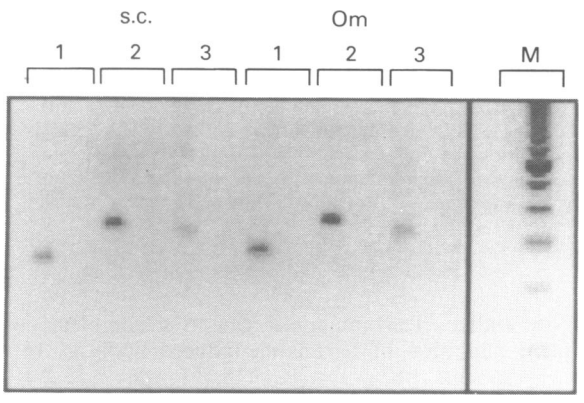

Figure 6 β_3-Adrenoceptor mRNA expression in subcutaneous (s.c.) and omental (Om) fat cells. The negatives of agarose gel electrophoresis pictures of β_1-(1), β_2-(2) and β_3-adrenoceptor (3) cDNA fragments amplified by PCR are shown. Each horizontal bracket delimits two lanes containing PCR samples obtained from RNA-treated (left lane), or untreated (right lane) with reverse transcriptase. Size markers (M) are the 123 bp DNA ladder (GIBCO BRL). Results ae representative of one of four experiments. The RNA samples were obtained from subcutaneous and omental adipocytes isolated from the same subject.

Figure 6. Four subjects were investigated. In fat cells of all subjects there was a positive expression of mRNA for all three mRNA subtypes. No attempts were made to quantitate each mRNA, since the PCR assay is not quantitative (Krief *et al.*, 1993). However, the corresponding lipolysis data with omental fat cells (Table 4) show that, as compared to isoprenaline, there was a full stimulation with dobutamine and terbutaline and a 40% and 60% stimulation, respectively, with CGP 12177 and BRL 37344.

Discussion

In this paper we provide pharmacological and molecular evidence for the existence of a functional β_3-adrenoceptor in human fat cells. A strong proof for the presence of β_3-receptors is constituted by the lipolytic action of high concentrations of the potent β_1- and β_2-receptor antagonist, CGP 12177. Analogous effects of this compound have previously been observed in rodent fat cells and were attributed to stimulation of the β_3-receptor (Mohell & Dicker, 1989; Feve *et al.*, 1991). CGP 12177-induced lipolysis was resistant to blockade by various β_1- and β_2-antagonists, which were used in a concentration (10^{-7} M) occupying >95% of β_1- and β_2-receptors. Neither the pD_2 nor the maximum levels of

Table 4 Lipolysis in fat cells where mRNA was determined

Agonist	*Glycerol release* (μmol g^{-1} lipid $2\,h^{-1}$)	pD_2	*Intrinsic activity*
0	0.63 ± 0.26	–	–
Isoprenaline	6.09 ± 1.06	10.52 ± 0.55	100
Terbutaline	6.44 ± 1.12	7.39 ± 0.17	102 ± 6
Dobutamine	6.52 ± 1.08	7.55 ± 0.33	105 ± 5
CGP 12177	2.25 ± 0.73	7.31 ± 0.53	40 ± 11
BRL 37344	3.41 ± 0.61	6.23 ± 0.27	62 ± 13

Four subjects were investigated. mRNA was measured using a PCR assay as described in Methods. All samples showed a clear expression of the three β-receptor subtypes. In parallel experiments, fat cells were incubated in the presence or absence (0) of increasing concentrations of the indicated agonists. The rate of lipolysis at maximum effective agonist concentration and the pD_2 were determined. % intrinsic activity of each agonist is related to the maximum lipolytic rate of isoprenaline.

CGP 12177-stimulated lipolysis – which could be considered to be mediated primarily by β_3-adrenoceptors – were affected by the concentrations of antagonists used. These observations correlate well with the markedly divergent pA_2 values (i.e., 2–3 log units) for selective β_1- and β_2-antagonists obtained with CGP 12177, as compared to dobutamine and terbutaline, respectively. It appears that CGP 12177-induced lipolysis in human fat cells is mediated mainly through β_3-adrenoceptors. Whether it is completely selective in this respect is unknown. Such a question can only be answered by using high affinity β_3-antagonists. Unfortunately, such agents do not exist at present.

Competition-binding experiments on isolated fat cells were performed using [^{125}I]-cyanopindolol to establish further the existence of β_3-adrenoceptors in human fat cells. The data are not reported since it was not possible to bind specifically to β_3-adrenoceptors. Non-specific binding is too high (> 50%) to label the receptor at the radioligand concentrations used. This is in accordance with previous binding studies on human fat cells (Langin *et al.*, 1991). Thus the use of available β-adrenoceptor radioligands is not feasible for a reliable characterization of the β_3-adrenoceptor binding sites in human fat cells. However, the mRNA analysis performed on the biological samples which we used for pharmacological experiments showed that the β_3-adrenoceptor transcripts are expressed in the same preparations of fat cells that have a lipolytic response to CGP 12177. Altogether, our data demonstrate the existence of β_3-adrenoceptors at the functional and molecular levels in human fat cells.

The higher sensitivity of our lipolysis assay (Wahrenberg *et al.*, 1992) may explain why previous investigators have not convincingly demonstrated the β_3-receptors on human isolated fat cells (Hollenga *et al.*, 1991; Langin *et al.*, 1991). The difference between the results may also partly be due to our use of diluted fat cell suspensions (1–2% v/v) instead of dense cell concentrations (10% v/v or higher) because the accumulation of endogenous metabolites may inhibit the lipolytic action of catecholamines (Kather, 1990). Finally, only subcutaneous adipocytes have previously been studied in man (Hollenga *et al.*, 1991; Langin *et al.*, 1991), although these cells have a lower metabolic activity, including lipolysis (Hamosh *et al.*, 1963; Fessler & Beck, 1965; Östman *et al.*, 1979) and β_1/β_2-receptor sensitivity (Hellmér *et al.*, 1992), than omental fat cells. In agreement with this, our present data show that the β_3-receptor agonistic effects of CGP 12177 are more pronounced in omental fat cells.

In brown fat cells of animals, BRL 37344 is a potent lipolytic agonist (Arch, 1989). This agonist was also lipolytic in human fat cells. In contrast to the findings with CGP 12177, the lipolytic effect of BRL 37344 could be inhibited by a blockade of β_1- and β_2-receptors, indicating an unselective lipolytic action. However, pA_2 for interaction between isoprenaline and BRL 37344, on the one hand, and blockers of β_1- and β_2-receptors, on the other hand differed by about 2 log units. This indicates that at least a part of the lipolytic effect of BRL 37344 is mediated by β_3-receptors in human fat cells. Again in the absence of a specific β_3-adrenoceptor antagonist, we cannot speculate about the relative contributions by the different β-receptor subtypes to the lipolytic action of BRL 37344.

In man, β_1-, β_2- and β_3-receptors all appear to be functionally coupled to lipolysis, as demonstrated in this paper. The relative contribution of each receptor subtype to the response remains to be established. It appears, however, that the β_3-receptor may be less well coupled to lipolysis than the other two receptor subtypes. Neither BRL 37344 nor CGP 12177 were full agonists, whereas the intrinsic activities of terbutaline and dobutamine were almost the same as that of isoprenaline. BRL 37344 had a much lower potency than isoprenaline, which is the converse of the situation in rat epididymal adipocytes, where BRL 37344 is a full agonist with high pD_2 (Hollenga *et al.*, 1990). It is possible that β_3-receptor coupling to adenylyl cyclase is different in man from other species, as previously suggested (Zaagsma & Nahorski, 1990). Indeed, there are structural differences in the coding parts of the genes for β_3-receptors in man (Emorine *et al.*, 1989), mouse (Nahmias *et al.*, 1991) and rat (Granneman *et al.*, 1991; Muzzin *et al.*, 1992). The physiological reason for the existence of three β-receptor subtypes in the same tissue is not yet known, but it may be linked to a difference in susceptibility of the β_1-, β_2- and β_3-receptors to hormonal and environmental regulation (Feve *et al.*, 1990; Granneman & Lahners, 1992). In human fat cells, for example, β_2-receptors are resistant to acute homologous desensitization, while β_1-receptors are rapidly desensitized (Arner *et al.*, 1991). Moreover, lipolytic resistance to catecholamines in man is due to reduced expression of β_2-receptors, but is not related to the β_1-receptor (Lönnqvist *et al.*, 1992). We have presently demonstrated regional differences in the β_3-receptor activity. These may suggest that the β_3-receptor is also independently regulated in human fat cells.

In conclusion, our data establish the existence in man of a functional β_3-adrenoceptor involved in the regulation of fat cell lipolysis. The existence of the receptor has been demonstrated both pharmacologically and at the mRNA level. Furthermore, our data also indicate that the β_3-receptor response may vary according to the adipose region, which suggests that its function may be subject to regulation. Finally, the data show that CGP 12177 is better than BRL 37344 as a tool for studying β_3-adrenoceptor in man.

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Vascular and anti-platelet actions of 1,2- and 1,3-glyceryl dinitrate

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1 The aim of this study was to investigate whether two metabolites of glyceryl trinitrate (GTN), 1,2 and 1,3-glyceryl dinitrate (1,2-GDN and 1,3-GDN) could account for the pharmacological effects of GTN. To this end the formation of nitric oxide (NO) from 1,2- and 1,3-GDN in the presence of bovine aortic smooth muscle cells (SMC) or endothelial cells (EC) was studied. The effects of various thiols on NO formation from these dinitrates was also evaluated.

2 1,2-GDN or 1,3-GDN (10^{-10}–10^{-5} M) caused a dose-dependent relaxation of rabbit aortic strips denuded of endothelium and precontracted with phenylephrine. The dinitrates were less than one tenth as potent as GTN.

3 Incubation of 1,2-GDN or 1,3-GDN (75–2400 μM) with SMC for 30 min led to a concentration-dependent increase in nitrite (NO_2^-) formation but this increase was less than that produced from GTN. Likewise incubation of 1,2-GDN or 1,3-GDN with N-acetylcysteine (NAC), glutathione (GSH) or thiosalicylic acid (TSA) (all at 1 mM) for 30 min at 37°C produced a concentration-dependent increase in NO_2^- formation.

4 Platelet aggregation induced by thrombin (40 mu ml^{-1}) was not modified by high concentrations of 1,2-GDN or 1,3-GDN (175–700 μM). However, aggregation was inhibited when platelets were exposed to 1,2-GDN or 1,3-GDN (700 μM) in the presence of SMC ($0.24–1.92 \times 10^5$ cells) or EC ($0.8–3.2 \times 10^5$ cells). These effects were abrogated by co-incubation with oxyhaemoglobin (OxyHb, 10 μM) indicating that they were due to NO release. The concentrations of the dinitrates required to inhibit platelet aggregation by 50% were about 15 times higher than for GTN in the presence of the same numbers of SMC or EC.

5 When NAC or TSA (both at 0.5 mM) were co-incubated with platelets for 3 min in the presence of 1,2-GDN or 1,3-GDN, a concentration-dependent inhibition of platelet aggregation was observed. These anti-platelet effects were abolished by co-incubation with OxyHb (10 μM). Glutathione had no potentiating effects.

6 Thus the dinitrate metabolites of GTN are metabolized to NO by SMC or EC and are acted upon by thiols to form NO at concentrations about 10 times higher than those of GTN. *In vivo*, after oral or intravenous GTN, GDN levels are reached which are more than 10 times higher than those of GTN. These data support the notion that part of the effects of GTN are due to the generation of NO from 1,2-GDN and 1,3-GDN by the cells of the vascular wall.

Keywords: Glyceryl trinitrate; glyceryl dinitrate; nitric oxide; oxyhaemoglobin; platelet aggregation; vasodilatation

Introduction

Bioconversion of glyceryl trinitrate (GTN) to nitric oxide (NO) is required for its vasodilator and antiplatelet effects and there is no doubt that this conversion is mainly enzymatic (Chung & Fung, 1990; Feelisch & Kelm, 1991; Salvemini *et al.*, 1992a). The enzyme(s) involved are still not known. Glutathione-S-transferase or cytochrome P_{450}, which are important in the biotransformation of GTN in the liver and kidney (Yeates *et al.*, 1989; Servent *et al.*, 1989), do not appear to be involved in the biotransformation of GTN to NO by smooth muscle cells (SMC) or endothelial cells (EC) (Gruetter & Lemke, 1985; Sakanashi *et al.*, 1991; Chung *et al.*, 1992; Salvemini *et al.*, 1993). There may, therefore, be different mechanisms for denitrification of GTN in the liver and kidney as compared to the vascular wall. In addition, NO can be formed from GTN by a non-enzymatic mechanism involving the interaction with a thiol. This may involve nucleophilic attack by a thiolate anion on the nitrogen atom of the ester group of GTN (Feelisch, 1991).

Besides the formation of NO or NO_2^- from GTN, metabolism of GTN gives rise to its two dinitrate metabolites, 1,2- and 1,3-glyceryl dinitrates (1,2- or 1,3-GDN). This bioconversion takes place not only in the liver but also in vascular smooth muscle (Kawamoto *et al.*, 1987), in the presence of haemoglobin or myoglobin (Bennett *et al.*, 1985; 1986) and in human plasma (Fung *et al.*, 1988; Posadas del Rio *et al.*, 1988; Chong & Fung, 1989; 1990). The half-lives of the dinitrates are much longer (40–60 min) than that of the parent compound (Bennett *et al.*, 1985; 1986). After oral administration, GTN is lost by first pass metabolism leading to systemic availability of 1,2-GDN or 1,3-GDN (Gumbleton & Benet, 1991). Both of these metabolites elicit vasodilatation in man, at concentrations similar to those that would be achieved in plasma following oral administration of GTN (Gumbleton & Benet, 1991). It was therefore proposed that the formation of these dinitrates could account for the vasodilator activity of GTN in man.

We have examined whether 1,2-GDN and 1,3-GDN like GTN are metabolized to NO in bovine aortic endothelial cells and smooth muscle cells and whether NO is formed from GDNs by the interaction with thiols. Our results support the concept that GDNs, after bioconversion to NO, contribute to the actions of GTN.

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Methods

Preparation of washed platelets

Human washed platelets were prepared as described by Radomski & Moncada (1983). The composition of the modified (Sneddon & Vane, 1988) Krebs bicarbonate buffer in which platelets were washed was (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.3, $MgSO_4$ 0.8, glucose 5.6 and $CaCl_2$ 1. Indomethacin (10 μM) was added to the final platelet suspension to prevent the formation of cyclooxygenase products. The platelet count was adjusted to approximately $1.5-2 \times 10^8$ ml^{-1}.

Preparation of smooth muscle or endothelial cells

Bovine aortic EC were harvested and grown on cytodex-3 microcarrier beads (Pharmacia/LKB, Ltd) in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum (Gibco) as described previously (deNucci et al., 1988). The approximate cell number was 3.5×10^7 ml^{-1} of beads. Bovine aortic SMC were characterized by the presence of specific alpha actin using a Sigma kit and were prepared as previously described (Mollace et al., 1991). Indomethacin (10 μM) was added to all final cell suspensions. Cell viability was more than 95% as assessed by the uptake of trypan blue.

Platelet aggregation

A suspension of washed platelets was incubated at 37°C for 4 min in a Payton dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1,000 r.p.m. and then stimulated with thrombin (40 mu ml^{-1}) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3 min incubation period with platelets, the inhibitory effects of 1,2-GDN or 1,3-GDN on platelet aggregation induced by thrombin were measured either alone or in the presence of SMC, EC, N-acetylcysteine (NAC), glutathione (GSH) or thiosalicylic acid (TSA). When required, OxyHb (10 μM) was added to the platelet mixture for the 3 min incubation period. When using cells or OxyHb, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission (Salvemini et al., 1989). Inhibition of platelet aggregation was calculated as described previously (Salvemini et al., 1989).

Nitrite analysis

Nitrite (NO_2^-) was measured by the Griess reaction. 1,2-GDN or 1,3-GDN (75–2400 μM) together with SMC were diluted in Krebs buffer containing indomethacin (10 μM) and superoxide dismutase (SOD) (100 u ml^{-1}) and were then stirred (37°C, 1,000 r.p.m.) for 30 min. The samples were centrifuged and each supernatant allowed to react with the Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) to form a chromophore absorbing at 546 nm. In some experiments, 1,2-GDN or 1,3-GDN (75–600 μM) were incubated in a shaking water bath for 30 min at 37°C in the presence of NAC, GSH or TSA (all at 1 mM) and then allowed to react with the Griess reagent. Nitrite concentration was determined with sodium nitrite as a standard. Results are expressed as nmol NO_2^- mg^{-1} protein or as nmol NO_2^- ml^{-1}. Protein concentrations were determined with bovine serum albumin as a standard (Lowry et al., 1951).

Organ bath experiments

The thoracic aorta of the rabbit was cut into rings 4 mm in width. The rings were cut open, denuded of endothelium and mounted in 20 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs buffer. The composition of the Krebs buffer was as follows (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.17, $CaCl_2.6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Changes in isometric tension were measured with Biegestab K30 type 351 transducers (Hugo Sachs Electronic) attached to MK II transducer coupler (Z.T.S., London, UK) and recorded with Linearcorder mark VII WR3101 (Graphtec). The tissues were equilibrated under resting tension of 2 g for 2 h and the Krebs buffer was changed every 15 min. Tissues were then washed and cumulative concentration-response curves to phenylephrine (0.03–10 μM) were produced. After 1.5 h of washing, the strips were contracted with phenylephrine (0.6–1 μM) to produce a 80–90% maximal contraction. Cumulative concentration-response curves to GTN, 1,2-GDN or 1,3-GDN (10^{-10}–10^{-5} M) were performed. Results are expressed as percentage change in the phenylephrine-induced tone.

Drugs used

Human thrombin, bovine serum albumin, phenylephrine (PHE), haemoglobin (from bovine blood), superoxide dismutase (from bovine erythrocytes), glutathione (GSH), N-acetylcysteine (NAC), thiosalicylic acid (TSA), indomethacin, sulphanilic acid, N-1-naphthyl ethylene diamine hydrochloride, Na_2CO_3, sodium nitrite and kits for characterizing alpha smooth muscle actin (procedure number S1H 903) were obtained from Sigma (Poole, Dorset). Oxyhaemoglobin (OxyHb) was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini et al., 1989). Glyceryl trinitrate (Nitronal) was obtained from Lipha Pharmaceuticals Ltd (West Drayton, Middlesex). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, Kent) and 1,2-glyceryl dinitrate (99.7% pure, 0.3% 1,3-GDN, no other contamination) or 1,3-glyceryl dinitrate (99.97% pure, 0.03% glycerol-1-mononitrate, no other contamination) were gifts from Dr M. Feelisch, Schwarz Pharma AG (Monheim, West Germany).

Statistics

Results are expressed as mean ± s.e.mean for (n) experiments. Student's unpaired t test was used to determine the significance of differences between means, and a P value of <0.05 was taken as significant.

Results

Effects on vascular smooth muscle

1,2-GDN and 1,3-GDN (10^{-10}–10^{-5} M) produced concentration-dependent relaxations of rabbit aortic strips denuded of endothelium (Figure 1). These dinitrates had at least one tenth of the potency of GTN. Thus, the concentrations required to relax the tissues by 50% of maximum (EC_{50}) were 2.5×10^{-8} M for GTN, 3.4×10^{-7} M for 1,2-GDN and 7.5×10^{-7} M for 1,3-GDN (Figure 1).

Nitrite analysis

Exposure of SMC to 1,2-GDN or 1,3-GDN (75–2400 μM) for 30 min at 37°C led to a concentration-dependent increase in NO_2^- formation (Figure 2). When compared to NO_2^- formed from GTN by SMC, the amount of NO_2^- formed from the dinitrates was much smaller (Figure 2). Boiling the cells for 15 min abolished the formation of NO_2^- ($n = 4$, not shown). Exposure of 1,2-GDN or 1,3-GDN (150–600 μM) to NAC, GSH or TSA (all at 1 mM) for 30 min in Krebs buffer at 37°C led to an approximate doubling of the formation of NO_2^- ($n = 4$, not shown). In this respect, GSH and TSA

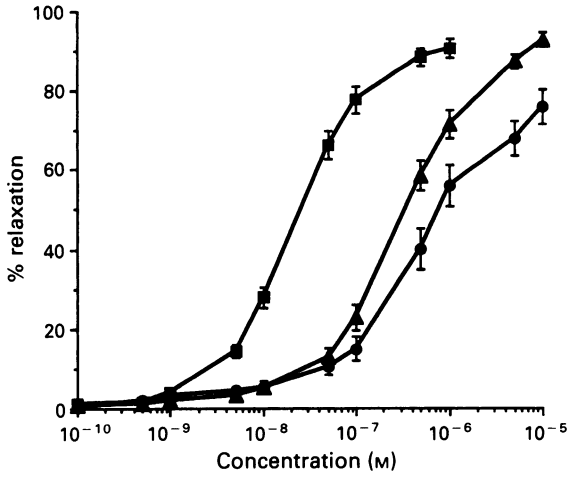

Figure 1 Comparison of the vasorelaxant effects of glyceryl trinitrate (GTN, ■), 1,2-glyceryl dinitrate (1,2-GDN, ▲) or 1,3-GDN (●) on rabbit aortic strips denuded of endothelium. The tissues were preconstricted with phenylephrine (5×10^{-6}–10^{-5} M) to produce approximately a 90–100% of maximum contraction. When the contraction was stable, a cumulative dose-response curve to GTN, 1,2-GDN or 1,3-GDN (all at 10^{-10}–10^{-5} M) was constructed. Results are expressed as % change in the phenylephrine-induced tone. Each point represents the mean ± s.e.mean of 4 experiments performed with aortic strips from different rabbits (average number of observations for each point >8).

were somewhat more potent than NAC. By themselves, the dinitrates did not produce NO_2^- ($n = 4$, not shown).

Effects on platelet aggregation

At concentrations up to 1 mM, 1,2-GDN or 1,3-GDN failed to inhibit platelet aggregation induced by thrombin (40 mu ml^{-1}, $n = 4$, not shown). However, when SMC (0.24–1.92×10^5 cells) or EC (0.8–3.2×10^5 cells) were incubated with platelets for 3 min in the presence of 1,2-GDN or 1,3-GDN (700 μM) inhibition of thrombin (40 mu ml^{-1})-induced platelet aggregation was observed (Figure 3a and b). This potentiating effect was abrogated by OxyHb (10 μM) indicating release of NO. Thus, the combined anti-platelet effect obtained with SMC (0.24×10^5 cells) and 1,2-GDN or

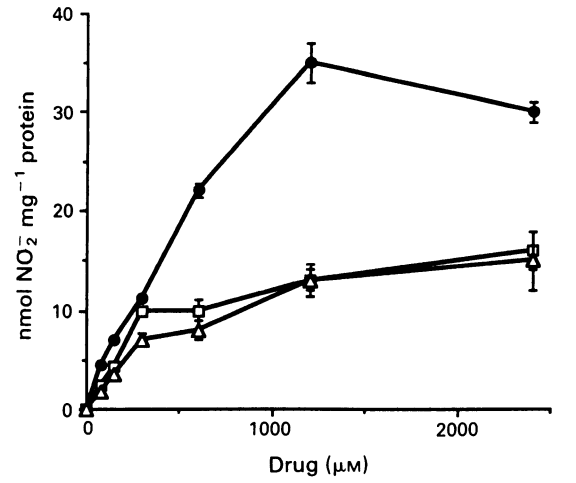

Figure 2 Exposure of SMC to 1,2-glyceryl dinitrate (1,2-GDN, □) or 1,3-GDN (△) (75–2400 μM) for 30 min at 37°C led to a concentration-dependent increase in the formation of NO_2^-. This release of NO_2^- was much smaller than that obtained when SMC were exposed to the same concentrations of glyceryl trinitrate (●). Results are expressed as nmol NO_2^- mg^{-1} protein. Each point is the mean ± s.e.mean of 4 experiments.

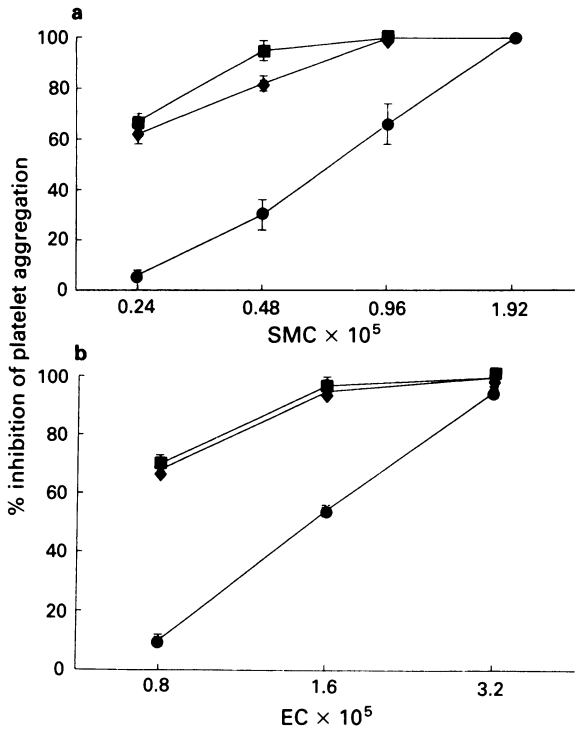

Figure 3 Inhibition of platelet aggregation induced by thrombin (40 mu ml^{-1}) by (a) SMC (0.24–1.92×10^5 cells, ●) or (b) EC (0.8–3.2×10^5 cells, ●) was substantially enhanced in the presence of 700 μM 1,2-glyceryl dinitrate (1,2-GDN, ◆) or 1,3-GDN (■). This effect was reversed by co-incubation with oxyhaemoglobin (10 μM). Results are expressed as % inhibition of platelet aggregation. Each point is the mean ± s.e.mean of 3 experiments.

1,3-GDN (700 μM) was reduced from 62 ± 4% or 67 ± 3% inhibition in the absence of OxyHb to 2 ± 1% or 3 ± 2% inhibition in the presence of OxyHb ($n = 4$, $P < 0.001$); the combined anti-platelet effect obtained with EC (0.8×10^5 cells) and 1,2-GDN or 1,3-GDN (700 μM) was reduced from 68 ± 2% or 70 ± 3% in the absence of OxyHb to 3 ± 2% or 4 ± 2% inhibition in the presence of OxyHb ($n = 4$, $P < 0.001$). The concentration required to inhibit platelet aggregation by 50% (IC_{50}) was 480 ± 20 μM or 750 ± 50 μM ($n = 4$) for 1,2- or 1,3-GDN in the presence of SMC (0.24×10^5 cells) and 600 ± 35 μM or 810 ± 35 μM ($n = 4$) for 1,2- or 1,3-GDN respectively in the presence of EC (0.4×10^5 cells).

Table 1 Effects of oxyhaemoglobin (OxyHb) on the combined anti-platelet effects of 1,2-glyceryl dinitrate (1,2-GDN) or 1,3-GDN and N-acetylcysteine (NAC), glutathione (GSH) or thiosalicylic acid (TSA)

	None	*NAC*	*GSH*	*TSA*
1,2-GDN	0	89 ± 1	8 ± 1	86 ± 6
1,2-GDN + OxyHb	0	4 ± 1*	3 ± 1	12 ± 4*
1,3-GDN	0	85 ± 2	7 ± 2	84 ± 5
1,3-GDN + OxyHb	0	6 ± 1*	2 ± 2	9 ± 2*

OxyHb (10 μM) reversed the inhibition of thrombin-induced platelet aggregation by 1,2-GDN or 1,3-GDN (both at 700 μM) in the presence of NAC or TSA; GSH (0.5 mM) did not potentiate the effects of 1,2-GDN or 1,3-GDN. Resutls are expressed as % inhibition of platelet aggregation. Each value is the mean ± s.e.mean of 4 experiments.
*$P < 0.01$ when compared to the values obtained in the absence of OxyHb.

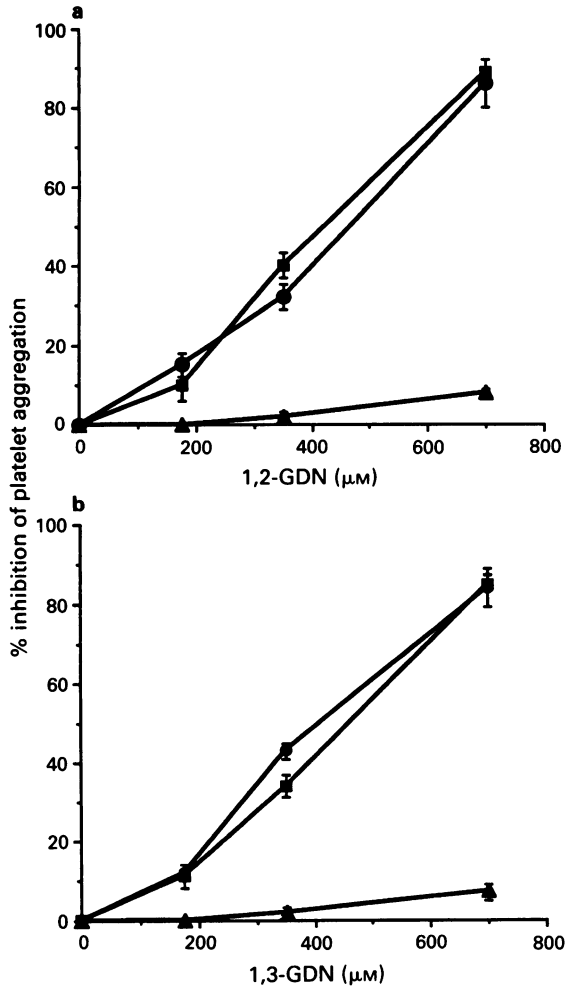

Figure 4 (a) 1,2-glyceryl dinitrate (1,2-GDN) or (b) 1,3-GDN at concentrations up to 700 μM did not inhibit thrombin-induced platelet aggregation. Incubation of the dinitrates (175–700 μM) with 0.5 mM N-acetylcysteine (■) or thiosalicylic acid (●) led to a concentration-dependent inhibition of platelet aggregation. Glutathione (▲) had no potentiating effect on either dinitrate (a and b). Results are expressed as % inhibition of platelet aggregation. Each point is the mean ± s.e.mean of 3 experiments.

Potentiation by thiols

NAC or TSA (both at 0.5 mM) potentiated the effects of 1,2-GDN or 1,3-GDN (175–700 μM) on thrombin-induced platelet aggregation in the absence of SMC or EC (Figure 4a and b). The IC_{50} was 422 ± 10 μM and 470 ± 10 μM for 1,2-GDN or 1,3-GDN in the presence of NAC and 480 ± 5 μM or 420 ± 15 μM for 1,2-GDN or 1,3-GDN in the presence of TSA. The inhibition of platelet aggregation observed with either dinitrate in the presence of NAC or TSA was abolished by co-incubation with OxyHb (10 μM; Table 1). In contrast to NAC or TSA, GSH (at 0.5 mM) had no effect on inhibition of platelet aggregation in the presence of the dinitrates ($n = 4$, Table 1).

Discussion

The activity of GTN and other organic nitrates such as isosorbide dinitrate (ISDN) require bioconversion to NO in order to elicit vasodilatation (Ignarro *et al.*, 1981; Murad, 1986) and inhibition of platelet aggregation (Benjamin *et al.*, 1991; Salvemini *et al.*, 1992a); they can therefore be considered as prodrugs. These effects of NO are the result of stimulation of the soluble guanylate cyclase and increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Mellion *et al.*, 1980; Ignarro *et al.*, 1981). The biological effects of NO are abrogated by OxyHb which oxidizes NO to nitrate (Haussmann & Werringloer, 1985). GTN has three nitrate groups and there is, therefore, the possibility of a cascade reaction generating NO in turn from GTN, then from GDN and finally from glyceryl mononitrate. The main finding of this study is that the second reaction can take place in endothelial cells and in vascular smooth muscle cells. Thus, the two metabolites of GTN, 1,2-GDN and 1,3-GDN are converted to NO by endothelial cells and vascular smooth muscle cells causing vasorelaxation and inhibition of thrombin-induced platelet aggregation. Earlier studies have shown a correlation between the vasorelaxant effects of GTN with increase in tissue levels of cyclic GMP and the formation of the 1,2-GDN and 1,3-GDN. Increase in cyclic GMP and appearance of GDNs preceded vasorelaxation (Brien *et al.*, 1986; 1988; Kawamoto *et al.*, 1990). Taken together the data suggest that the metabolites of GTN following their bioconversion to NO, play a role in the pharmacological action of their parent compound.

In our studies 1,2-GDN was found to be somewhat more potent than 1,3-GDN on vascular smooth muscle. Biotransformation of GTN in vascular smooth muscle preferentially gives rise to the 1,2-GDN but the ratio between 1,2-GDN/1,3-GDN varies depending upon the type of vascular smooth muscle tested (Brien *et al.*, 1988; Slack *et al.*, 1989; Kawamoto *et al.*, 1990). In man, oral intake of GTN leads to two fold higher levels of 1,2-GDN as compared to 1,3-GDN, indicating that of the two metabolites, 1,2-GDN is probably the most important (Kwon *et al.*, 1992). The concentrations at which the GDNs exerted their vasorelaxant and antiplatelet effects were 10–20 times higher than those of GTN (Salvemini *et al.*, 1992a). The reason for this lower activity is not known but may be related to the lower lipid solubility of the GDNs as compared to GTN (Fung, 1991). For example, the lipohilic isosorbide dinitrate is about ten times more potent than the more polar isosorbide-5-mononitrate (see Ahlner *et al.*, 1991 for review). The difference in lipid solubility and, therefore in potency is compensated for by the longer half-lives of the more polar metabolites. Thus, GTN has a very short half-life, (1–2 min) whereas the GDNs have half-lives of 30–40 min (see Ahlner *et al.*, 1991 for review). Following a constant intravenous infusion of GTN or application of GTN patch, the levels of GTN reach a steady state within minutes whereas the levels of the GDNs accumulate to a steady state within about 2–3 h (Lee *et al.*, 1990; Nakashima *et al.*, 1990). The resulting blood levels of GDNs are 10–20 times higher than those of GTN. Inhibition of platelet aggregation following intravenous infusion of GTN has been shown to be better correlated to the levels of GDNs than to the levels of GTN (Karlberg *et al.*, 1992). Thus, the results of our studies agree well with pharmacokinetic data supporting a role for GDNs in the effects of intravenous GTN.

The role of the GDNs becomes even more important following oral administration of GTN. As pointed out already by Needleman and coworkers (Needleman *et al.*, 1969; Needleman, 1975) and later confirmed by others (see Ahlner *et al.*, 1991 for review), oral GTN is completely removed by first pass metabolism leading to the formation of GDN metabolites. Following oral administration of GTN in doses giving prolonged (6–8 h) pharmacological effects, no plasma levels of GTN could be found (Nyberg & Westling, 1981; Kwon *et al.*, 1992). However, under these circumstances, the plasma levels of its metabolites were of the same order of magnitude as those which, when given intravenously, depressed blood pressure and increased pulse rate (Lau *et al.*, 1991; Gumbleton & Benet, 1991). Following oral GTN treatment the GDN metabolites should, therefore, be responsible for most of the clinical effects of GTN.

In the present study we have also provided evidence for release of NO from the GDNs following their interaction

with thiols. It was already known that GTN can generate NO both enzymatically in various cells and non-enzymatically by the action of thiols (Gruetter & Lemke, 1985; Sakanashi *et al.*, 1991; Chung & Fung, 1990; 1991; Feelisch & Kelm, 1991; Salvemini *et al.*, 1992a,b). Here we found that TSA and NAC but not GSH can release NO from the GDNs (as indicated by an OxyHb-sensitive inhibition of platelet aggregation). Glutathione is more potent than NAC in releasing NO_2^- from the GDNs but it failed to potentiate their anti-platelet effects, indicating that GSH does not form NO from the GDNs. Thus, the effects of GSH on NO and NO_2^- release from the dinitrates are similar to its effects on GTN (Feelisch & Noack, 1987; Feelisch *et al.*, 1988; Salvemini *et al.*, 1993). TSA produced equal amounts of NO_2^- to those produced by GSH, but in contrast to GSH it was as effective as NAC (which produced less NO_2^- than TSA) in potentiating the anti-platelet effects of the GDNs and thus in releasing NO. This indicates that as for GTN (Chung & Fung, 1991; Salvemini *et al.*, 1993), there is a preferential release of NO from the molecule of the GDNs and this preferential release depends on the type of thiol used.

Our study has therefore raised the possibility that bioconversion of the GDNs in EC or SMC to form the active species NO may contribute in part to the pharmacological actions of GTN. Furthermore the findings that thiols can also form NO from the metabolites of GTN reinforces the proposal that thiol-containing compounds with high catalytic effects with respect to NO formation may be useful for the management of GTN tolerance (Salvemini *et al.*, 1993).

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Study of the mechanism of the relaxant action of (+)-glaucine in rat vas deferens

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1 Effects of the aporphinoid alkaloid, (+)-glaucine, on rat vas deferens were investigated.

2 (+)-Glaucine (2–18 μM) competitively inhibited contractions induced by noradrenaline and methoxamine with a pA_2 value of about 6.

3 (+)-Glaucine (2 and 18 μM) did not change the accumulation of tritium during incubation of the vas deferens with [^{3}H]-noradrenaline.

4 (+)-Glaucine (0.3 nM–0.1 mM) inhibited specific [^{3}H]-prazosin binding to membranes from rat vas deferens with a pK_i value of 6.63, which is close to the pA_2 value obtained against noradrenaline and methoxamine in functional studies.

5 In electrically-stimulated rat vas deferens, (+)-glaucine (0.3–10 μM) enhanced twitch contractions and competitively antagonized the inhibitory effect of clonidine with a pA_2 value of 5.91.

6 In tissues incubated in depolarizing calcium-free high-potassium medium, (+)-glaucine (30–80 μM) inhibited Ca^{2+}-induced contractions with depression of the maximal response at higher doses and with a pD'_2 value of 3.65. Furthermore, (+)-glaucine (50 μM) did not modify basal ^{45}Ca uptake but strongly inhibited the influx of ^{45}Ca induced by K^+.

7 These results suggest that (+)-glaucine has non-selective α_1- and α_2-adrenoceptor blocking properties. At higher doses, (+)-glaucine shows calcium antagonist activity which may be responsible, at least in part, for the inhibition of the contractions induced by Ca^{2+} in calcium-free high-potassium medium.

Keywords: Rat vas deferens; (+)-glaucine; α_1-adrenoceptor blocking agents; calcium antagonist activity

Introduction

(+)-Glaucine [(S)-1,2,9,10-tetramethoxyaporphine] is an aporphinoid alkaloid isolated from the above-ground parts of *Glaucium flavum* Crantz (Papaveraceae) (Ivanov & Ivanova, 1958). It is structurally related to papaverine and displays a range of pharmacological actions including antitussive (Kasé *et al.*, 1983), analgesic and central nervous system depressor activities (Petkov *et al.*, 1979; Berthe *et al.*, 1983).

Like papaverine, (+)-glaucine relaxes vascular and non-vascular smooth muscle. The mechanism of this activity is unknown and seems to be different from that of papaverine (Cortés *et al.*, 1990). Several hypotheses have been advanced, including an inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase in intestinal smooth muscle (Petkov & Stancheva, 1980) and bovine aorta (Ivorra *et al.*, 1992). In rat uterus, (+)-glaucine shows relaxant activity, possibly related to inhibition of Ca^{2+} entry through potential-operated Ca^{2+} channels (Anselmi *et al.*, 1992). Similar Ca^{2+}-antagonist properties have been described in rat cerebral cortex, where (+)-glaucine inhibits [^{3}H]-(+)-*cis*-diltiazem binding (Ivorra *et al.*, 1992). The relaxant action of (+)-glaucine in rat aorta seems to be related to α_1-adrenoceptor blockade because it inhibits the contractile response induced by noradrenaline more strongly than that induced by KCl (Orallo *et al.*, 1991; Ivorra *et al.*, 1992). Results obtained in rat cerebral cortex (Loza *et al.*, 1991; Ivorra *et al.*, 1992), where (+)-glaucine blocks [^{3}H]-prazosin binding, are also in favour of an adrenoceptor blocking action.

In view of these reports and with the aim of elucidating the mechanism of the spasmolytic action of (+)-glaucine in non-vascular smooth muscle, we have studied the effect of this alkaloid on: (a) tension responses to noradrenaline, methoxamine and $CaCl_2$ (in calcium-free high-potassium medium), on neuronal uptake of [^{3}H]-noradrenaline and on ^{45}Ca influx (basal and K^+-induced) in rat vas deferens, (b) specific binding of [^{3}H]-prazosin in rat vas deferens cell membranes and (c) contractions in electrically-stimulated preparations.

Methods

Male Sprague-Dawley rats (250–300 g) were killed by a blow on the head and exsanguinated. Whole vasa deferentia were removed, placed in a Petri dish with Krebs bicarbonate solution [composition mM: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, disodium salt of ethylenediaminetetraacetic acid (EDTA) 0.03, ascorbic acid 0.56 and glucose, 11] and cleaned of connective tissue and blood vessels.

Contraction studies

General procedure The isolated organ was set up in an organ bath containing 10 ml Krebs bicarbonate solution thermoregulated at 37°C and bubbled with carbogen (95% O_2 + 5% CO_2). The preparation was equilibrated at a resting tension of 0.5 g for at least 1 h, during which the physiological solution was replaced every 10 min. Tension responses were measured by means of a Letica TRI 110 isometric transducer and recorded on a Letica Unigraph 1000-506 polygraph.

Postjunctional functional studies Cumulative concentration-response curves were obtained by the method of Van Ros-

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sum (1963) administering the next higher dose after a steady state level had been reached. In experiments with noradrenaline, four consecutive concentration-response curves were obtained at 60-min intervals to allow washout and to minimize the possibility of receptor desensitization; the first curve differed from the last three, which were reproducible. In the contralateral preparation, after obtaining two similar control curves with the agonist, tissues were incubated with (+)-glaucine for 10 min (a sufficient exposure time to achieve equilibrium) and a fourth curve was constructed. In experiments with methoxamine, successive concentration-response curves determined in one preparation were not reproducible. Control curves and (+)-glaucine curves were therefore constructed in different preparations. The alkaloid was administered 10 min before construction of the curve.

Calcium concentration-response curves were determined as follows. After an equilibration period of 1 h in Krebs bicarbonate solution, tissues were incubated for 30 min in calcium-free depolarizing Krebs bicarbonate solution (containing 50 mM KCl instead of the equivalent amount of NaCl). Calcium chloride (100 μM–30 mM) was then added to the bath in stepwise fashion. After two reproducible control concentration-response curves had been obtained, (+)-glaucine was added 10 min before initiation of a third curve.

Prejunctional functional studies Platinum ring electrodes were placed above and below the vas deferens, and continuous field stimulation was applied by 2 ms square wave pulses at supramaximal voltage (40–60 V) and a frequency of 0.1 Hz (Narco Sl-10 stimulator). In the first set of experiments, carried out when the twitch response to field stimulation had become stable, (+)-glaucine was added to the bath in a stepwise cumulative fashion in order to evaluate potentiation of the response. In the second set of experiments, cumulative clonidine concentration-response curves were obtained by Van Rossum's method (1963), following 10 min of exposure to a single dose of (+)-glaucine to minimize the possibility of receptor desensitization. The control response to clonidine was obtained in the contralateral preparation. The antagonist potency of (+)-glaucine against clonidine in each preparation was evaluated as pA_2 calculated from Schild plots (Arunlakshana & Schild, 1959).

[^{3}H]-noradrenaline uptake

After an initial 60-min equilibration period in Krebs bicarbonate solution containing β-oestradiol (10 μM) to block extraneuronal uptake, maintained at 37°C and bubbled with carbogen, the vas deferens was incubated in the same solution with 32 nM L-[7,8-^{3}H]-noradrenaline (specific activity 9.3 Ci mmol^{-1}) for 60 min. To investigate the action of (+)-glaucine, the alkaloid was added to the Krebs solution 15 min before the start and during the incubation with [^{3}H]-noradrenaline. At the end of each experiment, tissues were removed, blotted dry, weighed and digested in 1 ml H_2O_2 (110 volumes) at 115°C for 90 min. After cooling, 5 ml of Ready Safe HP Beckman was added and the radioactivity measured in a liquid scintillation counter (Beckman LS 3801).

^{45}Ca influx

Tissues were equilibrated for at least 60 min in Krebs bicarbonate solution (containing 0.2 mM instead of 2.5 mM $CaCl_2$) maintained at 37°C and bubbled with carbogen. Afterwards, the tissues were incubated for 5 min in a solution containing 0.18 μM ^{45}Ca (specific activity 28.60 mCi mg^{-1}) with or without K^+ (50 mM). To investigate the effect of (+)-glaucine on basal and induced ^{45}Ca uptake, the alkaloid was added to the bath 15 min before and during incubation with ^{45}Ca.

Preparations were then washed for 30 min in 250 ml of La^{3+} solution [composition mM: NaCl 119, KCl 4.7, tris(hydroxymethyl)-aminomethane 5, $MgSO_4$ 1.2, $LaCl_3$ 50 and glucose 11 (pH = 6.8)] in order to remove extracellular Ca^{2+} from the tissue. Tissues were then removed, blotted dry, weighed and digested in H_2O_2 and their radioactivity measured following the procedures described above.

[^{3}H]-prazosin binding

The potencies of drugs in competing for the specific [^{3}H]-prazosin binding were determined as described previously (Sallés & Badía, 1991). Crude membrane preparations obtained from a pool of six whole vasa deferentia were used in a single experiment. Briefly, tissues were homogenized in 10 ml of ice cold buffer (50 mM Tris-HCl, pH 7.5) with a polytron type homogenizer ($Rafer_{10/20}$, setting 6,2 × 15 s). The homogenate was filtered through a double layer of surgical gauze and centrifuged at 50,000 *g* for 20 min at 4°C. The resulting pellet was washed twice by resuspension and centrifugation under the same conditions. The final pellet was resuspended in incubation buffer to give a final protein concentration of approximately 2 mg ml^{-1}.

The potencies of (+)-glaucine, (±)-WB 4101 and prazosin in competing for specific [^{3}H]-prazosin binding sites were determined in triplicate by incubation of 100 μl of tissue preparations with a single concentration (0.2–0.3 nM) of [^{3}H]-prazosin (specific activity 76 Ci mmol^{-1}) in the presence or absence of 13–15 concentrations of drugs in a final volume of 0.5 ml. After 45 min of incubation at 25°C, the reaction was terminated by addition of 5 ml of the same buffer and rapid filtration over a glass fibre filter (Whatman GF/C) using a Brandel M24R cell harvester. The filters were washed 3 times with 5 ml of 50 mM Tris-HCl (pH 7.5) and subsequently dried at 65°C for 2 h. The radioactivity retained in the filters was determined in a liquid scintillation counter (LKB 1209 Rackbeta) with an efficiency of 50–60%. Phentolamine (10 μM) was used to define non-specific binding which was usually less than 25% of total binding. Membrane protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Expression and statistical analysis of results

Unless otherwise specified, results shown in the text and figures are expressed as means ± s.e.mean. Statistical differences between two means ($P<0.05$ or $P<0.01$) were determined by Student's two-tailed *t* test for paired or unpaired data.

Ligand binding data were analysed with a computerized curve-fitting programme (Graphpad Inplot). Competition data were first fitted to a one- and then a two-site model and *F*-test analysis was used to decide whether a model of one or two binding site fit was more appropriate ($P<0.05$). IC_{50} values were transformed into K_i values as described by Cheng & Prussoff (1973). Pseudo Hill coefficients (n_H), $pK_{i\ high}$ and $pK_{i\ low}$ (− log K_i for high or low affinity sites, respectively) were also determined.

In functional postsynaptic studies, contractile responses to constrictor agents (in the presence or absence of (+)-glaucine) are expressed as a percentage of the maximal response to the agonist in the absence of (+)-glaucine. In presynaptic studies, responses are expressed as a percentage of the control response to a single-stimulus pulse in the absence of (+)-glaucine or yohimbine.

Constrictor agent pD_2 values (negative $\log_{10}$ of the molar concentration of agonist required to elicit 50% of the maximal response) were calculated (Van Rossum, 1963). (+)-Glaucine pA_2 values were obtained according to Arunlakshana & Schild (1959), by linear regression of Schild plots derived from mean concentration-response curves; 95% confidence intervals for both pA_2 and slopes are included where appropriate (calculated according to Tallarida & Murray, 1987). Antagonist pD'_2 value (negative $\log_{10}$ of the molar concentration of antagonist required to cause a 50% depression of the maximal response of the agonist) was calculated

as the x-intercept of the regression of log (X-1) on $-\log$ [antagonist], where X is the ratio (obtained from mean concentration-response curves) of the maximal control response to the agonist over the maximal response to the agonist in the presence of the antagonist. Again, 95% confidence interval for pD'_2 value is included where appropriate.

[^{3}H]-noradrenaline uptake was calculated from the formula: [^{3}H]-noradrenaline uptake [nmol (kg wet tissue)$^{-1}$] = [counts per minute (c.p.m.) in tissue (wet tissue weight, kg)$^{-1}$] × [nmol [^{3}H]-noradrenaline in 1 litre solution (c.p.m. in 1 litre of solution)$^{-1}$].

^{45}Ca tissue uptake was calculated from the formula: ^{45}Ca uptake [nmol (kg wet tissue)$^{-1}$ = [cpm in tissue (wet tissue weight, kg)$^{-1}$] × [nmol ^{45}Ca in 1 litre solution (c.p.m. in 1 litre of solution)$^{-1}$]. Note that the numerator of the second factor in this expression is the concentration of ^{45}Ca, not the total Ca^{2+} concentration.

Drugs, chemicals and radioisotopes

The following drugs were used: (+)-glaucine hydrochloride (isolated from *G. flavum* in the Laboratory of Organic Chemistry of the University of Santiago de Compostela), (−)-noradrenaline bitartrate (Sigma), (±)-methoxamine hydrochloride (Gayoso-Wellcome), clonidine hydrochloride (Boehringer-Ingelheim), β-oestradiol (Sigma), prazosin hydrochloride (Pfizer), (−)-cocaine hydrochloride (Abelló), phentolamine hydrochloride (Ciba-Geigy), yohimbine hydrochloride (Sigma) and (±)-WB 4101 [2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride] (Research Biochemicals). The radioisotopes used were ^{45}Ca (New England Nuclear), L-[7,8-^{3}H]-noradrenaline (Amersham International) and [^{3}H]-prazosin (New England Nuclear).

(+)-Glaucine and cocaine were dissolved in de-ionized water immediately before use. β-Oestradiol was dissolved in 95% ethanol to make a stock solution of 10 mM, and aliquots of this solution were then diluted with de-ionized water prior to use. (−)-Noradrenaline bitartrate and (±)-methoxamine hydrochloride were prepared daily in de-ionized water from stock solutions (10 mM) kept at −20°C. Sodium bisulphite (0.2%) was added to the noradrenaline stock solution to prevent oxidation. Clonidine hydrochloride, phentolamine hydrochloride, yohimbine hydrochloride and prazosin hydrochloride were prepared daily from a stock solution (1 mM) and kept at −20°C.

Results

The preparation lacked spontaneous activity. Resting tone was unaffected by (+)-glaucine (0.5–80 μM) ($n = 5$).

Contraction studies

Noradrenaline elicited dose-related contractions. The pD_2 and maximal tension (mg) values were 5.46 ± 0.10 and 830 ± 60, respectively ($n = 5$). (+)-Glaucine (2–18 μM) shifted the concentration-response curve for noradrenaline to the right, with an increase of the maximum effect (Figure 1a). The pA_2 value was 5.88 (4.60 to 7.16) and the slope of the Schild plot [−0.95 (−2.54 to 0.63)] did not differ significantly from unity ($P > 0.05$, $n = 5$).

Methoxamine produced concentration-dependent contraction in the rat vas deferens. The pD_2 value was 5.13 ± 0.15 and the maximal tension (mg) reached was 710 ± 64 ($n = 5$). (+)-Glaucine (2–8 μM) caused a shift to the right of the concentration-response curve without depression of the maximum response (Figure 1b). The pA_2 value was 6.14 (4.95 to 7.34), which does not differ significantly from that obtained against noradrenaline ($P > 0.05$) and the slope [−0.97 (−2.46 to 0.51) did not differ significantly from unity ($P > 0.05$, $n = 5$).

In calcium-free high-potassium (50 mM) medium, addition of calcium to the bath induced a gradual increase in tension. The maximal tension reached was 500 ± 46 mg and the EC_{50} value (50% effective concentration) was 1.66 ± 0.14 mM ($n = 5$). (+)-Glaucine (30–80 μM) antagonized non-competitively the Ca^{2+}-induced contractions with depression of the

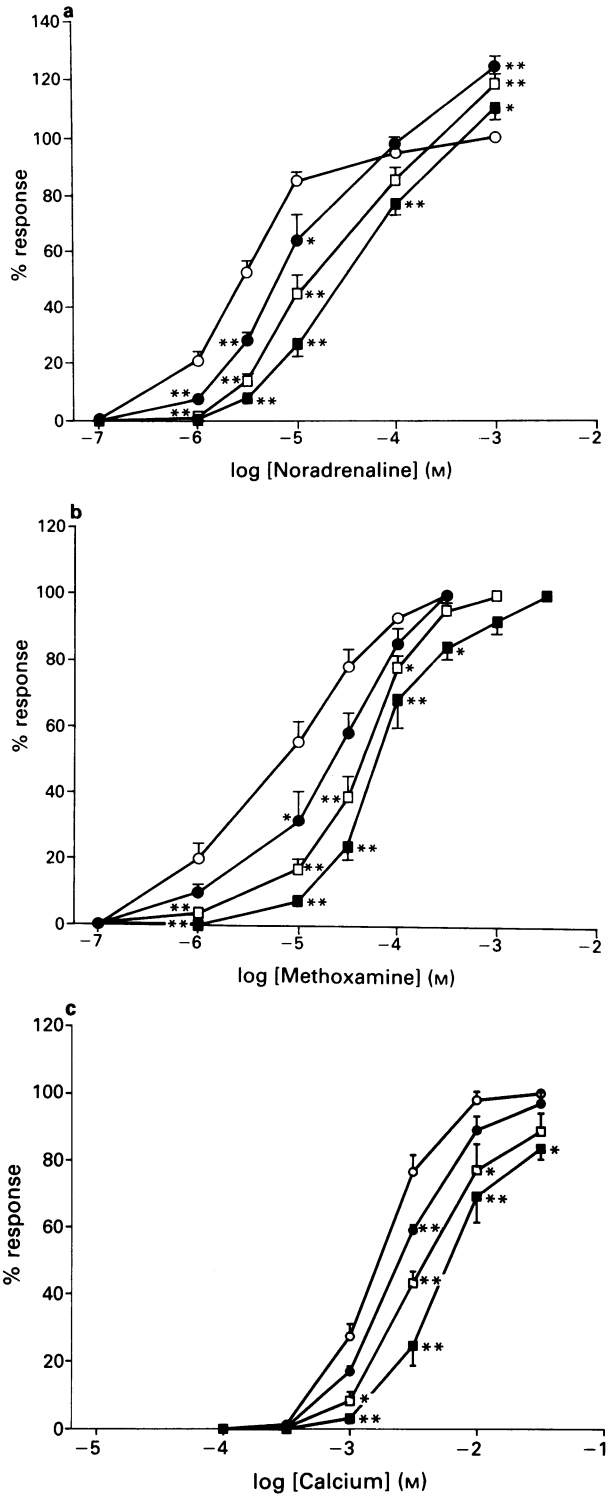

Figure 1 Cumulative concentration-response curves for (a) noradrenaline (○) in the absence or presence of (+)-glaucine (●, 2 μM; □, 6 μM and ■, 18 μM); (b) methoxamine (○) in the absence or presence of (+)-glaucine (●, 2 μM; □, 4 μM and ■, 8 μM); (c) Ca^{2+} in calcium-free high-potassium solution (○) in the absence or presence of (+)-glaucine (●, 30 μM; □, 50 μM and ■ 80 μM). Each point represents the mean value ± s.e.mean (indicated by vertical bars) from 5 experiments. Level of statistical significance with respect to control curves: $^{**}P < 0.01$ or $^{*}P < 0.05$.

maximal response at higher doses (Figure 1c). The pD'_2 value was 3.65 (3.13 to 4.18, $n = 5$).

Low frequency stimulation of the intramural axons of the rat vas deferens (0.1 Hz) produced regular contractions (476 ± 27 mg) ($n = 5$). The preparation was very stable with no spontaneous changes in resting tension, and the contractions remained constant for at least 3–4 h. (+)-Glaucine (0.3–10 μM) dose-dependently enhanced the twitch contractions induced by continuous field stimulation, without increasing basal tension. The maximum percentage increase (with respect to the control response to a single pulse in the absence of (+)-glaucine) was 41.3 ± 4.3%, and the EC_{50} (concentration required to reach 50% of the maximum effect) was 0.46 ± 0.02 μM.

Yohimbine (50 nM–0.5 μM) did not affect twitch contractions, whereas at the dose of 1 μM it inhibited twitch contractions by 17.0 ± 2.5% ($P < 0.05$, $n = 5$). At the same dosage, yohimbine (1 μM) did not antagonize the potentiation of twitch contractions induced by (+)-glaucine; the EC_{50} was 0.48 ± 0.06 μM, which does not differ significantly from the value obtained in the absence of yohimbine ($P > 0.05$, $n = 5$).

Electrically-induced contractions of whole vas deferens were inhibited by low concentrations of clonidine (1–30 nM) with a pD_2 value of 8.52 ± 0.16 ($n = 5$). (+)-Glaucine (0.5–3 μM) shifted the clonidine concentration-response curves to the right, without depression of the maximal response. The pA_2 value [5.91 (4.81 to 7.00)] was similar to those obtained in postsynaptic functional studies ($P > 0.05$, $n = 5$) and the value of Schild plot slope, − 1.28 (− 5.64 to 3.07), did not differ significantly from unity ($P > 0.05$, $n = 5$) (Figure 2).

[^{3}H]-noradrenaline uptake

Control tissues accumulated an amount of radioactivity corresponding to 206 ± 31 nmol kg^{-1} ($n = 5$) during a 60 min period of exposure to [^{3}H]-noradrenaline. This accumulation was markedly inhibited by cocaine 10 μM (93.6 ± 7.2 nmol kg^{-1}; $P < 0.01$, $n = 5$). (+)-Glaucine (2 and 18 μM) did not change the accumulation of tritiated material (203 ± 19 and 198 ± 21 nmol kg^{-1}, respectively; $P > 0.05$ with respect to control, $n = 8$).

^{45}Ca Influx

Basal uptake of ^{45}Ca by rat vas deferens during a 5 min incubation period was 37.1 ± 1.7 nmol kg^{-1}. (+)-Glaucine (10 and 50 μM) did not affect this value significantly (41.8 ± 1.4 and 42.0 ± 1.3 nmol kg^{-1}, respectively; $P > 0.05$, $n = 5$).

High K^+ (50 mM) significantly increased ^{45}Ca uptake (116.8 ± 5.7 nmol kg^{-1}; $P < 0.01$, $n = 5$). (+)-Glaucine (50 μM), added 15 min before high K^+, considerably reduced this value (67.8 ± 2.7 nmol kg^{-1}; $P < 0.01$, $n = 5$). A lower dose of (+)-glaucine (10 μM) had no significant inhibitory effect (112.2 ± 6.5; $P > 0.05$, $n = 5$).

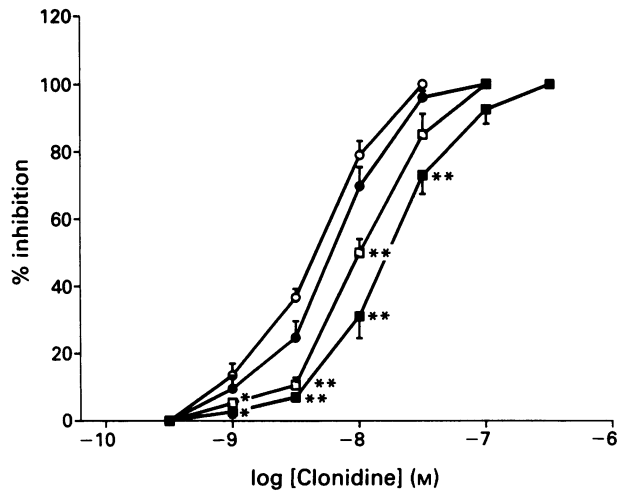

Figure 2 Concentration-response curves for the inhibitory effect of clonidine (○) on electrically evoked twitch contractions of rat vas deferens and its antagonism by (+)-glaucine (●, 0.5 μM; □, 1 μM and ■, 3 μM). Each point represents the mean ± s.e.mean of 5 experiments. Level of statistical significance with respect to curves without (+)-glaucine: $^{**}P < 0.01$ or $^{*}P < 0.05$.

Table 1 Inhibition of specific [^{3}H]-prazosin binding to α_1-adrenoceptors of whole rat vas deferens by antagonists

Antagonist	n	n_H	$pK_{i\ high}$	$pK_{i\ low}$	$\%R_{high}$
Prazosin	5	0.97 ± 0.08	9.43 ± 0.03	–	100
(±)-WB 4101	5	0.70 ± 0.04	9.89 ± 0.03	8.59 ± 0.13	37.4
(+)-Glaucine	6	0.92 ± 0.04	6.63 ± 0.10	–	100

Data are expressed as means ± s.e.mean of n experiments and carried out with a membrane pool from 6 vasa deferentia.

[^{3}H]-prazosin binding

Specific [^{3}H]-prazosin binding to membranes from whole rat vas deferens was inhibited by (+)-glaucine (0.3 nM–0.1 mM), prazosin (0.03 nM–0.1 μM) and (±)-WB 4101 (3 pM–3 μM). Unlabelled prazosin and (+)-glaucine inhibited specific [^{3}H]-prazosin binding in a monophasic manner with a single pK_i value for each drug (Table 1). In contrast, the inhibition curves for (±)-WB 4101 were shallow and analysis revealed that data were better fitted by a two site model. In these experiments the absolute value of [^{3}H]-prazosin binding averaged 2391 ± 90 d.p.m. and non-specific binding in presence of phentolamine (10 μM) averaged 579 ± 45 d.p.m. in the same preparations ($n = 48$).

Discussion

In the present work, we have investigated the relaxant effect of (+)-glaucine on rat vas deferens. Our results show that (+)-glaucine inhibits the contractions induced by noradrenaline and methoxamine more strongly than those induced by Ca^{2+} in calcium-free high-potassium medium. This may be due to an antagonism at postjunctional α_1-adrenoceptors because (+)-glaucine caused a shift to the right of the noradrenaline and methoxamine concentration-response curves, without depression of the maximal response. The slope values in Schild plots were not significantly different from unity, suggesting competitive antagonism. Furthermore, the pA_2 values of (+)-glaucine against methoxamine (a selective α_1-adrenoceptor agonist) and noradrenaline (an α_1- and α_2-adrenoceptor agonist) (Starke *et al.*, 1975) were similar, in accordance with the view that the alkaloid interacts with α_1-adrenoceptors. Similar results have been reported by Orallo *et al.* (1991) in rat aorta.

(+)-Glaucine increased the maximal response to noradrenaline. This phenomenon, which has been described previously for certain α-adrenoceptor antagonists (Jurkiewicz & Jurkiewicz, 1976; Roquebert *et al.*, 1981), could be due to an interference with the neuronal noradrenaline uptake process (Furchgott, 1972; Kenakin, 1985) because it was not observed with noradrenaline in preparations preincubated with cocaine (data not shown) or with methoxamine, which is not taken up into noradrenergic neurones. However, (+)-glaucine did not reduce the accumulation of tritium in tissues exposed to [^{3}H]-noradrenaline and, hence, presumably did not significantly inhibit this uptake mechanism. Other mechanisms are therefore implicated, such as a sensitization to the effects of noradrenaline (see Jurkiewicz & Jurkiewicz, 1976).

There is increasing evidence for the existence of different

α_1-adrenoceptor subtypes in several tissues, including the rat vas deferens (Morrow & Creese, 1986; Gross *et al.*, 1988; Hanft & Gross, 1989; Hanft *et al.*, 1989; Sallés & Badía, 1991). In order to investigate in greater depth whether (+)-glaucine was able to block the α_1-adrenoceptor and to discriminate between these binding sites, radioligand binding assays were performed. Our results indicate that (+)-glaucine, like prazosin, interacts with [^{3}H]-prazosin binding in a monophasic manner whereas the curves for (±)-WB 4101 revealed the existence of two binding sites. These results confirm and extend previous results obtained in rat cerebral cortex (Loza *et al.*, 1991; Ivorra *et al.*, 1992). The pK_i value for (+)-glaucine inhibition corresponded approximately to the pA_2 value obtained in functional studies.

Besides blocking α_1-adrenoceptors, (+)-glaucine may also have α_2-adrenoceptor antagonistic properties. Therefore, we evaluated the effects of (+)-glaucine on contraction and cumulative clonidine concentration-response curves in the electrically-stimulated rat vas deferens. It has been shown that noradrenaline and ATP (or a related nucleotide) are postganglionic sympathetic co-transmitters in rat vas deferens (Mallard *et al.*, 1992) and that under our experimental conditions (low frequencies and pulse trains of short duration) the twitch contractions are mainly due to ATP release (see Von Kügelgen & Starke, 1991), which can be inhibited presynaptically by agents such as clonidine, via activation of α_2-adrenoceptors (Doxey *et al.*, 1977; Drew, 1977; MacDonald & McGrath, 1980), possibly α_{2D}-adrenoceptors (Smith & Docherty, 1992). Our results shows that (+)-glaucine enhances the contractions of electrically-stimulated rat vas deferens, reverses (data not shown) and competitively antagonizes the inhibition produced by clonidine, with a pA_2 very similar to those obtained against noradrenaline and methoxamine in non-stimulated rat vas deferens. This suggests that (+)-glaucine is equally effective in blocking presynaptic (α_2) and postsynaptic (α_1) adrenoceptors. The mechanism of enhancement of neurogenic twitches by (+)-glaucine is not clear and may be related to: (a) an increase of the noradrenaline and/or ATP concentration in the synaptic biophase (via presynaptic α_2-adrenoceptor blockade and/or glaucine-related reduction in neurotransmitter degradation or ATP uptake); (b) sensitization to the effects of noradrenaline and/or ATP (see above). However, the α_2-adrenoceptor blockade hypothesis is unlikely because yohimbine (1 μM), which on its own inhibited twitch contractions (Drew, 1977), did not modify the enhancement of twitches by (+)-glaucine.

(+)-Glaucine at higher doses non-competitively antagonized contractions induced by Ca^{2+} which suggests that the alkaloid may also display an action on the cell membrane, blocking calcium channels and/or opening K^+ channels. The inhibitory effect does not seem to be due to the opening of K^+ channels, since cromakalim and other potassium channel openers do not inhibit contractions induced by Ca^{2+} in calcium-free high-potassium medium at concentrations greater than about 30 mM in vascular and non-vascular smooth muscle (Hamilton *et al.*, 1986; Weir & Weston, 1986; Clapham & Wilson, 1987; for review see Cook & Quast, 1990). An action of (+)-glaucine on calcium channels is illustrated by the experiments involving ^{45}Ca uptake. Basal ^{45}Ca uptake –i.e. the amount of calcium entering by means of leak channels (Van Breemen & Saida, 1989) – was unchanged by the addition of (+)-glaucine, whereas high concentrations of (+)-glaucine (50 μM) strongly inhibited the uptake of ^{45}Ca induced by K^+. These results suggest that (+)-glaucine may inhibit contractions induced by Ca^{2+} (in calcium-free high-potassium medium) at least in part by blocking transmembrane calcium influx through voltage-dependent calcium channels.

In conclusion, (+)-glaucine has been characterized as an agent with weak relaxant effect on rat vas deferens smooth muscle. Our results suggest that the selective inhibition of noradrenaline- and methoxamine-induced contractions is likely to be due to its α_1-adrenoceptor blocking properties, whereas the inhibition of Ca^{2+}-induced contractions may be due, at least in part, to its Ca^{2+}-entry blocking properties.

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Adenosine receptor-mediated modulation of acetylcholine release from rat striatal synaptosomes

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1 The effects of A_1 and A_{2a} adenosine receptor agonists on the veratridine-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from rat striatal synaptosomes was investigated by use of the A_1-selective agonist, **R**-PIA and the 185 fold selective A_{2a} agonist, CGS 21680. The effects of NECA, which is equipotent at both receptor subtypes, were also studied.

2 The evoked release of [^{3}H]-ACh was significantly enhanced by the A_{2a} agonist CGS 21680 but decreased by the A_1 agonist, **R**-PIA. The effects of NECA were dependent on the concentration used, with high concentrations inhibiting and low concentrations enhancing the evoked release of [^{3}H]-ACh. In the absence of any antagonists, the rank order of potency for these three drugs on increasing [^{3}H]-ACh release was CGS 21680 > NECA > **R**-PIA.

3 The stimulatory effects of CGS 21680 and low NECA concentrations on evoked [^{3}H]-ACh release were antagonized by the A_{2a} receptor antagonists, CP66,713 (300 nM) and CGS 15943A (50 nM) whilst the inhibitory effects of **R**-PIA were reversed by the selective A_1 antagonist, DPCPX (4 nM). In the presence of DPCPX, NECA greatly enhanced the evoked release of [^{3}H]-ACh at all concentrations studied when, during such A_1 receptor blockade, the rank order of potency was NECA $\gg$ CGS 21680 > **R**-PIA.

4 These results demonstrate that both A_1 and A_{2a} adenosine receptors modulate the veratridine-evoked release of [^{3}H]-ACh from rat striatal synaptosomes.

Keywords: Acetylcholine release; A_{2a} adenosine receptors; CGS 21680; CP66,713; CGS 15943A; rat striatum; neuromodulation

Introduction

Adenosine is a potent modulator of neurotransmitter release in both the peripheral and central nervous systems (Dunwiddie, 1985; Snyder, 1985; Fredholm & Dunwiddie, 1988; Williams, 1989). Adenosine exerts its neuromodulatory actions via four major receptor subtypes, A_1, A_{2a}, A_{2b} and A_3 which have been characterized according to their pharmacological profile, their effects on adenylate cyclase and their organ or tissue distribution (see Abbracchio *et al.*, 1993).

In the rat, mouse, guinea-pig and human brain the localization of high affinity A_{2a} receptors has been confined to the striatum, nucleus accumbens, olfactory tubercle and lateral segment of the globus pallidus (Jarvis *et al.*, 1989; Parkinson & Fredholm, 1990; Wan *et al.*, 1990; Martinez-Mir *et al.*, 1991; James *et al.*, 1992). These excitatory A_{2a} receptors have been shown to stimulate adenosine 3':5'-cyclic monophosphate (cyclic AMP) production in both striatal membranes and PC12 cells (Brown *et al.*, 1990; Hide *et al.*, 1992) and enhance the release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from striatal nerve terminals and the skeletal neuromuscular junction of the rat (Brown *et al.*, 1990; Correra de Sá *et al.*, 1991). A_{2a} adenosine receptors also increase hippocampal excitability (Sebastião & Ribeiro, 1992), enhance the release of excitatory amino acids from ischaemic cerebral cortex of the rat (Simpson *et al.*, 1992), and regulate cardiovascular function via actions in the nucleus tractus solitarius (Barraco & Phillis, 1991).

However, recent *in situ* hybridisation studies in the dog, rat and human brain have revealed, that while the cloned A_{2a} adenosine receptor (RDC8) can be localized to the caudate, putamen and nucleus accumbens, this receptor is only present on the medium sized neurones (Schiffman *et al.*, 1990; 1991a) and not on either substance P containing nor ACh containing nerves (Schiffman *et al.*, 1991b). In an independent study, Fink *et al.* (1992) suggested that the cloned rat A_{2a} receptor (DT-35), which has considerable sequence homology with RDC8, is expressed exclusively in a subpopulation of striatal neurones that also express dopamine D_2 receptors.

In the light of these findings, it was necessary to characterize the A_{2a} receptor responsible for the stimulation of acetylcholine release in the striatum. The 180 fold selective (over the A_1 receptor) A_{2a} agonist, CGS 21680, was used since it has also been reported to have no effect on A_{2b} receptors (Hutchinson *et al.*, 1989; Lupica *et al.*, 1990). If the presence of such a receptor on cholinergic nerves could be confirmed, it would suggest that more than one A_{2a} subtype exists in the striatum.

Methods

Preparation of crude synaptosomal fraction (P_2)

Wistar rats were killed by a blow to the head, decapitated and the brains quickly removed into ice-cold 0.32 M sucrose. The striata were dissected free and crude synaptosomal fractions (P_2) prepared according to the method of Gray & Whittaker (1962). The P_2 pellet was then resuspended in ice-cold standard reaction mixture (SRM) of the following composition (mM): NaCl 125, KCl 4.75, $MgCl_2$ 1.4, $CaCl_2$ 2.0, HEPES 20.0 (pH 7.4) glucose 10.0. The SRM contained physostigmine 100 μM throughout. The synaptosomes were incubated with 1 μM [methyl-^{3}H]-choline chloride (2.5 μCi ml^{-1}) for 30 min at 37°C. The concentration of labelled choline used is well below the K_m for the low affinity uptake and has been shown to be sodium and hemicholinium dependent (Pittel *et al.*, 1990). After radiolabelling, the synaptosomes were washed three times with ice-cold SRM, centrifuging (10,000 *g*, 2 min, 4°C) between washes. Adenosine deaminase (4 u ml^{-1}) was added after the final resuspension to reduce the effects of endogenous adenosine. The resuspended synaptosomes were kept on ice and 200–250 μl samples pipetted out when required.

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Immunoaffinity purification of cholinergic nerve terminals

Cholinergic nerve terminals were affinity purified from rat striatum using sheep anti-(Chol-1) serum and a mouse anti-(sheep IgG) immunoadsorbent as previously described (Richardson *et al.*, 1984; Richardson, 1986). The yield of cholinergic nerve terminals is expressed as units (1 nmol h^{-1} at 37°C) of choline acetyltransferase (EC 2.3.1.6) activity and amounted to approximately 10% of the initial activity present. The purity of terminals used in these experiments was routinely assessed by measuring the % recovery of choline acetyltransferase and lactate dehydrogenase (EC 1.1.1.27). The preparations used showed a 7.0 ± 1.0 ($n = 3$) fold greater yield of choline acetyltransferase over lactate dehydrogenase, which corresponds to cholinergic nerve terminals of very high purity (Richardson, 1981). All the experiments were performed with nerve terminals still attached to the solid phase immunoadsorbent.

Release of radiolabelled ACh and choline

A_1- and A_2-adenosine receptor agonists and/or antagonists were added to microcentrifuge tubes immediately prior to each reaction, while SRM only was added to control (veratridine-only) and basal samples.

Portions (220–250 µl) of the P_2 fraction were added to each tube at the onset of each reaction and the samples incubated at 37°C for 2 min, the final volume being 250 µl. For basal efflux, ethanol, the vehicle for veratridine, was then added to each sample while in evoked-release samples, veratridine (75 µM) was added. The microcentrifuge tubes were then gently inverted and the sample returned to the water bath for a further 2 min. The reaction was stopped by centrifugation at 10,000 *g* for 2 min at 0°C, 75 µl samples (representing the total release of radiolabelled compounds) of the supernatant were removed for scintillation counting. [^{3}H]-ACh was extracted from the remaining supernatant as described below. Total radioactivity remaining in the pellet was measured by solubilizing the pellets overnight in 0.2 ml of 20% Triton X-100. The pellets were then resuspended and counted on a Packard Tricarb liquid scintillation counter.

In desensitization experiments, half of the immunoaffinity purified cholinergic nerve terminals derived from 600 mg tissue were preincubated with 5'-N-ethylcarboxamide adenosine (NECA, 100 pM) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 4 nM) for 2 min prior to veratridine stimulation as above. The other half of the preparation was incubated with NECA (100 pM) and DPCPX (4 mM) for 10 min prior to stimulation. After their 10 min incubation with NECA, the terminals were washed and resuspended in fresh SRM containing the same concentrations of NECA and DPCPX as before and then incubated with NECA for a further 2 min before stimulation with veratridine.

Extraction of [^{3}H]-ACh

Radiolabelled ACh was extracted from the supernatant by a modified version of the choline kinase method described by Pittel *et al.* (1990). In these experiments, 75 µl of the reaction supernatant was removed to fresh microcentrifuge tubes and 300 µl of 50 mM glycylglycine buffer (pH 8.5) containing (mM): adenosine 5' triphosphate (ATP) 50.0, $MgCl_2$ 1.2 and 0.08 u of choline kinase ml^{-1} added. The final volume was 375 µl. The microcentrifuge tubes were then gently vortexed and incubated at 37°C for 30 min. The phosphorylation process was stopped immediately by placing the tubes on ice and then adding 700 µl of heptanone containing tetraphenylboron (10 mg ml^{-1}). To extract the unwanted phosphorylated choline into the aqueous layer and to separate the two phases that were produced, the samples were thoroughly shaken and vortexed for 5 s before being centrifuged at 10,000 *g* for 3 min; 600 µl of the organic layer, containing the [^{3}H]-ACh, was removed and added to 600 µl 1 M hydrochloric acid to back extract the [^{3}H]-ACh. Then 500 µl of the acid containing [^{3}H]-ACh was counted in 8 ml of scintillant (Emulsifier Safe, this represents total [^{3}H]-ACh release). [^{3}H]-ACh release in each tube was expressed as a fraction of the total radioactivity of each sample (i.e. [^{3}H]-ACh released divided by total label released + total label remaining in pellet). The amount of veratridine-evoked release was obtained after subtracting the appropriate basal values (i.e. without veratridine). The effects of increasing concentrations of A_1 and A_{2a} receptor agonists were assessed by comparing the fraction release of [^{3}H]-ACh evoked by veratridine in their presence, with the fractional release of [^{3}H]-ACh evoked by veratridine in their absence. In those experiments involving antagonists, the control tubes also included the antagonists.

Drugs and chemicals

5'-N-ethylcarboxamide adenosine (NECA), **R**-N^6-phenylisopropyladenosine (**R**-PIA), adenosine deaminase, physostigmine veratridine, ATP, glycylglycine, heptanone and tetraphenylboron were all obtained from Sigma. (2-[*p*-(2-Carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidine adenosine) (CGS 21680) and 1,3-dipropyl-8-cyclo-pentylxanthine (DPCPX) were obtained from Research Biochemicals Incorporated. 4-Amino-1-phenyl-[1,2,4] triazolo [4,3-a] quinoxaline (CP66,713) was a generous gift from Dr R. Sarges, Pfizer Central Research, CT, U.S.A. Stock solutions of CP66,713 and DPCPX were dissolved in dimethylsulphoxide (DMSO, Sigma), the final DMSO concentration in reaction tubes being 0.02%. 9-Chloro-2-(2-furanyl)-5, 6-dihydro-1,2,4-triazolo [1,5-c] quinazoline-5-imine (CGS 15493A) was a generous gift from Ciba Geigy; stock solutions were dissolved in ethanol. Salts used in SRM and other buffers were obtained from Fisons Laboratory Supplies and [methyl-^{3}H]-choline chloride (specific activity 79.3 Ci $mmol^{-1}$) was purchased from Amersham.

Statistics

All results are given as mean ± s.e.mean and n equals the number of individual experiments. Results were compared by a two-tailed Student's t test.

Results

In control experiments, addition of veratridine (75 µM) to synaptosomes significantly enhanced the efflux of [^{3}H]-ACh. In the presence of veratridine the fractional release of [^{3}H]-ACh increased from a basal level of 0.099 ± 0.008 to 0.150 ± 0.007 ($P < 0.001$, $n = 7$). The effects of increasing concentrations of the A_1 and A_2 adenosine receptor agonists, **R**-PIA, NECA and CGS 21680 on veratridine-evoked [^{3}H]-ACh release from rat striatal synaptosomes is shown in Figure 1. The highly selective A_{2a}-adenosine receptor agonists, CGS 21680, produced a dose-dependent increase in the evoked efflux of [^{3}H]-ACh, its effects were maximal at 10^{-10} M and statistically significant at concentrations as low as 10^{-11} M ($n = 3$, $P < 0.01$). The potentiation of [^{3}H]-ACh release was less marked with higher concentrations ($\geq 10^{-7}$ M) of CGS 21680. Similarly, NECA, which is equipotent at A_1 and A_2 adenosine receptors, produced a significant increase in the veratridine-evoked release of [^{3}H]-ACh; this enhancement was statistically significant at concentrations of 10^{-10} M or less ($n = 4$, $P < 0.01$). Unlike CGS 21680, which is 185 fold selective for A_{2a} adenosine receptors, NECA (10^{-5} M) decreased the release of [^{3}H]-ACh (not shown).

Conversely, the A_1-adenosine receptor agonist, **R**-PIA produced a decrease in the release of [^{3}H]-ACh which was statistically significant at all the concentrations studied; its effects were maximal at 10^{-5} M where the release of [^{3}H]-ACh was reduced by 66 ± 8.6% ($n = 3$, $P < 0.001$).

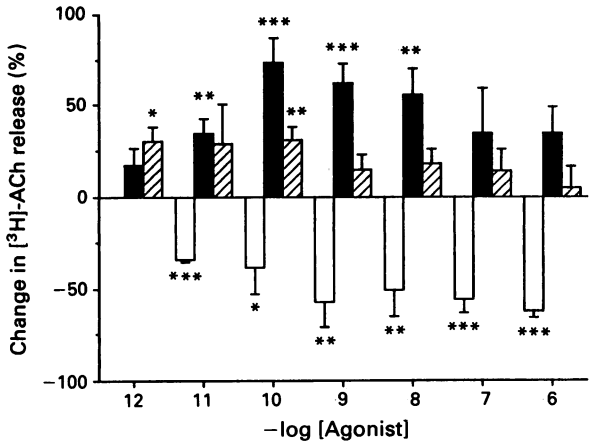

Figure 1 Modulation of veratridine-evoked [^{3}H]-ACh release by adenosine agonists. The results are expressed as % change in [^{3}H]-ACh release when compared to control incubations without agonists, and are means ± s.e.mean. CGS 21680 (solid columns, $n = 3$), R-PIA (open columns, $n = 3$), NECA (hatched columns, $n = 5$). Significant difference from control: $*P<0.05$, $**P<0.01$, $***P<0.001$. For abbreviations, see text.

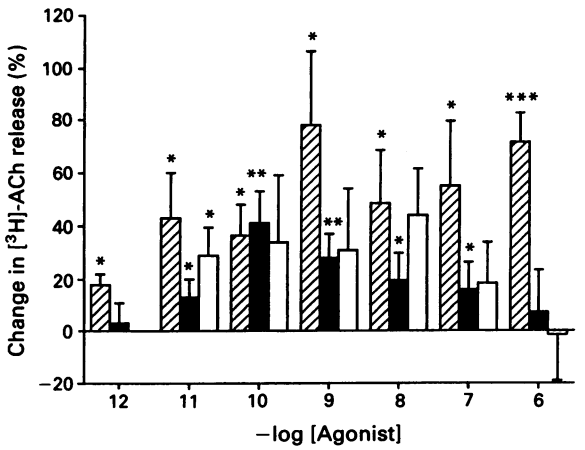

Figure 2 Modulation of veratridine-evoked [^{3}H]-ACh release by adenosine agonists in the presence of DPCPX. DPCPX (4 nM) was included in all incubations and the ability of CGS 21680 (solid columns, $n = 6$), R-PIA (open columns, $n = 4$) and NECA (hatched columns, $n = 3$) to modulate the release determined as described. Significant difference from control as in Figure 1. For abbreviations, see text.

A_1 adenosine receptor antagonism

The results from similar experiments, repeated in the presence of the 750 fold selective A_1 adenosine receptor antagonist, DPCPX are shown in Figure 2. Preincubation of the synaptosomes with DPCPX (4 nM) for 2 min prior to veratridine stimulation had little effect on the enhancement of [^{3}H]-ACh release by CGS 21680 but the stimulatory effect of NECA, which is equipotent at A_1 receptors under control conditions, was greatly augmented; under these circumstances NECA significantly enhanced the release of labelled ACh at all the concentrations studied (see Figure 2). In the presence of DPCPX, R-PIA actually produced an increase in the veratridine-evoked release of [^{3}H]-ACh. Indeed, only when the concentration of R-PIA was greater than 10^{-6} M was there any decrease in the release of [^{3}H]-ACh.

A_2 adenosine receptor antagonism

The effects of 2 min preincubation with 300 nM CP66,713 on A_1 and A_{2a} mediated adenosine responses are shown in Figure 3. CP66,713 significantly impaired the ability of CGS 21680 to enhance [^{3}H]-ACh release. Indeed, when low concentrations of the A_{2a} agonist were used some inhibition, which was not statistically significant, was observed. However, CGS 21680 was able to enhance the release of [^{3}H]-ACh at concentrations greater than 10^{-8} M. In these experiments, the maximal increase in [^{3}H]-ACh release (27.4 ± 6.0%) was obtained when 10^{-7} M CGS 21680 was used. Similar results were obtained with the more potent but less selective A_2-adenosine receptor antagonist, CGS 15943A. As shown in Figure 4, CGS 15943A (50 nM) inhibited the excitatory effect of CGS 21680 on [^{3}H]-ACh release. In fact, low concentrations of CGS 21680 actually inhibited the release of [^{3}H]-ACh; however, higher concentrations increased the evoked release of [^{3}H]-ACh although the enhancement was less marked than with CGS 21680 alone.

In the presence of 300 nM CP66,713, NECA was unable to enhance the veratridine-evoked release of [^{3}H]-ACh; in fact, under these experimental conditions, NECA inhibited the efflux of [^{3}H]-ACh at all the concentrations studied although this inhibition was not statistically significant (Figure 3). The inhibitory effects of R-PIA on [^{3}H]-ACh release were little affected by A_{2a} receptor blockade, until the concentration R-PIA reached 10^{-6} M, then the inhibition by R-PIA in the presence of CP66,713 was greater than observed with R-PIA

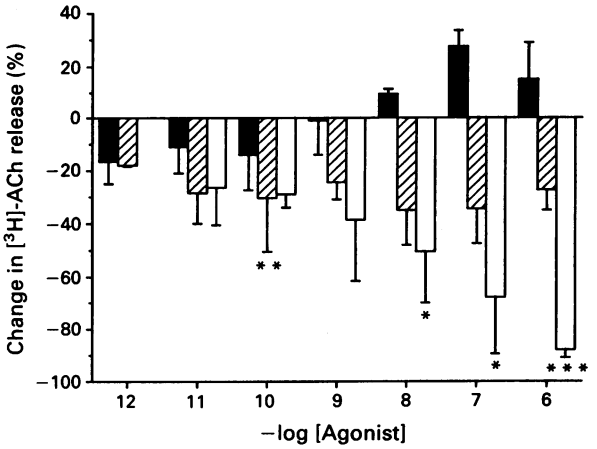

Figure 3 Modulation of veratridine-evoked [^{3}H]-ACh release by adenosine agonists in the presence of CP66,713. CP66,713 (300 nM) was included in all incubations and the ability of CGS 21680 (solid columns, $n = 3$), R-PIA (open columns, $n = 3$) and NECA (hatched columns, $n = 3$) to modulate the release determined as described. Significant difference from control as in Figure 1. For abbreviations, see text.

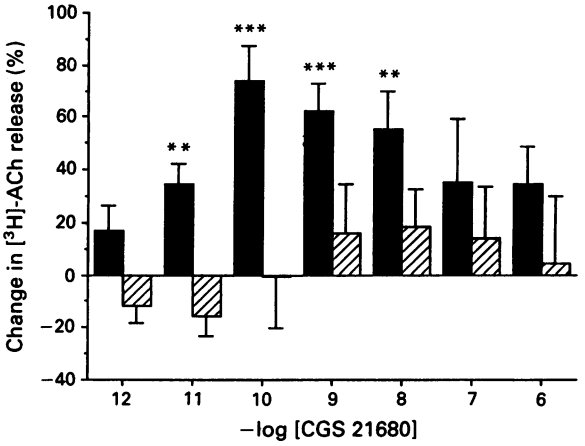

Figure 4 Modulation of veratridine-evoked [^{3}H]-ACh release by CGS 21680 in the presence of CGS 15943. The ability of CGS 21680 to modulate [^{3}H]-ACh release was determined as described, in the presence (hatched columns) or absence (solid columns) of 50 nM CGS 15943. The results are means ± s.e.mean of 3 experiments. Significant difference from control as in Figure 1. For abbreviations, see text.

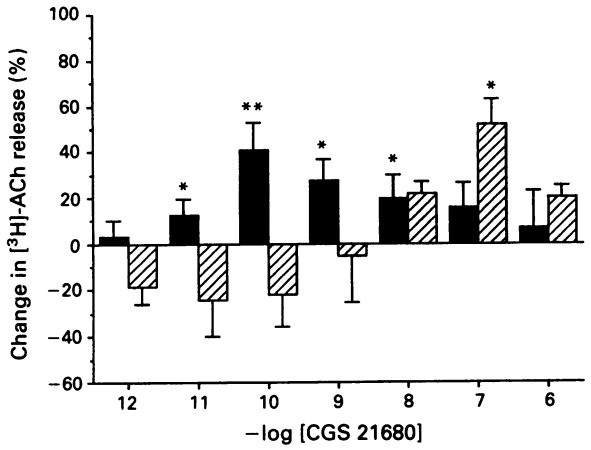

Figure 5 Modulation of veratridine-evoked [^{3}H]-ACh release by CGS 21680 in the presence of DPCPX and CP66,713. The ability of CGS 21680 to modulate [^{3}H]-ACh release was determined as described, in the presence of 4 nM DPCPX (solid columns) and with both 4 nM DPCPX and 300 nM CP66,713 (hatched columns). The results are means ± s.e.mean of 3 experiments. Significant difference between the two conditions: $*P<0.05$, $**P<0.01$. For abbreviations, see text.

alone (c.f. Figure 1). In these experiments, maximal inhibition of [^{3}H]-ACh release occurred at 10^{-6} M **R**-PIA where inhibition was 87 ± 3.4%.

A_1 and A_{2a} adenosine receptor antagonism

In order to assess the effects of CGS 21680 on A_{2a} receptors during A_1 receptor blockade, the effects of CGS 21680 on [^{3}H]-ACh release in the presence of DPCPX (4 nM) and CP66,713 (300 nM) were studied. Under these conditions CGS 21680 was unable to potentiate the evoked efflux of [^{3}H]-ACh until concentrations exceeded 10^{-9} M. Unlike the control experiments where the effects of CGS 21680 were maximal at 10^{-10} M, under these conditions the maximum increase in [^{3}H]-ACh release was obtained at 10^{-7} M CGS 21680 and the maximal enhancement of [^{3}H]-ACh overflow was 51.9 ± 10.7%. At low concentrations of CGS 21680 the release of labelled acetylcholine was actually reduced although this reduction was not statistically significant (see Figure 5).

Desensitization experiments

In the presence of the A_1 antagonist DPCPX (4 nM), the release of [^{3}H]-ACh from immunoaffinity purified cholinergic nerve terminals was enhanced by 23.8 ± 12.4% when NECA (10^{-10} M) was added 2 min prior to addition of veratridine (75 μM). However, preincubation with the same concentration of NECA for 10 min prior to exposure with veratridine and NECA (10^{-10} M) produced a statistically significant decrease in the evoked release of [^{3}H]-ACh. This decrease in the evoked release of [^{3}H]-ACh after 10 min pre-exposure to NECA was seen in all experiments ($n = 4$) despite the fact that DPCPX was present; the mean inhibition was 30.2 ± 13.9%.

Discussion

The data presented in this paper demonstrate that there are A_1 and A_{2a} adenosine receptors, present on the synaptosomal membrane, that can modulate the release of [^{3}H]-ACh from rat striatal synaptosomes. Figure 1 shows quite clearly that while the A_1 selective agent **R**-PIA inhibited [^{3}H]-ACh efflux, the release was stimulated by the A_{2a} selective agonist, CGS 21680. The equipotent A_1/A_2 agonist, NECA, had an intermediate effect which appeared as a low level stimulation of [^{3}H]-ACh release.

In an attempt to analyse this system in greater detail the 740 fold A_1 selective antagonist DPCPX (K_D for A_1 0.5 nM, A_2 340 nM, Bruns *et al.*, 1986) was used. This concentration of DPCPX had little effect on the stimulation by CGS 21680, other than a slight reduction in the maximal stimulation of release. This reduction may reflect a reduced ability of the A_{2a} receptor to stimulate ACh release in the absence of inhibitory A_1 receptor function; however further work would be required to justify such a hypothesis. The most dramatic effect of DPCPX was seen in its abolition of the inhibitory effect of **R**-PIA. Thus the inhibition of ACh release observed with **R**-PIA was reversed. Indeed a variable potentiation of release was seen. DPCPX also augmented the stimulatory effect of NECA, particularly at agonist concentrations greater than 10^{-10} M.

The presence of DPCPX allowed us to determine an apparent relative order of potency of the three agonists at the A_{2a} receptor. From Figure 2 it appears that the order was NECA>>CGS 21680>**R**-PIA. It remains to be seen whether this is significantly different from the order observed when studying A_{2a} receptor modulation of striatal GABA release where the rank order of potency of these agonists was CGS 21680> NECA>**R**-PIA (Kirk & Richardson, 1993).

In the presence of the poorly selective A_{2a} antagonists CP 66,173 (13 fold, Sarges *et al.*, 1990) and CGS 15943A (7 fold, Williams, 1991), the inhibitory effect of **R**-PIA was slightly augmented at high concentrations. This would be consistent with **R**-PIA acting on A_2 receptors at concentrations greater than 10^{-7} M. Similarly, the inhibitory effect of NECA predominated in the presence of such A_2 antagonists, while the stimulatory effect of CGS 21680 was considerably reduced. CGS 21680 was eventually, at much higher concentrations, able to overcome the A_{2a} blockade and enhance the release of [^{3}H]-ACh but the maximum enhancement was much less than that seen when CGS 21680 was used on its own. This is probably because at the higher concentrations required to overcome A_{2a} blockade, the CGS 21680 was also acting at A_1 adenosine receptors. This view is supported by the fact that, in the presence of DPCPX and CP66,713 the maximum enhancement of [^{3}H]-ACh release produced by CGS 21680 was around 52%, substantially more than that seen in the presence of CP66,713 (about 27%) or CGS 15943A (around 20%) alone.

In the presence of DPCPX, CP66,713 was able to abolish the stimulatory effect of CGS 21680 at agonist concentrations less than 10^{-7} M. The apparent shift in EC_{50} values for CGS 21680 (approximately 500 fold in the presence of 4 nM DPCPX and 300 nM CP66,713) implied an affinity of CP66,713 of approximately 10^9 M^{-1}. This is seven fold higher than that estimated from binding studies (James & Richardson, 1993), but the current estimation may have been complicated by the presence of the A_1 receptor.

Since the EC_{50} of CGS 21680 mediated stimulation of [^{3}H]-ACh release was low (50 pM) compared to other studies (0.1–10 nM, Hutchinson *et al.*, 1989; Jarvis *et al.*, 1989; Correra de Sá *et al.*, 1991; Sebastião & Ribeiro, 1992) we investigated whether this A_{2a} receptor showed desensitization (Porter *et al.*, 1988; Ramkumar *et al.*, 1991). Our results support the view that desensitization of this receptor readily occurs, since in simple *in vitro* experiments, preincubation of the synaptosomes with low concentrations of NECA (10^{-10} M) for 10 min, prevented NECA from enhancing the release of [^{3}H]-ACh from immunoaffinity purified nerve terminals. Indeed in desensitized immunoaffinity purified terminals we found that NECA inhibited the release of [^{3}H]-ACh despite the fact that DPCPX (4 nM) was present. While this may be due to the fact that, at this concentration, DPCPX occupies only around 88% of the A_1 receptors and thus NECA may be able to mediate its inhibitory effects via the unoccupied

remainder; it is also possible that NECA mediates these DPCPX-resistant inhibitory actions via the newly cloned and characterized A_3 receptor (Zhou *et al.*, 1993). The role of this novel receptor, which is found in greater numbers in the peripheral nervous system than in the striatum requires further study. This inhibition of [^{3}H]-ACh release is unlikely to be the result of uncoupling of the A_{2a} receptor from G_s since neither a change in receptor number nor an alteration in the coupling of the receptor to its excitatory G-protein are believed to underly the mechanism involved during desensitization (Porter *et al.*, 1988; Ramkumar *et al.*, 1991).

A_{2a} receptor desensitization would occur much more readily when endogenous adenosine levels were allowed to rise (Linden, 1989) and may have occurred in studies where no adenosine deaminase was present (Correra de Sá *et al.*, 1991; Sebastião & Ribeiro, 1992). Receptor desensitization may also be one explanation why Brown *et al.* (1990) were able to detect an increase in the release of ACh from purified terminals only with much higher concentrations of NECA (10^{-7} M). Of course it may also be possible that Brown *et al.* (1990) having already desensitized the high affinity A_{2a} receptor were investigating the effects of NECA on an A_2 receptor with much lower affinity.

In any case, all the data presented are consistent with the presence of both A_1 and A_{2a} receptors on striatal cholinergic nerve terminals. The present findings are in close agreement with previous studies from our laboratory which demonstrated that A_{2a} activation by NECA (in the presence of the A_1-antagonist DPCPX) could enhance the evoked release of ACh by around 50%. These current findings therefore strengthen the view that A_{2a} receptors are present on cholinergic nerve terminals where they increase cyclic AMP production; an effect which has been shown to copurify with immunoaffinity purified cholinergic nerve endings (Brown *et al.*, 1990). In addition [^{3}H]-CGS 21680 binding sites have been shown to be present on immunoaffinity purified striatal cholinergic membranes (James & Richardson, 1993). Further evidence to suggest that CGS 21680 acts on cholinergic nerve endings comes from behavioural studies where CGS 21680 has been shown to inhibit the apomorphine-induced turning of unilaterally 6-hydroxydopamine-lesioned rats in an atropine-dependent manner (Vellucci *et al.*, 1993).

It therefore appears that there are two A_{2a}-like striatal receptors, both of which bind CGS 21680. One has been cloned and shown to be present in the GABA-metenkephalin neurones (Schiffman *et al.*, 1991b), where it is able to inhibit [^{3}H]-GABA release (Kirk & Richardson, 1993). The other is present in cholinergic neurones, was not detected by *in situ* hybridization using probes derived from the cloned receptor, and is able to stimulate [^{3}H]-ACh release. Given the increasing number of reports of CGS 21680 effects in non-striatal neurones (Correra de Sá *et al.*, 1991; Barraco & Phillis, 1991; Sebastião & Ribeiro, 1992; Simpson *et al.*, 1992), it may be that one A_{2a}-like receptor is widely distributed, whereas the other is localized to the striatum. Such a situation has been described in human brain (James *et al.*, 1992).

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Relative contributions of direct and indirect mechanisms mediating endothelin-induced contraction of guinea-pig trachea

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1 The present study was undertaken to determine the mechanism of action of endothelin-1 (ET-1)-induced contraction of the guinea-pig isolated trachea.

2 ET-1 (1 nM–0.3 μM) produces a concentration-dependent contraction of guinea-pig trachea with an EC_{50} of approximately 25 nM. The combination of the peptidoleukotriene receptor antagonist, SK&F 104353 (10 μM) and the H_1-histamine receptor antagonist, mepyramine (10 μM), which abolishes antigen-induced contraction in guinea-pig trachea, was without effect on ET-1 concentration-response curves. Furthermore, the platelet-activating factor (PAF) receptor antagonist, WEB 2086, (1 or 10 μM) did not inhibit ET-induced contraction.

3 ET-1 (0.3 μM) did not stimulate histamine or immunoreactive peptidoleukotriene release from guinea-pig isolated trachea.

4 The release of various prostanoids from guinea-pig trachea was increased significantly by ET-1 (0.3 μM); the profile of release was prostaglandin D_2 (PGD_2) = PGE_2 = 6-keto $PGF_{1\alpha}$ (PGI_2 metabolite) > thromboxane B_2 = $PGF_{2\alpha}$ >> 9α, 11β PGF_2 (PGD_2 metabolite). ET-1-induced release of prostaglandins, which was about 30% of that elicited by antigen in sensitized tissues, was not affected by epithelium removal and was observed in tissues from which the smooth muscle had been removed. Previous studies in our laboratory indicated that indomethacin potentiated contraction produced by high concentrations of ET-1, whereas a thromboxane receptor antagonist was without appreciable effect on ET-1 concentration-response curves.

5 Pretreatment of tissues for 1 h with capsaicin (10 μM), which depletes different sensory neurones, produced a small, but significant, inhibitory effect on ET-1 concentration-response curves in the presence but not the absence of the epithelium. The combination of the NK_1 tachykinin receptor antagonist, CP-96,345 (0.1 μM), and the NK_2 tachykinin receptor antagonist, SR 48968 (0.1 μM), was without effect on ET-1 concentration-response curves but substantially antagonized capsaicin-induced contraction.

6 The present data suggest that in guinea-pig isolated trachea, ET-1 produces contraction predominantly via a direct mechanism: there is no significant contribution of the release of histamine, leukotrienes, PAF, or tachykinins (acting on NK_1 or NK_2 receptors). Although ET-1 evokes the release of an array of prostanoids from the trachea they do not appear to have a major influence on the contractile response.

Keywords: Endothelin-1; guinea-pig trachea; SK&F 104353; mepyramine; WEB 2086; capsaicin; thiorphan; tachykinin receptor antagonists; neutral endopeptidase; epithelium removal; prostanoid release

Introduction

In 1988, Yanagisawa and co-workers isolated, cloned and characterized pharmacologically a novel, potent 21-amino acid vasoconstrictor peptide, named endothelin (ET), that was released from porcine cultured endothelial cells (Yanigasawa *et al.*, 1988). Of the 3 different forms of ET that are now known to exist, ET-1 is the original form isolated from porcine, and subsequently also from human, endothelial cells. In addition to possessing potent vasoconstricting properties, ET-1 produces a plethora of activities in a variety of systems including the respiratory tract (Yanagisawa & Masaki, 1989). Specific binding sites for ET have been detected in airways, particularly the smooth muscle, of various species including man (Power *et al.*, 1989; Turner *et al.*, 1989; Kanse *et al.*, 1989; Hemsén *et al.*, 1990; Henry *et al.*, 1990). Furthermore, ET-1 is a potent contractile agonist of mammalian airways both *in vitro* and *in vivo* (Payne & Whittle, 1988; Uchida *et al.*, 1988; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989; Turner *et al.*, 1989; Maggi *et al.*, 1989; 1990; Hay, 1990; Hemsén *et al.*, 1990; Matsuse *et al.*, 1990).

In guinea-pig isolated trachea it has been proposed that ET-1 produces contraction predominantly directly via an interaction with a specific receptor, stimulation of phosphatidylinositol turnover and subsequent release of intracellular calcium (Hay, 1989). There is conflicting information on the relative contribution of indirect mechanisms to the contraction produced by ET-1 in guinea-pig trachea. For example, a role for released histamine and peptidoleukotrienes from mast cells has been proposed in preliminary reports (Ninomiya *et al.*, 1989; Nomura *et al.*, 1990), although another study found no evidence for a contribution from these mediators (Hay, 1989). In addition, there are discrepancies regarding the functional role of prostanoids in contractions of isolated airway smooth muscle elicited by ET-1 (Maggi *et al.*, 1989; Filep *et al.*, 1990; Hay, 1990; Henry *et al.*, 1990; Sarriá *et al.*, 1990). To attempt to clarify the role of prostanoids, and also histamine and peptidoleukotrienes in mediating ET-1-induced contraction in guinea-pig trachea, in the present study the ability of ET-1 to release these mediators was measured directly.

The epithelium has been shown to inhibit ET-1-induced contractions in guinea-pig isolated trachea (Hay, 1989) and bronchus (Maggi *et al.*, 1989). The effect was inhibited by phosphoramidon, an inhibitor of neutral endopeptidase (NEP) (Hudgin *et al.*, 1981) and was attributed to the

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epithelium acting as a significant site of metabolism for ET-1 (Hay, 1989). The ETs have been shown to be good substrates for NEP (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991). However, the possibility exists that the modulatory influence of the epithelium may reflect the release of epithelium-derived tachykinins, e.g. substance P, which are substrates for neutral endopeptidase (Skidgel *et al.*, 1984; Hooper & Turner, 1985; Hooper *et al.*, 1985) so that their effects would be potentiated by inhibitors of this enzyme, such as phosphoramidon (Fine *et al.*, 1989; Frossard *et al.*, 1989). To study this possibility the effect of capsaicin, which depletes sensory substance P-containing neurones (Buck & Burks, 1986; Maggi & Meli, 1988) on ET-1-induced contractions in guinea-pig trachea with and without epithelium was examined. In addition the influence of the potent and selective antagonists at NK_1 receptors (CP-96,345; Snider *et al.*, 1991) and NK_2 receptors (SR 48968; Advenier *et al.*, 1991) on contractions produced by ET-1 was investigated.

Accordingly, the purpose of the present study was to investigate further and clarify the mechanism(s) of action (direct versus indirect) of ET-1 in guinea-pig isolated trachea. A preliminary account of the results has been presented by Hay & Undem (1991).

Methods

Contraction studies

Tissue preparation Tracheae were removed from male Hartley guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.; 450–750 g body weight). In some experiments the guinea-pigs were actively sensitized to ovalbumin with three intraperitoneal injections (10 mg kg^{-1}) on days 1, 3, and 5. The animals were killed beginning 21 days after the last injection. The tissues were placed in modified Krebs-Henseleit solution which was gassed with 95% O_2:5% CO_2 and maintained at 37°C. The composition of the Krebs solution was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.5. Following careful removal of adherent fat and connective tissue, the trachea was cut open along its longitudinal axis, directly opposite the smooth muscle and strips consisting of two adjacent cartilage rings were prepared. The tissue preparations were then placed in 10 ml water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FT03C force-displacement transducers. Mechanical responses were recorded isometrically by multi-channel polygraphs. Tissues were equilibrated under 1.5 g resting load for at least 1 h, and washed every 15 min with fresh Krebs-Henseleit solution before the start of each experiment.

In some experiments, the epithelium was removed mechanically from alternate strips of trachea by gently rubbing the luminal surface with a cotton-tipped applicator. We have demonstrated previously that this procedure effectively removes the epithelium from guinea-pig trachea without producing obvious damage to the underlying mucosal and smooth muscle layers, and does not alter the basic mechanical properties of the tissue (Hay *et al.*, 1986; Fedan *et al.*, 1988).

Concentration-response curves Concentration-response curves for ET-1 were obtained by its cumulative addition to the organ bath in three fold increments according to the technique of Van Rossum (1963). In most experiments examining the effects of drugs, tissues were exposed to these agents for 30 min before addition of contractile agonists. In studies examining the effects of capsaicin, tissues were exposed to this drug for 1 h then washed thoroughly over a period of 45 min before construction of ET-1 concentration-response curves. In experiments examining the effects of the tachykinin receptor antagonists on ET-1- and capsaicin-induced contractions, tissues were incubated with CP-96,345 and SR 48968 for 30 min and 120 min, respectively, before starting the agonist concentration-response curves. All experiments were conducted in the presence of 5 μM indomethacin which was present throughout the study.

After the equilibration period, and before construction of concentration-response curves, tissues were exposed to 10 μM carbachol. Following plateau of this reference contraction, tissues were washed several times over 15–30 min until the tension returned to baseline level. The preparations were then left for at least 30 min before the start of the experiment.

Measurement of mediator release

Guinea-pig tracheas were isolated as outlined above, cut into 12 rings and incubated in 2 ml of Krebs-Henseleit solution, which was gassed with 95% O_2/5% CO_2 and maintained at 37°C. The physiological buffer was replaced at 15 min intervals for 90 min. Following this equilibration period, 2 ml of Krebs-Henseleit containing or lacking ET-1 (1 nM, 10 nM or 0.3 μM), was added for 15 min. After this time the supernatant was taken to assay histamine, prostanoid and immunoreactive leukotriene C_4 (i-LT) release. In addition, to determine the total tissue content of histamine, 2 ml of 0.4 N perchloric acid was added to the tissue, which was then placed in a boiling water bath for 15 min; the supernatant fluid was assayed to measure the total histamine content. The studies examining mediator release were conducted in the absence of indomethacin and generally employed epithelium-containing tissues. However, in some experiments the epithelium was removed from 6 rings (as described above) with the remaining 6 rings serving as paired controls. Similarly, the effect of ET-1 was compared to specific antigen challenge in a paired fashion by treating 6 rings with ovalbumin and 6 rings with ET-1. In one set of experiments the trachealis was dissected from the tissues with the aid of a dissecting microscope. The effect of ET-1 on mediator release from the non-trachealis portion of the trachea was then evaluated.

Histamine was assayed by the automated fluorometric technique described by Siraganian (1974). Prostaglandin release into the supernatant fluid was assayed by combined gas chromatography (negative ion chemical ionization) mass spectrophotometry (GC/MS) as described previously (Hubbard *et al.*, 1986). Pepidoleukotriene released from the trachea was assayed by the radioimmunoassay outlined previously (Undem *et al.*, 1987). The limit of sensitivity of this assay is approximately 10–25 pg, as defined by that amount required to inhibit [^{3}H]-LTC_4 binding by 10%. The anti-peptidoleukotriene antibody is highly selective with little affinity (crossreactivity $<1\%$) for a variety of heterologous eicosanoids. The antibody does not, however, distinguish markedly between leukotriene C_4 (LTC_4), LTD_4, and LTE_4. Standard curves with authentic LTC_4, LTD_4 and LTE_4 were parallel; the amounts of LTC_4, LTD_4 and LTE_4 required to inhibit [^{3}H]-LTC_4 binding by 50% were found to be approximately 0.4, 0.5 and 0.6 pmol 0.1 ml^{-1}, respectively.

Analysis of data

Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction. Geometric mean EC_{50} values were calculated from linear regression analyses of data. Results for control- and treated-tissues were analysed for differences in both the EC_{50} s and also the maximum contractile response produced by ET-1. The prostanoids released from the trachea are expressed as pg g^{-1} wet weight of tissue, whereas the release of i-LT and histamine are given as ng g^{-1} and μg g^{-1} or % total histamine content, respectively. All data are given as the mean ± s.e.mean. Statistical analysis was conducted by 2-tailed Student's *t* test for paired or unpaired samples. Where appropriate; a probability value less than 0.05 was regarded as significant.

Drugs

The following drugs were used: endothelin-1 (human, porcine) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Calbiochem Corp. (Le Jolla, CA, U.S.A.). SK&F 104353 (2(S)-hydroxy-3(R)-(2-carboxyethylthio)-3-[2-(8-phenyloctyl) phenyl]-propanoic acid) was synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Carbachol, capsaicin, indomethacin, tetrodotoxin and mepyramine were obtained from Sigma Chemical Co. WEB 2086 {3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone} was a gift from Boehringer Ingelheim (Richfield, CT, U.S.A.) and CP-96,345 {(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine} and SR 48968 {(S)-N-methyl-*N*[4-acetylamino-4 phenylpiperidino)-2-(3,4-dichlorophenyl) butyl] benzamide} were generously provided by ICI pharmaceuticals (Wilmington, DE, U.S.A.).

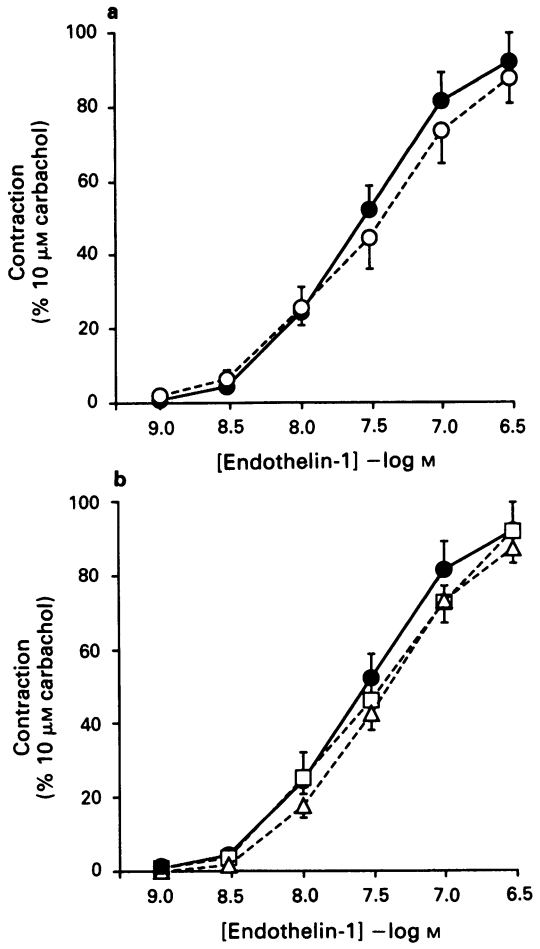

Figure 1 Effects of (a) the combination of the leukotriene receptor antagonist, SK&F 104353 (10 μM) and the H_1-histamine receptor antagonist, mepyramine (10 μM), and (b) the PAF receptor antagonist, WEB 2086 (1 or 10 μM), on endothelin-1 (ET-1) concentration-response curves in epithelium-containing guinea-pig trachea. Results are expressed as a percentage of the response to 10 μM carbachol and are the mean ± s.e.mean of 7 experiments. (a): (●) control; (○) SK&F 104353 and mepyramine; (b): (●) control; (□) 1 μM WEB 2086; (△) 10 μM WEB 2086. Studies were conducted in the presence of 5 μM indomethacin.

Results

Endothelin-1 (ET-1) (1 nM–0.3 μM) produced a concentration-dependent contraction of epithelium-containing (intact) guinea-pig isolated trachea with an EC_{50} of 23.3 nM. The maximum contractile response elicited by ET-1 (0.3 μM) was 78.5 ± 5.4% ($n = 15$) of that induced by 10 μM carbachol.

Effects of receptor antagonists and tetrodotoxin (TTX)

The combination of the leukotriene receptor antagonist, SK&F 104353 (10 μM), and the H_1-histamine receptor antagonist, mepyramine (10 μM), was without effect on ET-1 concentration-response curves (Figure 1a). In addition, the PAF antagonist, WEB 2086 (1 or 10 μM), did not significantly affect contractions induced by ET-1 (Figure 1b). In confirmation of previous findings in guinea-pig epithelium-intact trachea (Maggi *et al.*, 1989), TTX (1 μM) did not inhibit ET-1-induced contractions (data not shown).

Release of mediators

The release of histamine from the guinea-pig trachea was measured, as outlined in Methods, under basal conditions and following addition of 0.3 μM ET-1 for 15 min. This concentration of ET-1, which produces the maximum or close to the maximum contractile response, did not stimulate the release of histamine above basal levels. Thus, the spontaneous release and that elicited in the presence of ET-1 were both <2 ng, which represents <0.5% of the total histamine content of 5.1 ± 0.3 μg g^{-1} tissue ($n = 11$, Table 1). Furthermore, in trachea obtained from guinea-pigs actively sensitized with ovalbumin, ET-1 (0.3 μM) did not stimulate histamine release (Table 1). In contrast, ovalbumin (0.01 mg ml^{-1})

Table 1 Endothelin-1 (ET-1)-induced mediator release from the guinea-pig trachea[a]

Mediator	*Spontaneous release* (pg g^{-1})	*ET-1-induced release (net)* (pg g^{-1})	n
Histamine	Not detectable[b] (<0.5% total)	Not detectable (<0.5% total)	11
i-LT	Not detectable[b]	Not detectable	4
PGD_2	496 ± 210	4120 ± 573*	13
9α, 11β-PGF_2	7 ± 6	36 ± 12*	13
PGE_2	565 ± 100	3276 ± 381*	13
$PGF_{2\alpha}$	166 ± 35	879 ± 113*	13
TxB_2	241 ± 74	1036 ± 168*	13
6-keto $PGF_{1\alpha}$	397 ± 69	2632 ± 318*	13

[a]Tracheae were isolated from naive ($n = 6$) or activity sensitized ($n = 7$) guinea-pigs (see Methods). The basal release of mediators and that evoked by the addition of ET-1 (0.3 μM) was quantified. There was no appreciable differences in the release of mediators from naive and actively sensitized trachea and, therefore, the values were pooled. These data are derived from the results illustrated in Figures 2a and 2b.

[b]The amount of histamine and immunoreactive leukotriene C_4 (i-LT) released from the trachea was below our limits of detection. The histamine assay is sensitive to about 1 ng per sample (5–10 ng g^{-1} in our experiments); the limit of detection for i-LT was about 5 ng g^{-1}.

*Statistically significantly different from spontaneous release; $P < 0.05$.

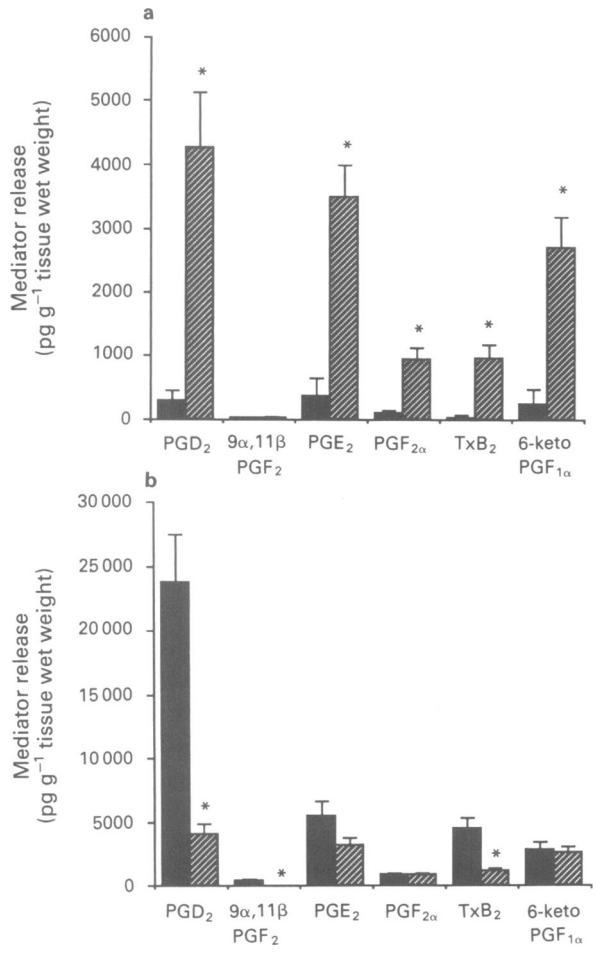

Figure 2 (a) Effects of endothelin-1 (ET-1, 10 nM or 0.3 μM) on prostanoid release from epithelium-containing guinea-pig trachea, and (b) comparison of the effects of ET-1 (0.3 μM) or ovalbumin (0.01 mg ml^{-1}) on prostanoid release from paired, epithelium-containing trachea removed from guinea-pigs actively sensitized with ovalbumin. Data are given as pg g^{-1} tissue wet weight and are the mean ± s.e.mean. (a) Solid columns: 10 nM ET-1, $n = 3$; hatched columns: 0.3 μM ET-1: $n = 6$. (b) Solid columns: ovalbumin, $n = 7$; hatched columns ET-1, $n = 7$. Prostanoid levels were measured by gas chromatography-mass spectrophotometry. Note the different scales for the y-axes of (a) and (b).

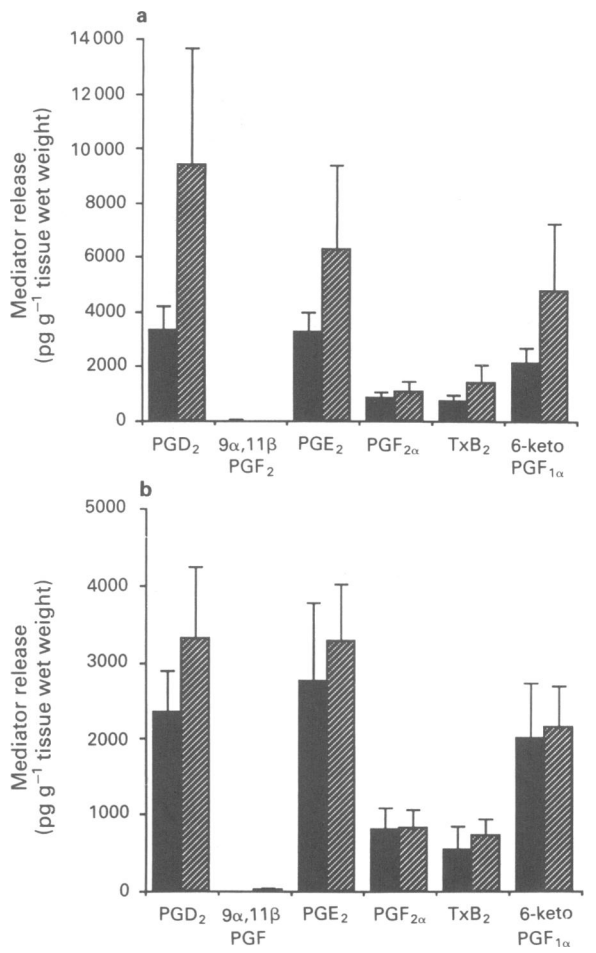

Figure 3 (a) Effect of epithelium removal on prostanoid released from paired guinea-pig tracheae induced by 0.3 μM endothelin-1 (ET-1) and (b) prostanoid release elicited by 0.3 μM ET-1 from epithelium-containing guinea-pig trachea with or without the smooth muscle (i.e. predominantly cartilage). Data are given as pg g^{-1} tissue wet weight and are the mean ± s.e.mean. (a) Solid columns: + epithelium, $n = 4$; hatched columns − epithelium, $n = 4$. (b) Solid columns: preparations without smooth muscle, $n = 4$; hatched columns: preparations containing smooth muscle, $n = 4$–6. Prostanoid levels were measured by gas chromatography-mass spectrophotometry.

induced the release of 12.9 ± 3.2% ($n = 7$) of the total histamine stores. ET-1 (0.3 μM) also failed to elicit i-LT release from the trachea. However, it should be noted that we could not detect i-LT release much below 5 ng g^{-1} of trachea. Nevertheless, ovalbumin (0.01 mg ml^{-1}) evoked the release of detectable amounts of i-LT, averaging 12.6 ± 2.6 ng g^{-1} ($n = 6$).

In epithelium-intact guinea-pig trachea, ET-1 (0.3 μM) stimulated the release of a variety of prostanoids, measured using GC/MS techniques (Figure 2a, Table 1). The profile of release was: $PGD_2 = PGE_2 = 6$ keto $PGF_{1α}$ (PGI_2 metabolite) $> PGF_{2α} = TxB_2 >> 9α$, 11β PGF_2 (PGD_2 metabolite). The threshold concentration of ET-1 to elicit prostanoid release appeared to be about 10 nM, as 1 nM ET-1 was without effect in two preparations (data not shown), whereas 10 nM elicited a small but detectable release of most of the postanoids; the magnitude of the release was less than 10% of that produced by 0.3 μM ET-1 (Figure 2a).

As indicated in Figure 2b a comparison was made of the ability of ovalbumin (0.01 mg ml^{-1}, a maximally effective concentration) and ET-1 (0.3 μM) to stimulate prostanoid release from trachea taken from guinea-pigs that were actively sensitized with ovalbumin. Two main observations were apparent from this study. First, the total prostanoid release induced by ovalbumin (37,588 pg g^{-1} tissue net weight) was about three fold higher than that elicited by ET-1 (11,635 pg g^{-1} tissue net weight). Secondly, the profile of prostanoid release produced by ovalbumin ($PGD_2 >> PGE_2 = TXB_2 > 6$ keto $PGF_{1α} > PGF_{2α} > 9α$, 11β PGF_2) was different from that observed with ET-1 (see above).

The influence of the epithelium on ET-1-induced prostanoid release was analysed. In the four tissues studied, epithelium removal did not inhibit the prostanoid release induced by ET-1 (0.3 μM) (Figure 3a); furthermore, the relative profile of release of the individual prostanoids was not significantly altered. The total amount of prostanoid release in epithelium-denuded tissues (22,952 pg g^{-1} tissue net weight) was more than two fold higher than that observed in epithelium-containing preparations (10,302 pg g^{-1} tissue net weight); due to the intraexperimental variability these differences were not statistically significant, $P > 0.3$.

The influence of the smooth muscle (trachealis) on ET-1-induced prostanoid release was studied. In preparations from which the smooth muscle had been carefully removed, ET-1 (0.3 μM) elicited prostanoid release which was very similar, both in terms of the absolute and relative amounts of the individual prostanoids, to that observed in preparations containing smooth muscle (Figure 3b).

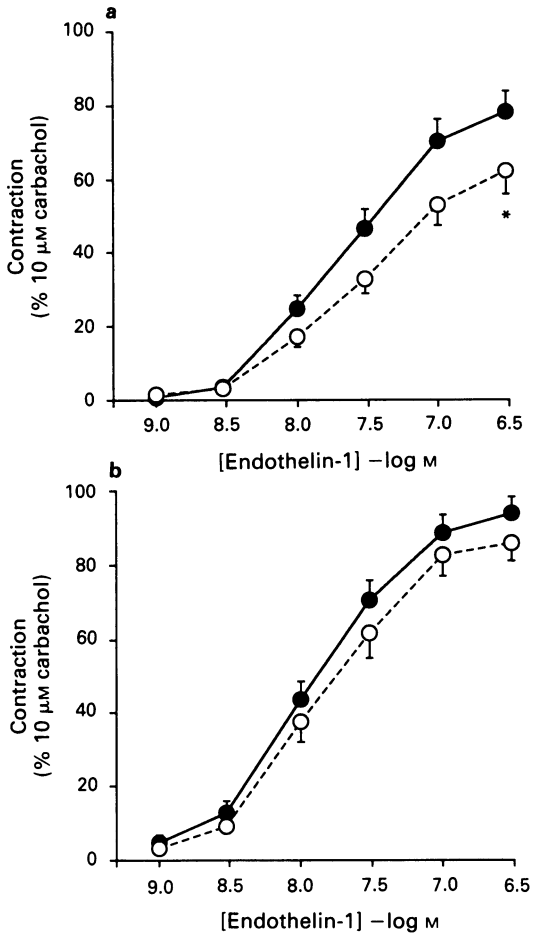

Figure 4 Effect of capsaicin (10 μM; 1 h exposure) on endothelin-1 (ET-1) concentration-response curves in guinea-pig trachea in the presence (a) and the absence (b) of the epithelium. Results are expressed as a percentage of response to 10 μM carbachol and are the mean ± s.e.mean of 15 experiments; (●) control; (○) plus capsaicin. Studies were conducted in the presence of 5 μM indomethacin. *$P<0.05$.

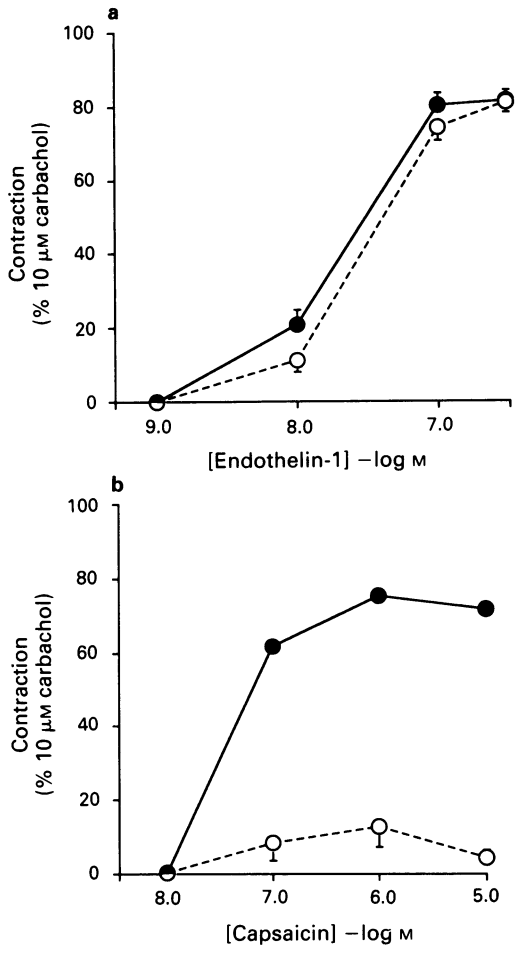

Figure 5 Effect of the combination of the NK_1 tachykinin receptor antagonist CP 96,345 (0.1 μM) and the NK_2 tachykinin receptor antagonist SR 48968 (0.1 μM) on (a) endothelin-1 (ET-1) and (b) capsaicin concentration-response curves in epithelium-containing guinea-pig trachea. Results are expressed as a percentage of the response to 10 μM carbachol and are the mean ± s.e.mean of 6 experiments; (●) = control; (○) plus CP 96,345 and SR 48968. Studies were conducted in the presence of 5 μM indomethacin.

Effects of the epithelium, capsaicin and tachykinin receptor antagonists on ET-1-induced contractions

We tested the hypothesis that ET-1 releases tachykinins from capsaicin-sensitive nerve fibres; these experiments were conducted in the presence of 5 μM indomethacin. Addition of capsaicin (10 μM) resulted in a significant elevation in tone which returned to baseline after approximately 45–60 min. Pretreatment for 1 h with capsaicin (10 μM), followed by washout over 30–45 min, abolishes the response to subsequent exposure to capsaicin, as well as the response to stimulation of tachykinin-containing nerves in the trachea (Ellis & Undem, 1990). Treatment of the tissues with capsaicin in this fashion had a slight, but significant inhibitory effect on ET-1 concentration-response curves in epithelium-containing tissues with an approximately 20% reduction in the maximum response to 0.3 μM ET-1. In contrast, capsaicin had no effect on the ET-1-induced contractions in epithelium-denuded preparations (Figure 4). Capsaicin (10 μM, 1 h pretreatment) had no effect on carbachol concentration-response curves in either the presence or absence of the epithelium (data not shown).

It was observed that the combination of the NK_1 tachykinin receptor antagonist, CP 96,345 (0.1 μM) and the NK_2 tachykinin receptor antagonist, SR48986 (0.1 μM) was without effect on ET-1 concentration-response curves in epithelium-containing guinea-pig trachea (Figure 5a). In contrast, the combination of these antagonists virtually abolished the contractile response to capsaicin (Figure 5b).

Discussion

In a previous paper it was concluded that in guinea-pig trachea, ET-1 produces contraction predominantly via a direct mechanism (Hay, 1990). Thus, atropine, mepyramine, SK&F 104353 (the peptidoleukotriene receptor antagonist) and SQ 29,548 (the thromboxane receptor antagonist) were without significant effect on ET-1-induced contraction (Hay, 1990). The data from the present study provide further evidence in support of the above postulate in that the combination of mepyramine (10 μM) and SK&F 104353 (10 μM) did not inhibit the contraction induced by ET-1. This experiment was performed as it had been noted previously that, either agent alone produced only partial inhibition of the antigen-induced, mast-cell dependent contraction in guinea-pig isolated trachea, whereas the combination of the two antagonists actually abolished the response (Hay *et al.*, 1987). The lack of effect of the combination of mepyramine and SK&F 104353 against contraction to ET-1 suggests that the release of histamine and peptidoleukotrienes from mast cells are not involved in this response. This is supported by the lack of ability of ET-1 to stimulate the release of histamine or peptidoleukotrienes from trachea. The levels of histamine

or i-LTC_4 released basally or in the presence of a concentration of ET-1 which produces the maximum or close to the maximum contraction, 0.3 μM, contrasted with the substantial release from trachea isolated from actively sensitized guinea-pigs induced by a maximally effective concentration of antigen. These data, considered along with our previous findings that the thromboxane receptor antagonist, SQ 29,548 had no effect, and indomethacin slightly enhanced ET-induced contractions of the isolated trachea (Hay, 1990) support the hypothesis that ET contracts the trachea primarily via a direct effect on the trachealis. Additional support of this can be found from experiments in which intra-arterial injection of ET in guinea-pig perfused lung did not stimulate the release of histamine into the lung effluent (Touvay *et al.*, 1990). Furthermore, mepyramine did not influence ET-induced bronchoconstriction in guinea-pigs *in vivo* (Lagente *et al.*, 1989; Nambu *et al.*, 1990). In addition, in the present study, WEB 2086, the potent, selective PAF receptor antagonist (Casals-Stenzel, 1987a,b) was without affect on the response produced by ET-1.

In contrast to the above data, there are some reports that ET-1 produces contraction of guinea-pig trachea, in part, via an indirect mechanism involving the release of secondary mediators. For example, a PAF antagonist, BN 52021, attenuated ET-1-induced contraction of guinea-pig trachea and also bronchi (Battistini *et al.*, 1990); although no data were shown, these authors indicated that similar results were obtained with WEB 2086, the PAF antagonist used in the present study. Accordingly, it was proposed that PAF may mediate, in part, ET-induced contraction in guinea-pig airways. It is difficult to envisage a prominent role for PAF in mediating ET-1-induced contraction of guinea-pig isolated trachea in view of the unresponsiveness of this preparation to the contractile effects of PAF that is generally observed (Vargaftig *et al.*, 1980; Prancan *et al.*, 1982; Cerrina *et al.*, 1983). Furthermore, autoradiography or binding studies revealed few if any specific binding sites for PAF in guinea-pig trachea (Hwang *et al.*, 1983; Goldie *et al.*, 1990). Filep and co-workers concluded, following studies examining the effects of various receptor antagonists, that ET-1-induced contraction and thromboxane release in guinea-pig trachea were, in part, mediated by the release of peptidoleukotrienes and PAF (Filep *et al.*, 1991b). It has been reported that a histamine receptor antagonist alone inhibited ET-induced contraction in this tissue (Ninomiya *et al.*, 1989; Nomura *et al.*, 1990). Furthermore, based on the effects of thromboxane receptor antagonists, there is some discrepancy as to the role of thromboxane in the response produced by ET-1 in guinea-pig isolated trachea (Hay, 1990; Filep *et al.*, 1990). A recent study indicated that ET-1 stimulated the release of histamine from guinea-pig pulmonary, but not peritoneal mast cells (Uchida *et al.*, 1992). The reason(s) for the differences between some of the findings from the above studies and the data of the present study is unknown.

ET was an efficacious stimulator of prostanoid production in the trachea. Interestingly, PGD_2, along with PGE_2, was the major prostanoid produced. PGD_2 is synthesized by mast cells (Lewis *et al.*, 1982), and is released from the guinea-pig trachea by antigenic stimulation (Undem *et al.*, 1988). If ET-1 is stimulating the mast cell to synthesize PGD_2 it would appear to be doing so in the absence of degranulation and histamine release. Alternatively, cells other than mast cells may be synthesizing the PGD_2 in the guinea-pig trachea. The cell types stimulated by ET-1 to synthesize the prostanoids cannot be discerned from our results. Nevertheless, the findings with epithelium-denuded trachea, and trachealis-free trachea indicate that neither the cells associated with the epithelium or the smooth muscle are obligatory for prostanoid production. ET-1 potently stimulates 6-keto $PGF_{1\alpha}$ from cultured bovine aortic endothelial cells with an EC_{50} of 3 nM (Filep *et al.*, 1991a). In contrast, 10 nM ET-1 was reported to be without effect on the release of $PGF_{2\alpha}$, and also histamine, from guinea-pig trachealis smooth muscle (Nagai *et al.*, 1991), although Filep and co-workers observed that ET-1 stimulated thromboxane release from guinea-pig trachea (Filep *et al.*, 1990; 1991b). Furthermore, ET-1 (0.1 μM – 10 μM) stimulated the release of the cyclo-oxygenase products PGD_2, PGE_2 and TxB_2 and also 15-HETE, but not 6-keto $PGF_{1\alpha}$ or the peptidoleukotrienes, from human cultured nasal mucosa (Wu *et al.*, 1992). It is noteworthy that the greatest effect was on the release of PGD_2 and it was also observed that the ET-1-induced release of eicosanoids was not affected by a PAF receptor antagonist (Wu *et al.*, 1992).

Notwithstanding the relatively minor effects of the cyclo-oxygenase inhibitor, indomethacin, on ET-induced contractions of the isolated trachea (Maggi *et al.*, 1989; Hay, 1990; Henry *et al.*, 1990; Sarriá *et al.*, 1990), cyclo-oxygenase products may contribute to the effects of ET on the airways *in vivo*. Indeed, from the limited *in vivo* studies conducted to date, there seems to be a consensus that ET-1 produces bronchoconstriction in guinea-pigs, in part, indirectly by the release of secondary mediators, of which the predominant one appears to be thromboxane. For example, the bronchoconstriction elicited by intravenous administration of ET was essentially abolished by pretreatment with a cyclo-oxygenase inhibitor (Payne & Whittle, 1988; Macquin-Mavier *et al.*, 1989) or a thromboxane A_2 synthase inhibitor (Nambu *et al.*, 1990). In addition, indomethacin, or the PAF antagonist, BN 52021, produced approximately 50–60% inhibition of aerosolized ET-induced bronchoconstriction in guinea-pigs (Lagente *et al.*, 1989). Furthermore, it is possible that released prostanoids contribute significantly to effects of the ETs in airways other than bronchoconstriction.

Previous studies from our laboratory indicated that epithelium removal potentiates the response to ET-1 in guinea-pig trachea (Hay, 1989). Phosphoramidon, the NEP inhibitor (Hudgin *et al.*, 1981), inhibited the potentiating effect of epithelium removal on endothelin-induced contraction, suggesting that the phenomenon was due to the removal of an epithelium-derived phosphoramidon-sensitive peptidase, presumably NEP, that normally metabolizes endothelin (Hay, 1989). The members of the ET family have been shown to be good substrates for NEP (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991). Other researchers have recently provided further data in support of a modulatory role for epithelium-derived NEP on ET-1-induced contraction in guinea-pig isolated trachea (Noguchi *et al.*, 1991; Di Maria *et al.*, 1992; Yamaguchi *et al.*, 1992). For example, recombinant human NEP decreased ET-1-induced contraction (Di Maria *et al.*, 1992). Alternatively, however, the effects could be, in part, due to ET-1 releasing from the epithelium a peptide, e.g. a tachykinin such as substance P, that is metabolized by NEP. Substance P-containing nerves have been localized in the airway epithelium of several species including guinea-pigs (Lundberg *et al.*, 1984). To examine this other postulate the effects of capsaicin, which depletes afferent sensory neurones (Buck & Burks, 1986; Maggi & Meli, 1988), on ET-1-induced responses was examined. Capsaicin pretreatment produced a statistically significant inhibition of ET-1-induced contraction in guinea-pig trachea with an intact epithelium but not in preparations in which the epithelium had been removed. This suggests that in intact preparations a component of the response to ET-1 is due to the release of a substance from capsaicin-sensitive nerves. However, the contribution is a relatively minor one as capsaicin produced only approximately a 20% reduction in the maximum contraction produced by ET-1. The lack of effect of the combination of the potent and selective NK_1 and NK_2 receptor antagonists, CP-96,345 (Snider *et al.*, 1991) and SR 48968 (Advenier *et al.*, 1991), respectively, on ET-1-induced contraction in epithelium-containing guinea-pig trachea, at concentrations which essentially abolished contraction elicited by capsaicin, suggests that the inhibitory effect of capsaicin in these tissues is not due to the depletion of a substance (e.g. substance P or neurokinin A) which activates NK_1 or NK_2 tachykinin receptors. It is more likely

that the effect of capsaicin involved the release of another substance acting through another receptor system. Alternatively, the effect of capsaicin may not be related to its recognized ability to deplete afferent sensory neurones (Buck & Burks, 1986; Maggi & Meli, 1988); it is not due to a non-specific action on smooth muscle responsiveness as the inhibitory effect on responses to ET-1 was observed only in epithelium-containing tissues and capsaicin was without effect on carbachol-induced contraction in either the presence or absence of the epithelium. It is of interest that Yamaguchi and co-workers reported recently that phosphoramidon potentiated the response to ET-1 in guinea-pig trachea even after treatment of tissues with capsaicin (Yamaguchi *et al.*, 1992).

In summary, the present results provide further evidence that in guinea-pig isolated trachea, ET-1 produces contraction predominantly via a direct mechanism; there is no significant contribution of released histamine, leukotrienes, or PAF. There is circumstantial evidence of a contribution, albeit minor, of an epithelium-derived substance(s) that is released from capsaicin-sensitive sensory neurones. However, this does not appear to involve tachykinins acting on NK_1 or NK_2 receptors. ET-1 stimulates the release of various prostanoids, most notably PGD_2, PGE_2 and prostacylin, but only at relatively high concentrations ($\geqslant 10$ nM). Although these prostanoids do not appear to play a major role in ET-induced contraction of the isolated trachea, they may contribute to the overall pulmonary response to ET-1 exposure in the guinea-pig.

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Aminoguanidine selectively inhibits inducible nitric oxide synthase

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1 Endotoxin induces nitric oxide synthase in vascular tissue, including rat main pulmonary artery. Currently available agents that cause inhibition of nitric oxide synthase are relatively non-selective between the constitutive and inducible forms of the enzyme.

2 Aminoguanidine caused a dose-dependent increase in phenylephrine-induced tension in intact and endothelium-denuded pulmonary artery rings from endotoxin-treated rats, but had no effect on sham-treated controls.

3 Contraction caused by aminoguanidine in endothelium-denuded vessels from endotoxin-treated rats was unaffected by indomethacin (10 μM), and by cimetidine and mepyramine (both 10 μM), excluding an effect of aminoguanidine mediated by arachidonic acid metabolites or histamine.

4 Contraction caused by aminoguanidine in endothelium-denuded vessels from endotoxin-treated rats was abolished by L-arginine (2 mM) and L-N^G-monomethyl arginine (300 μM), but unaffected by D-arginine and D-N^G-monomethyl arginine, suggesting that its action is mediated by the L-arginine/nitric oxide pathway.

5 Aminoguanidine had no effect on acetylcholine-induced relaxation of intact vessels from sham-treated rats. However, relaxation of artery rings from endotoxin-treated rats by L-arginine was competitively inhibited by aminoguanidine.

6 These results in isolated main pulmonary arteries of the rat confirm previous reports that aminoguanidine is a selective inhibitor of inducible nitric oxide synthase.

Keywords: Nitric oxide; NO synthase; aminoguanidine; endotoxin; pulmonary arteries

Introduction

Endotoxin is an initiator of the sepsis syndrome (Bone, 1991) which is characterized by systemic vasodilatation, a diminished response to vasoconstrictors and hypotension (Suffredini *et al.*, 1989; Parrillo *et al.*, 1990). In contrast, clinical sepsis is associated with pulmonary hypertension (Zapol *et al.*, 1977) and in animal models endotoxaemia causes a loss of hypoxic pulmonary vasoconstrictors (Weir *et al.*, 1976).

Endotoxin leads to induction of a calcium-independent NO synthase in endothelium (Radomski *et al.*, 1990) and vascular smooth muscle (Rees *et al.*, 1990; Fleming *et al.*, 1991). Isolated arteries from rats treated *in vivo* with *E. coli* lipopolysaccharide show impaired responsiveness to vasoconstrictors, such as catecholamines and potassium chloride (Wakabayashi *et al.*, 1987). Induction of NO synthase with overproduction of the vasodilator NO has been demonstrated in the vascular smooth muscle of rat thoracic aorta *ex vivo* and is thought to underlie this phenomenon (Julou-Schaeffer *et al.*, 1991). A combination of three cytokines (interleukin-1β, interferon γ and tumour necrosis factor α) and endotoxin induce NO synthase in cultured rat pulmonary artery smooth muscle (Nakayama *et al.*, 1992). In isolated endotoxin-treated main pulmonary arteries of the rat NO synthase inhibitors reverse vascular hyporesponsiveness to phenylephrine (Griffiths *et al.*, 1992).

Aminoguanidine incorporates the guanido group of L-arginine linked to hydrazine. In cultured pancreatic islet cells, aminoguanidine and L-N^G-monomethyl arginine (L-NMMA) are equipotent inhibitors of interleukin-1-induced nitrite and guanosine 3':5'-cyclic monophosphate (cyclic GMP) production (Corbett *et al.*, 1992). When [^{3}H]-L-arginine conversion to citrulline in endotoxin-stimulated cultured macrophages was used to assay activity of the inducible enzyme, aminoguanidine was approximately seven times more potent than L-NMMA (Tilton *et al.*, 1993). However, L-NMMA was 15 times more potent than aminoguanidine at inhibiting constitutive NO synthase isolated from rat brain (Tilton *et al.*, 1993). Aminoguanidine was 40 fold less potent in increasing mean arterial pressure in anaesthetized rats (Corbett *et al.*, 1992) and 30 fold less potent in contracting porcine isolated splenic arteries (Lot & Wilson, 1992). These observations suggest significant selectivity for aminoguanidine in inhibiting the inducible, rather than the constitutive form of neuronal and vascular NO synthase.

Aminoguanidine has effects on several enzyme systems; it interferes with non-enzymic glycosylation (Edelstein & Brownlee, 1992), leading to its investigation as a potential treatment for the complications of diabetes. Furthermore, it inhibits diamine oxidase which contributes to inactivation *in vivo* of histamine (Ohrui *et al.*, 1992), a dilator of rat isolated pulmonary arteries (Szarek *et al.*, 1992). Recent work in the rat isolated lung suggests that whilst aminoguanidine inhibits NO synthase, it also increases angiotensin II-mediated prostacyclin release although the mechanism underlying this is unclear (Eaton *et al.*, 1993).

The aim of this study was therefore to test the hypothesis that aminoguanidine is a selective inhibitor of inducible NO synthase and that this accounts for its actions on endotoxin-treated tissues.

Methods

Tissue preparation

Male Wistar rats (250–300 g) were treated 4 h before they were killed by cervical dislocation, with either *Salmonella*

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enteritidis endotoxin (20 mg kg^{-1}, i.p.) or saline vehicle (sham: 3 ml kg^{-1}). The main pulmonary arteries were dissected, cut into rings 2 mm long, mounted over a pair of rigid wires and suspended in 2 ml organ baths containing warmed (37°C) oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. One wire was fixed and the other attached to a force transducer (FT.03 Grass Instruments, Quincy, U.S.A.). Changes in isometric force were recorded on a polygraph (Grass Model 7). After 15 min equilibration the rings were contracted with KCl (40 mM) and relaxed to a uniform baseline tension (500 mg) by washing with KH solution. Rings were left to equilibrate in the bath for a total of 60 min and washed every 20 min.

To test the selectivity of aminoguanidine for vessels from endotoxin-treated animals

Four rings were obtained from each animal. The endothelium was removed from two by gentle abrasion over a roughened needle and successful denudation subsequently confirmed by failure of tissue preparations to relax to acetylcholine (100 μM) after contraction with phenylephrine (EC_{90} of phenylephrine: 1 μM). After further equilibration (30 min), aminoguanidine (100 μM) or vehicle (distilled water 20 μl) were added to an endothelium-denuded and an intact ring; 15 min later a concentration-response curve to phenylephrine (PE, 1 nM to 10 μM) was constructed. Results are expressed as a percentage of the pretreatment contraction to PE (1 μM).

To test the hypothesis that aminoguanidine acts by inhibiting NO synthase

A concentration-response curve to PE was constructed in endothelium-denuded rings from endotoxin-treated rats and the EC_{50} calculated for each ring. One hour later, each ring was contracted with its EC_{50} of PE and concentration-response curves constructed by addition of aminoguanidine (1 μM to 30 mM) or vehicle. Series of identical experiments was performed in the presence of indomethacin (10 μM), or cimetidine and mepyramine (both 100 μM). Cimetidine and mepyramine (both 100 μM) abolished histamine-induced vasodilatation of pre-contracted intact pulmonary arteries (data not shown).

In a further set of experiments, L-arginine (2 mM), D-arginine (2 mM), L-N^G-monomethyl arginine (L-NMMA 300 μM) or D-N^G-monomethyl arginine (D-NMMA 300 μM) were added to preparations 15 min prior to contraction of the rings with their EC_{50} of PE. L-NMMA (300 μM) had previously been shown to produce the maximum increase in tone of precontracted pulmonary artery rings (data not shown). Dose-response curves to aminoguanidine were constructed as before. Results are expressed as a percentage of the contraction to the EC_{50} of PE.

To confirm that aminoguanidine has no effect on constitutive NO synthase

Arterties from untreated rats were incubated for 15 min with aminoguanidine (300 μM) or vehicle. After preincubation with PE (1 μM), an acetylcholine concentration-response curve was constructed (1 nM to 300 μM). Results are expressed as a percentage of the contraction induced by PE.

To test the hypothesis that aminoguanidine is a competitive inhibitor of inducible NO synthase in rat main pulmonary artery

A concentration-response curve to PE was constructed in endothelium-denuded rings from endotoxin-treated and sham-treated rats. After an equilibration period (60 min) an equal number of endothelium-denuded and intact rings were contracted with PE (1 μM) and the tension produced was manually adjusted (by minimal stretching of the preparation) to the maximum tension previously achieved by the phenylephrine contraction-response curve. Relaxation-response curves were constructed with L-arginine (0.1 μM to 300 μM).

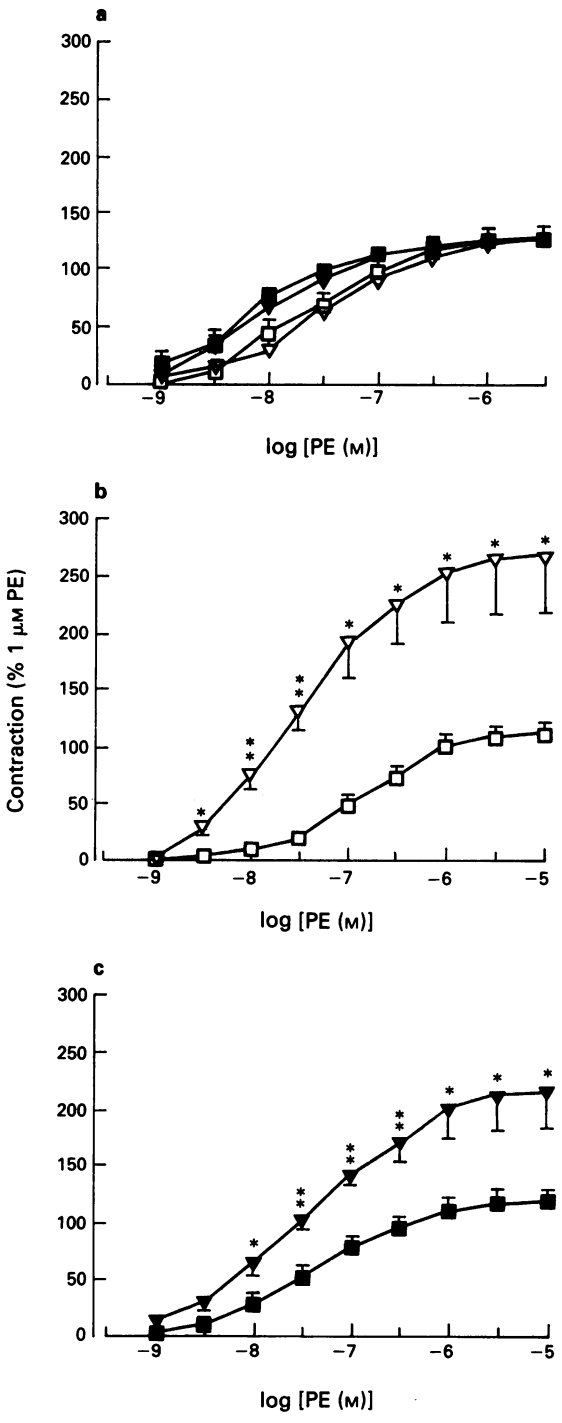

Figure 1 (a) Contraction by phenylephrine in endothelium-denuded (solid symbols) and intact (open symbols) main pulmonary artery rings, from sham-treated rats, in the presence (▼,▽)) and absence (■,□) of aminoguanidine (100 μM). (b) Contraction by phenylephrine in intact main pulmonary artery rings, from endotoxin-treated rats, in the presence (▽) and absence (□) of aminoguanidine (100 μM). (c) Contraction by phenylephrine in endothelium-denuded main pulmonary artery rings, from endotoxin-treated rats, in the presence (▼) and absence (■) of aminoguanidine (100 μM). Results are expressed as mean ± s.e.mean of six observations and indicate percentage of tension produced by phenylephrine (1 μM). *$P<0.05$; **$P<0.01$.

Endothelium-denuded vessels from endotoxin-treated rats were incubated for 15 min with aminoguanidine (300 μM) after equilibration. Aminoguanidine-treated rings were contracted with their EC_{50} of PE and the tension produced was adjusted (as above), so that the preparations were under equivalent tension following PE (1 μM) and aminoguanidine plus their EC_{50} of PE. Concentration-response curves were constructed with L- and D-arginine (0.1 μM to 300 μM). Results are expressed as a percentage of the manually adjusted precontraction.

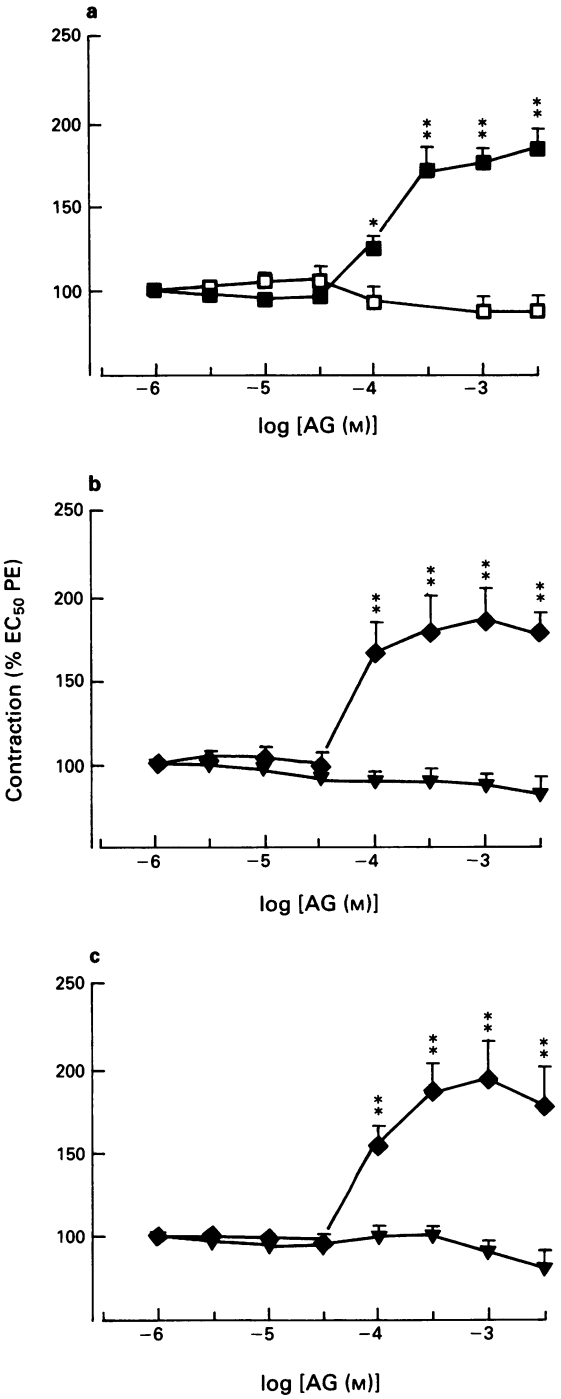

Figure 2 (a) Contraction by aminoguanidine (AG) (■) or vehicle (□) of endothelium-denuded main pulmonary artery rings, from endotoxin-treated rats, following contraction by the ring's EC_{50} of phenylephrine. (b) Contraction by aminoguanidine of endothelium-denuded main pulmonary artery rings, from endotoxin-treated rats, following contraction by the ring's EC_{50} of phenylephrine in the presence of L-arginine (▼; 2 mM) or D-arginine (◆; 2 mM). (c) Contraction by aminoguanidine of endothelium-denuded main pulmonary artery rings, from endotoxin-treated rats, following contraction by the ring's EC_{50} of phenylephrine in the presence of L-N^G-monomethyl-arginine (▼; 300 μM) or D-N^G-monomethyl-arginine (◆; 300 μM). Results are expressed as mean ± s.e.mean of six observations and indicate percentage of tension produced by an EC_{50} of phenylephrine for each ring. $^*P<0.05$; $^{**}P<0.01$.

Drugs

Acetylcholine chloride, aminoguanidine hemisulphate, D-arginine hydrochloride, L-arginine hydrochloride, indomethacin, lipopolysaccharide from *Salmonella enteritidis* (code number L6011), D-N^G-monomethyl-arginine acetate, L-N^G-monomethyl-arginine acetate and L-phenylephrine hydrochloride were obtained from Sigma, Poole, Dorset, UK; cimetidine hydrochloride from Smith-Kline and Beecham, Welwyn Garden City, UK; and mepyramine mesylate from Rhone-Poulenc, Dagenham, UK.

Statistics

Results are expressed as mean ± s.e.mean. Comparisons between means are made by Student's unpaired *t* test. $P<0.05$ was considered to be significant for all tests. All values for EC_{50} and IC_{50} were obtained from sigmoid logistic curves (Graph PAD Inplot, Graph PAD Software, San Diego, CA, U.S.A.).

Results

Effects of aminoguanidine on phenylephrine-induced contraction in pulmonary artery rings from sham and endotoxin-treated rats

Concentration-response curves to PE in arteries with and without endothelium from sham and endotoxin-treated rats are shown in Figure 1 and the corresponding EC_{50} and maximal contraction values are given in Table 1. Aminoguanidine had no significant effect on vessels from endotoxin-untreated rats, but enhanced PE-induced contraction in intact and, to a lesser extent, in endothelium-denuded arteries.

Effects of indomethacin, mepyramine and cimetidine, L- and D-arginine and L- and D-NMMA on aminoguanidine-induced contraction in endothelium-denuded pulmonary artery rings from endotoxin-treated rats

Aminoguanidine caused a dose-dependent contraction of endothelium-denuded vessels from endotoxin-treated rats (Figure 2a). Aminoguanidine-induced contraction required up to 1 h to reach a plateau. Pretreatment with either indomethacin (10 μM), cimetidine and mepyramine (both 100 μM), D-arginine (2 mM) or D-NMMA (300 μM) had no significant effect on the response to aminoguanidine (Table 2). Pretreatment with L-arginine (2 mM) or L-NMMA (300 μM) abolished the contractile response to aminoguanidine (Figure 2b and c).

Effects of aminoguanidine (300 μM) on acetylcholine-induced relaxation in rat pulmonary arteries

There was no difference in relaxation to acetylcholine in vessels from sham-treated rats pretreated with aminoguanidine or vehicle (IC_{50} $7.41 \pm 0.24 \times 10^{-8}$ M cf. $7.94 \pm 0.15 \times 10^{-8}$ M; maximal relaxation 93.2 ± 1.87% cf. 94.3 ± 0.56%, $n = 6$, Figure 3).

Effects of aminoguanidine (300 μM) on L- and D-arginine-induced relaxation of intact pulmonary artery rings from endotoxin-treated rats

L-Arginine caused dose-dependent relaxation of endothelium-denuded vessels from rats pretreated with endotoxin (IC_{50}

Table 1 Effects of aminoguanidine (AG; 100 μM) on phenylephrine-induced contraction in endothelium-intact (E+) and denuded (E−) pulmonary arteries from sham and endotoxin-treated (LPS) rats

Rat treatment	*E*	*AG* (100 μM)	EC_{50} (M)	T_{max} (%)
Sham	+	−	$2.57 \pm 0.23 \times 10^{-8}$	129 ± 11
Sham	+	+	$1.95 \pm 0.20 \times 10^{-8}$ NS	125 ± 4 NS
Sham	−	−	$9.12 \pm 0.07 \times 10^{-9}$	125 ± 6
Sham	−	+	$7.94 \pm 0.21 \times 10^{-9}$ NS	126 ± 2 NS
LPS	+	−	$1.66 \pm 0.11 \times 10^{-7}$	113 ± 8
LPS	+	+	$1.58 \pm 0.26 \times 10^{-8}$ *	275 ± 57 *
LPS	−	−	$3.02 \pm 0.24 \times 10^{-8}$	118 ± 11
LPS	−	+	$2.57 \pm 0.26 \times 10^{-8}$ NS	215 ± 33 *

T_{max} = maximum tension developed, expressed as a percentage of the pretreatment contraction by phenylephrine (1 μM). Values are the mean ± s.e.mean of six experiments.
*$P<0.05$, NS not significant ($P<0.05$) comparing vessels exposed to aminoguanidine with controls.

Table 2 Effects of indomethacin (10 μM), mepyramine and cimetidine (both 100 μM), L- and D-arginine (2 mM) and L- and D-N^G-monomethyl arginine (L- and D-NMMA, 300 μM), on aminoguinadine-induced contraction in endothelium-denuded pulmonary artery rings from endotoxin-treated rats

Treatment	EC_{50} (M)	T_{max} (%)
AG	$1.51 \pm 0.17 \times 10^{-4}$	196 ± 10
AG + indomethacin	$1.35 \pm 0.09 \times 10^{-4}$ NS	198 ± 11 NS
AG + mepyramine and cimetidine	$1.82 \pm 0.11 \times 10^{-4}$ NS	219 ± 13 NS
AG + D-arginine	$7.41 \pm 0.05 \times 10^{-4}$ NS	193 ± 21 NS
AG + D-NMMA	$9.77 \pm 0.12 \times 10^{-5}$ NS	205 ± 18 NS

T_{max} = maximum tension developed, expressed as a percentage of the pretreatment contraction by each ring's EC_{50} of phenylephrine. Values are the mean ± s.e.mean of six experiments.
NS not significant; $P>0.05$ comparing vessels exposed to aminoguanidine alone with those pretreated as described.

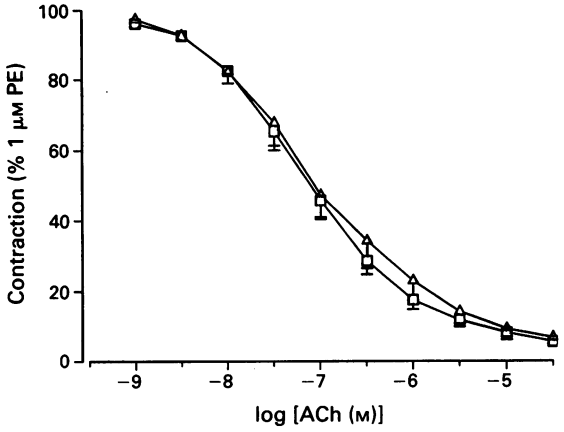

Figure 3 Relaxation by acetylcholine of intact pulmonary artery rings from sham-treated rats in the absence (□) and presence of aminoguanidine (300 μM; ▽). Results are expressed as mean ± s.e.mean of six observations and indicate percentage of tension produced by phenylephrine (PE, 1 μM).

$2.45 \pm 0.17 \times 10^{-5}$ M; maximal relaxation 58 ± 10%; $n = 5$), but had no effect on vessels from sham-treated rats (results not shown). L- but not D-arginine caused dose-dependent relaxation of arteries from endotoxin-treated rats in the presence of aminoguanidine (300 μM) [IC_{50} $2.09 \pm 0.25 \times 10^{-5}$ M, $P<0.05$; maximal relaxation 81 ± 5.3% NS; $n = 5$, compared with L-arginine-induced relaxation in the absence of aminoguanidine] (Figure 4).

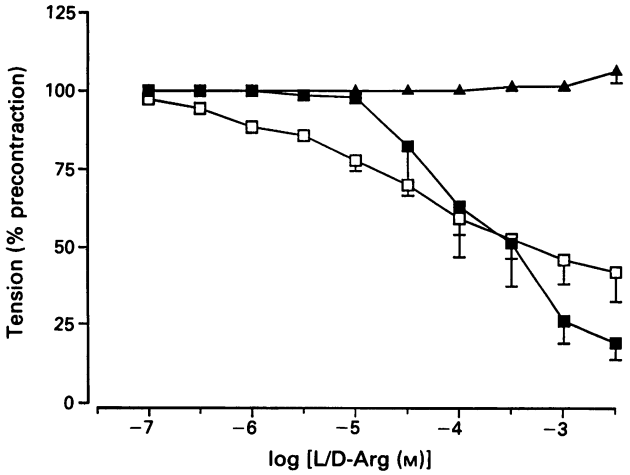

Figure 4 Relaxation by L-arginine (■) or D-arginine (▼) of pulmonary artery rings from endotoxin-treated rats in the presence or absence (□) of aminoguanidine (300 μM). Results are expressed as mean ± s.e.mean of five observations and indicate percentage of the adjusted phenylephrine induced precontraction; *$P<0.05$, **$P<0.01$.

Discussion

Previous studies using an identical rat model have demonstrated induction of NO synthase with over-production of NO, in endothelium-denuded thoracic aorta (Julou-Schaeffer *et al.*, 1990) and main pulmonary artery (Griffiths *et al.*, 1992). Aminoguanidine had no demonstrable effect on phenylephrine-induced contraction or acetylcholine-induced relaxation in arteries from sham-treated rats, providing no evidence of inhibition of the constitutive enzyme in rat main pulmonary artery. Previous studies have cited an increase in systemic arterial pressure as evidence of inhibition of vascular constitutive NO synthase in anaesthetized rats (Corbett *et al.*,

1992) and have demonstrated inhibition by aminoguanidine of the constitutive enzyme from rat cerebella (Tilton *et al.*, 1993). Following endothelial removal, confirmed functionally by failure of vessels to relax to acetylcholine, maximal phenylephrine-induced contraction in arteries from endotoxin-treated rats was increased by aminoguanidine (100 μM) consistent with inhibition of inducible NO synthase in vascular smooth muscle. The failure of aminoguanidine to increase significantly sensitivity to phenylephrine in endothelium-denuded but not intact rings may be due to increased endothelial production of NO following endotoxin treatment. This may be accounted for by induction of NO synthase in vascular endothelial cells, as has been demonstrated *in vitro* (Radomski *et al.*, 1990). Increased production of NO from the constitutive enzyme has also been demonstrated in the rat *in vivo* following intravenous endotoxin administration (Szabo *et al.*, 1993). Failure of aminoguanidine to penetrate the endothelial cell and gain access to the constitutive enzyme cannot be excluded on the basis of these data.

Aminoguanidine produced a slowly-developing dose-dependent increase in tone in endothelium-denuded arteries from endotoxin-treated animals, consistent with inhibition of NO that is continuously being produced by the inducible enzyme. Aminoguanidine infusion in a rat isolated lung preparation is associated with increased angiotensin II-mediated prostacyclin release (Eaton *et al.*, 1993). The lack of effect of indomethacin (10 μM) on aminoguanidine-induced contraction suggests that the latter are unlikely to be mediated or attenuated by products of cyclo-oxygenase. A significant effect of histamine on the vascular actions of aminoguanidine may be suggested because the latter inhibits diamine oxidase, which inactivates histamine *in vivo* (Ohrui *et al.*, 1992). However, mepyramine and cimetidine (both 100 μM) did not significantly alter aminoguanidine-induced vasoconstriction.

L-NMMA but not D-NMMA is a specific inhibitor of NO formation from L-arginine (Rees *et al.*, 1989). Our aim was to prevent aminoguanidine from increasing tone by inhibiting NO synthase maximally with L-NMMA. Whilst L-NMMA alone abolished the aminoguanidine-induced contraction, other factors may have a similar maximum increase in phenylephrine-induced tone to that produced by aminoguanidine, which would *per se* be expected to diminish the effect of another vasoconstrictor regardless of its mechanism of action. Furthermore, it has been suggested that L-NMMA may provide additional substrate for the enzyme via conversion to L-citrulline in endothelial cells (Hecker *et al.*, 1990). We propose that complete reversal of the effects of aminoguanidine by this dose of L-NMMA suggests that the two agents are acting at the same locus.

L-Arginine, but not D-arginine, is the substrate for NO synthase (Palmer *et al.*, 1988). An excess of L-arginine abolished the aminoguanidine-induced contraction, whilst D-arginine had no effect. In order to demonstrate competition between L-arginine and aminoguanidine, the effects of aminoguanidine on L-arginine-induced relaxation of precontracted artery rings from endotoxin-treated rats were studied. L-Arginine caused a dose-dependent relaxation of arteries from endotoxin-treated animals. This is consistent with evidence from studies in rat thoracic aorta that demonstrated activity of the inducible enzyme to be limited by the supply of extracellular L-arginine (Schini & Vanhoutte, 1991; Julou-Schaeffer *et al.*, 1991). Aminoguanidine (300 μM) significantly decreased the sensitivity of endotoxin-treated artery rings to L-arginine-induced relaxation, but had no significant effect on maximal relaxation. These data suggest that aminoguanidine inhibits inducible NO synthase.

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Comparison of the effects of EXP3174, an angiotensin II antagonist and enalaprilat on myocardial infarct size in anaesthetized dogs

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1 In order to determine whether the renin-angiotensin system is involved in myocardial ischaemia-reperfusion injury, we investigated and compared the effects on infarct size of two different drugs which interfere with this system, i.e., an angiotensin II (AT_1) antagonist, EXP3174, and an angiotensin I-converting enzyme inhibitor (ACEI), enalaprilat in a canine model of ischaemia-reperfusion.

2 EXP3174 (0.1 mg kg^{-1}, i.v. followed by 0.02 mg kg^{-1} h^{-1} for 5.5 h) and enalaprilat (0.3 mg kg^{-1}, i.v. followed by 0.06 mg kg^{-1} h^{-1} for 5.5 h) were used in doses inducing a similar level of inhibition (87 ± 4 and 91 ± 3%, respectively) of the pressor responses to angiotensin I. Control animals received saline.

3 Infarct size and area at risk were quantified by *ex vivo* dual coronary perfusion with triphenyltetrazolium chloride and monastral blue dye. Regional myocardial blood flows (ischaemic and nonischaemic, endocardial, epicardial) were assessed by the radioactive microsphere technique.

4 Both EXP3174 and enalaprilat induced a decrease in mean arterial blood pressure. However, non significant changes in regional myocardial blood flows, whether ischaemic or nonischaemic, were observed after administration of either the ACEI or the AT_1 antagonist.

5 The size of the area at risk was similar in the three groups. By direct comparison, there were no significant differences between infarct sizes in the three groups. Furthermore, there was a close inverse relationship between infarct size and transmural mean collateral blood flow in controls, and none of the treatments altered this correlation. Thus, neither EXP3174 nor enalaprilat limited infarct size.

6 These results indicate that activation of the renin-angiotensin system does not contribute to myocyte death in this canine ischaemia/reperfusion model.

Keywords: Angiotensin II; renin-angiotensin system; coronary circulation; infarct size; enalaprilat; EXP3174; AT_1 antagonist; regional myocardial blood flow

Introduction

Among the numerous factors contributing to ischaemia-induced myocardial damage during coronary artery occlusion, the increase in sympathetic tone and the release of catecholamines are known to play a major role (Schömig *et al.*, 1984; Heusch, 1990). However, ischaemia-induced activation of the renin-angiotensin system may also contribute (Ertl, 1987; Ambrosioni & Borghi, 1989). Indeed, angiotensin II has potent coronary vasoconstrictor properties (Heyndrickx *et al.*, 1976) as well as mild positive inotropic effects (Kobayashi *et al.*, 1978) which may jeopardize the ischaemic myocardium by reducing the oxygen supply while increasing the myocardial oxygen demand.

It has been shown that blockade of the renin-angiotensin system by captopril (Ertl *et al.*, 1982), enalapril (Lefer & Peck, 1984; Hock *et al.*, 1985), and ramipril (Martorana *et al.*, 1990) produces a beneficial effect in myocardial ischaemia. However, this cardioprotective effect of angiotensin I-converting enzyme inhibitors (ACEIs) is not universally recognized, as Liang *et al.* (1982) and Daniell *et al.* (1984) have failed to demonstrate any myocardial protection with ACEIs. But, it must be stressed that the pharmacological approach using ACEIs to investigate the effects of the renin-angiotensin system on myocardial ischaemia is indirect and non specific. Indeed, it is not known whether the results obtained are the direct consequence of the blockade of angiotensin II production, or rather that of some of the indirect effects of ACEIs. For example, ACEIs might enhance the cardioprotective effects of endogenous bradykinin (Linder & Heusch, 1990; Martorana *et al.*, 1990; Baumgarten *et al.*, 1993). In addition, sulphydryl containing ACEIs such as captopril may exert cardioprotective effects during ischaemia and reperfusion through their capacity to scavenge oxygen-derived free-radicals (Westlin & Mullane, 1988; Przyklenk & Kloner, 1989; Grover *et al.*, 1991). Thus, the role of the renin-angiotensin system in the myocardial damage induced by ischaemia and reperfusion cannot be evaluated on the sole basis of the results obtained with ACEIs. In this regard, the recent development of non-peptide angiotensin II receptor antagonists should provide a more direct tool for the investigation of the role of this system in myocardial ischaemia/reperfusion injury.

Losartan (DuP 753) is a recently developed nonpeptide angiotensin II (AT_1) antagonist which, unlike peptide antagonists such as saralasin, does not appear to possess partial agonist properties (Chiu *et al.*, 1991). In the rat, losartan generates an active metabolite, EXP3174, which is most likely to be responsible for part of the pharmacological effects of the drug (Wong *et al.*, 1990). In contrast, the haemodynamic effects of losartan in the dog are limited, probably as a result of the weak biotransformation of losartan into EXP3174 in this species (Wong *et al.*, 1991).

The aim of the present study was to evaluate more directly the role of the renin-angiotensin system in ischaemia-reperfusion injury in dogs. For this purpose, we investigated the effects of EXP3174, the active metabolite of losartan, on infarct size in a model of ischaemia-reperfusion. Furthermore, these effects were compared to those of an ACEI, enalaprilat, in order to determine whether the nonspecific properties of ACEIs do or do not add to the potential beneficial effects of renin-angiotensin system blockade on

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infarct size. Since the renin-angiotensin system plays only a minimal role in the control of vascular tone in normotensive dogs, this study was carried out in dogs pretreated with frusemide in order to activate their renin-angiotensin systems.

Methods

The animal instrumentation and the ensuing experiments were performed in accordance with the official regulations of the French Ministry of Agriculture. Furthermore, in order to assess regional myocardial blood flow and infarct size, we followed the recommendations of the NHLBI cooperative study (Reimer *et al.*, 1985).

Experimental preparation

Experiments were performed on mongrel dogs of either sex, weighing 15–25 kg and in which the renin-angiotensin system was activated by prior treatment with frusemide (two separate i.v. bolus doses of 20 mg kg^{-1} per day for two consecutive days before the experiment). Animals were anaesthetized with 35 mg kg^{-1} i.v. sodium pentobarbitone and ventilated by a Harvard respirator with room air supplemented with low-flow oxygen. Arterial blood gases were maintained within physiological range by adjusting ventilatory variables as needed. A saline-filled catheter was inserted into the right femoral artery and advanced in the ascending thoracic aorta for measurement of arterial blood pressure and for withdrawal of reference arterial blood samples required for determining tissue flow. Another catheter was inserted in the right femoral vein for injection of drugs. A left thoracotomy was performed in the fourth intercostal space and the heart was suspended in a pericardial cradle. A saline-filled catheter was placed into the left atrial appendage for subsequent injections of radioactive microspheres. The left circumflex coronary artery was isolated distal to the atrial appendage but proximal to its first marginal branch and was encircled with a silk suture so that the artery could be occluded with an atraumatic Schwartz vascular clamp. Any dog that developed ventricular fibrillation during either occlusion or reperfusion was cardioverted, if possible, using a Lifepack 8 defibrillator (Physiocontrol, Redmond, WA, U.S.A.). Arterial blood pressure and lead II of the electrocardiogram were continuously monitored on a Gould Recorder 2200 S (Gould Instruments Inc., Cleveland, OH, U.S.A.). Heart rate was calculated from the blood pressure recordings. The animals were allowed to rest at least 20 min before the first microsphere injection.

Measurement of regional myocardial blood flows

The distribution of regional myocardial tissue flow was assessed by the radioactive microspheres technique. At the times indicated in the experimental protocol, approximately 2×10^6 microspheres (diameter 15 ± 3 μm), labelled with ^{46}Sc, ^{103}Ru or ^{141}Ce (New England Nuclear, Boston, Massachusetts, U.S.A.), were injected through the catheter into the left atrial appendage, followed by a 10 ml saline flush. Beginning just before and continuing for 60 s after injection, a reference sample of arterial blood was withdrawn from the femoral artery at a rate of 9 ml min^{-1}. Reference blood samples were collected in 6 separate aliquots in order to ensure that all radioactivity had been cleared from the circulation at the end of the sampling.

Experimental protocol

Infarcts were produced by a 90 min occlusion of the left circumflex coronary artery followed by a 4 h reperfusion. Regional myocardial blood flow was measured before drug administration, 10 min into occlusion and 60 min into reperfusion. At the completion of the surgical preparation, animals were randomly assigned to one of the three experimental groups (saline, enalaprilat or EXP3174). Ten minutes before the end of reperfusion, the pressor response to i.v. administration of angiotensin 1 (300 ng) (Sigma Chimie, la Verpillère, France) was assessed, then the heart was excised.

Postmorten studies

Area at risk and infarct size The anatomical boundaries of the previously ischaemic and nonischaemic vascular beds were defined by dual perfusion of dyes at physiological pressure (120 mmHg) in the left main coronary artery and in the previously occluded circumflex artery (Reimer *et al.*, 1985). The perfusion fluid was sodium phosphate buffer (pH = 7.4) plus 7% dextran (87.000 mol. wt., Sigma Chimie) to which either 1% triphenyltetrazolium chloride (TTC, Sigma Chimie) (for the previously occluded circumflex artery), or 3% monastral blue dye (Sigma Chimie) (left main artery) was added. The heart was then fixed by immersion for 3 days in a large volume of phosphate-buffered formalin. The fixed left ventricle was then sectioned into eight transverse slices which were weighed. Five slices were used for the measurements of area at risk (previously occluded vascular bed) and of infarct size. The boundaries of the area at risk and infarct (TTC negative area) were then traced; the tracings were digitized using a HP scanner (Hewlett-Packard, Les Ulis, France) interfaced to a Macintosh computer, and the area at risk and infarct were quantitated with image analysis software.

Regional blood flows Flow measurements were made in the three remaining slices. These slices were divided into nonischaemic and central ischaemic zones. Lateral and septal border zones were eliminated to avoid measurements from samples containing a mixture of nonischaemic and ischaemic tissue. Each sample was further subdivided into subendocardial, midmyocardial and subepicardial thirds. Regional myocardial blood flows were calculated according to the formula:

$$\dot{Q}\,m = (\dot{Q}\,r \times Cm)/Cr$$

where $\dot{Q}$ m = myocardial blood flow (ml min^{-1}); $\dot{Q}$ r = reference blood flow (ml min^{-1}); Cm = counts min^{-1} in tissue sample; Cr = counts min^{-1} in reference blood sample. Myocardial blood flows were expressed relative to the tissue sample weight (ml min^{-1} g^{-1}).

Drugs

EXP3174 and enalaprilat were a gift of Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A. Enalaprilat was dissolved in sterile saline and EXP3174 was dissolved in a mixture of 5% $NaHCO_3$ and 5% dextrose. EXP3174 (0.1 mg kg^{-1}) and enalaprilat (0.3 mg kg^{-1}) were administered as an i.v. bolus immediately after the first microsphere injection (i.e. 30 min before ischaemia), followed by a continuous i.v. infusion (0.02 and 0.06 mg kg^{-1} h^{-1} for EXP 3174 and enalaprilat, respectively) which lasted until the end of the experiment. The doses of EXP3174 and enalaprilat used in the present study were chosen on the basis of our prior experiments in anaesthetized dogs, as those inducing a similar decrease in mean arterial pressure and a similar inhibition of the pressor response to angiotensin I (Richard *et al.*, 1993).

Statistical analysis of data

All values are expressed as mean ± s.e.mean. Comparisons of regional myocardial blood flows, heart rate and mean arterial pressure values were made by a one-way analysis of variance for repeated measures, followed by a Student-Newman-Keuls test for multiple pairwise comparisons. Comparisons of area at risk and infarct size (expressed as percentage of the area at risk as well as percentage of the left ventricle) were made by a one-way analysis of variance. Furthermore, in order to control for the variability in infarct size due to collateral

flow, infarct size was compared among groups by an analysis of covariance with size as a dependent variable and collateral flow as a covariate. A P value <0.05 was considered statistically significant.

Results

Thirty-two dogs initially entered this study. Eight dogs had to be excluded ($n = 1$, 2 and 5 in the control, enalaprilat and EXP3174 groups, respectively) because they developed intractable ventricular fibrillation. Consequently, results are presented for 24 dogs ($n = 8$ in each group).

Effect of intravenous angiotensin I on mean arterial pressure

Intravenous injection of angiotensin I (300 ng) increased mean arterial pressure by 17 ± 3% in control dogs. This pressor response was reduced by 87 ± 4% and 91 ± 3% by EXP3174 and enalaprilat, respectively.

Haemodynamic data

Table 1 shows heart rate values measured in the three groups throughout this study. Baseline heart rate values which were similar in the three groups, were not affected by any of the three treatments.

Table 1 also shows mean arterial pressure values measured in the three groups throughout the study. There were no significant differences between the baseline values of mean arterial pressure in the three groups. In control dogs, no significant change occurred at any time. Before coronary occlusion, enalaprilat and EXP3174 induced a decrease in mean arterial pressure which was reinforced by ischaemia. However, at the end of occlusion and during reperfusion, there were no significant differences in mean arterial pressure values between animals treated with saline, enalaprilat or EXP3174.

Regional myocardial blood flows

Table 2 shows regional myocardial blood flow values in the nonischaemic zones. These regional blood flows were not significantly different between the three groups at any time. However there was a small trend toward higher regional flows during occlusion and reperfusion in the animals treated with EXP3174 and during reperfusion in the animals treated with enalaprilat.

Table 3 shows regional myocardial blood flow values in the ischaemic zones. Coronary occlusion significantly reduced regional blood flows in the three groups. At reperfusion, regional blood flows returned to values that were not significantly different from the preocclusion ones, except in EXP3174-treated dogs were epicardial blood flow values were significantly greater ($P<0.05$). However, there was never any significant difference between regional myocardial blood flow values among the three groups of animals.

Infarct size

The size of area at risk, an important baseline predictor of infarct size, was similar in the three groups and averaged 38.2 ± 1.4% of the left ventricle in controls, 38.7 ± 1.6% in the enalaprilat-treated group, and 39.9 ± 1.1% in the EXP 3174-treated group (Figure 1). Analysis of infarct size (Figure 1), expressed either as a percentage of the left ventricle (14.5 ± 2.8% in the control group, 16.5 ± 3.0% in the enalaprilat-treated group, 20.1 ± 3.5% in the EXP3174-treated group) or as a percentage of the area at risk (36.9 ± 6.6% in the control group, 42.1 ± 7.0% in the enalaprilat-treated group, 49.8 ± 8.5% in the EXP3174-treated group) indicated no significant differences among the three groups. Thus, by direct group comparison, neither of the treatments was able to limit infarct size. Inasmuch as it is well known that variations in infarct size may be due to variations in collateral blood flow (Reimer *et al.*, 1985), we also investigated the relation between infarct size and collateral blood flow (Figure 2). There was a close inverse relation between infarct

Table 1 Heart rate and mean arterial pressure values at baseline, before ischaemia, 10 and 90 min into ischaemia and 60 min into reperfusion in the three groups of animals

	Group	*Baseline*	*Before ischaemia*	*10 min ischaemia*	*90 min ischaemia*	*60 min reperfusion*
Heart rate (beats min^{-1})	Saline	133 ± 7	133 ± 7	133 ± 5	129 ± 8	123 ± 6
	Enalaprilat	137 ± 8	132 ± 8	133 ± 8	131 ± 9	123 ± 9
	EXP3174	134 ± 4	128 ± 6	135 ± 5	140 ± 15	127 ± 7
Mean arterial pressure (mmHg)	Saline	89 ± 6	88 ± 6	83 ± 7	77 ± 7	76 ± 8
	Enalaprilat	96 ± 5	72 ± 6*	59 ± 4**	71 ± 7	73 ± 5
	EXP3174	86 ± 2	76 ± 4*	63 ± 4**	76 ± 6	75 ± 7

Values are mean ± s.e.mean.
Value significantly different (*$P<0.05$; **$P<0.01$) from corresponding saline value.

Table 2 Regional (Endo: endocardial, mid: middle, Epi: epicardial) and transmural (Trans) myocardial blood flows (ml min^{-1} g^{-1}) values in the nonischaemic zones before ischaemia, 10 min into ischaemia and 60 min into reperfusion in the three groups of animals

		Endo	*Mid*	*Epi*	*Trans*
Saline	Before ischaemia	0.69 ± 0.05	0.62 ± 0.05	0.57 ± 0.05	0.63 ± 0.05
	10 min ischaemia	0.82 ± 0.06	0.71 ± 0.05	0.66 ± 0.05	0.73 ± 0.06
	60 min reperfusion	0.76 ± 0.10	0.68 ± 0.09	0.62 ± 0.08	0.69 ± 0.09
Enalaprialt	Before ischaemia	0.89 ± 0.08	0.84 ± 0.08	0.77 ± 0.09	0.83 ± 0.08
	10 min ischaemia	0.85 ± 0.13	0.81 ± 0.13	0.79 ± 0.13	0.82 ± 0.13
	60 min reperfusion	0.98 ± 0.10	0.98 ± 0.11	0.98 ± 0.13	0.98 ± 0.11
EXP3174	Before ischaemia	0.76 ± 0.07	0.70 ± 0.07	0.66 ± 0.06	0.71 ± 0.07
	10 min ischaemia	0.93 ± 0.09	0.85 ± 0.08	0.84 ± 0.07	0.88 ± 0.08
	60 min reperfusion	1.00 ± 0.04	0.93 ± 0.03	0.89 ± 0.03	0.94 ± 0.03

Values are mean ± s.e.mean.

Table 3 Regional (Endo: endocardial, mid: middle, Epi: epicardial) and transmural (Trans) myocardial blood flows (ml min^{-1} g^{-1}) values in the ischaemic zones before ischaemia, at 10 min into ischaemia and at 60 min into reperfusion in the three groups of animals

		Endo	*Mid*	*Epi*	*Trans*
Saline	Before ischaemia	0.78 ± 0.08	0.71 ± 0.08	0.68 ± 0.10	0.73 ± 0.08
	10 min ischaemia	0.04 ± 0.01[ab]	0.08 ± 0.02[ab]	0.16 ± 0.03[ab]	0.09 ± 0.02[ab]
	60 min reperfusion	0.86 ± 0.16	0.85 ± 0.12	0.79 ± 0.13	0.84 ± 0.08
Enalaprialt	Before ischaemia	0.93 ± 0.07	0.87 ± 0.07	0.80 ± 0.07	0.87 ± 0.07
	10 min ischaemia	0.04 ± 0.02[ab]	0.08 ± 0.02[ab]	0.15 ± 0.03[ab]	0.09 ± 0.02[ab]
	60 min reperfusion	1.27 ± 0.36	1.17 ± 0.25	0.86 ± 0.12	1.09 ± 0.21
EXP3174	Before ischaemia	0.86 ± 0.08	0.82 ± 0.08	0.72 ± 0.08	0.79 ± 0.07
	10 min ischaemia	0.04 ± 0.02[ab]	0.06 ± 0.02[ab]	0.11 ± 0.03[ab]	0.06 ± 0.02[ab]
	60 min reperfusion	0.69 ± 0.19	0.96 ± 0.11	1.17 ± 0.08[ac]	0.93 ± 0.09

Values are mean ± s.e.mean.
[a]Value significantly different ($P<0.01$) from before occlusion value.
[b]Value significantly different ($P<0.01$) from 60 min reperfusion value.
[c]Value significantly different ($P<0.01$) from 60 min reperfusion value in saline- and enalaprilat-treated dogs.

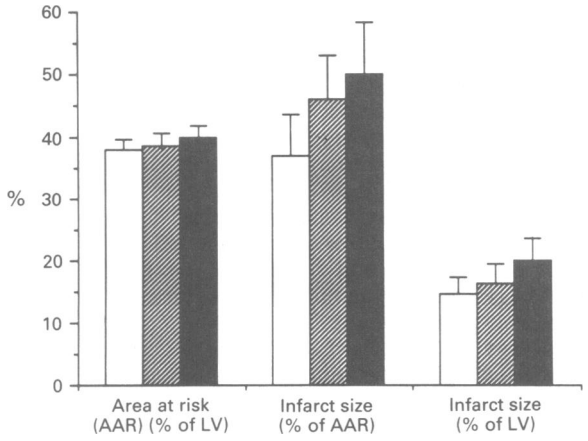

Figure 1 Area at risk and infarct size (expressed as percentage of area at risk and as percentage of left ventricle). Open columns, control; hatched columns, enalaprilat; solid columns, EXP3174.

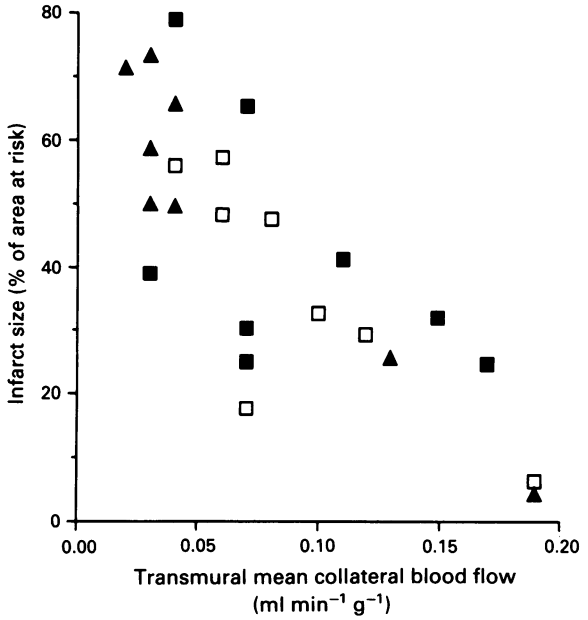

Figure 2 Plot of transmural collateral blood flow versus infarct size in saline (□)- enalaprilat (■)- and EXP3174 (▲)-treated dogs.

size and collateral blood flow in controls. Neither enalaprilat nor EXP3174 altered this relationship, suggesting that none of these treatments affected infarct size. This visual observation was confirmed by an analysis of covariance with collateral blood flow as an independent variable and infarct size as a dependent variable ($F = 0.35$; P = NS). Thus clearly neither of the treatments limited infarct size, even when the variation due to collateral flow was controlled.

Discussion

In this study, intravenous administration of enalaprilat induced a marked inhibition (91%) of the pressor response to angiotensin I, indicating an almost complete angiotensin-converting enzyme inhibition. Similarly, administration of EXP3174 resulted in a strong inhibition (87%) of the response to angiotensin I, demonstrating an almost complete blockade of the AT_1 receptor responsible for the pressor effects of angiotensin II, in agreement with the results of Wong *et al.* (1991) in conscious dogs. In addition, the fact that a similar blockade of angiotensin I pressor responses was achieved with EXP3174 and with enalaprilat indicates that the effects of the two drugs were investigated at an identical level of inhibition of the renin-angiotensin system.

The renin-angiotensin system plays a minor role in the regulation of mean arterial pressure (Laubie *et al.*, 1984) and coronary blood flow (Noguchi *et al.*, 1985; Gerard *et al.*, 1990) in normal dogs. Thus in this study this system was activated by a frusemide-pretreatment. As a result, EXP3174 and enalaprilat decreased mean arterial pressure whereas at the same doses and for the same level of inhibition of the renin-angiotensin system, these compounds do not exhibit any effect in non frusemide-pretreated dogs (Richard *et al.*, 1993). The decrease in mean arterial pressure induced by both EXP3174 and enalaprilat is related to the inhibition of the vasoconstrictor (Liang *et al.*, 1982) and sympathetic system facilitating (Clough *et al.*, 1982) properties of angiotensin II. This hypotension induced by both treatments was reinforced after 10 min of coronary occlusion, whereas in control dogs ischaemia did not induce any modification of mean arterial pressure. Thus, this result confirms the role of the renin-angiotensin system in the control of blood pressure in frusemide-treated dogs. In contrast, Ertl *et al.* (1983) reported that the renin-angiotensin system does not contribute importantly to the control of blood pressure following coronary artery occlusion. However, it must be stressed that the latter experiments were performed with saralasin, a peptide angiotensin II antagonist which exhibits a significant intrinsic partial agonist activity, mimicking the actions of angiotensin II itself (Chiu *et al.*, 1991).

In our experiments, the hypotension induced by either EXP3174 or enalaprilat was transient. At the end of the ischaemic period, no significant difference in arterial pressure was seen among the three groups. Comparison of the values obtained after 10 and 90 min of ischaemia revealed that this was due both to a decrease in blood pressure in controls and to an increase in blood pressure in treated animals. The reason for the increase in arterial pressure (despite persistent

blockade of the pressor response to angiotensin II) is not clear, but could be due to ischaemia-induced compensatory mechanisms (such as sympathetic stimulation), which could compete with the decrease in arterial pressure resulting from the inhibition of the renin-angiotensin system.

In our experimental model, no significant changes in regional myocardial blood flows were observed after administration of either the ACEI or the angiotensin II AT_1 antagonist. Furthermore, and despite the decrease in mean arterial pressure observed after 10 min of coronary artery occlusion, neither drug induced a decrease in collateral blood flow. The lack of effects of EXP3174 and enalaprilat on regional myocardial blood flows is in agreement with previous studies which showed (a) that these regional flows and their distribution (between epicardial and endocardial layers and between nonischaemic and iscahemic zones) are not modified by four ACEIs in anaesthetized dogs submitted to repeated coronary occlusions (Berdeaux *et al.*, 1987), and (b) that in anaesthetized open-chest dogs with or without activation of the renin-angiotensin system, no change in regional myocardial blood flow occurs after intravenous administration of enalaprilat and EXP3174 in nonischaemic conditions (Richard *et al.*, 1993). Thus our present data indicate that the renin-angiotensin system does not play a major role in the regulation of myocardial blood flows in the canine model of ischaemia-reperfusion in confirmation of what has been observed in the above mentioned studies.

The fact that neither enalaprilat nor EXP3174 limited infarct size in our study demonstrates that angiotensin II is not importantly involved in myocardial ischaemia-reperfusion injury. In this respect, our results are in agreement with those of other studies performed with ACEIs (Liang *et al.*, 1982; Daniell *et al.*, 1984; De Lorgeril *et al.*, 1992). In contrast, other investigators have reported an increase in regional myocardial blood flows and a limitation of infarct size with captopril in dogs (Ertl *et al.*, 1982). However, several experiments suggest that the anti-ischaemic effect of the sulphydryl-containing ACEI captopril could be due to non specific effects of this drug, such as an inhibition of oxygen free radical-dependent reperfusion injury (Westlin & Mullane, 1988; Chopra *et al.*, 1992) and/or an interference with arachidonic acid metabolism (Van Gilst *et al.*, 1987). However, in some experimental models of ischaemia, acute administration of non sulphydryl-containing ACEIs also limited infarct size in rats (Hock *et al.*, 1985) and in cats (Lefer & Peck, 1984). Differences in animal models, duration and severity of ischaemia, occurrence of reperfusion, and drug concentrations could contribute to these conflicting results. Finally it must be stressed that exclusion of dogs from the study because of the development of intractable ventricular fibrillation did not lead to an underestimation of infarct size in the control group as only one of the excluded animals belonged to that group.

Several experiments have suggested that the anti-ischaemic effects of non-sulphydryl containing ACEIs could entirely be related to their capacity to potentiate the cardioprotective effects of bradykinin (Becker *et al.*, 1989; Martorana *et al.*, 1990; Van Gilst *et al.*, 1992; Baumgarten *et al.*, 1993). For example, Martorana *et al.* (1990) have shown that the infarct size-limiting effect of ramipril in a dog model of 6 h permanent ischaemia was abolished by a bradykinin antagonist. However, our experiments do not support this hypothesis since enalaprilat did not induce any limitation of infarct size. The discrepancies between the results of Martorana *et al.* (1990) and ours could be explained by differences in the models used. In the former study, experiments were performed in non-salt depleted dogs, i.e., a situation of low renin-angiotensin system activation. Also, in that study, the ACEI was administered into the coronary artery, thus avoiding the systemic effects of the drug. Finally, it must be stressed that Martorana *et al.* (1990) did not measure collateral blood flow. Thus, it is possible that the observed beneficial effect of ramipril in this preparation was due to undetected differences in the level of collateral blood flow between the different experimental groups.

In conclusion, neither EXP3174 nor enalaprilat limited infarct size in frusemide-pretreated dogs. Thus, it appears that the renin-angiotensin system does not significantly contribute to myocyte death in the canine ischaemia-reperfusion model.

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Time-related increases in cardiac concentrations of doxorubicinol could interact with doxorubicin to depress myocardial contractile function

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1 The present study evaluated the time-dependency of acute anthracycline cardiotoxicity by varying the duration of exposure of rabbit isolated atria to doxorubicin and determing changes (1) in contraction and relaxation and (2) in atrial concentrations of doxorubicin and its C-13 hydroxy metabolite, doxorubicinol.

2 Following addition of doxorubicin (175 μM) to atria, contractility (dF/dt), muscle stiffness (resting force, RF) and relaxation (90% relaxation time, 90% RT) were monitored for a 3.5 h period.

3 Doxorubicin (175 μM) progressively diminished mechanical function (decreased dF/dt, increased RF and prolonged 90% RT) over 3 h. Doxorubicinol (1.8 μM), however, failed to produce time-related cardiac dysfunction; it depressed contractile function and increased muscle stiffness during the first 30 min without causing additional cardiac dysfunction during the remaining 3 h of observation. Doxorubicinol had no effect on 90% RT.

4 During treatment with doxorubicin, atria contained considerably more doxorubicin than doxorubicinol (ratio of doxorubicin to doxorubicinol ranged from 778 to 74 at 0.5 and 3 h, respectively). Elevations of doxorubicin and doxorubicinol in atria paralleled the degree of dysfunction of both contraction and relaxation; increases in muscle stiffness, however, were more closely associated with increases of doxorubicinol than doxorubicin.

5 To probe the relation between cardiac doxorubicinol and myocardial dysfunction further, without confounding effects of cardiac doxorubicin, concentration-response experiments with doxorubicinol (0.9–7.2 μM) were conducted.

6 Plots of doxorubicinol concentrations in atria vs contractility indicated that the cardiac concentration of doxorubicinol, at which contractility is reduced by 50%, is five fold lower in doxorubicin-treated than in doxorubicinol-treated preparations. Thus, doxorubicin and doxorubicinol appear to interact to depress contractile function.

7 Cardiac concentrations of both doxorubicin and doxorubicinol, as observed in these studies, were found to stimulate markedly Ca^{2+} release from isolated SR vesicles, but 3 μM doxorubicinol promoted a 15 fold greater release rate than 3 μM doxorubicin.

8 Our observations coupled with the previously reported finding that doxorubicinol inhibits Ca^{2+} loading of SR, suggests that doxorubicinol accumulation in heart contributes to the time-dependent component of doxorubicin cardiotoxicity, through a mechanism that could involve perturbations of Ca^{2+} homeostasis.

Keywords: Doxorubicin; doxorubicinol; cardiotoxicity; sarcoplasmic reticulum; Ca^{2+} release; Ca^{2+} loading; kinetics; dysfunction of contraction and relaxation

Introduction

Myocardial preparations are highly susceptible to the cardiotoxic effects of anthracyclines such as doxorubicin and daunorubicin. *In vitro* studies, while clearly demonstrating the concentration-related nature of the toxicity, also suggest that duration of exposure to an anthracycline may be a major determinant of cardiac dysfunction. Studies in rat Langendorff preparations (Miwa *et al.*, 1986; Pelikan *et al.*, 1986) and in guinea-pig isolated atria (Hagane *et al.*, 1988) showed progressively greater inhibition of cardiac function following doxorubicin treatment during 60 or 180 min observation periods. The above-mentioned studies, however, were not designed to characterize or address the time-dependent component of doxorubicin cardiotoxicity; the relation between exposure time to anthracyclines and acute cardiac dysfunction remains to be investigated.

The demonstration by Olson *et al.* (1988) that cardiac tissue can metabolize doxorubicin to a highly potent cardiotoxin, doxorubicinol, has raised the possibility that anthracycline toxicity over time could be related to this myocardial metabolism. Thus, it is important to determine whether (1) the heart synthesizes sufficient concentrations of doxorubicinol to impair contraction and relaxation and whether (2) the rate of doxorubicinol synthesis parallels the rate of development of myocardial dysfunction. It is also relevant to determine if treatment with doxorubicinol alone produces time-dependent cardiotoxic effects, as would be expected if doxorubicin and doxorubicinol exert toxicities by similar mechanisms. Differing mechanisms of toxicity could result in antagonistic or synergistic interactions between parent anthracycline and metabolite.

To address such issues, our study investigated the relation between time-dependent alterations in atrial contraction and

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relaxation and rates of change of doxorubicin or doxorubicinol in isolated atria treated with either doxorubicin or doxorubicinol. Abilities of these compounds to affect Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles were also compared, to provide additional information about how doxorubicin or its metabolite, doxorubicinol, could impair cardiac function. This paper describes major differences between cardiac effects of doxorubicin and doxorubicinol, which were especially prominent during prolonged exposure to the agents.

Methods

Isolated atria preparations

Experiments were conducted in accordance with the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Adult (2.5–3.5 kg) New Zealand white rabbits of either sex were killed by captive bolt discharge to the cranium. Immediately, a median sternotomy was performed, and the heart removed within 30 s. After placing the heart in iced Krebs-bicarbonate buffer (see below), the left atrium was dissected free, and divided into two thin strips (100 mg each); each muscle strip was placed in a thermojacketed, 25 ml, muscle bath (maintained at 30°C) containing Krebs-bicarbonate buffer composition, mM: NaCl 127, $CaCl_2$ 2.5, KCl 2.3, $NaHCO_3$ 25, KH_2PO_4 1.3, glucose 5.6; the buffer was continuously bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4.

The muscle was attached to an isometric force transducer and stretched to a resting tension of 0.5 g. Muscles were electrically stimulated (S88 stimulator, Grass Medical Instruments, Quincy, MA, U.S.A.) with a square wave pulse (3 ms in duration) at a voltage 10% above threshold voltage of the muscle. Atrial strips were stimulated at 1 Hz and allowed to stabilize for 90–180 min. Experiments were conducted with muscles contracting at a frequency of 2 Hz. Cardiac functional variables examined for each muscle preparation were resting force (RF; mg), maximal rate of rise of force (dF/dt; $g\,s^{-1}$), and 90% relaxation time (90% RT; time for peak developed force to decrease by 90%; ms). Variables evaluated were recorded using high speed (100 mm s^{-1}) oscillographic tracings (Gould 4200S oscillographic recorder) and data analyzed using a Buxco Pulsatile Analyzer (Buxco Electronics, Inc., Sharon, CT, U.S.A.).

Experimental design

Atrial effects of doxorubicin or doxorubicinol were determined 30, 90 and 150 and 210 min after addition of doxorubicin (175 μM) or doxorubicinol (1.8 μM) to the bath. Preliminary trials had shown that the above concentration of either agent produced only a slight, but statistically significant depression of contractile function during a 30 min treatment period, thereby allowing sufficient latitude for each agent to express its ability to compromise cardiac function further during the final 3 h of the experiment. Concentration-response relationships to doxorubicinol were investigated by increasing the concentration of doxorubicinol in the bath in a stepwise fashion (0.9, 1.8, 3.6, 7.2 μM) and measuring functional variables 30 min after each increase (i.e., doxorubinicol was added at times 0, 60, 120 and 180 min; cardiac function was assessed 30, 90, 150 and 210 min after beginning the experiment).

Cardiac concentrations of doxorubicinol were determined at various bath concentrations (i.e., 0.9, 1.8, 3.6, 7.2 μM) in atrial strips treated as described above for concentration-response studies. Each strip was removed for assay after a 30 min exposure to a specific doxorubinicol concentration. Cardiac concentrations of doxorubicin and doxorubicinol were determined at 30 min intervals (for 210 min) after adding doxorubicin (175 μM) to atrial strips.

Assay for doxorubicin and doxorubicinol

After removal from the bath, the atrial strip was homogenized with a polytron (Brinkman) for 30 s in 3 ml of iced saline saturated with ammonium sulphate; daunorubicin (500 ng) was then added as an internal standard. The homogenate was extracted with chloroform:isopropanol (5 ml of a 50:50 (v/v) mixture), vortexed for 3 min, and centrifuged at 1000 *g* for 10 min. The organic phase (which contained the anthracycline) was removed and evaporated under N_2 at room temperature. The residue was resuspended in 500 μl methanol and analyzed with a high performance liquid chromatography (h.p.l.c.) system that included a phenyl reversed phase column (4 micron, Radial-Pak, Waters), a programmable infusion pump to control the gradient of the mobile phase, and a fluorescent detector (Kratos; excitation wavelength = 470 nm, emission wavelength = 550 nm). The mobile phase, which initially contained 72% ammonium formate buffer (16 mM; pH 4.0) and 28% acetonitrile (v/v), was changed to 66% ammonium formate and 34% acetonitrile at 6.5 min, and returned to 72% ammonium formate and 28% acetonitrile at 11.5 min. Concentrations of doxorubicin and doxorubicinol were determined from standard curves generated by adding known amounts of doxorubicin and doxorubicinol to atrial tissue treated exactly as above.

Preparation of cardiac SR vesicles

Canine cardiac sarcoplasmic reticulum (SR) vesicles were prepared by a modification of the method of Harigaya & Schwartz (1969). Mongrel dogs of either sex were killed with sodium pentobarbitone. The heart was rapidly removed from the chest and perfused with ice-cold saline. Fat, atrial and right ventricular tissues were discarded. The remaining tissue (i.e., left ventricular free wall and septum) was minced in a food processor (Waring); 40 g of minced tissue was added to 120 ml of buffer (0.9% NaCl, 10 mM Tris maleate, pH 6.8). The buffered tissue was vortexed, homogenized (Brinkman polytron; three 20 s intervals, setting of 4) and centrifuged for 20 min at 4000 *g* at 4°C. Supernatant was collected, filtered through 2 layers of cheesecloth and centrifuged at 8000 *g* for 20 min. The resulting supernatant was centrifuged at 40,000 *g* for 30 min. The pellet (40,000 *g*) was resuspended in buffer (pH 6.8, 0.9% NaCl, 10 mM Tris maleate, 0.3 M sucrose) to achieve a final concentration of approximately 20–25 mg protein ml^{-1}, then stored in liquid N_2. Protein concentrations were determined as described by Lowry *et al.* (1951).

Assay for SR calcium release

The metallochromic indicator, antipyralazo III, was used to measure calcium release according to the technique of Palade & Vergra (1982). Ca^{2+} concentration was determined by subtracting absorbences measured simultaneously at two different wavelengths (absorbence at 710 nm minus absorbence at 790 nm) with an HP 8450A u.v./visible diode array spectrophotometer (Hewlett Packard, Avondale, PA, U.S.A.). Procedures were performed at 32°C; 15 μl (338 μg) cardiac microsomes (22.5 mg protein ml^{-1}) were added to 0.985 ml of a buffered (pH 7.0) solution containing (mM): antipyralazo III 0.3, MOPS (3-[N-morpholino]propanesulphonic acid) 20, KH_2PO_4 50, KCl 5, $MgCl_2$ 2 and ATP 2. Thereafter, calcium chloride (5 nmol Ca^{2+}) was added to load the microsomes with Ca^{2+}; the process was repeated 15 times. Loaded microsomes were then exposed to varying concentrations of doxorubicin or doxorubicinol and rates of calcium release were determined.

To compare effects associated with atrial tissue levels of anthracyclines (doxorubicin or doxorubicinol) with effects of

anthracyclines in isolated subcellular preparations, we converted tissue levels from μg anthracycline per g wet weight to μmol anthracycline per μl of aqueous solution. Because cardiac tissue has a density very similar to that of water (1 g ml^{-1}), a tissue doxorubicinol concentration of 1 μg g^{-1} wet weight is assumed to be equivalent to 1 μg ml^{-1} or 1.8 μM (molecular weight of doxorubicinol is 540).

Materials

Doxorubicin was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Doxorubicinol was synthesized from doxorubicin according to the technique of Takanashi & Bachur (1976). Identity and purity were confirmed by h.p.l.c. as previously described (Olson *et al.*, 1988).

Statistics

One way analysis of variance for unpaired data or randomized block analysis of variance for paired data was used to analyze effects of doxorubicin or doxorubicinol on cardiac mechanical variables as a function of duration of exposure or concentration of anthracycline. Specific contrasts among groups (comparisons at different time points) were made by Duncan's New Multiple Range test. EC_{50}'s (tissue concentation of anthracycline associated with a 50% reduction in measured effect) were compared by probit analysis. Probabilities less than 5% were considered statistically significant ($P<0.05$).

Results

Effect of anthracycline vehicle on cardiac function

Rabbit atria contracting at 2 Hz were stable following addition of 0.9% NaCl to the muscle bath during the 210 min study period (control; Table 1); no substantial changes occurred in any cardiac functional variables at any of the times studied. The largest reductions noted in mean dF/dt were a 6% diminution at 150 min and a 13% decrease at 210 min.

Time-related effects of doxorubicin on cardiac function

Doxorubicin (175 μM) decreased myocardial contractility (dF/dt), impaired cardiac relaxation (90% relaxation time; 90% RT), and increased muscle stiffness (resting force; RF) in a time-related manner (Figures 1 and 2). There was little change in mean dF/dt after a 30 min exposure to doxorubicin (86 ± 11% of pre-doxorubicin value); however, a 90 min exposure decreased dF/dt by more than 50% (48 ± 12% of pre-doxorubicin value), and a 210 min exposure reduced dF/dt by 67 ± 6%. Myocardial relaxation was markedly impaired; 90% RT more than doubled (220 ± 12% of pre-doxorubicin value) during exposure to doxorubicin for 210 min (Figure 2). Resting force, which was not increased after treatment with doxorubicin for 90 min, was significantly elevated at 150 min and further increased at 210 min (136 ± 12% of pre-doxorubicin values) (Figure 2).

Table 1 Changes in dF/dt, RF and 90% RT as a function of time after adding vehicle (0.9% NaCl) to isometrically contracting rabbit atria

Cardiac variable	*Time of exposure to vehicle* 30 min	90 min	150 min	210 min
dF/dt	106 ± 5	101 ± 8	94 ± 10	87 ± 11
RF	101 ± 2	99 ± 2	99 ± 2	100 ± 2
90% RT	102 ± 2	101 ± 3	99 ± 4	98 ± 4

Values are mean ± s.e.mean of 7 experiments and are expressed as percentages of pre-vehicle values. dF/dt, maximum rate of rise of force; RF, resting force-force exerted by atria when not actively contracting (at rest); 90% RT, 90% relaxation time (time for peak developed force to decrease by 90%); for additional information, see Methods.

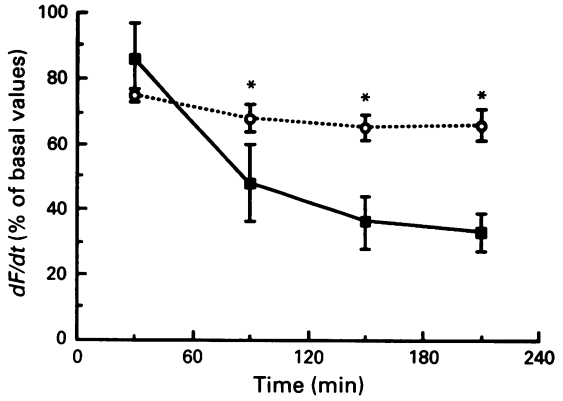

Figure 1 Effects of doxorubicin (Dox ■; 175 μM) and doxorubicinol (Doxol ○; 1.8 μM) on cardiac contractility (dF/dt) as a function of time after adding either agent to isometrically contracting rabbit atria. The x-axis shows duration of treatment with Dox or Doxol; the y-axis shows dF/dt, expressed as percentages of pre-Dox or pre-Doxol values. Data are mean ± s.e.mean ($n = 4$). *$P<0.05$ (for Dox vs Doxol, using 1-way analysis of variance and Duncan's New Multiple Range test).

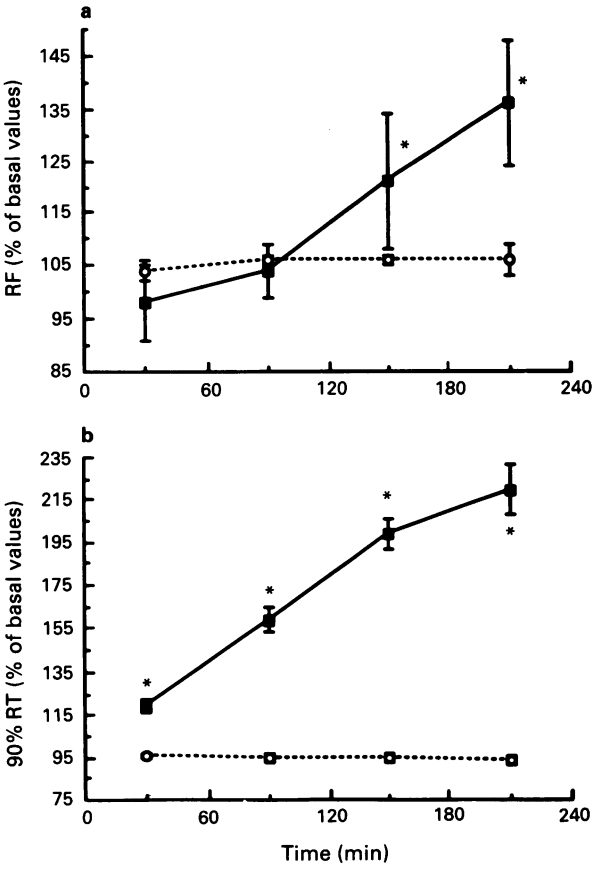

Figure 2 Effects of doxorubicin (Dox ■; 175 μM) and doxorubicinol (Doxol ○; 1.8 μM) on resting force (RF, a) and 90% relaxation time (90% RT, b) as a function of time after adding either agent to isometrically contracting rabbit atria. The x-axis shows duration of treatments; the y-axis shows RF and 90% RT, expressed as percentages of pre-Dox or pre-Doxol values. Data are mean ± s.e.mean ($n = 4$). *$P<0.05$ (for Dox vs Doxol, via 1-way analysis of variance and Duncan's New Multiple Range test).

Table 2 Atrial doxorubicin and doxorubicinol levels following treatment with doxorubicin

	Time (min)						
	30	*60*	*90*	*120*	*150*	*180*	*210*
Dox	70	110	207	183	240	229	239
(s.e.mean, $n = 3$)	26	24	20	24	32	37	34
Doxol	0.09	0.60	0.72	1.57	1.91	3.09	2.62
(s.e.mean, $n = 5$)	0.05	0.04	0.10	0.20	0.35	0.81	0.43
Dox/Doxol	778	183	287	116	126	74	91
% Dox	99.9	99.5	99.7	99.1	99.2	98.7	98.9
% Doxol	0.13	0.54	0.34	0.87	0.79	1.34	1.08
% Dox max	24	40	78	68	98	100	100
(s.e.mean, $n = 3$)	8	6	4	5	8	0	0
% Doxol max	4	18	20	47	57	81	100
(s.e.mean, $n = 5$)	2	3	2	7	11	12	0

Doxorubicin (dox) concentration expressed as μg per g wet weight of tissue, doxorubicinol (doxol) concentration expressed as μg per g wet weight of tissue.
% Dox = % of anthracycline that is Dox (% Dox = Dox/(Dox + Doxol)).
% Doxol = % of anthracycline that is Doxol (% Doxol = Doxol/(Dox + Doxol)).
% max Dox = Dox concentration at each time/highest concentration of Dox during each time course.
% max Doxol = Doxol concentration at each time/highest concentration of Doxol during each time course.

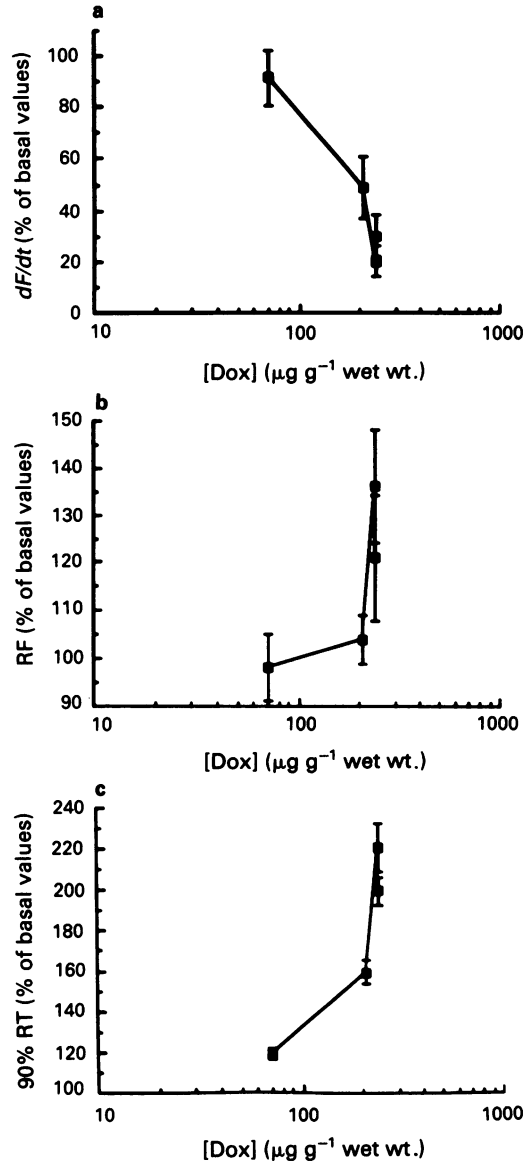

Figure 3 Relationship of atrial doxorubicin (Dox) concentration and changes in cardiac variables (dF/dt, a; resting force, RF, b; 90% relaxation time, 90% RT, c) in rabbit atria treated with 175 μM Dox. The x-axis shows mean ($n = 3$) atrial Dox concentrations; the y-axis shows mean ± s.e.mean ($n = 4$) values of cardiac mechanical variables after 30, 90, 150 and 210 min of treatment with Dox; mechanical variables are expressed as percentages of pre-Dox values.

Time-related effects of doxorubicinol on cardiac function

Unlike doxorubicin, no substantial time-related effects were observed during treatment with doxorubicinol (1.8 μM) for 210 min. Doxorubicinol (1.8 μM) treatment did decrease dF/dt by 25 ± 2% at 30 min and by 32 ± 4% at 90 min, but no further decrease in dF/dt occurred over the next 120 min (i.e., between 90 and 210 min, mean dF/dt decreased by 2%) (Figure 1). Similarly, doxorubicinol significantly increased RF (4 ± 2%; $P < 0.05$) after 30 min, without further increasing RF during the ensuing 180 min (between 30 and 210 min) (Figure 2). In contrast to the ability of doxorubicin to prolong 90% RT, doxorubicinol did not alter 90% RT (Figure 2).

Effects of time on atrial concentrations of doxorubicin and doxorubicinol following exposure to doxorubicin

Treatment of atrial strips with doxorubicin (175 μM) for 210 min produced a time-related increase in atrial concentrations of doxorubicinol (Table 2). Atrial doxorubicin level attained 78% of peak value during the first 90 min of exposure to doxorubicin and increased only 3.4 fold between 30 and 210 min of exposure to doxorubicin (70 to 239 μg g^{-1} wet weight; Table 2). By contrast, doxorubicinol concentration increased 35 fold between 30 and 180 min of exposure (0.088 μg g^{-1} wet weight at 30 min to 3.09 μg g^{-1} wet weight at 180 min), with the most rapid rate of increase occurring between 30 and 90 min (i.e., doxorubicinol level increased more than 8 fold; from 0.088 to 0.715 μg g^{-1} wet weight). During the course of study, the concentration of doxorubicinol increased approximately 10 fold relative to the concentration of doxorubicin ([doxorubicin]/[doxorubicinol] decreased from 778 at 30 min to 74 at 180 min). Nonetheless, on an absolute basis, doxorubicin always predominated over doxorubicinol, never constituting less than 98.7% of the anthracycline (doxorubicin and doxorubicinol) present in cardiac tissue.

Relation between atrial concentrations of doxorubicin and doxorubicinol and changes in cardiac function

Treatment with doxorubicin for 210 min produced alterations of cardiac functional variables (dF/dt, RF, 90% RT) that were directly related to cardiac concentrations of both doxorubicin and doxorubicinol (Figures 3 and 4). The most pronounced effects on functional variables were observed when tissue doxorubicin levels were between 100 and 300 μg g^{-1} wet weight. At a tissue level of 200 μg doxorubicin g^{-1}

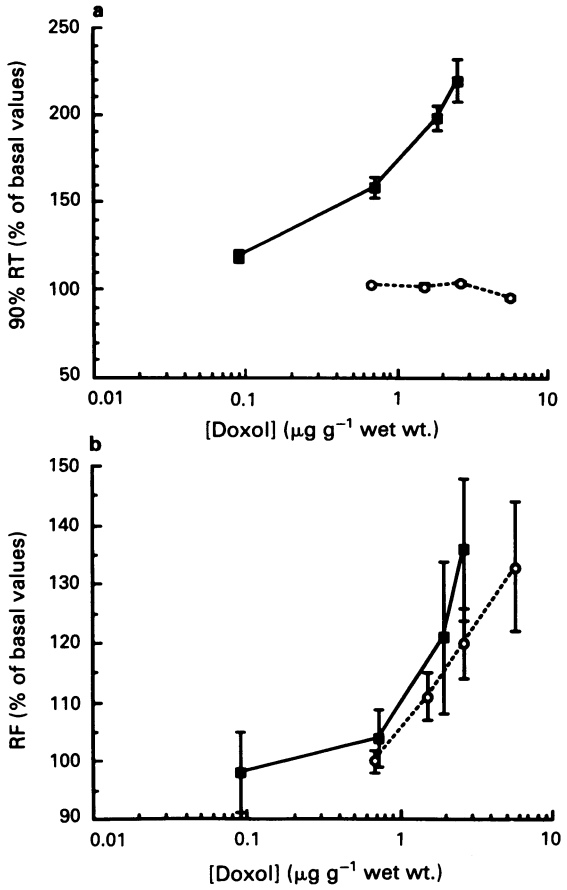

Figure 4 Relationship of diastolic dysfunction and atrial doxorubicinol (Doxol) concentration. Changes in diastolic function (90% relaxation time, 90% RT, a; resting force, RF, b) were evaluated in rabbit isolated atrial strips following exposure to either doxorubicin (Dox; ■; 175 μM) or to doxorubicinol (Doxol; ○). Atrial Doxol concentrations were measured in similarly treated atrial strips. The x-axis shows mean atrial Doxol concentrations (expressed as μg Doxol g^{-1} wet weight) resulting from exposure to Dox for 30, 90, 150 and 210 min (■, $n = 3$) or from treatment with a range of Doxol concentrations (0.9, 1.8, 3.6 and 7.2 μM; ○, $n = 3$); the y-axis shows corresponding mean values of RF and 90% RT, expressed as a percentage of the value immediately prior to addition of Dox ($n = 4$) or Doxol ($n = 7$) to the muscle bath. Data are mean ± s.e.mean.

wet weight, dF/dt had already declined by 50%, whereas RF and 90% RT were just beginning to rise sharply.

Exposure of atria to various concentrations of doxorubicinol (0.5, 1.0, 2.0 and 4.0 μg ml^{-1}) led to increases of doxorubicinol in the atrial strips (0.66 ± 0.10, 1.53 ± 0.20, 2.68 ± 0.52 and 5.72 ± 0.71 μg g^{-1} wet weight, respectively). These data allowed determination of the relation between mechanical dysfunction and concentration of doxorubicinol in heart, without the confounding influence of doxorubicin concentration (Figures 4 and 5). Cardiac doxorubicinol concentration, while unrelated to 90% RT (Figure 4), paralleled the depression of dF/dt (Figure 5) and increase in RF (Figure 4) observed during treatment with doxorubicinol. The functional variable that appeared most sensitive to cardiac doxorubicinol level was dF/dt, which was inhibited by 18 ± 5% ($P < 0.05$) when the atrial doxorubicinol level was 0.66 ± 0.10 μg g^{-1} wet weight (Figure 5). Resting force was not significantly increased until the doxorubicinol level was 2.3 fold higher (1.53 ± 0.20 μg g^{-1} wet weight; RF increased by 11 ± 4%; $P < 0.05$). By contrast, treatment with doxorubicinol never increased 90% RT (Figure 4), not even when atrial doxorubicinol level reached 5.72 ± 0.71 μg g^{-1} wet weight (a level associated with a 54 ± 8% decrease in dF/dt and a 33 ± 11% increase of resting force).

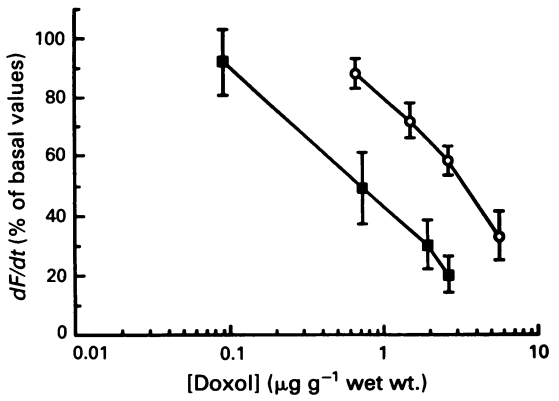

Figure 5 Relationship of contractile dysfunction and atrial doxorubicinol (Doxol) concentration. Changes in contractile function (dF/dt) were measured in isometrically contracting rabbit atrial strips following exposure to either doxorubicin (Dox; ■) or to doxorubicinol (Doxol; ○). Atrial Doxol concentrations were measured in other similarly treated atrial strips. The x-axis shows mean atrial Doxol concentrations (expressed as μg Doxol g^{-1} wet weight) resulting from exposure to Dox (175 μM) for 30, 90, 150 and 210 min (■, $n = 3$) or from treatment with a range of Doxol concentrations (0.9, 1.8, 3.6 and 7.2 μM; ○, $n = 3$); the y-axis shows corresponding mean values of dF/dt, expressed as percentages of values obtained immediately prior to addition of Dox ($n = 4$) or Doxol ($n = 7$) to the muscle bath. Data are mean ± s.e.mean.

The degree of contractile dysfunction associated with any particular concentration of cardiac doxorubicinol depended upon whether the level was elevated by treatment with doxorubicin or treatment with doxorubicinol (Figure 5). The two treatments produced parallel tissue concentration-response relations, allowing for comparisons of EC_{50} (atrial concentration of doxorubicinol associated with a 50% reduction in dF/dt); EC_{50} associated with doxorubicinol treatment was 5 fold greater than the EC_{50} due to doxorubicin treatment (0.7 vs 3.5 μg g^{-1} wet weight).

Effects of doxorubicin and doxorubicinol on SR Ca^{2+} release

Atrial levels of doxorubicin and doxorubicinol resulting from incubation with 175 μM doxorubicin (Table 2) provided the basis for selecting anthracycline concentrations (3 and 10 μM) to study in our experimental model of sarcoplasmic reticulum (SR) calcium release. Doxorubicinol and doxorubicin both caused a concentration-related release of calcium from isolated SR vesicles (Table 3). Doxorubicinol, however, was more potent than doxorubicin, producing a 3 and 15 fold greater stimulation of calcium release at 10 and 3 μM, respectively ($P < 0.01$).

Because anthracyclines can enhance the sensitivity of the Ca^{2+} release channel of SR to Ca^{2+} (increase Ca^{2+} induced-

Table 3 Effects of doxorubicin or doxorubicinol on maximum rates of calcium release from sarcoplasmic reticulum vesicles (SR) from canine cardiac muscle

	Concentration	
Anthracycline	3 μM	10 μM
Doxorubicin	0.8 ± 0.4	59 ± 7
Doxorubicinol	12.0 ± 1.4*	165 ± 29*

Values (nmol Ca^{2+} mg^{-1} protein min^{-1}) are mean ± s.e. mean; $n = 6$ at 3 μM; $n = 4$ at 10 μM. Amount of free Ca^{2+} (nmol) in reaction cuvettes did not differ at the time that the anthracycline was added to release SR Ca^{2+}.
*Indicates significant differences ($P < 0.01$) between doxorubicin and doxorubicinol at each concentration.

Table 4 Doxorubicin (Dox) and doxorubicinol (Doxol) contributions[1] to sarcoplasmic reticulum (SR) dysfunction in Dox-treated atria: estimates based on atrial concentrations of Dox and Doxol and their abilities to cause subcellular dysfunction

	Dox or Doxol contribution to Ca^{2+} release by SR in working atria				
	Potency factor[2]	*Atrial concentration-ratio (Dox/Doxol)* or (Doxol/Dox)‡*		*Percentage contribution*[3]	
Anthracycline	(SR Ca release)	30 min	180 min	30 min	180 min
Dox	0.07	778*	74*	100%	96%
Doxol	15	1/778‡	1/74‡	0%	4%
	Dox or Doxol contributions to inhibition of SR Ca^{2+} ATP-ase in working atria				
	Potency factor[2]	*Atrial concentration-ratio (Dox/Doxol)* or (Doxol/Dox)‡*		*Percentage contribution*[3]	
Anthracycline	(ATP-ase inhibition)	30 min	180 min	30 min	180 min
Dox	0.01	778*	74*	98%	35%
Doxol	100	1/778‡	1/74‡	2%	65%

[1]Assumes direct relationship between cardiac concentration of Dox or Doxol in working atria and effects of these agents on subcellular function.

[2]Potency factors (PF) (ratios of relative potencies to stimulate Ca^{2+} release (data from Table 3) or to inhibit Ca^{2+} ATP-ase (data from Boucek *et al.*, 1987b)); PF_{Dox} = potency of Dox/potency of Doxol; PF_{Doxol} = potency of Doxol/potency of Dox.

[3]Percentage contribution (PC) for Dox or Doxol was computed as follows: $PC_{Dox} = RC_{Dox}/(RC_{Dox} + RC_{Doxol}) \times 100$. $PC_{Doxol} = RC_{Doxol}/(RC_{Doxol} + RC_{Dox}) \times 100$. (RC, in above formula, is defined as the product of potency factor and atrial concentration-ratio. For example, RC for Dox on Ca^{2+} release at 180 min is $0.07 \times 74 = 5$).

Ca^{2+} release; Pessah *et al.*, 1990), it was necessary to control carefully the concentration of free Ca^{2+} during introduction of anthracyclines; no significant differences occurred among groups (nmol free $Ca^{2+} = 1.70 \pm 0.12$, 1.60 ± 0.09, 1.57 ± 0.05 and 1.56 ± 0.07 in doxorubicin 3 μM, doxorubicinol 3 μM, doxorubicin 10 μM and doxorubicinol 10 μM groups, respectively).

Non-anthracyclines that alter Ca^{2+} handling by SR: contractile effects over time

To determine whether non-anthracyclines that affect SR Ca^{2+} availability also cause time-related inhibition of contraction, we added 1 mM caffeine or 20 μM ruthenium red to isolated atria and monitored the cardiac mechanical effects over time (protocol identical to studies with doxorubicin and doxorubicinol). Both caffeine and ruthenium red decreased contractility from pre-drug values by 30 min ($25 \pm 5\%$ and $17 \pm 3\%$, respectively). Neither agent, however, caused further time-related effects on contraction ($13 \pm 6\%$ and $14 \pm 4\%$ decrease in contractility from pre-drug values 210 min after addition of caffeine and ruthenium red, respectively).

Discussion

Doxorubicinol, the C-13 OH metabolite of doxorubicin, was considerably more potent that doxorubicin in depressing mechanical function of rabbit isolated atria. Within the first 30 min of treatment, both doxorubicin (175 μM) and doxorubicinol (1.8 μM) produced a similar degree of contractile depression. However, only doxorubicin caused progressive myocardial dysfunction during the remaining 3 h of the experiment; doxorubicinol failed to produce time-dependent cardiodepressant effects.

Treatment with doxorubicin for 30 min lowered dF/dt by 14%, and lengthened 90% RT by 19%, without changing RF (Figures 1 and 2), whereas a 3.5 h exposure reduced dF/dt by 67%, prolonged 90% RT by 220% and elevated RF by 36%. By contrast, treatment with doxorubicinol (1.8 μM) decreased dF/dt by 25% (no major effect on RF or 90% RT) within 30 min, without further altering cardiac variables during the final 3 h of the experiment (Figures 1 and 2). Thus, despite nearly identical chemical structures (dissimilar only at C-13 moeity—doxorubicin contains a ketone, doxorubicinol an OH group), doxorubicin and doxorubicinol differed markedly in their abilities to produce time-related cardiotoxic effects.

Time-dependent dysfunction could possibly reflect progressive destabilization of *in vitro* preparations. Isolated hearts, for example, may be susceptible to hypoxia or acidosis, owing to ineffective delivery of oxygen or removal of metabolic waste. Such problems, however, would be unlikely in thin strips of muscle (atrial preparations less than 1 mm thick). Also, at 30°C, our muscle preparations are quite stable over time (Table 1); higher temperatures are associated with greater mechanical instability, perhaps owing to imbalances in oxygen supply and demand.

The relationship between time-dependent dysfunction and alteration of cytoplasmic Ca^{2+} needs to be considered. For example, increases in cytoplasmic Ca^{2+} have been reported to provoke myocardial ischaemia (Hearse *et al.*, 1977; Nayler *et al.*, 1979; Carter *et al.*, 1986). However, in our preparation, caffeine (1 mM), which rapidly depressed contractility, failed to produce significant time-related changes, despite its well-known ability to release SR Ca^{2+} (Palade, 1987; Pessah *et al.*, 1987) and increase muscle stiffness (Sutko *et al.*, 1986; Schouten, 1990). Similarly, ruthenium red, a negatively inotropic agent that inhibits Ca^{2+} release from SR (Zimanyi & Pessah, 1991), inhibited contractility within 30 min but failed to depress contractility further over time (Results). Thus, it appears that neither continuous impairment of SR function nor negative inotropic actions alone can account for the time-dependent cardiotoxicity of doxorubicin.

Time-dependency could arise from effects of doxorubicin at multiple subcellular sites (Boucek *et al.*, 1987b; Olson *et al.*, 1981; Floreani & Carpandeo, 1989; Averbuch *et al.*, 1988; Olson & Mushlin, 1990; Gaudiano & Koch, 1991), as a result of differences in time-constants for events mediated by doxorubicin-receptor interactions. Cardiac functional status at any particular time would then reflect a dynamic blending of early, intermediate, and delayed toxic effects of doxorubicin. Influences of anthracyclines on gene expression (selective decrease in synthesis of myofibrillar proteins) have been postulated to contribute to the time-dependent nature of chronic anthracycline cardiotoxicity (Ito *et al.*, 1990), but genetic lesions would seem unlikely to contribute to the pattern of acute cardiotoxicity observed in our preparation.

Our data indicate that doxorubicinol would not be expected to mediate time-related components of doxorubicin cardiotoxicity if such effects were merely caused by SR Ca^{2+} release. Concentrations of doxorubicinol in heart were always quite low, 74 fold–778 fold lower than cardiac concentra-

tions of doxorubicin (Table 2). On this basis, for doxorubicin and doxorubicinol to make equal contributions to dysfunction associated with doxorubicin treatment, doxorubicinol would need to be 74 fold to 778 fold more potent than doxorubicin to impair subcellular functions (Table 4). Our studies with isolated SR, however, indicate that at most, doxorubicinol is 3 to 15 fold more potent than doxorubicin as a stimulator of Ca^{2+} release (Table 3). Therefore, we would not expect the metabolite to disrupt mechanical function via releasing Ca^{2+} from SR, even after prolonged exposure to doxorubicin. Instead, we would predict that doxorubicin, the levels of which in working heart can markedly alter the ability of SR to release Ca^{2+} (Tables 3 and 4), may account for more than 96% of any toxic effect due to enhanced release of SR Ca^{2+} (Table 4).

Doxorubicinol, on the other hand, is an extremely potent inhibitor of various ATPase activities, being nearly 100 times more potent than doxorubicin in inhibiting Ca^{2+}-Mg ATPase and Ca^{2+} loading activities of SR (Boucek *et al.*, 1987b). Such subcellular actions of doxorubicinol (e.g., inhibition of cytoplasmic Ca^{2+} uptake by SR) may lead to mechanical dysfunction (impaired relaxation or diminished contractility) in the working heart, especially as cardiac levels of metabolite increase 8 to 10 fold during prolonged exposure to doxorubicin (Table 2). The analysis in Table 4 suggests that doxorubicinol could be as important as doxorubicin in mediating dysfunction caused by an impairment of Ca^{2+} uptake into SR. Based on this reasoning, we would predict that rising concentrations of C-13 hydroxy metabolite in heart could progressively amplify mechanical effects related to doxorubicin-induced release of SR Ca^{2+}, accounting, at least in part, for the time-dependent component of doxorubicin cardiotoxicity.

Degree of muscle stiffness (RF) was more clearly associated with the cardiac concentration of doxorubicinol than doxorubicin (Figure 4). Indeed, the source of doxorubicinol (whether from treatment with doxorubicin or doxorubicinol) was relatively unimportant as a predictor of the degree of muscle stiffness occurring at any particular concentration of doxorubicinol in heart. The largest increases in RF in doxorubicin-treated preparations, occurred after levels of doxorubicin had peaked and while levels of doxorubicinol were progressively rising. The subcellular basis for the increases in RF is unclear, but our data militate against enhanced release of SR Ca^{2+} as the single, predominant mechanism. Because muscle stiffness reflects the amount of Ca^{2+} at the myofibrillar apparatus during quiescence, agents that interfere with the clearance of Ca^{2+} from cytoplasm can readily influence RF. As mentioned above, doxorubicinol, at least in subcellular systems, inhibits the three primary mechanisms involved in clearing Ca^{2+} from the myofibrils; namely sarcolemmal Ca^{2+} ATPase (Harada *et al.*, 1990), Na/Ca^{2+} exchange (Boucek *et al.*, 1987a) and SR Ca^{2+}-Mg ATPase (Boucek *et al.*, 1987b). Thus, doxorubicinol may decrease cardiac compliance by inhibiting sequestration of Ca^{2+} into SR and/or by interfering with processes that translocate Ca^{2+} from intracellular to extracellular sites.

Impairment of myocardial relaxation (90% RT) appeared to be closely related to the cardiac concentration of doxorubicin but not doxorubicinol (Figure 4). Consistent with this finding, treatment with doxorubicin (175 μM) markedly increased the duration of contraction, whereas doxorubicinol (0.9 μM–7.2 μM) never altered contractile duration (Figure 2). (The highest doxorubicinol concentration that could be used in our study, however, was nearly 25 fold below the doxorubicin concentration because doxorubicinol was a much more potent negative inotrope than doxorubicin.) From a mechanistic viewpoint, impairment of relaxation following treatment with doxorubicin appears to be either (1) independent of the level of doxorubicinol in heart or (2) dependent upon an interaction between doxorubicinol and doxorubicin in heart (Figure 4). In addition, the ability of an anthracycline to impair cardiac relaxation seems to depend more upon its ability to stimulate Ca^{2+} release from SR than upon its propensity to inhibit mechanisms that remove Ca^{2+} from the vincinity of the contractile apparatus (Table 4).

Contractile dysfunction (decreased dF/dt) was directly related to the concentration of doxorubicinol in heart (Figure 5). The degree of contractile depression, however, was highly dependent on the source of cardiac doxorubicinol (Figure 5). The tissue concentration-response curve for doxorubicinol, using data from doxorubicin-treated preparations, was shifted to the left relative to the curve obtained from doxorubicinol treated muscles ($EC_{50} = 0.75$ vs $3.5\ \mu g\ g^{-1}$ wet weight). This concentration-response analysis suggests that doxorubicin and doxorubicinol may interact to depress contractility. Such an interaction may occur as doxorubicin diminishes the SR pool of activator Ca^{2+} by enhancing SR Ca^{2+} release, and doxorubicinol decreases the amount of activator Ca^{2+} through inhibition of cytoplasmic Ca^{2+} uptake into SR.

While the knowledge of tissue concentrations of drugs or metabolites (e.g., doxorubicinol) may provide critical information about pharmacological mechanisms, basing inferences exclusively on tissue levels can be misleading. Such information, for example, tells us nothing about the intracellular location or distribution of doxorubicinol or the concentration of doxorubicinol at putative sites of toxicity (e.g., sarcoplasmic reticulum, sarcolemma, or mitochondria). Cardiac doxorubicinol levels measured after doxorubicinol treatment, could reflect intramyocardial doxorubicinol as well as doxorubicinol adsorbed to nonmyocardial tissue or to outer surfaces of myocytes, whereas cardiac doxorubicinol levels measured after treatment with doxorubicin most likely represent intracellular doxorubicinol, generated by intracardiac anthracycline reductase. As a result, a component of the myocardial dysfunction observed during treatment with doxorubicinol could arise from effects on the exterior surface of the sarcolemma (e.g., ion channels) rather than from (or in addition to) effects at intracellular sites. Further studies will be needed to identify specific, functionally important receptors for doxorubicin and doxorubicinol and to quantify the anthracycline present at such receptors following treatment of cardiac preparations with doxorubicin or doxorubicinol.

In conclusion, our data suggest that the time-related formation of the potent cardiotoxin, doxorubicinol, may contribute to the time-dependency of doxorubicin cardiotoxicity in an isolated, working heart preparation. Owing to its relative abundance in heart, doxorubicin would appear to make a much larger contribution than doxorubicinol to mechanical dysfunction resulting from anthracycline-induced release of Ca^{2+} from SR. On the other hand, doxorubicinol, being considerably more potent that doxorubicin to inhibiting processes that remove Ca^{2+} from the contractile apparatus, could play a role in myocardial dysfunction stemming from impaired clearance of cytoplasmic Ca^{2+}. Thus, our data indicate that time-related increases of doxorubicinol in heart, even in the presence of an unchanging cardiac concentration of doxorubicin, could conceivably amplify the functional impact of doxorubicin-induced release of SR Ca^{2+}. Doxorubicin and doxorubicinol may thereby act in concert to promote cardiac dysfunction *in vitro*.

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Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M_3 muscarinic receptors

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1 The regulation of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) during exposure to carbachol was measured directly in canine cultured tracheal smooth muscle cells (TSMCs) loaded with fura-2. Stimulation of muscarinic cholinoceptors (muscarinic AChRs) by carbachol produced a dose-dependent rise in $[Ca^{2+}]_i$ which was followed by a stable plateau phase. The EC_{50} values of carbachol for the peak and sustained plateau responses were 0.34 and 0.33 μM, respectively.

2 Atropine (10 μM) prevented all the responses to carbachol, and when added during a response to carbachol, significantly, but not completely decreased $[Ca^{2+}]_i$ within 5 s. Therefore, the changes in $[Ca^{2+}]_i$ by carbachol were mediated through the muscarinic AChRs.

3 AF-DX 116 (a selective M_2 antagonist) and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP, a selective M_3 antagonist) inhibited the carbachol-stimulated increase in $[Ca^{2+}]_i$ with pK_B values of 6.4 and 9.4, respectively, corresponding to low affinity for AF-DX 116 and high affinity for 4-DAMP in antagonizing this response.

4 The plateau elevation of $[Ca^{2+}]_i$ was dependent on the presence of external Ca^{2+}. Removal of Ca^{2+} by the addition of 2 mM EGTA caused the $[Ca^{2+}]_i$ to decline rapidly to the resting level. In the absence of external Ca^{2+}, only an initial transient peak of $[Ca^{2+}]_i$ was seen which then declined to the resting level; the sustained elevation of $[Ca^{2+}]_i$ could then be evoked by the addition of Ca^{2+} (1.8 mM) in the continued presence of carbachol.

5 Ca^{2+} influx was required for the changes of $[Ca^{2+}]_i$, since the Ca^{2+}-channel blockers, diltiazem (10 μM), nifedipine (10 μM), verapamil (10 μM) and Ni^{2+} (5 mM), decreased both the initial and sustained elevation of $[Ca^{2+}]_i$ in response to carbachol. These Ca^{2+}-channel blockers also decreased the sustained elevation of $[Ca^{2+}]_i$ when applied during the plateau phase.

6 In conclusion, we have demonstrated that the initial detectable increase in carbachol-stimulated $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from internal stores, followed by the flux of external Ca^{2+} into the cells. This influx of extracellular Ca^{2+} partially involves an L-type Ca^{2+}-channel. M_3 muscarinic receptors appear to mediate the Ca^{2+} mobilization in canine TSMCs.

Keywords: Tracheal smooth muscle cells; Ca^{2+}; muscarinic receptors; Ca^{2+}-channel blockers

Introduction

Stimulation of muscarinic cholinoceptor (muscarinic AChRs) by agonists evokes a contractile response in several smooth muscles. The mechanism of stimulus-response coupling has been extensively studied in tracheal smooth muscle (Farley & Miles, 1978; Park & Rasmussen, 1986; Grandordy *et al.*, 1986; Gunst & Bandyopadhyay, 1989; Yang *et al.*, 1991a). In the trachea, stimulation of muscarinic AChRs activates phospholipase C, via a cholera toxin-sensitive G-protein (Gp) (Yang *et al.*, 1991a), which hydrolyzes phosphoinositide, leading to the formation of inositol-1,4,5-triphosphate (IP_3) (Baron *et al.*, 1984; Grandordy *et al.*, 1986; Meurs *et al.*, 1988; Chilvers *et al.*, 1989; Roffel *et al.*, 1990; Yang *et al.*, 1991a,b). This in turn stimulates the release of Ca^{2+} from its internal stores, triggering contraction of tracheal smooth muscle (Hashimoto *et al.*, 1985). Therefore, IP_3-mediated Ca^{2+} release appears to be an important fact in the contractile response, particularly during the developmental stage of force in the tracheal smooth muscle. There is a high metabolic flux of phosphoinositide during contraction (Baron *et al.*, 1989). Muscarinic agonists induce increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration and in force in the tracheal smooth muscle under Ca^{2+}-free conditions (Takuwa *et al.*, 1987), but muscarinic-induced contractions are markedly slower to develop under conditions where phosphoinositide hydrolysis and IP_3 accumulation are inhibited by phorbol ester (Baba *et al.*, 1989).

In contrast, entry of Ca^{2+} from the external environment into the vascular smooth muscle during agonist stimulation plays an important role in maintaining the plateau phase of a prolonged contraction. This plateau phase disappears in a Ca^{2+}-free medium (Cauvin & Van Breemen, 1985). Other studies also indicate that the initial increase of $[Ca^{2+}]_i$ is indeed transient, but that the decline does not fall to the resting level in the presence of external Ca^{2+} (Merrit & Rink, 1987; Shieh *et al.*, 1991). Therefore, other mechanisms may operate during the prolonged response. Putney (1986) proposed that the two phases of increase in $[Ca^{2+}]_i$ may be intimately linked: IP_3 first stimulates the release of Ca^{2+} from the internal stores and the depletion of these stores then becomes the signal for the entry of Ca^{2+} from outside the cell.

In our laboratory, both M_2 and M_3 receptor subtypes have been shown to exist in canine TSMCs (Yang, 1991) that are coupled to the inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation and to inositol phosphate (IP) accumulation, respectively (Yang *et al.*, 1991a). Furthermore, the M_3 receptor subtype has been demonstrated to couple to Ca^{2+} mobilization in SH-SY5Y and Lan-1 human neuroblastoma cells (Murphy *et al.*, 1991; Fatatis *et al.*, 1992). Therefore, these experiments were carried out to determine whether activation of M_3 receptors in canine TSMCs results in an increase of $[Ca^{2+}]_i$.

Defining the cellular mechanisms that mediate a prolonged muscle contraction is important in understanding tracheal smooth muscle function; therefore we have undertaken these

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studies to clarify in part the nature of changes in $[Ca^{2+}]_i$ during continued exposure to carbacahol. The purpose of the present study was two fold: to investigate the changes in $[Ca^{2+}]_i$ during exposure to carbachol and to assess the contribution of Ca^{2+} from various sources to these changes in $[Ca^{2+}]_i$ in cultured TSMCs. The results show that the changes in $[Ca^{2+}]_i$ during the occupation of muscarinic AChRs by carbachol are due to an immediate, but transient release of Ca^{2+} from the internal stores as well as to the entry of Ca^{2+} from outside the cells, persisting as long as the muscarinic AChRs are continuously exposed to carbachol. The extent of Ca^{2+} influx through Ca^{2+}-channels, depends, in part, on the Ca^{2+} concentration outside the cells. Furthermore, we have pharmacological evidence that the muscarinic AChR subtype which is coupled to Ca^{2+} mobilization is the M_3 receptor type in canine TSMCs.

Methods

Animals

Mongrel dogs, 10–20 kg of either sex were purchased from a local supplier and used throughout this study. Dogs were housed indoors in the animal facilities under automatically controlled temperature and light cycle conditions and were fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with pentobarbitone (30 mg kg^{-1}, intravenously) and 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 previously reported (Yang, 1991; Yang *et al.*, 1991a,b). 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 material 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 cells were counted and diluted with DMEM/F-12 to a final concentration of 5×10^5 cells ml^{-1}. The cells (2 ml/well) were plated onto 6-well culture plates containing glass coverslips coated with collagen. The medium was changed after 24 h and then every 3 days. After 5 days in culture, the cells were moved 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 an indirect immunofluorescence method (Gown *et al.*, 1985). Over 95% of the cells were smooth muscle cells.

Measurement of intracellular Ca^{2+} levels

$[Ca^{2+}]_i$ was measured in confluent monolayers with the calcium-sensitive dye fura-2/AM as described by Grynkiewicz *et al.* (1985). Upon confluence, the cells were cultured in DMEM/F-12 with 1% FBS for one day before measurements were taken. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μM fura-2/AM and were incubated at 37°C for 45 min. At the end of the loading period, the coverslips were washed twice wth the physiological buffer solution (PBS) containing (mM): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10 and glucose 10, pH 7.4. The cells were then incubated in PBS for another 30 min to complete dye deesterification. The coverslip was inserted into a quartz cuvette at an angle of approximately 45° to the excitation beam and placed in the temperature controlled holder of a SLM 8000C spectrofluorometer. Continuous stirring was achieved with a magnetic stirrer.

Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at emission wavelength 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) to the fluorescence at the two wavelengths was computed and used to calculate changes in $[Ca^{2+}]_i$. The ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by lysing the cells with ionomycin (10 μM) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in a Ca^{2+}-free PBS, respectively. Values obtained were 14.09, 0.96, and 22.07 for R_{max}, R_{min}, and β, respectively. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz *et al.*, 1985).

Analysis of data

The EC_{50} of carbachol for stimulating $[Ca^{2+}]_i$ was estimated by Graph Pad programme (Graph Pad, San Diego, California, U.S.A.). The dissociation constants (K_B) of muscarinic antagonists were estimated by the method of Furchgott (1972), from their ability to antagonize carbachol-mediated increase in $[Ca^{2+}]_i$.

The data are expressed as the mean ± s.e.mean of at least four experiments with statistical comparisons based on a two-tailed Student's paired 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 obtained from Boehringer Mannheim (Germany). Fura-2/AM was ordered from Molecular Probes Inc (Eugene, OR, U.S.A.). AF-DX 116 (11-{[-2[(diethylamino)methyl]-1-piperidinyl) acetyl }-5, 11-dihydro-6H-pyrido [2,3-b] [1,4]benzodiazepine-6-one) was a gift from Dr K. Noll at Dr Karl Thomae, Germany. 4-Diphenylacetoxy-*N*-methylpiperidine (4-DAMP) was purchased from Research Biochemicals Inc (Natick, MA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St Louis, MO, U.S.A.).

Results

Effects of carbachol on $[Ca^{2+}]_i$

Stimulation of muscarinic AChRs by carbachol (100 μM) rapidly generated a peak and then a sustained increase in $[Ca^{2+}]_i$, as measured directly in cultured canine TSMCs loaded with fura-2 in the presence of external Ca^{2+} (Figure 1). The calculated resting value of $[Ca^{2+}]_i$ was 105 ± 28 nM ($n = 36$). The initial transient $[Ca^{2+}]_i$ peak (318 ± 25 nM, $n = 18$) was reached within 30 s and was followed by a sustained elevation of $[Ca^{2+}]_i$ for at least 5 min in the presence of carbachol. During prolonged carbachol application, $[Ca^{2+}]_i$ was significantly higher (282 ± 29 nM, $n = 18$, $P<0.001$) than its resting value. When the potent muscarinic antagonist, atropine (10 μM), was added during the plateau phase, the $[Ca^{2+}]_i$ decreased to 165 ± 30 nM ($n = 20$) within 60 s. This level is substantially higher than that of the resting level ($P<0.01$). Both the peak and plateau of $[Ca^{2+}]_i$ in-

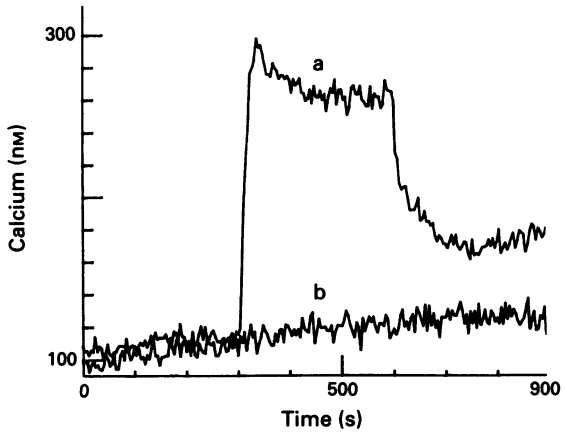

Figure 1 Effect of carbachol on $[Ca^{2+}]_i$ in canine tracheal smooth muscle cells (TSMCs). Confluent cultures of canine TSMCs on glass coverslips were loaded with 5 μM fura-2 and fluorescent measurement of $[Ca^{2+}]_i$ was carried out in a dual excitation wavelength spectrofluorometer, with excitation at 340 and 380 nm. Trace (a) cell cultures were added with carbachol (100 μM) at 5 min after incubation. The initial transient $[Ca^{2+}]_i$ rose from 95 nM to 298 nM in 30 s. This level was sustained with only a slight decline during the presence of carbachol. After 5 min of carbachol exposure, atropine (10 μM) was added, whereupon the $[Ca^{2+}]_i$ decreased from the plateau level to 165 nM within 60 s. Trace (b) atropine (10 μM) was added at the beginning. After 5 min incubation, carbachol (100 μM) was added, no change in $[Ca^{2+}]_i$ was seen.

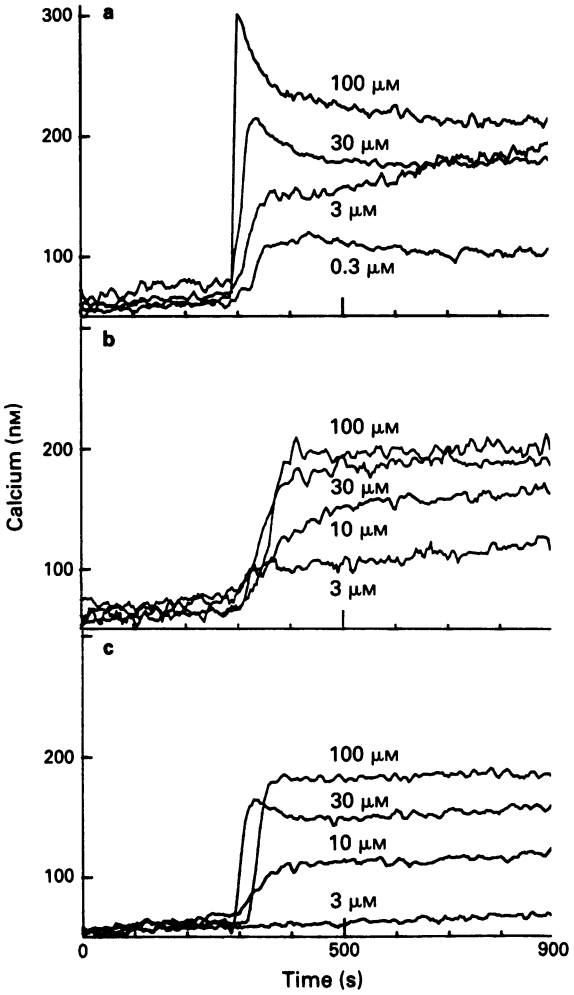

Figure 2 Competitive antagonism of carbachol-mediated increase in $[Ca^{2+}]_i$ by (a) control, (b) AF-DX 116, and (c) 4-DAMP. Tracheal smooth muscle cells (TSCMs) were incubated in the presence of AF-DX 116 (10 μM) or 4-DAMP (10 nM) for 5 min before the addition of increasing concentrations of carbachol as indicated for each trace. For abbreviations, see text.

crease were abolished when atropine was added before carbachol, suggesting that the increase of $[Ca^{2+}]_i$ after carbachol exposure required occupation of muscarinic AChRs.

Effect of muscarinic antagonists on $[Ca^{2+}]_i$

The carbachol-stimulated effect on $[Ca^{2+}]_i$ was dose-dependent (Figure 2). The biphasic responses became less pronounced as the carbachol concentration was reduced (Figure 2). The half-maximal effect of carbachol (EC_{50}) for the initial peak was 0.33 μM (Figure 3) and 0.34 μM for the sustained phase.

In order to differentiate the muscarinic AChR subtype that is coupled to changes in $[Ca^{2+}]_i$, we compared the affinity of selective M_2 and M_3 antagonists, AF-DX 116 and 4-DAMP, on the inhibition of carbachol-induced increase in $[Ca^{2+}]_i$. As shown in Figure 2, preincubation of fura-2-loaded TSMCs with these antagonists inhibited both the initial peak and the sustained phase of changes in $[Ca^{2+}]_i$. The concentration-effect relationship of carbachol was shifted to the right in a nearly parallel fashion, upon addition of AF-DX 116 (10 μM) and 4-DAMP (10 nM) (see Figure 3). The EC_{50} values of carbachol were increased from 0.33 μM to 12 and 16 μM in the presence of AF-DX 116 and 4-DAMP, respectively. The pK_B values of AF-DX 116 and 4-DAMP for antagonizing the carbachol-induced $[Ca^{2+}]_i$ were 6.4 and 9.4, respectively, which were in good agreement with the low affinity for AF-DX 116 and high affinity for 4-DAMP in this response (see review, Hulme *et al.*, 1990).

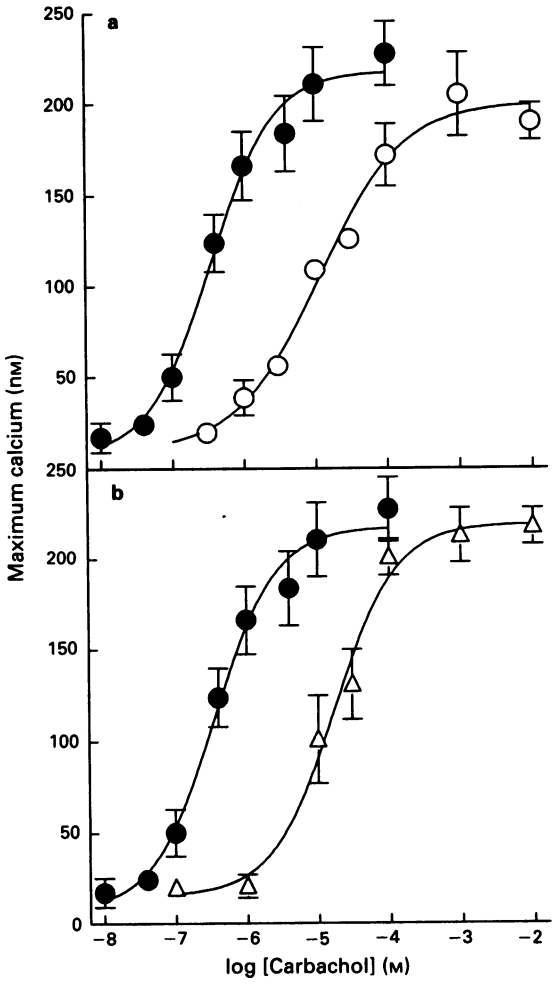

Figure 3 The concentration-response curves of carbachol for the increase in $[Ca^{2+}]_i$ by (●) control, (○) AF-DX 116, 10 μM, and (△) 4-DAMP, 10 nM. Each point represents the mean ± s.e.mean of ten measurements.

Effect of external Ca^{2+}

To define the mechanisms of the changes in the initial and sustained elevation of $[Ca^{2+}]_i$ in response to carbachol, experiments were carried out to examine the changes in $[Ca^{2+}]_i$ induced by carbachol with or without external Ca^{2+}. As shown in Figure 4. Trace a indicates the increase in $[Ca^{2+}]_i$ when TSMCs were exposed to carbachol (100 μM) in the presence of external Ca^{2+} (1.8 mM). As soon as carbachol was added, an immediate increase in $[Ca^{2+}]_i$ was seen and reached a peak (297 ± 35 nM, $n = 9$) within a few seconds. The peak was followed by a short, but sharp decline (249 ± 42 nM, $n = 9$) which became gradual during the continuous exposure to carbachol. Removal of the external Ca^{2+} by the addition of 2 mM EGTA during the sustained phase of $[Ca^{2+}]_i$ caused an abrupt decline to its resting level within 10 s. Trace b represents the response of TSMCs exposed to carbachol in the absence of external Ca^{2+}. The profile shows an immediate transient increase in $[Ca^{2+}]_i$ similar to trace a, but of a lesser magnitude (197 ± 17 nM, $n = 9$). The peak rapidly decayed within 10 s to the resting level. There was no sustained elevation of $[Ca^{2+}]_i$ in the absence of external Ca^{2+} during exposure to carbachol. When Ca^{2+} (1.8 mM) was added 10 min after the addition of carbachol to the solution, an immediate and sustained increase in $[Ca^{2+}]_i$ (255 ± 30 nM, $n = 9$) was observed, reaching a level equal to the sustained elevation of $[Ca^{2+}]_i$ in TSMCs of trace a. These results suggest that the changes in $[Ca^{2+}]_i$ caused by carbachol in canine TSMCs required two sources of Ca^{2+}: (1) stored intracellular Ca^{2+} that is immediately but transiently mobilized upon receptor occupation; and (2) an influx of external Ca^{2+} into the cells that accounts for the sustained high level of $[Ca^{2+}]_i$. These processes occur simultaneously but may also be separated under appropriate conditions, as shown in Figure 4.

Because the sustained increase of $[Ca^{2+}]_i$ required external Ca^{2+}, we explored this effect as a function of the external Ca^{2+} concentration. Typical results are shown in Figure 5, where TSMCs were exposed to carbachol in the presence of 1.8 (trace a), 1.0 (trace b), 0.2 (trace c) and 0 (Ca^{2+}-free; trace d) mM Ca^{2+}, respectively. All traces showed a similar trend, although the level was dependent on the external Ca^{2+} concentration. These results suggest that Ca^{2+} influx during the occupation of muscarinic AChRs is both immediate and persistent.

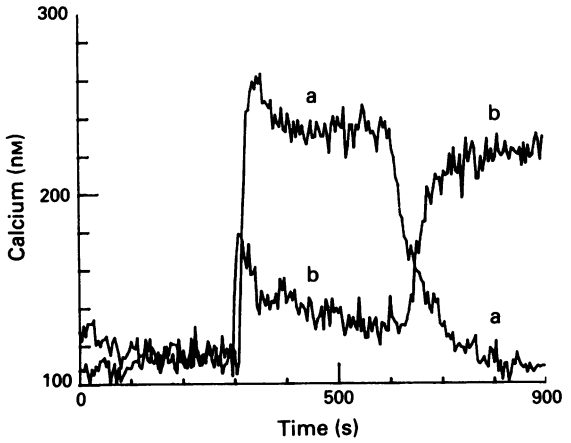

Figure 4 Effect of external Ca^{2+} on carbachol-stimulated changes in $[Ca^{2+}]_i$. Trace (a): cells were stimulated by adding carbachol (100 μM) to the buffer containing 1.8 nM Ca^{2+}. An immediate increase in $[Ca^{2+}]_i$ was seen which was followed by a sustained plateau level of $[Ca^{2+}]_i$. The plateau level decreased sharply when 2 mM EGTA was added during the sustained plateau level in the presence of carbachol. Trace (b): cells were incubated in the absence of external Ca^{2+} and stimulated by carbachol (100 μM). The profile shows an initial transient increase of $[Ca^{2+}]_i$ similar to trace (a) but to a substantially smaller degree. The sustained plateau $[Ca^{2+}]_i$ was not seen in the absence of the external Ca^{2+}. However, when Ca^{2+} (1.8 mM) was added 10 min after the addition of carbachol, a sustained increase of $[Ca^{2+}]_i$ occurred, reaching a level equal to the sustained elevation of $[Ca^{2+}]_i$ in tracheal smooth muscle cells observed in trace (a).

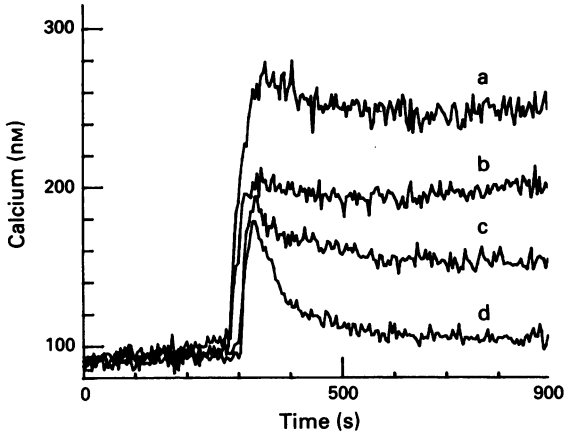

Figure 5 Carbachol-induced changes in $[Ca^{2+}]_i$ in tracheal smooth muscle cells (TSMCs) vary with external Ca^{2+} concentrations. Traces were obtained when TSMCs were stimulated with carbachol (100 μM) in the presence of external Ca^{2+} equal to 1.8 mM (trace a), 1.0 mM (trace b), 0.2 mM (trace c), or Ca^{2+}-free (trace d). All traces show a similar trend except for the changes in $[Ca^{2+}]_i$ levels which are dependent on the external concentrations.

Effects of calcium-channel blockers on $[Ca^{2+}]_i$

Such a sustained elevation in $[Ca^{2+}]_i$ probably involves Ca^{2+}-channels which may be operated by depolarization (Takuwa *et al.*, 1987). We therefore assessed the effect of Ca^{2+}-channel blockers on the carbachol-stimulated changes in $[Ca^{2+}]_i$. The Ca^{2+}-channel antagonists, diltiazem, nifedipine, and verapamil, were added either before or after carbachol (Table 1). The carbachol-stimulated initial transient increase of $[Ca^{2+}]_i$ was significantly reduced by diltiazem (10 μM), nifedipine (10 μM), or verapamil (10 μM). The initial transient increase of $[Ca^{2+}]_i$ decreased from 318 ± 25 nM in the control to 226 ± 18 nM with diltiazem, to 180 ± 23 nM with nifedipine, and to 232 ± 20 nM with verapamil ($n = 9$), as well as decreased the plateau phase of $[Ca^{2+}]_i$ from 282 ± 29 nM to 196 ± 15, 151 ± 17, and 185 ± 17 nM, ($n = 9$), respectively, when the Ca^{2+}-channel blockers were applied before the addition of carbachol. When diltiazem, nifedipine, or verapamil was added to the solution during the sustained plateau

Table 1 Effects of diltiazem, nifedipine, verapamil, and Ni^{2+} on carbachol-stimulated changes in $[Ca^{2+}]_i$

	Carbachol/Blocker		*Blocker/Carbachol*	
Drug	*Peak*	*Plateau*	*Peak*	*Plateau*
Control	318 ± 25	282 ± 29		
Diltiazem	315 ± 16	248 ± 13	226 ± 18*	196 ± 15*
Nifedipine	323 ± 19	184 ± 19*	180 ± 23*	151 ± 17*
Verapamil	302 ± 21	192 ± 18*	232 ± 20*	185 ± 17*
Ni^{2+}	295 ± 32	153 ± 11*	188 ± 15*	151 ± 16*

TSMCs were incubated in the presence of external Ca^{2+} (1.8 mM). Diltiazem (10 μM), nifedipine (10 μM), verapamil (10 μM) and Ni^{2+} (5 mM) were added to the buffer before (Blocker/Carbachol) or during response (Carbachol/Blocker) to carbachol (100 μM). Data are expressed as concentration of $[Ca^{2+}]_i$ in nM.

*$P < 0.01$ as compared with non-treated cells induced by carbachol.

phase, the sustained elevation of $[Ca^{2+}]_i$ decreased from 282 ± 29 nM to 248 ± 13 nM with diltiazem, to 185 ± 19 nM with nifedipine, and to 192 ± 18 nM with verapamil ($n = 9$). Ni^{2+} (5 mM), a transition metal ion that blocks voltage-gated Ca^{2+}-channels, also decreased the initial transient $[Ca^{2+}]_i$ from 318 ± 25 to 188 ± 15 nM ($n = 11$) as well as the sustained elevation of $[Ca^{2+}]_i$ from 282 ± 29 to 151 ± 11 nM ($n = 11$) (Table 1), when added before carbachol. Addition of 5 mM Ni^{2+} during the plateau phase abolished the sustained $[Ca^{2+}]_i$ level evoked by carbachol. If Ni^{2+} was added before carbachol, the response resembled that seen in a Ca^{2+}-free solution in Figure 4 (Table 1).

Discussion

Smooth muscle contraction is initiated by agonists which bind to a receptor and induce an increase in $[Ca^{2+}]_i$. Calcium release from internal stores and influx from external sources play an important role in the regulation of $[Ca^{2+}]_i$ (Bolton, 1979; Van Breeman & Saida, 1989). The present study demonstrates that muscarinic AChR-mediated increase in $[Ca^{2+}]_i$ is also due to calcium release from the internal stores and to an influx from the external source. Although both influx and intracellular release of calcium appear to be initiated simultaneously, intracellular release triggers a rapid peak in $[Ca^{2+}]_i$ which is transient even in the continued presence of carbachol. In contrast, the influx component of $[Ca^{2+}]_i$ reaches its maximum more slowly, is of lower magnitude, and persists as long as carbachol is present. These changes in $[Ca^{2+}]_i$ were directly determined in cultured canine TSMCs by use of the calcium-sensitive dye, fura-2.

Carbachol produced a dose-dependent, biphasic increase in $[Ca^{2+}]_i$ which displayed an initial transient peak and a sustained plateau phase. However, the peak is only apparent at higher concentrations of carbachol. The differences in Ca^{2+} mobilization can be due to varying extents of receptor occupancy by muscarinic agonists (Al-Hassani *et al.*, 1993). Thus low concentrations of agonists may not be potent enough to stimulate the release of $[Ca^{2+}]_i$, leaving extracellular Ca^{2+} influx as the primary source of Ca^{2+} for activation of tracheal smooth muscle contraction. Both the initial peak and the plateau phase were prevented by the addition of the muscarinic antagonist, atropine. However, once $[Ca^{2+}]_i$ is initiated by carbachol, even atropine did not completely reverse the effect (Figure 1), while depletion of external Ca^{2+} did (Figure 4). Thus it appears that some event is initiated by carbachol and is continuing to occur in the presence of atropine. This event still involves an influx of extracellular Ca^{2+} since EGTA completely abolishes this response. This clearly indicates that these changes in $[Ca^{2+}]_i$ were due to the association of carbachol with specific muscarinic AChRs.

Our results demonstrate that the muscarinic AChRs coupled to $[Ca^{2+}]_i$, have a low affinity for AF-DX 116 ($pK_B = 6.4$) and high affinity for 4-DAMP ($pK_B = 9.4$). Therefore, they can be classified as M_3 muscarinic receptors (Hulme *et al.*, 1990). These results are consistent with an M_3 receptor being linked to the rise in $[Ca^{2+}]_i$ (Murphy *et al.*, 1991) and are in good agreement with our previous report that activation of M_3 muscarinic receptors leads to generation of IPs and muscle contraction (Yang *et al.*, 1991a).

In the absence of external Ca^{2+}, carbachol produced a rapid increase in $[Ca^{2+}]_i$ which declined to the resting level within 10 s. An initial transient elevation of $[Ca^{2+}]_i$ still occurred in the absence of any external Ca^{2+} but to a substantially smaller extent than that measured in the presence of external Ca^{2+}. This smaller elevation of $[Ca^{2+}]_i$ can be taken as representative of the IP_3-mediated release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1989). In tracheal smooth muscle, it is known that stimulation of muscarinic AChRs by agonists results in the activation of phospholipase C which hydrolyzes phosphoinositide and leads to the formation of IP_3 (Baron *et al.*, 1984; Grandordy *et al.*, 1986; Meurs *et al.*, 1988; Chilvers *et al.*, 1989; Roffel *et al.*, 1990; Yang *et al.*, 1991a,b). Carbachol-stimulated IP_3 accumulation was maximized occuring within 5 to 10 s and then fell to or below the basal levels within 60 s. This profile is very simliar to the time course described for the initial transient $[Ca^{2+}]_i$ induced by carbachol in canine TSMCs.

An important observation was that the addition of 1.8 mM external Ca^{2+} to the Ca^{2+}-free medium rapidly brought $[Ca^{2+}]_i$ to the sustained plateau level after $[Ca^{2+}]_i$ had returned to the resting level as the cells continued to be exposed to carbachol. It appears that muscarinic AChR occupation by carbachol and the presence of external Ca^{2+} are necessary and sufficient conditions for Ca^{2+} influx. However, the mechanism regulating this influx was obscure. Our data provide evidence that the two phases of the carbachol-stimulated increase in $[Ca^{2+}]_i$ are mediated by two different mechanisms. An increase in intracellular Ca^{2+} concentration is generally caused by its release from internal stores or by entry through the cell membrane from an external source (Bolton, 1979; Van Breemen & Saida, 1989). Calcium entry through the cell membrane can be mediated via voltage- and receptor-operated calcium channels. The initial transient $[Ca^{2+}]_i$ has been shown to be dependent on both internal and external Ca^{2+} since it was attenuated but not abolished by calcium channel blockers. These organic Ca^{2+}-channel blockers did not attenuate the rise in $[Ca^{2+}]_i$ induced by carbachol at a concentration of 1 μM, though at a 30 μM concentration they caused a reduction of both the peak and plateau phase of $[Ca^{2+}]_i$ (Fatatis *et al.*, 1992). In this study, diltiazem, nifedipine, and verapamil only partially attenuated and Ni^{2+} completely reversed the plateau phase at a concentration of 10 μM, when they were applied after the initiation of $[Ca^{2+}]_i$ response by carbachol. Not only Ni^{2+}, but all other Ca^{2+}-channel antagonists inhibited the plateau phase of $[Ca^{2+}]_i$, when these antagonists were added before the addition of carbachol. This indicates that the sustained plateau of $[Ca^{2+}]_i$ is mediated by Ca^{2+} influx through those L-type calcium channels which are sensitive to calcium channel blockers. These changes in $[Ca^{2+}]_i$ implicated in the regulation of smooth muscle contraction have been demonstrated (Bolton, 1979; Takuwa *et al.*, 1987; Baba *et al.*, 1989; Al-Hassani *et al.*, 1993). It has been suggested that the initial rise in $[Ca^{2+}]_i$ which occurs during force development is due to the release of intracellular Ca^{2+}, whereas the plateau phase of $[Ca^{2+}]_i$ and of tension in tracheal smooth muscle result from an interplay between increases in the influx of extracellular Ca^{2+} and the release of Ca^{2+} from internal stores (Takuwa *et al.*, 1987).

A number of other characteristics of the sustained elevation of $[Ca^{2+}]_i$ were observed. Ni^{2+} appears to block both voltage-sensitive Ca^{2+} channels (Tsien, 1983) and receptor-operated Ca^{2+} entry in platelets (Hallam & Rink, 1985). When Ni^{2+} is added before carbachol, it is able to abrogate the sustained plateau of $[Ca^{2+}]_i$, but the initial transient $[Ca^{2+}]_i$ is not completely abolished. Addition of Ni^{2+} during the sustained plateau of $[Ca^{2+}]_i$ evoked by carbachol caused the $[Ca^{2+}]_i$ to decline immediately to the resting level and was consistent with blockage of Ca^{2+} entry (Tsien, 1983).

In conclusion, our data show that muscarinic receptor-stimulated canine tracheal smooth muscle contraction is mediated by Ca^{2+} release from the internal stores and by the entry of extracellular Ca^{2+} through calcium channels. These results suggest that the initial peak results from IP_3-mediated Ca^{2+} release from intracellular stores and that sustained elevation of $[Ca^{2+}]_i$ is due to an agonist-dependent influx of Ca^{2+} from external sources. These changes in $[Ca^{2+}]_i$ relate to the generation of tension in the canine tracheal smooth muscle (Takuwa *et al.*, 1987; Al-Hassani *et al.*, 1993).

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Capsaicin-induced relaxation in the rat isolated external urethral sphincter: characterization of the vanilloid receptor and mediation by CGRP

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1 The potential role of capsaicin-sensitive nerves in the relaxation of the rat external urethral sphincter (REUS) was evaluated by demonstrating the existence of specific vanilloid (capsaicin) receptors and by investigating the sensory neurotransmitter(s) putatively involved in this relaxation.

2 Capsaicin (1 μM) relaxed REUS strips precontracted with noradrenaline (NA) (0.1 mM). This effect underwent desensitization and it was absent in preparations taken from adult capsaicin-pretreated rats.

3 Capsaicin-induced relaxation of NA-precontracted REUS was mimicked by calcitonin gene-related peptide (CGRP, 0.3–10 μM), but not by substance P (1 μM), vasoactive intestinal polypeptide (VIP, 1 μM), α-β methylene ATP (10 μM), γ-aminobutyric acid (GABA, 3 mM) or galanin (1 μM). A cross-tachyphylaxis between capsaicin (1 μM) and CGRP (1 μM) was observed. Both capsaicin and CGRP-induced relaxation were partially antagonized by the proposed CGRP antagonist, CGRP (8-37) (10 μM).

4 Electrical field stimulation (EFS, 2.5 Hz, 60 V, 1 ms, trains of 5 s every 5 min) of REUS evoked a contraction characterized by a largely adrenergic slowly developing tonic contraction with superimposed fast twitches due to the striated component of the strips. Both capsaicin (1 μM) and CGRP (0.01–1 μM) produced an almost complete inhibition of EFS-induced tonic contraction. A cross-tachyphylaxis between capsaicin and CGRP was observed. Furthermore, these inhibitory actions were unaffected by CGRP (8-37) (10 μM).

5 [^{3}H]-resiniferatoxin displayed specific, saturable binding to rat urethral membranes. Data were consistent with a single site with a K_d of 105 pM and a B_{max} of 40 fmol mg^{-1} protein. This binding was inhibited by capsaicin with a K_i of 0.6 μM and it was reduced by approximately 80% in preparations taken from rats that had undergone surgical ablation of the major pelvic ganglion 4 days earlier.

6 In conclusion we have demonstrated the existence of vanilloid receptors on capsaicin-sensitive nerves innervating the rat urethra mainly through the major pelvic ganglion. The activation of this set of nerves could lead to a local release of CGRP that in turn elicits a remarkable urethral relaxation. Such a mechanism could be of relevance in physiological conditions to facilitate urine expulsion during micturition and in pathological conditions to help removal of noxious stimuli following mechanical/chemical irritation of the lower urinary tract.

Keywords: Neurogenic inflammation; sensory neuropeptides; micturition; resiniferatoxin

Introduction

The rat urethra has a rather complex anatomical organization with bundles of striated muscle fibres interlaced with smooth muscle (El-Badawi & Schenk, 1974) and a dense network of autonomic and somatic innervation (Watanabe & Yamamoto, 1979). Noradrenergic nerves are thought to be the major excitatory input to the urethra (Levin & Wein, 1979; Gosling, 1986; Chen & Brading, 1992) determining the continence of the bladder outlet in the periods between micturitions. However, during urination, the urethral sphincter has to relax to allow urine expulsion and a series of neural mechanism(s) should be targeted to contrast sympathetic tone. Recent reports indicate that one of these mechanisms could be the generation and release of nitric oxide from nonadrenergic-noncholinergic nerves (Persson *et al.*, 1992; Parlani *et al.*, 1993). Immunohistochemical studies combined with retrograde tracing techniques indicate the presence in the urethral wall of nerves containing substantial amounts of calcitonin gene-related peptide (CGRP) and tachykinins (Su *et al.*, 1986). These nerves have their cell bodies in dorsal root ganglia (L6-S1) and their CGRP content is substantially reduced following systemic capsaicin desensitization (Su *et al.*, 1986). Activation of these capsaicin-sensitive primary afferents can be involved in the neurogenic inflammation that follows mechanical irritation of the urethral meatus (Nordling *et al.*, 1990; Abelli *et al.*, 1991). The aims of this investigation were: (a) to assess whether or not the activation of capsaicin-sensitive nerves can relax urethral strips precontracted by exogenous noradrenaline or electrical field stimulation of sympathetic nerves; (b) to investigate the putative neurotransmitter involved in such inhibitory effects and (c) to demonstrate the existence of vanilloid (capsaicin) receptors in rat urethral tissues by using a binding assay with the tritiated ultrapotent capsaicin analogue, resiniferatoxin (RTX) (Szallasi & Blumberg, 1990).

Methods

Organ bath studies

Male albino rats (Wistar-Morini strain, 360–400 g) were killed by cervical dislocation. An incision was made in the lower abdomen; the bladder was exposed and the proximal urethra was freed from the surrounding tissues. The pubic bone was cut and then the external urethral sphincter (EUS) was disconnected from the perineal muscles and removed *in toto*. Thereafter, the preparation was placed in oxygenated Krebs solution and strips (approximately 7 mm by 3 mm) were taken from the middle region of the EUS (Parlani *et al.*, 1992). Samples were mounted in a 5 ml organ bath containing Krebs solution (37°C) continuously aerated with a mix-

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ture of 96% O_2 and 4% CO_2. The Krebs solution contained (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2 and glucose 11. The preparations were connected with a silk thread to an isometric strain gauge under a constant load of 1 g. Contractile activity was recorded by means of a Basile 7050 unirecord polygraph. The preparations were allowed to equilibrate for at least 60 min. Based on preliminary experiments assessing the concentration-response curve to the motor effect of noradrenaline (NA), 0.1 mM was chosen as a supramaximal concentration of the adrenoceptor agonist. Exposure to NA (0.1 mM) produced a contraction that reached a steady state within 5–15 min and then lasted for at least an additional 30–45 min. The effect of drugs was studied when a stable tone was established. The inhibitory actions were measured as mg of relaxation or % of inhibition of the NA (0.1 mM)-induced contractile response before exposure to drugs. In other experiments electrical field stimulation (EFS) of the EUS was carried out by means of two platinum wire electrodes placed at the top and at the bottom of the organ bath and connected to a Grass S11 stimulator. Square wave pulses were delivered at 60 V, 2.5 Hz (trains of 5 s every 5 min). The pulse width was 1 ms. A scale of the frequency (range between 1.5 and 5 Hz) revealed that 2.5 Hz was the frequency triggering maximal responses. This frequency of EFS was selected to evaluate the effect of pharmacological interventions. After three consecutive reproducible responses (accepted variation $<10\%$) had been obtained, drugs were added to the organ bath. The effect of drugs was evaluated on the subsequent response (5 min later) and expressed as % inhibition of the basal contraction.

To prevent desensitization, CGRP concentration-response curves were carried out in a non-cumulative manner. In each preparation a maximum of three concentrations was tested.

An incubation time of 10 min was selected to study the effect of the putative CGRP antagonist, CGRP (8-37). In each preparation the effect of the antagonist was tested on one type of contraction (NA or EFS) and one type of agonist (capsaicin or CGRP or papaverine). Statistical comparison was performed between control and treated group.

Surgical procedures

Denervation of the EUS was carried out by bilateral removal of the major pelvic ganglion, as described previously (Conte *et al.*, 1989). After the surgical operation, the rats were kept for 4 days in separate cages to allow nerve degeneration. To prevent infections, each rat received amikacin (2.5 mg kg^{-1}, i.m.) and gentamicin (2 mg kg^{-1}, i.p.) once daily.

Systemic capsaicin desensitization

Capsaicin (50 mg kg^{-1}, dissolved in a vehicle containing 10% Tween 80, 10% ethanol and 80% saline) was administered subcutaneously 4 days before the experiment. Control rats received the vehicle as described previously (Santicioli *et al.*, 1985).

[^{3}H]-RTX binding assay

Binding experiments were carried out with a crude, post-nuclear membrane fraction obtained from urethra of Sprague-Dawley rats as described for other tissues (Szallasi & Blumberg, 1990; 1992). Briefly, the urethra was disrupted by the aid of a Polytron tissue homogenizer in ice-cold buffer (pH 7.4), containing (in mM): KCl 5, NaCl 5.8, $MgCl_2$ 2, $CaCl_2$ 0.75, sucrose 137 and HEPES 10. The homogenate was centrifuged for 10 min at 1 000 *g* (4°C), the pellet discarded, and the supernatant was then centrifuged at 35,000 *g* for 30 min. The resulting pellet was resuspended in the above described buffer and stored at − 20°C until assayed. Aliquots (100 μg) of the membrane protein in 0.5 ml of this buffer containing 0.25 mg ml^{-1} bovine serum albumin (Sigma, Cohn fraction V) were incubated in triplicate with [^{3}H]-RTX and nonradioactive ligands at 37°C for 30 min. Nonspecific binding was determined in the presence of 100 nM nonradioactive RTX. After the binding reaction had been terminated by chilling the assay mixture on ice, 100 μg $alpha_1$-acid glycoprotein was added to each tube to reduce nonspecific binding (Szallasi *et al.*, 1992). Bound and free [^{3}H]-RTX were then separated by centrifuging the membranes in a Beckman 12 microfuge, and the counts in the resulting pellet determined by scintillation counting.

Binding data from saturation experiments with increasing concentrations (7–500 pM) of radioactive ligand were analysed by the collection of computer programmes LIGAND (Biosoft Cambridge, UK); inhibitory constants were determined by the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Drugs

Drugs used were: noradrenaline, atropine sulphate (Fluka), acetylcholine, papaverine, GABA, L-NOARG, α,β-methylene ATP (Sigma), capsaicin (Serva), CGRP, SP, NKA, VIP, galanin, hCGRP(8-37) (Peninsula), phentolamine (Ciba Geigy), tetrodotoxin (Sankyo), amikacin (Bristol), gentamicin (Shering), pentothal sodium (Abbott). A stock solution (10 mM) of capsaicin was prepared in absolute ethanol and then appropriate dilutions in distilled water were made; all other drugs were dissolved directly in distilled water.

[^{3}H]-RTX (37 Ci $mmol^{-1}$) was synthesized by the Chemical Synthesis and Analysis Laboratory; NCI-FCRDC, Frederick, MD, U.S.A. Nonradioactive RTX and resiniferonol 9,13,14-orthophenylacetate (ROPA) were purchased from LC Services (Woburn, MA, U.S.A.).

Statistics

All data in the text are means ± s.e. Statistical evaluation was performed by using Student's *t* test for paired or unpaired data when applicable or by analysis of variance (ANOVA) followed by Tukey's test.

Results

Effect of capsaicin on noradrenaline-induced tonic contraction of rat external urethral sphincter

In basal conditions specimens of rat external urethral sphincter were either quiescent (about 30%) or displayed a small-amplitude (20–100 mg) contractile activity (about 70%). After 30 min of incubation all preparations exhibited no spontaneous contractile activity. In these conditions, the administration of capsaicin (1 μM) or CGRP (0.3 μM) produced a small relaxation of 42 ± 12 mg ($n = 7$) and 35 ± 8 mg ($n = 6$), respectively. On the other hand, substance P (10 μM) was ineffective ($n = 4$), while neurokinin A (10 μM) elicited a small and transient contraction the amplitude of which was 61 ± 10 mg ($n = 6$).

The administration of noradrenaline (NA) (0.1 mM) elicited a contraction with an amplitude of 351 ± 20 mg ($n = 25$) reaching a steady-state within 5–15 min. The NA-induced tonic contraction remained stable for at least 30–45 min ($n = 6$) and it was abolished by phentolamine (3 μM, $n = 4$), but was unaffected by atropine (3 μM, $n = 4$) or tetrodotoxin (0.6 μM, $n = 4$).

Administration of capsaicin (1 μM) during NA-induced tonic contraction resulted in a rapid relaxation reaching a maximal effect (82 ± 5 mg corresponding to an inhibition of 32 ± 3%, $n = 13$) within 1–2 min (Figure 1). Capsaicin-induced relaxation lasted for at least 15–20 min and then the urethral tone slowly returned toward the previous contracted level. A second (or in one case a third) administration of capsaicin (1 μM) was without any relaxant effect, suggesting

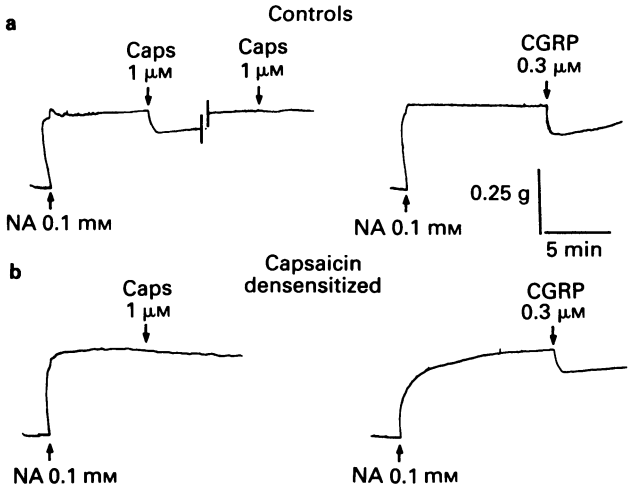

Figure 1 Typical tracings showing the effect of capsaicin (Caps) and calcitonin gene-related peptide (CGRP) on noradrenaline (NA)-induced tonic contraction of control (a) and systemic capsaicin-desensitized (b) rats. In (a) is also shown the desensitization that occurred with a second application of capsaicin. Tension and time scales apply to all tracings. In the left upper tracings the two bars indicate a lag of about 20 min.

desensitization ($n = 4$) (Figure 1). Capsaicin-induced relaxation was unaffected by atropine (3 μM, $n = 4$) or tetrodotoxin (0.6 μM, $n = 4$).

In some experiments, external urethral sphincters obtained from adult capsaicin-pretreated rats (50 mg kg^{-1}, s.c., 4 days before) were used. In these preparations the motor responses to NA were normal (396 ± 47 mg, $n = 6$, NS as compared to control), while the administration of capsaicin (1 μM) produced only negligible relaxation (13 ± 8 mg corresponding to 3 ± 2% inhibition, $P < 0.001$ as compared to control rats, $n = 6$) (Figure 1). In urethral preparations from capsaicin-pretreated rats, capsaicin (1 μM) had no effect on basal tone ($n = 7$).

In a further set of experiments the ability of capsaicin to relax NA-precontracted urethral strips taken from rats in which the major pelvic ganglia had been surgically removed 4 days before, has been evaluated. Capsaicin (1 μM)-induced relaxation was significantly reduced as compared to urethral tissues taken from normal rat (41 ± 19 mg and 16 ± 3% inhibition $n = 4$; $P < 0.001$ vs. control rats, $n = 4$). On the other hand the motor response to NA and the relaxation induced by CGRP (0.3 μM) or papaverine (10 μM) were similar in control and in denervated preparations ($n = 4$).

Mimicking by CGRP of capsaicin-induced relaxation of NA-precontracted EUS: antagonism by CGRP(8-37)

To obtain some insight into the neurotransmitter(s) involved in capsaicin-induced relaxation of NA-precontracted rat external urethral sphincter, the potential relaxant properties of various putative neurotransmitters were assessed. No relaxation of NA-induced tone was obtained with vasoactive intestinal polypeptide (VIP, 1 μM, $n = 4$), substance P (1 μM, $n = 4$), α-β-methylene-ATP (10 μM, $n = 4$), (γ-aminobutyric acid) GABA (3 mM, $n = 4$) or galanin (1 μM, $n = 4$). On the other hand a clear and remarkable relaxation (114 ± 24 mg corresponding to an inhibition of 35 ± 7%) was elicited by CGRP (0.3 μM, $n = 5$) (Figure 1). CGRP-induced relaxation was concentration (0.3–1–10 μM)-dependent with an ED_{50} of 0.94 μM (0.91–0.97) ($n = 6$). Relaxant response to CGRP (0.3 μM) was also observed in preparations taken from adult capsaicin pretreated rats (Figure 1). CGRP (1 μM)-induced relaxation underwent desensitization and a second application on the same NA-induced hypertone was without effect ($n = 4$) (Figure 2). Interestingly, a cross-tachyphylaxis between CGRP and capsaicin (1 μM) also occurred the latter drug being ineffective when administered following CGRP (1 μM) (Figure 2a) ($n = 4$). Control experiments indicated that a previous similar relaxation of NA-induced tone with papaverine (10 μM) did not prevent the relaxant effect of capsaicin (1 μM) (Figure 2b, $n = 3$).

The proposed selective CGRP antagonist, CGRP (8-37) (10 μM) (Chiba *et al.*, 1989) significantly reduced the relaxation induced by CGRP (0.3 μM) or by capsaicin (1 μM), but not that by papaverine (10 or 100 μM), of NA-induced tonic contraction (Table 1).

Effect of capsaicin or CGRP on electrical field stimulation induced tonic contraction of EUS

Electrical field stimulation (2.5 Hz, 60 V, 1 ms, trains of 5 s every 5 min) induced a contractile response characterized by two components (Figure 3): a slowly developing tonic contraction on which were superimposed a series of twitches due to the activation of the striated component of the sphincter. The tonic component was abolished by tetrodotoxin (0.3 μM, $n = 4$) and largely reduced (84 ± 7% inhibition) by phentolamine (3 μM, $n = 5$).

Capsaicin (1 μM) or CGRP (1 μM) produced an almost complete inhibition of EFS-induced tonic contraction (95 ± 6%, $n = 8$, and 81 ± 8%, $n = 4$, for capsaicin and CGRP, respectively, Figure 3a,b). Capsaicin-induced inhibition could be evoked only once in each preparation ($n = 4$) and it was significantly reduced in rats desensitized to capsaicin (16 ± 7%, $n = 6$, $P < 0.001$, Figure 3c). In these latter preparations the inhibitory action of CGRP (1 μM) was normal (91 ± 5% of inhibition; $n = 4$, Figure 3d). The inhibitory effect of CGRP (1 μM) on EFS-induced tonic contraction underwent desensitization and a cross-desensitization with capsaicin (1 μM) was observed (Figure 4, $n = 4$). CGRP-induced relaxa-

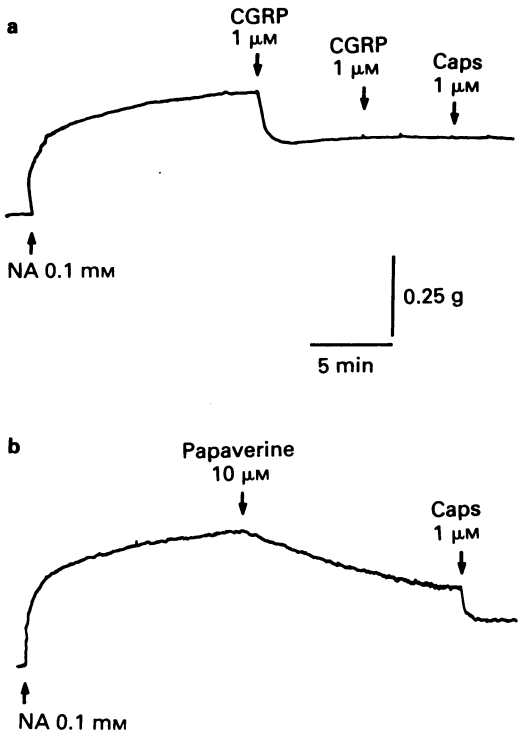

Figure 2 Typical tracings showing the desensitization of the calcitonin gene-related peptide (CGRP) relaxant response and the cross-desensitization with capsaicin (Caps) (a). Panel (b) shows the specificity of this cross-desensitization: papaverine (at an equieffective concentration to CGRP) did not modify capsaicin-induced relaxation. Tension and time scales apply to all tracings.

Table 1 Effect of calcitonin gene-related peptide (CGRP (8-37)) (10 μM) on relaxation of noradrenaline (NA)-precontracted EUS induced by CGRP, capsaicin or papaverine

			Control		*+ hCGRP (8-37)*	
Drug	(μM)	n	*Relaxation* (mg)	*% inhibition*	*Relaxation* (mg)	*% inhibition*
CGRP	0.3	4	110 ± 18	38 ± 8	44 ± 13*	13 ± 3*
Capsaicin	1	6	88 ± 4	30 ± 3	36 ± 4**	14 ± 2**
Papaverine	10	6	153 ± 23	45 ± 6	152 ± 25	37 ± 11

Inhibition relate to the % reduction of the amplitude of the contraction. Each value is the mean ± s.e.mean.
*$P<0.05$ vs control; **$P<0.01$ vs control.

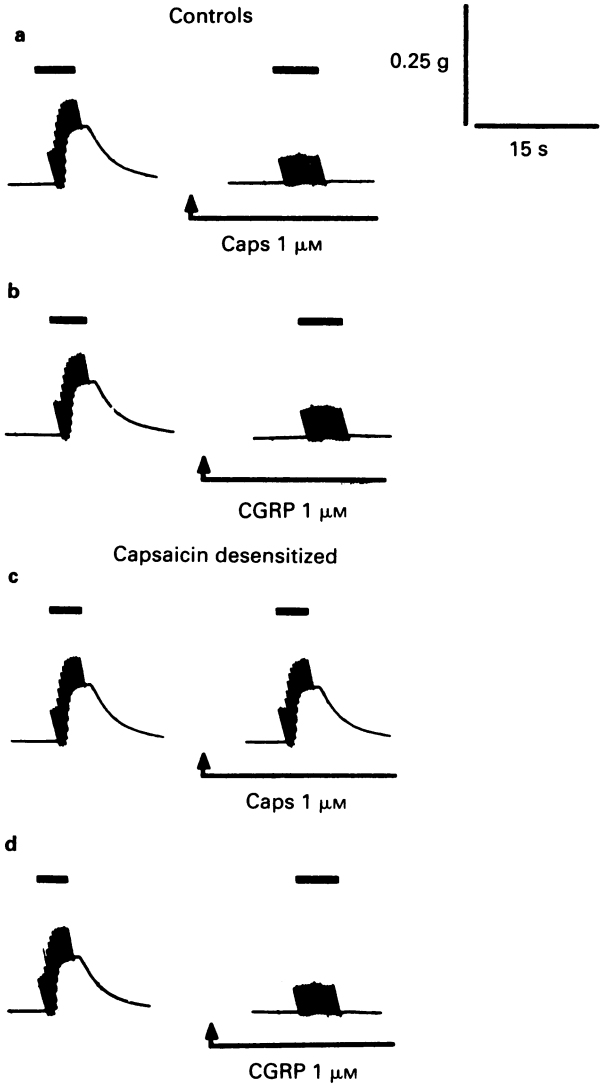

Figure 3 Inhibitory effect of capsaicin (Caps) (a) and calcitonin gene-related peptide (CGRP) (b) on EFS-induced contraction of rat external urethral sphincter. In (c) and (d) the effects obtained in systemic capsaicin-pretreated rats are shown. EFS (2.5 Hz, 60 V, 1 ms, trains of 5 s every 5 min) was applied at the black horizontal bars. Tension and time scales apply to all tracings.

tion was concentration (0.01–0.1–1 μM)-dependent with an ED_{50} of 44 nM (16–120) ($n=6$). The inhibitory effect of capsaicin (1 μM) or CGRP (0.3–1 μM) were unaffected by CGRP (8-37) at a concentration of 10 μM ($n=4$ for each substance). CGRP (8-37) was also ineffective against a concentration of CGRP (0.01 μM) that produced an inhibition of EFS-induced contraction (30 ± 2%) similar to that exerted by CGRP (0.3 μM) on NA-induced contraction ($n=4$).

Characterization of vanilloid (capsaicin) receptor in rat urethral tissues

[^{3}H]-RTX displayed specific, saturable binding to rat urethral membranes. Nonspecific binding at the K_d value represented approximately 30% of the total. Scatchard analysis of the data was consistent with a single component possessing a K_d of 105 ± 26 pM and a B_{max} of 40 ± 6 fmol mg^{-1} protein (3 determinations; mean ± s.e.mean, Figure 5). The Hill coefficient was approaching 1, suggesting that the binding sites are not cooperative. The curvilinear analysis of the data by the LIGAND programme confirmed the one-site model suggested by the Scatchard plot. Capsaicin inhibited binding with a K_i of 0.6 ± 0.3 μM (range) indicating a 6 000 fold lower potency than that of RTX (not shown). Resiniferonol 9,13,14-orthophenylacetate (ROPA), the C-20 de-esterified parent compound of RTX which lacks vanilloid-like activity (Szallasi *et al.*, 1989), by contrast, did not inhibit binding up to 1 μM. In the urethra of rats that had undergone surgical ablation of major pelvic ganglia 4 days earlier, specific [^{3}H]-RTX binding was reduced by approximately 80% (2 determinations).

Discussion

In the rat urethra the existence of capsaicin-sensitive neuropeptide-containing sensory nerves have been firmly established by immunohistochemical techniques (Su *et al.*, 1986). Capsaicin-sensitive primary afferents subserve a dual sensory and efferent function with potential physiological and pathological relevance (Maggi & Meli, 1988). Stimulation of these urethral sensory nerves can trigger a series of reflexes important for the urethra-bladder coordination during micturition (Conte *et al.*, 1989; Maggi, 1993). At the same time the local release of sensory neuropeptides can produce neurogenic inflammation (Abelli *et al.*, 1991) and relax the bladder outlet thus facilitating urine expulsion (Andersson *et al.*, 1990). Following excessive mechanical or chemical irritation of the urethral meatus a hyperreactivity of this neural system could, at least in principle, lead to dyssynergia between bladder and urethral function and to incontinence due to reduced tone of the urethral sphincter.

In the present study, we have characterized further this sensory innervation of the EUS with a binding and functional pharmacological approach. The functional analysis was carried out on EUS precontracted by increasing the sympathetic tone by stimulation at pre- (EFS) or post- (exogenous NA) junctional level.

First, we have demonstrated the existence in urethral membranes of specific binding sites for [^{3}H]-RTX. RTX is an ultrapotent analogue of capsaicin that allowed the characterization of vanilloid (capsaicin) receptors in the soma (dorsal root ganglia) and central arms (spinal cord) of this subgroup of sensory nerves (Szallasi & Blumberg, 1990). Recently, using a modified assay in which the nonspecific binding is reduced by adding alpha-1acid glycoprotein, a plasma protein that binds RTX to the assay mixture (Szallasi & Blumberg, 1992), we have been able to demonstrate vanil-

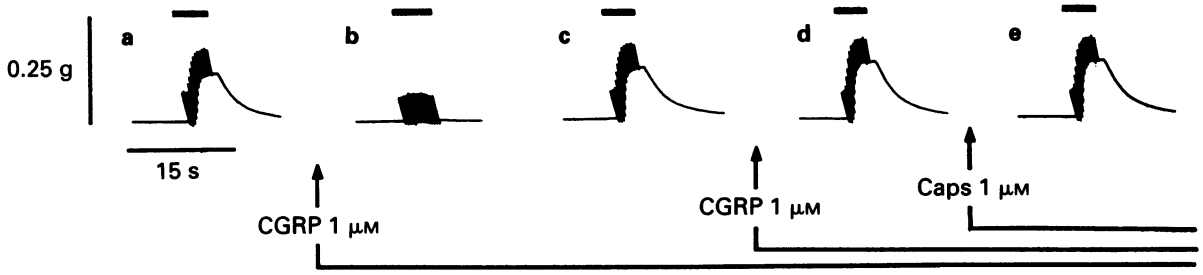

Figure 4 Typical experiment showing the cross-desensitization between calcitonin gene-related peptide (CGRP) and capsaicin (Caps)-induced inhibition of EFS-induced tonic contraction of EUS. In (a) is shown the control response that was completely abolished by a first application of CGRP (b). In these conditions a normal response to EFS could be readily restored by addition of L-N^G-nitro-arginine 100 μM (c). At this point a second application of CGRP (d) or of capsaicin (e) had no inhibitory effects.

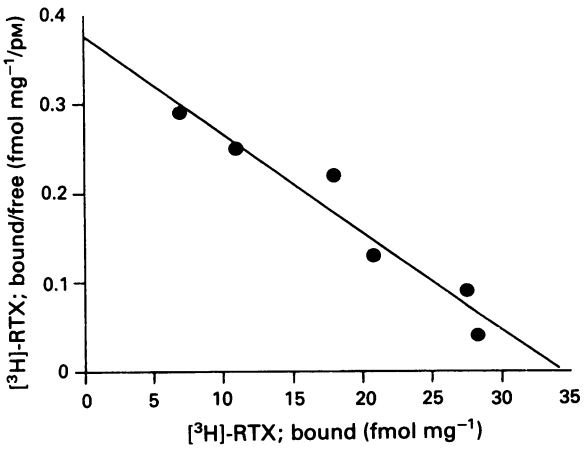

Figure 5 Scatchard plot of specific [^{3}H]-resiniferatoxin ([^{3}H]-RTX) binding by rat urethral membranes. Points represent mean values from a single experiment. An additional two experiments gave similar results. The line was fitted using the LIGAND programme.

loid receptors (i.e. specific [^{3}H]-RTX binding sites) in the urinary bladder (Szallasi *et al.*, 1993) and in the rat urethra (this study). The specificity of the [^{3}H]-RTX binding sites is confirmed by their competitive displacement by capsaicin but not by ROPA, a diterpene derivative of RTX that is devoid of stimulating properties for the vanilloid receptor (Szallasi *et al.*, 1989). Moreover, specific [^{3}H]-RTX binding was largely reduced (by approximately 80%) in urethral samples taken from rats that underwent resection of the major pelvic ganglion. This finding suggests that the majority of vanilloid receptors in the urethra are expressed on nerves that travel through the major pelvic ganglion. The remaining approximately 20% of specific [^{3}H]-RTX binding suggests that vanilloid receptors could also be present on some fibres of other nerves projecting to the urethra such as the pudendal and/or the hypogastric.

The affinity of the vanilloid receptor for RTX and capsaicin in the urethra (105 pM and 0.5 μM respectively) is similar to that determined in the urinary bladder (30 pM and 0.5 μM) (Szallasi *et al.*, 1993). Likewise, both urethral and urinary bladder membranes bound [^{3}H]-RTX in a non-cooperative fashion. This behaviour contrasts to the apparent positive cooperativity of [^{3}H]-RTX binding in dorsal root ganglia, as well as dorsal horn membranes (Szallasi & Blumberg, 1993). These findings suggest that vanilloid receptors in the bladder and the urethra are probably identical.

In good agreement with binding data, capsaicin at a concentration of 1 μM (that is, about two times the K_i for the vanilloid receptor) elicited a remarkable relaxation of EUS precontracted by adding exogenous noradrenaline or by EFS. Similar results were obtained in the female rat urethra (Andersson *et al.*, 1990) and in the male rat proximal urethra (Maggi *et al.*, 1987). Specificity of this relaxant effect of capsaicin was confirmed by its desensitization (the response could be evoked only once in each preparation), by its absence in preparations taken from systemic capsaicin-pretreated animals and by its reduction in urethral strips from ganglionectomized animals. Experiments designed to identify the putative neurotransmitter(s) that mediates these inhibitory responses to capsaicin ruled out the involvement of VIP, GABA, galanin, purines and substance P, since none of these endogenous substances exerted relaxant effect on NA-precontracted EUS. On the other hand, exogenous CGRP mimicked the capsaicin inhibitory response both on NA- and on EFS-induced urethral hypertone as well as in resting conditions. As expected, the inhibitory effects of exogenous CGRP (that should be unrelated to the presence of sensory nerves) were unaffected in EUS obtained from capsaicin-pretreated or ganglionectomized rats.

Since CGRP is usually co-stored with tachykinins in sensory nerves (Maggi & Meli, 1988), it could be hypothesized that the functional response to capsaicin could be the resultant of the corelease of several sensory neuropeptides. However, substance P has been shown to contract slightly longitudinal and circular strips of female rat urethra (Andersson *et al.*, 1990) and in our hands neurokinin A, but not substance P, had a small contractile effect on basal tone (this study). These motor responses to tachykinins could, in principle, slightly counteract the CGRP-induced relaxation and explain the tendentially minor action of capsaicin, as compared to CGRP, on NA-induced contraction. However, when the urethral tone was increased by means of EFS, a complete inhibition of the tonic response was obtained either with capsaicin or with CGRP. In the female rat urethra, CGRP was equieffective with capsaicin in circular but not in longitudinal urethral strips (Andersson *et al.*, 1990). As a whole, we believe that tachykinins have a very marginal (if any) role and that CGRP release can fully explain capsaicin-induced relaxation of NA-precontracted EUS. This hypothesis is further indicated by two other evidence: (a) a cross-desensitization was noticed between CGRP and capsaicin-induced relaxation and (b) CGRP (8-37) inhibited in a significant manner both capsaicin- and CGRP-induced relaxation of NA-contracted REUS. The peptide fragment CGRP (8-37) has been characterized as a competitive antagonist of at least a subclass of CGRP receptor (Chiba *et al.*, 1989; Maggi *et al.*, 1991; Barthò *et al.*, 1991). It is interesting to note that CGRP (8-37) had no antagonist action on the inhibition by capsaicin or CGRP (either 0.01, 0.3 or 1 μM) of EFS-induced tonic contraction. CGRP is significantly more effective in relaxing EFS- than NA-induced contraction of EUS. It is tempting to speculate that CGRP could antagonize EFS-induced contraction also through prejunctional (CGRP (8-37) resistant) receptors. Further experiments are obviously necessary to assess the potential involvement in EFS- (but not NA-)induced motor responses of a CGRP

(8-37)-resistant receptor, such as the proposed $CGRP_2$ subtype (Quirion *et al.*, 1993).

In conclusion we have demonstrated the existence of vanilloid receptors on capsaicin-sensitive nerve endings (mainly passing through major pelvic ganglia) in the rat EUS. We postulate that their activation leads to CGRP release, that in turn elicits a remarkable urethral relaxation. Such a mechanism could be of relevance in functional antagonism of sympathetic tone during micturition and could help in removal of noxious stimuli following chemical or mechanical irritation of urethra and the bladder. Further studies are necessary to evaluate whether a dysfunction of this system could also be involved in urinary pathologies such as urinary incontinence or detrusor instability.

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Nitric oxide modulation of calcium-activated potassium channels in postganglionic neurones of avian cultured ciliary ganglia

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1 A study has been made of the modulation of calcium-activated potassium channels in cultured neurones of avian ciliary ganglia by sodium nitroprusside and L-arginine.

2 Sodium nitroprusside (100 μM) reduced the net outward current by 22 ± 1% at 4.8 ms (mean ± s.e.mean) and 25 ± 1% at 350 ms during a test depolarization to + 40 mV from a holding potential of − 40 mV. The outward current remained reduced for the duration of the recording following a single application of sodium nitroprusside. These effects did not occur if the influx of calcium ions was first blocked with Cd^{2+} (500 μM). Application of ferrocyanide (100 μM) reduced the net outward current by only 6 ± 3% at 350 ms during a test depolarization to + 40 mV.

3 L-Arginine (270 μM) reduced the net outward current on average by 19 ± 2% at 4.8 ms and 22 ± 2% at 350 ms during a test depolarization to + 40 mV. The current remained in this reduced state for the duration of the recording following a single application of L-arginine. These effects were reduced to 11 ± 1% at 4.8 ms and 11 ± 2% at 350 ms in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM).

4 In order to alleviate the dependence of calcium-activated potassium channels ($I_{k(Ca)}$) on the inward flux of calcium ions, the patch-clamp pipettes were filled with a solution containing 100 μM $CaCl_2$, and the Ca^{2+} in the bathing solution was replaced with EGTA. Under these conditions sodium nitroprusside reduced the total outward current during a depolarizing pulse of + 40 mV by 9 ± 1% at 4.8 ms and by 36 ± 3% at 350 ms. L-Arginine (270 μM) reduced this current under the same conditions by 9 ± 1% at 4.8 ms and by 35 ± 2% at 350 ms.

5 Calcium-activated potassium currents were sensitive to apamin (50 nM), as this reduced the outward current by 23 ± 3% at 350 ms when a high calcium-containing pipette was used during a depolarizing command to + 40 mV. L-Arginine still decreased the outward current in the presence of apamin (50 nM), by 5 ± 1% at 4.8 ms and by 19 ± 2% at 350 ms, indicating that L-arginine could reduce an apamin-insensitive $I_{k(Ca)}$.

6 Calcium-activated potassium currents were also sensitive to charybdotoxin (10 nM), as this reduced the outward current by 34 ± 4% at 350 ms when a high calcium-containing pipette was used during a depolarizing command to + 40 mV. L-Arginine still decreased the outward current in the presence of charybdotoxin, by 6 ± 1% at 4.8 ms and 12 ± 4% at 350 ms, showing that L-arginine could reduce a charybdotoxin-insensitive $I_{k(Ca)}$.

7 The present results indicate that NO-synthase in ciliary ganglia can modulate $I_{k(Ca)}$ by a method which is independent of the action of NO on the calcium channels. The $I_{k(Ca)}$ is decreased significantly at 4.8 ms into a depolarizing pulse, at a time that would decrease the rate of repolarization of the action potential. $I_{k(Ca)}$ is also reduced at longer times (350 ms), indicating an affect on the inactivating process.

Keywords: Nitric oxide; calcium-activated potassium channels; postganglionic neurones; avian cultured ciliary ganglia

Introduction

Nitric oxide (NO) acts as a transmitter in the nervous system as well as a possible retrograde messenger from the neurones to the synapses which impinge upon them (Bredt & Snyder, 1992). For example, the inhibitory junction potential in the gastrointestinal tract (Bennett *et al.*, 1966) is likely to be due to the release of NO from the enteric inhibitory neurones in the gut (for a review see Rand, 1992). In the hippocampus, long-term potentiation (LTP) of transmission at the synapses between Schaffer collaterals and CA1 pyramidal neurones may be due in part to the action of NO on nerve terminals after its synthesis in the pyramidal neurones consequent on the influx of calcium ions through NMDA receptors and the activation of NO-synthase (Böhme *et al.*, 1991; for a review see Bredt & Snyder, 1992). It has recently been discovered that NO-synthase is present in the avian ciliary ganglion (Scott *et al.*, 1992) and that the ganglion possesses a form of long-term potentiation (Scott & Bennett, 1992; 1993a; for a review see Kuba & Kumamoto, 1990). Because the ganglion has giant calyciform nerve terminals (de Lorenzo, 1960), it offers an ideal preparation for the analysis of both the mechanisms of LTP and also how this might be initiated, maintained or otherwise modulated by NO.

One way in which LTP might be initiated involves a decrease in current through potassium channels that participate in the repolarization of the action potential; such a decrease has the effect of increasing the duration of the action potential and so extends the duration of calcium influx in the nerve terminal, thus increasing transmitter release (Klein *et al.*, 1982). Over-expression of potassium channels has been shown to lead to an increase in the rate of repolarization of the action potential in nerve terminals, with a consequent decrease in the duration of the action potential and in calcium influx, so decreasing transmitter release (Kaang *et al.*, 1992). Repolarization of the action potential is in part due to the opening of fast calcium-activated potassium channels (I_c) in sympathetic neurones (Lancaster & Pennefather, 1987; Marsh & Brown, 1991) and in the hip-

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pocampus (Lancaster & Adams, 1986; Storm, 1987; Yoshida *et al.*, 1991). In chick ciliary ganglion neurones, Dryer *et al.* (1991) showed that $I_{k(Ca)}$ contributes to the late phase of spike repolarization and after-hyperpolarization and by blocking this channel activity they showed that the action potential duration was broadened. In the present work a study has been made of the effects of NO on calcium-activated potassium channels with the idea that if $I_{k(Ca)}$ is blocked by NO this may form the basis for the induction of enhanced transmitter release observed during LTP.

Methods

Tissue culture

Ciliary ganglia were dissected from 9–11 day old chick embryos in Hanks Balanced Salt Solution and then incubated for 15–20 min at 37°C in 1 ml of Ca^{2+}, Mg^{2+}-free Hanks Balanced Salt Solution containing 0.3% bovine serum albumen (BSA). Following the incubation period, the cells were dissociated in the incubation media by repeated (6–8) trituration through a Pasteur pipette, and the remaining clumps of ganglia tissue were allowed to settle to the bottom. The supernatant containing the cell suspension was then removed and stored aside. Then 1 ml of 'Ciliary ganglion medium (CGM)' containing Dulbecco's Modified Eagles Media (DMEM, ICN Biomedicals) supplemented with 2 mM glutamine, 1.00 u ml^{-1} penicillin, 50 mg ml^{-1} streptomycin, 10% heat-inactivated horse serum (CSL) and 2% chick embryo extract was added to the remaining ganglia tissue and triturated again (6–8 times) through the Pasteur pipette. This procedure of trituration was repeated 3 more times in CGM giving a single-cell suspension in 5 ml. If required, this suspension was further diluted with CGM to give a concentration of 1 ganglia per well when plated onto 13 mm glass coverslips previously coated with poly-L-lysine in 24-well plates (ICN Biochemicals) and the culture maintained at 37°C in a 5% CO_2 in air water-saturated atmosphere until required. The neurones were used for whole-cell recording within 6 to 24 h after plating, when they have few processes. These cells were chosen to minimize difficulties involving spatial non-uniformities of membrane potential arising from inadequate space clamp; if these occurred the results were excluded.

Solutions

Electrodes for forming gigaohm seals with the cell membranes of ciliary neurones were fabricated from boro-silicate

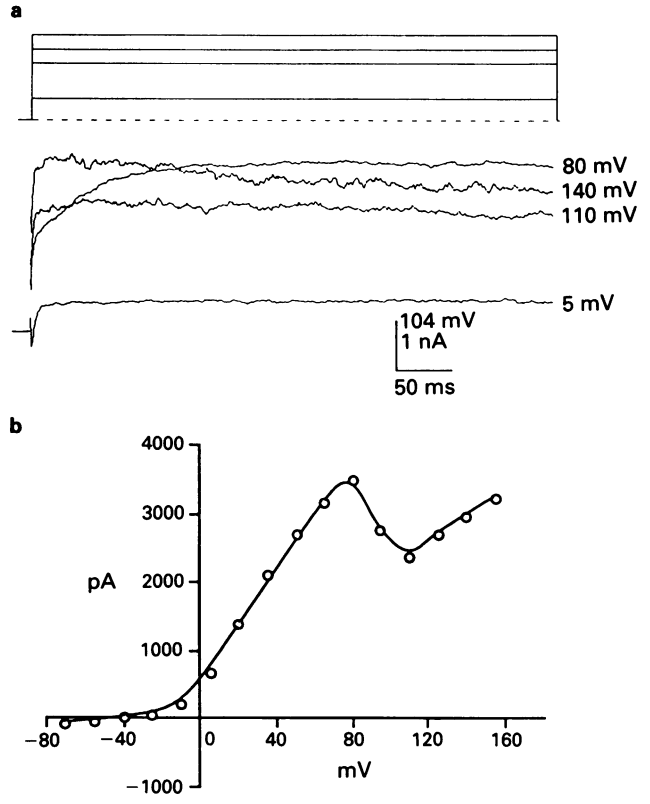

Figure 1 (a) Shows set of voltage-clamp currents for a ciliary neurone during command pulses of 470 ms duration to the different levels indicated by the rectangular traces above; the holding potential (Vh) was −40 mV. (b) Shows the current-voltage relation determined from the currents at the end of the traces for the neurone in (a).

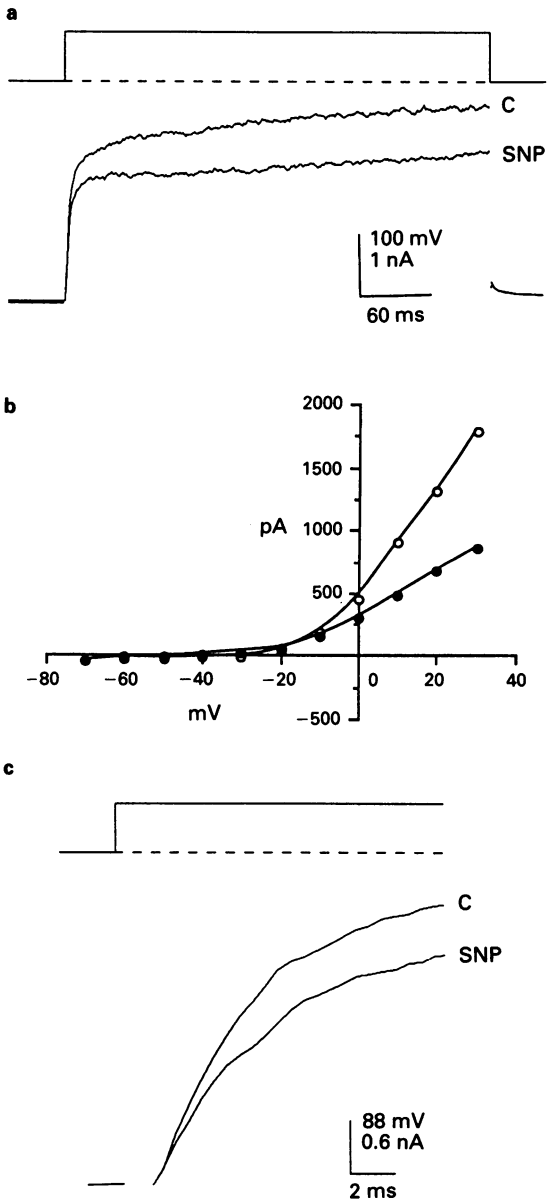

Figure 2 The effect of sodium nitroprusside (100 μM) on the current amplitude during voltage-clamp of the ciliary neurone soma. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration (indicated by the rectangular trace) to +40 mV from a Vh of −40 mV both before (C) and after sodium nitroprusside (SNP) application. (b) Shows the current-voltage relation before (○) and after sodium nitroprusside (●) application; the currents were measured at 350 ms into the command potential. (c) Shows the effect of SNP on the voltage-clamp current for the same condition as in (a), on an expanded time scale.

capillary tubing and had resistances of 3–4 MΩ when filled with internal solution (Hamill *et al.*, 1981). For the whole-cell recording described in this work, the pipette solution consisted of (mM): KCl 115, $MgCl_2$ 1, HEPES 40, GTP 2, ATP 1 with KOH added to give a pH of 7.2–7.3. The bathing solution consisted of (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 10 with NaOH added to give a pH of 7.2–7.3. Variations on these solutions involved addition of EGTA to the pipette or bath solution, addition of $CdCl_2$ to the bath solution or omission of $CaCl_2$ from the bath solution. Such changes are noted in the appropriate legends. All experiments were at room temperature (18–21°C).

Recording

Whole-cell patch-clamp techniques were used (Fenwick *et al.*, 1982) to voltage-clamp cells. Prior to forming seals the pipette current due to liquid junction potentials within the electrode and bath were nulled and the pipette capacitance compensated. After establishing a seal, the patch membrane was broken under slight negative pressure by a short (0.5 ms) 1.5 V pulse. This negative pressure, preventing the reforming of the membrane was maintained throughout the experiment. Ion currents were recorded via an Axopatch-IC amplifier and a labmaster interface on an IBM-AT using pCLAMP (Axon Instruments) software. Currents filtered at 1 kHz were sampled at 1.67 kHz.

If there were indications of incomplete voltage clamping, due to inadequate space clamp, then the results were rejected. Voltage-clamp control was determined according to several criteria: firstly, smooth voltage-dependent current activation; secondly, lack of excessive delay in onset of current; thirdly, onset and offset kinetics that were dependent on voltage but not on the amplitude of the current.

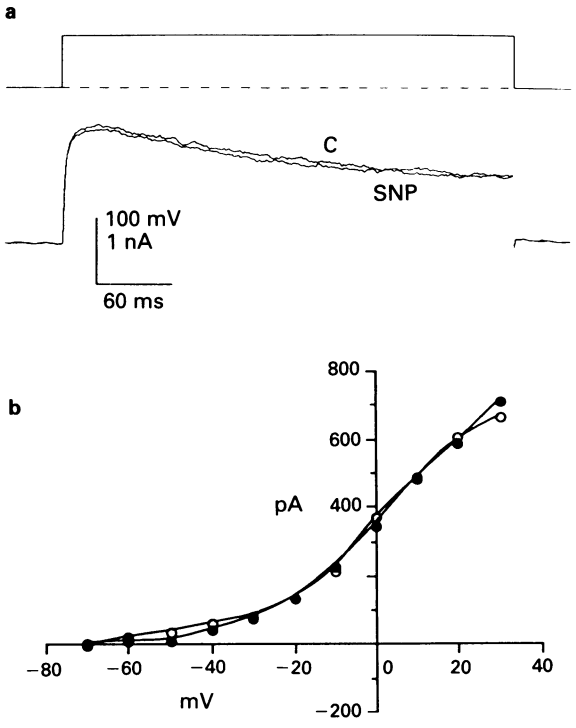

Figure 3 The effect of sodium nitroprusside (100 μM) on the delayed rectifier current (I_k). (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and after sodium nitroprusside (SNP) application. (b) Shows the current-voltage relation before (○) and after sodium nitroprusside (●) application; the currents were measured at 350 ms into the command potential. There was 500 μM Cd^{2+} in a calcium-free bath and EGTA (10 mM) substituted for KCl (10 mM) in the pipette solution.

Drug application

Drugs which were dissolved in recording media were applied by pressure ejection (2–7 kPa) from micropipettes with tip diameters of about 10 μm. These pipettes were positioned about 20 to 50 μm from the neurone cell body during a puff application and then removed. The neurones were required to show stable current magnitudes for at least 20 s before the application of drug, otherwise the results were rejected.

For recording drug effects two methods were used. The first method involved evoking currents by applying a command potential of + 40 mV from a holding potential of − 40 mV. During repetitive stimulation of this nature, drugs were applied as described above and their effect was determined by calculating the difference in current before and after the drug application. The second method involved recording from a different neurone to produce current versus voltage graphs by applying depolarizing command potentials over a range of voltages.

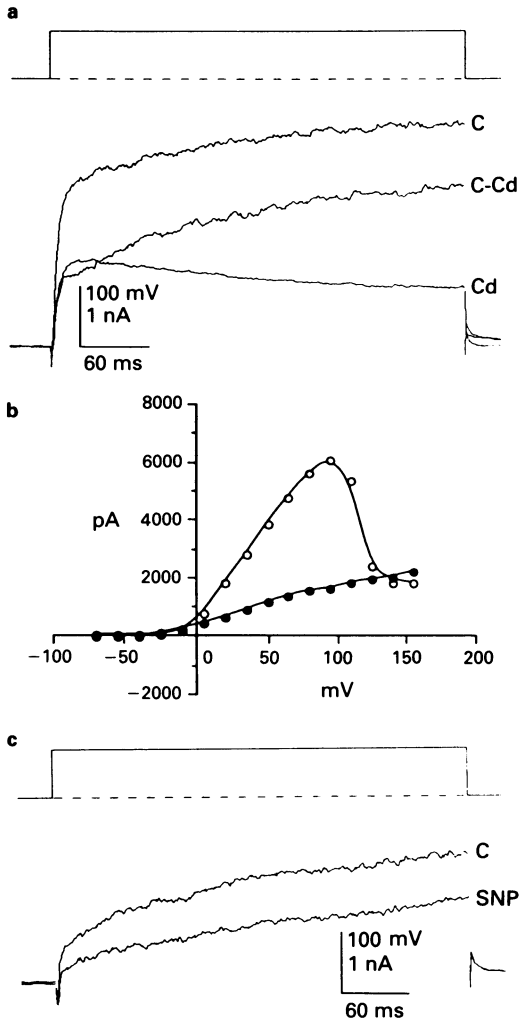

Figure 4 Separation of the total outward current in a ciliary neurone into delayed rectifier (I_k) and calcium-activated potassium ($I_{k(Ca)}$) components. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV before (C) and during the application of 500 μM Cd^{2+} (Cd); the middle trace representing $I_{k(Ca)}$ (C-Cd) is the difference between the currents before and after Cd^{2+}. (b) Shows the current-voltage relation for the current (I_k) remaining during the application of Cd^{2+} (●), as well as the current-voltage relation for the current ($I_{k(Ca)}$; ○) due to subtracting the current (I_k) remaining after Cd^{2+} from the control current (not shown). (c) Shows the effect of sodium nitroprusside (100 μM) on $I_{k(Ca)}$ after the I_k component has been subtracted from the total outward current from Figure 2a.

Drugs

Drugs obtained from Sigma were: L-arginine, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) and apamin. Sodium nitroprusside was obtained from Ajax Chemicals, potassium ferrocyanide from B.D.H. Chemicals and charybdotoxin from NPS Pharmaceuticals (formerly Natural Product Sciences, Inc.), Salt Lake City, UTAH 84108, U.S.A.

Statistics

Data are expressed as mean ± s.e.mean and n represents the number of experiments. The significance of the difference between the n pairs of observations made before and after application of a drug was calculated by Student's t test. P values of 0.05 or less were considered to represent significant differences.

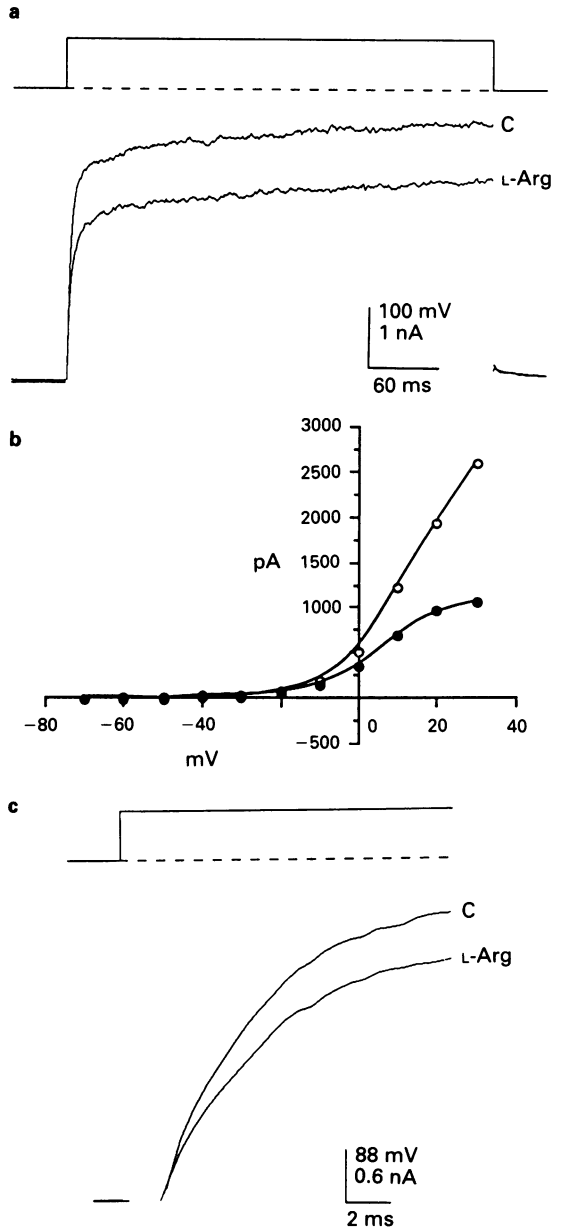

Figure 5 The effect of L-arginine (270 μM) on the current amplitude during voltage-clamp of the ciliary neurone soma. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and after L-arginine (L-Arg) application. (b) Shows the current-voltage relation before (○) and after L-arginine (●) application; the currents were measured at 350 ms into the command potential. (c) Shows the effect of L-arginine on the voltage-clamp current for the same condition as in (a), on an expanded time scale.

Results

Depolarizing voltage-command steps of 470 ms duration from holding potential (Vh) of − 40 mV gave outward currents of the kind shown in Figure 1a in all ciliary neurones studied (n = 15). The current increases in amplitude up to depolarizations of 80 mV; thereafter the current declines to reach a minimum at about 110 mV from which it increases again (Figure 1b). This N-shaped current-voltage curve is due to the decrease in the calcium-activated potassium outward current ($I_{k(Ca)}$) that occurs for depolarizing commands that approach the calcium equilibrium potential (see for example Bennett *et al.*, 1992). The further increase in current at higher depolarizations is primarily due to the delayed rectifier (I_k) (Bennett *et al.*, 1991).

Application of sodium nitroprusside (SNP; 100 μM) from a puffer pipette decreased the amplitude of the outward current during command steps over a wide range of voltages (Figure 2b); this reduction amounted to 22 ± 1% (n = 32) at 4.8 ms and 25 ± 1% at 350 ms for a command voltage of + 40 mV; both of these changes were very significant (Figure 2a,c). In order to check that SNP was not having an effect through the action of ferrocyanide, this was applied to the ganglion through a puffer pipette. Ferrocyanide (100 μM) decreased the amplitude of the current by only 6 ± 3% at 350 ms (n = 8; recording not shown), indicating that it was predominantly NO that modulated the channel activity.

From Figure 1 we know that the total current represented in Figure 2 is due primarily to two types of current, namely $I_{k(Ca)}$ and the I_k. The effect of SNP (100 μM) on I_k was investigated by blocking the influx of calcium ions with Cd^{2+} (500 μM) in the recording bath, so blocking $I_{k(Ca)}$ (Bennett *et al.*, 1991). Under these conditons, the application of SNP did not affect the size of the current at any command potential (Figure 3b); at a command potential of + 40 mV SNP decreased the I_k by 1 ± 3% at 350 ms (n = 7; Figure 3a),

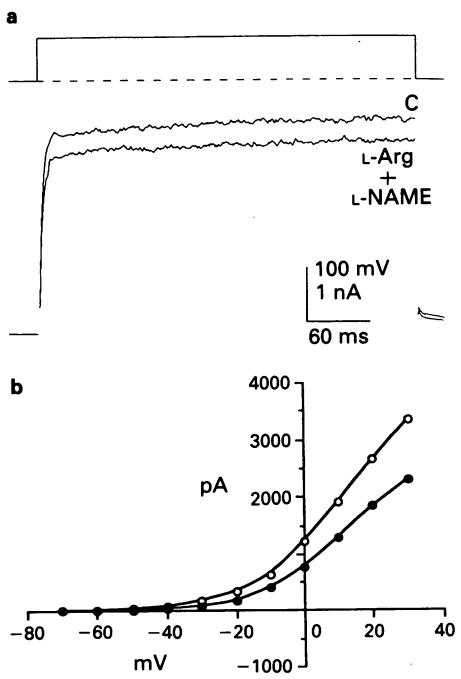

Figure 6 The effect of L-arginine (270 μM) in the presence of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 100 μM) on the current amplitude, during voltage-clamp of the ciliary neurone soma. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and after L-arginine (L-Arg + L-NAME) application. (b) Shows the current-voltage relation before (○) and after L-arginine (●) application; the currents were measured at 350 ms into the command potential. L-NAME (100 μM) was added to the bath.

which was not significant. Thus the effect of SNP on the total outward current is due to its effect on $I_{k(Ca)}$.

The proportion of the total outward current due to I_k can be determined by blocking $I_{k(Ca)}$ with 500 μM Cd^{2+}. Figure 4a shows that at a command potential of + 40 mV, the I_k amounted to about 30% of the outward current at 350 ms; subtraction of this gives a measure of the large $I_{k(Ca)}$ current. According to this analysis $I_{k(Ca)}$ provided most of the outward current in the voltage-range from 0 mV to + 120 mV (Figure 4b). In order to obtain a measure of the effect of SNP on $I_{k(Ca)}$ at a command potential of + 40 mV, the following procedure was adopted: the I_k measured in the experiment of Figure 4a was subtracted from both the control and SNP-treated currents given in the experiment of Figure 2a; the resulting currents are shown in Figure 4c. When the average I_k over 4 experiments was subtracted from the average control of SNP treated cells in 32 experiments, the decrease in $I_{k(Ca)}$ due to SNP was 45% at 350 ms.

L-Arginine (270 μM) was applied to voltage-clamped ganglion cells in order to test whether endogenous nitric oxide was being produced by the nitric oxide synthase system present in the ganglion (Figure 5). The current-voltage curve for different command voltages showed that L-arginine reduced the current over the entire range of depolarizing

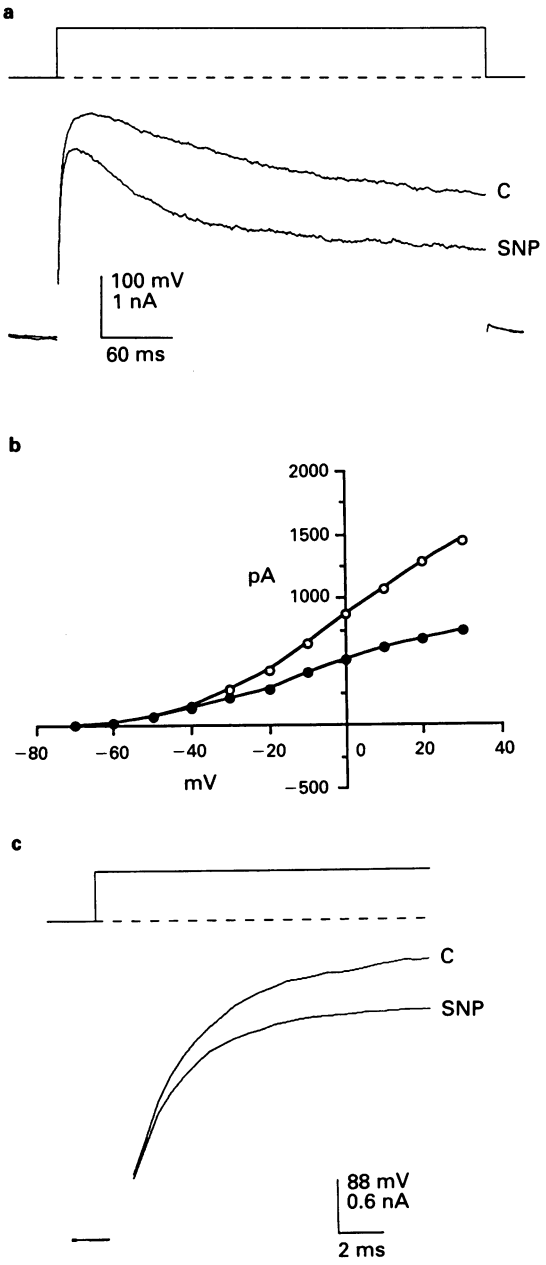

Figure 7 The effect of sodium nitroprusside (100 μM) on $I_{k(Ca)}$ during voltage-clamp of the ciliary neurone soma, under the conditions of no calcium currents. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during sodium nitroprusside (SNP) application. (b) Shows the current-voltage relation before (○) and after sodium nitroprusside (●) application; the currents were measured at 350 ms into the command potential. (c) Shows the effect of SNP on the voltage-clamp current for the same condition as in (a), on an expanded time scale. There was no Ca^{2+} in the bath solution, with 10 mM EGTA substituted for 10 mM NaCl in the bath; 100 μM $CaCl_2$ was added to the patch-pipette solution.

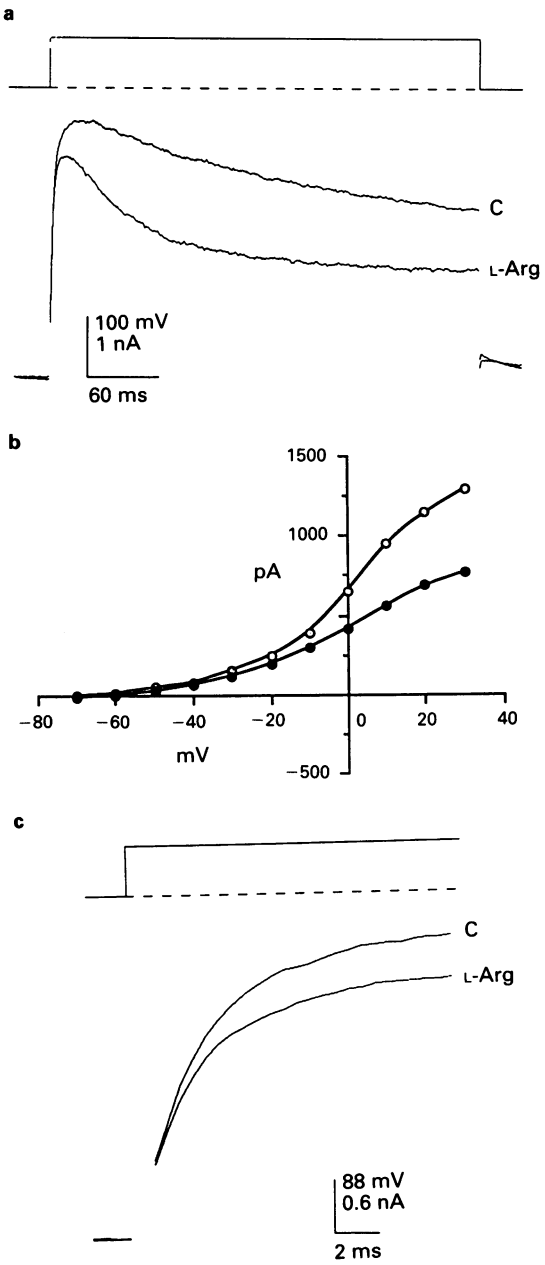

Figure 8 The effect of L-arginine (270 μM) on $I_{k(Ca)}$ during voltage-clamp of the ciliary neurone soma, under the conditions of no calcium currents. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during L-arginine (L-Arg) application. (b) Shows the current-voltage relation before (○) and after L-arginine (●) application; the currents were measured at 350 ms into the command potential. (c) Shows the effect of L-arginine on the voltage-clamp current for the same condition as in (a), on an expanded time scale. There was no Ca^{2+} in the bath solution with 10 mM EGTA substituted for 10 mM NaCl in the bath; 100 μM $CaCl_2$ was added to the patch-pipette solution.

command voltages (Figure 5b). At a command voltage of + 40 mV the current was significantly reduced by 19 ± 2% at 4.8 ms ($n = 15$; Figure 5c) and 22 ± 2% at 350 ms (Figure 5a). L-NAME (100 μM), a competitive inhibitor of NO-synthase was added to the bath in order to show that L-arginine was working via NO-synthase. Under these conditions L-arginine decreased the total outward current by a reduced amount, namely 11 ± 1% at 4.8 ms and 11 ± 2% at 350 ms ($n = 12$; Figure 6).

It has already been shown that NO can decrease the transient and sustained calcium currents in this ganglion (Khurana & Bennett, 1993), so the decrease in $I_{k(Ca)}$ in the presence of NO may be due to the decrease in calcium influx. In order to test whether the $I_{k(Ca)}$ could be directly modulated by NO, a calcium-free soution with 10 mM EGTA was added to the bath to prevent the influx of calcium. The patch-clamp pipette was then filled with a solution that contained 100 μM $CaCl_2$ to activate $I_{k(Ca)}$ directly. Under these conditions a large $I_{k(Ca)}$ could be generated, as much of the outward current could be blocked with both apamin and charybdotoxin (CTx) (see below). When either SNP (100 μM) or L-arginine (270 μM) were applied, the outward current was decreased over the entire range of positive command-potentials studied (Figures 7b and 8b). At a command potential of + 40 mV SNP significantly reduced the current by 9 ± 1% at 4.8 ms and by 36 ± 3% at 350 ms ($n = 6$; Figure 7a,c) and L-arginine reduced the current by 9 ± 1% at 4.8 ms and by 35 ± 2% at 470 ms ($n = 27$; Figure 8a,c).

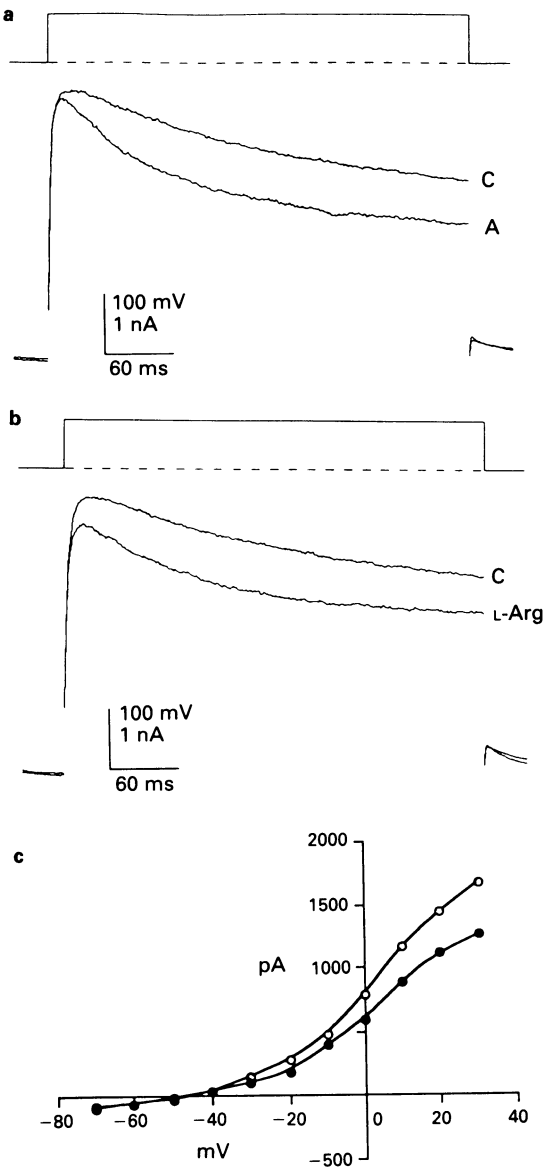

Figure 9 The effect of L-arginine (270 μM) on $I_{k(Ca)}$ in the presence of apamin (50 nM) during voltage-clamp of the ciliary neurone soma, under the conditions of no calcium currents. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during apamin (A) application. (b) Shows the current-voltage currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during L-arginine (L-Arg) application, in the presence of apamin (50 nM). (c) Shows the current-voltage relation before (○) and after L-arginine (●) application in the presence of apamin (50 nM); the currents were measured at 350 ms into the command potential. There was no Ca^{2+} in the bath solution with 10 mM EGTA substituted for 10 mM NaCl in the bath; 100 μM $CaCl_2$ was added to the patch-pipette solution.

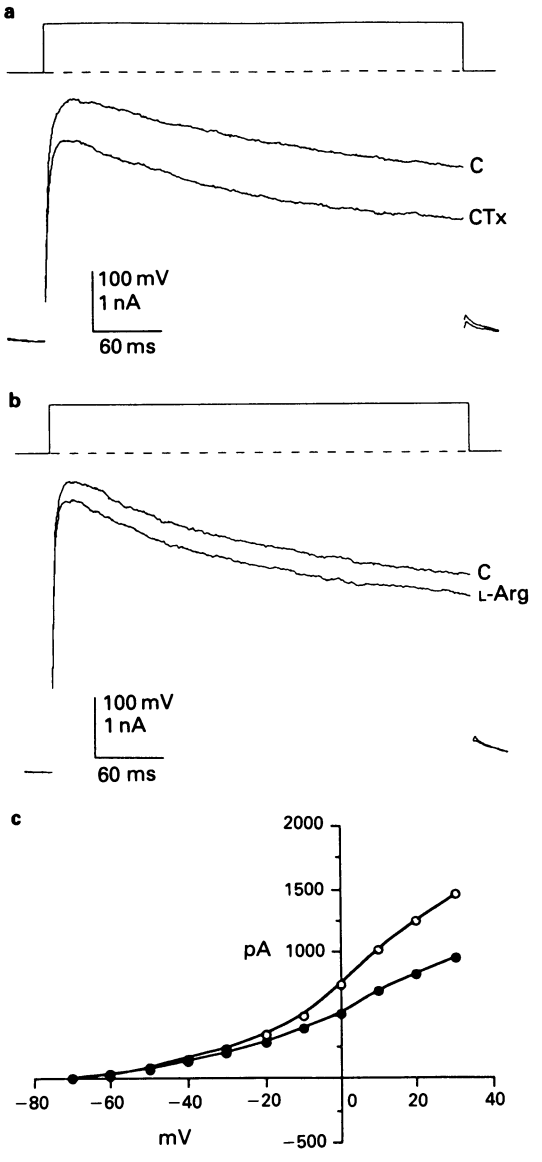

Figure 10 The effect of L-arginine (270 μM) on $I_{k(Ca)}$ in the presence of charybdotoxin (10 nM) during voltage-clamp of the ciliary neurone soma, under the conditions of no calcium currents. (a) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during charybdotoxin (CTx) application. (b) Shows the voltage-clamp currents evoked by command potentials of 360 ms duration to + 40 mV from a Vh of − 40 mV both before (C) and during L-arginine (L-Arg) application, in the presence of charybdotoxin (10 nM). (c) Shows the current-voltage relation before (○) and after L-arginine (●) application in the presence of charybdotoxin (10 nM); the currents were measured at 350 ms into the command potential. There was no Ca^{2+} in the bath solution with 10 mM EGTA substituted for 10 mM NaCl in the bath; 100 μM $CaCl_2$ was added to the patch-pipette solution.

In order to determine if the action of NO on $I_{k(Ca)}$ was specific to the apamin-sensitive (SK type) low conductance $I_{k(Ca)}$ (Latorre *et al.*, 1989; Dreyer, 1990) and/or to the charybdotoxin (CTx)-sensitive (BK or maxi-K type) high conductance $I_{k(Ca)}$ (Latorre *et al.*, 1989; Dreyer, 1990), these substances were used to block the channels and the effects of L-arginine then determined. At a command potential of + 40 mV, application of apamin (50 nM) significantly reduced the current by 23 ± 3% ($n = 7$) at 350 ms indicating the presence of SK type $I_{k(Ca)}$ (Figure 9a). Application of L-arginine (270 μM) under these conditions still significantly reduced the current by 5 ± 1% at 4.8 ms ($n = 11$) and by 19 ± 2% at 350 ms (Figure 9b). This effect of L-arginine occurred over the entire range of positive command potentials studied (Figure 9c). Application of CTx (10 nM) also significantly reduced the current in response to a command potential of + 40 mV by 34 ± 4% at 350 ms ($n = 7$; Figure 10a). In the presence of 10 nM CTx, application of L-arginine (270 μM) further significantly reduced the current by 6 ± 1% at 4.8 ms ($n = 8$) and by 12 ± 4% at 350 ms (Figure 10b). The reduction of current in the presence of CTx also occurred over the entire range of positive command potentials studied (Figure 10c). These results imply that NO acts on both the apamin-insensitive and the CTx-insensitive $I_{k(Ca)}$.

Discussion

The ciliary ganglion is known to possess a number of different potassium channel types, which are likely to participate in both the repolarization and after-hyperpolarization phases of the action potential. In the neurone soma there are delayed rectifier channels, calcium-activated potassium channels, inward rectifier potassium channels, cholinoceptor agonist activated channels, fast-transient potassium channels, and sodium-activated potassium channels (Bennett *et al.*, 1991). A number of different potassium channels have also been identified in the calyciform nerve terminal: these include delayed rectifier channels, inward rectifier channels and calcium-activated potassium channels (Bennett & Ho, 1991; Fletcher & Chiappinelli, 1992). The identity of the type of $I_{k(Ca)}$ is not clear, although an $I_{k(Ca)}$ is blocked by tetraethylammonium ions (TEA; Bennett & Ho, 1992), and it is known that the fast $I_{k(Ca)}$, I_C, which contributes to the repolarization of the action potential is very sensitive to TEA in autonomic ganglia (Lancaster & Pennefather, 1987; Marsh & Brown, 1991). The $I_{k(Ca)}$ studied in the present work consisted of a charybdotoxin-sensitive component and an apamin-sensitive component. The former is likely to be the I_C current (Dryer *et al.*, 1991). Apamin is known to suppress the $I_{k(Ca)}$ involved in the after-hyperpolarization of the action potential but not the $I_{k(Ca)}$ involved in the repolarization of the action potential in autonomic neurones (Tanaka *et al.*, 1986; Kawai & Watanabe, 1986; Dryer *et al.*, 1991). Taken together with the present observations that show NO can depress both apamin and charybdotoxin-sensitive $I_{k(Ca)}$ at times comparable to that of the repolarization phase of the action potential at about 5 ms (Dryer *et al.*, 1991), it seems likely that both the I_C current involved in the repolarization of the action potential as well as the apamin-sensitive current involved in the after-hyperpolarization are depressed.

There is considerable evidence to suggest that changes in I_C occur in nerve terminals under physiological conditions which lead to changes in transmitter release. For example, somatostatin inhibits transmitter release from pituitary cells by stimulating a maxi-K channel, probably responsible for the I_C current, through a protein dephosphorylation mechanism, leading to an increased rate of repolarization during the action potential and so a decrease in calcium entry (White *et al.*, 1991). In *Hermissenda*, behavioural changes elicited by associative conditioning have focussed on the reduction of I_C in the nerve terminal that are brought about by the phosphorylation of a G protein (Nelson *et al.*, 1990). At the amphibian neuromuscular junction charybdotoxin-sensitive channels normally decrease the duration of the terminal action potential and thereby determine the extent of calcium entry into the terminal, and therefore the amount of transmitter released (Robitaille & Charlton, 1992). This process is facilitated by the I_C channels being placed in the vicinity of the high density of calcium-channels that are strategically positioned at the release sites (Roberts *et al.*, 1990; Gola *et al.*, 1990; Robitaille & Charlton, 1992).

NO blocks high-voltage threshold calcium channels in the ciliary ganglion (Khurana & Bennett, 1993) as well as blocking calcium influx in smooth muscle (Magliola & Jones, 1990; Clapp & Gurney, 1991). Any action of NO in directly depressing I_C during the time of the repolarization of the action potential at about 5 ms (Dryer *et al.*, 1991), and therefore potentially increasing the duration of nerve terminal action potentials, calcium influx and transmitter release, may therefore be offset by NO directly depressing calcium influx. However, it is not clear at present whether the high-voltage calcium channels that are blocked by NO are the calcium channels that are strategically placed at the release sites of nerve terminals and are involved in transmitter release by the nerve impulse. These channels are different from the high-voltage threshold L or N-type calcium channels (Fox *et al.*, 1987) at least in the case of the calyciform nerve terminal of the avian ciliary ganglion (Stanley, 1991).

In the present work, the calcium-dependent potassium currents were depressed to a much greater extent by L-arginine at 4.8 ms in normal calcium, than by L-arginine at this time when a calcium-free solution was used with high-calcium containing pipettes. The larger depression in the former case is probably due to NO blocking both calcium entry as well as having a direct effect on the activation of the potassium channels. At 350 ms the calcium-dependent potassium currents are substantially reduced by L-arginine independent of whether calcium enters these cells through the calcium channels or not. Inactivation of the potassium channels therefore appears to be especially sensitive to NO.

The mechanism of action of NO in the ganglion, by which it decreases $I_{k(Ca)}$, may in part involve elevation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Addition of 8-Br-cyclic GMP to the ganglion enhances synaptic transmission (Scott & Bennett, 1993b) indicating a possible role for endogenous guanylate cyclase in the action of NO in increasing the efficacy of transmission in the ganglion (Scott & Bennett, 1993b). NO can elevate levels of cyclic GMP in smooth muscle cells to produce a hyperpolarization that closes voltage-sensitive calcium channels (Cornwell & Lincoln, 1989). It seems unlikely that this occurs in the ciliary ganglion, as NO only seems to have a depressing effect on potassium channels and this would not lead to hyperpolarization of the terminals with a consequent closing of voltage-sensitive calcium channels. It remains to be determined however whether the effects of NO on the potassium channels of the ciliary ganglion soma are the same as those on potassium channels of the calyciform nerve terminals.

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Selective inhibition of basal but not agonist-stimulated activity of nitric oxide in rat aorta by N^G-monomethyl-L-arginine

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1 Two inhibitors of nitric oxide synthase, N^G-monomethyl-L-arginine (L-NMMA, 1–100 μM) and N^G-nitro-L-arginine (L-NOARG, 3–300 μM), each produced a concentration-dependent augmentation of phenylephrine-induced tone in endothelium-containing but not endothelium-denuded rings of rat aorta. Pretreatment with L-arginine (10 mM) prevented the augmentation of tone induced by L-NOARG and L-NMMA.

2 Following induction of sub-maximal tone with phenylephrine in endothelium-containing rings, acetylcholine (1 nM–3 μM) induced relaxations which were inhibited in a concentration-dependent manner by L-NOARG (10–100 μM).

3 In contrast to the action of L-NOARG, L-NMMA (100–1000 μM) had no effect on acetylcholine-induced relaxations. L-NMMA (100–300 μM) also had no effect on the endothelium-dependent relaxant actions of ATP (0.1–100 μM), whereas L-NOARG (100 μM) produced powerful blockade.

4 Unexpectedly, pretreatment with L-NMMA (30–300 μM), as with the endogenous substrate L-arginine (10 μM–10 mM), inhibited in a concentration-dependent manner the ability of L-NOARG (30 μM) to block acetylcholine-induced relaxation.

5 The ability of L-NOARG to augment phenylephrine-induced tone and inhibit relaxation by acetylcholine and ATP in endothelium-containing rings is consistent with blockade of basal and agonist-stimulated production of nitric oxide, respectively.

6 The ability of L-NMMA to augment phenylephrine-induced tone without affecting relaxation to acetylcholine or ATP in endothelium-containing rings suggests a selective ability to block basal but not agonist-stimulated production of nitric oxide in rat aorta.

Keywords: EDRF; nitric oxide; nitric oxide synthase; L-arginine; N^G-monomethyl-L-arginine; N^G-nitro-L-arginine; acetylcholine; adenosine 5′-triphosphate; endothelium

Introduction

The vascular endothelium is known to produce a powerful substance, endothelium-derived relaxing factor (EDRF), which dilates blood vessels and inhibits platelet aggregation (Furchgott & Zawadzki, 1980; Azuma *et al.*, 1986). There is increasing evidence that EDRF is nitric oxide (Palmer *et al.*, 1987) or a closely related nitrosothiol (Myers *et al.*, 1990). It is now widely accepted that nitric oxide is derived from either of the guanidino nitrogens of the amino acid, L-arginine (Palmer *et al.*, 1988), following activation of the enzyme nitric oxide synthase. Structural modification at one of the guanidino nitrogens of L-arginine has led to the development of a number of compounds, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1989a, 1990; Moore *et al.*, 1990), that competitively inhibit nitric oxide synthase. These agents inhibit endothelium-dependent vasodilatation *in vitro* and have a powerful hypertensive effect *in vivo*, indicating an important role for nitric oxide in the regulation of systemic blood pressure (Rees *et al.*, 1989b).

Synthesis of nitric oxide is not, however, restricted to the vascular endothelium. For example, neurones in the peripheral (Gillespie *et al.*, 1989) and central nervous system (Garthwaite *et al.*, 1988) manufacture nitric oxide and use it as a neurotransmitter to relax smooth muscle and modulate the activity of other neurones, respectively. Furthermore, cells of the immune system, most notably macrophages, can produce massive quantitites of nitric oxide (Stuehr & Marletta, 1987; Iyengar *et al.*, 1987) following activation by bacterial endotoxin or cytokines such as interleukin-1, tumour necrosis factor and interferon -γ, and use this substance not as an intercellular messenger, but as a means of killing invading microorganisms. Smooth muscle cells in the vascular wall have a similar capacity to synthesize large quantities of nitric oxide following such immunological stimulation, and this is believed to account for the profound fall in vascular tone associated with endotoxin shock (Kilbourn *et al.*, 1990; Gray *et al.*, 1991).

It is now recognised that nitric oxide synthase constitutes a family of related isoenzymes: that in endothelium is mainly particulate in nature (Förstermann *et al.*, 1991) whereas the form in neurones is soluble (Knowles *et al.*, 1989; Bredt *et al.*, 1990), but both are constitutively present, are activated by calcium and calmodulin, and require NADPH, FAD, FMN and tetrahydrobiopterin as co-factors. In contrast, the nitric oxide synthase in macrophages (Marletta *et al.*, 1988; Kwon *et al.*, 1989; Stuehr *et al.*, 1990) and vascular smooth muscle (Busse & Mulsch, 1990) is not normally present, but is synthesized only after stimulation by the immunological mediators described above. This form requires the same co-factors as the constitutive forms of the enzyme, but does not require calcium and calmodulin for activity.

In the vascular endothelial cell, the production of nitric oxide is subject to complex control: it is stimulated by a large number of biological mediators such as acetylcholine, peptides and purine nucleotides (Furchgott & Zawadzki, 1980; Furchgott, 1984) and by the physical shearing force of flowing blood (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986). In addition, experiments demonstrating endothelium-dependent depression of vasoconstriction (Eglème *et al.*, 1984; Martin *et al.*, 1986), and of falls in arterial guanosine 3′:5′-cyclic monophosphate (cyclic GMP) content following endothelial denudation (Rapoport & Murad, 1983) provide evidence for basal release of nitric oxide from endothelial cells (Martin, 1988). Recent reports suggest differences in the mechanisms responsible for basal and agonist-stimulated production of

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nitric oxide. For example, in the perfused vascular bed of the rabbit ear, L-NAME inhibits both basal and acetylcholine-induced production of nitric oxide, but only in the former case is blockade reversed by L-arginine (Randall & Griffith, 1991). Furthermore, in the isolated coronary artery of the greyhound, thimerosal, an inhibitor of acetyl-coA lysolecithin acyltansferase, has a complex action, transiently producing endothelium-dependent relaxation and then blocking agonist-stimulated but not basal production of nitric oxide (Crack & Cocks, 1992).

In this study, we have examined separately the effects of L-NMMA and L-NOARG on basal and agonist-stimulated production of nitric oxide in rat isolated aorta. While L-NOARG inhibits both basal and agonist-stimulated production of nitric oxide in this tissue, we describe the unexpected finding that L-NMMA selectively blocks basal but not agonist-stimulated production. A preliminary account of these results has already been published (Frew *et al.*, 1993).

Methods

Preparation of aortic rings and tension recording

The preparation of aortic rings for tension recording was essentially similar to that described by Martin *et al.* (1986). Briefly, female Wistar rats weighing 200–250 g were killed by stunning and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings with a razor blade slicing device. Endothelial cells were removed from some rings by gently rubbing the intimal surface with a moist wooden stick for 30–60 s. Successful removal of the endothelium from aortic rings was confirmed later by the inability of acetylcholine (1 μM) to elicit relaxation, and in some experiments histological examination of endothelial integrity was performed using a silver staining technique (Poole *et al.*, 1958). The aortic rings were mounted under 1 g resting tension on stainless steel hooks in 12 ml organ baths, and bathed at 37°C in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FT03C transducers and displayed on a Grass polygraph, model 7. Tissues were allowed to equilibrate for 90 min before experiments were begun, during which time the resting tension was re-adjusted to 1 g if required.

Experimental protocols

Basal activity of nitric oxide was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine-induced vasoconstriction (Martin *et al.*, 1986). In these experiments contraction to phenylephrine was measured on endothelium-containing and endothelium-denuded rings of rat aorta cumulatively over the full concentration effect range (0.1 nM–0.1 mM). When the effects of L-NMMA or L-NOARG, two inhibitors of nitric oxide synthase, were to be examined on basal release of nitric oxide, they were added 10 min before the addition of phenylephrine. In some experiments, the ability of L-arginine to interfere with the actions of L-NMMA and L-NOARG was examined. Two protocols were adopted for these experiments: firstly, L-arginine was added 10 min before addition of L-NMMA or L-NOARG to determine if it could protect against blockade; and secondly, once blockade had been established to L-NMMA or L-NOARG, the tissues were washed and re-equilibrated with L-NMMA or L-NOARG and then treated with L-arginine for 10 min before addition of phenylephrine to determine if blockade could be reversed.

Agonist-stimulated activity of nitric oxide was assessed by measuring the relaxation to acetylcholine (10 nM–10 μM) or ATP (0.1–100 μM) on endothelium-containing rings of rat aorta following induction of sub-maximal phenylephrine-induced tone. When the effects of L-NMMA or L-NOARG were to be examined, they were added 10 min before induction of tone with phenylephrine. Since L-NMMA and L-NOARG both enhance the induction of tone in endothelium-containing rings it was necessary in these experiments to lower the concentration of phenylephrine used so that the level of tone achieved was similar to that obtained in the absence of either agent. In some experiments, the ability of L-arginine or L-NMMA to interfere with the blocking actions of L-NOARG on acetylcholine-induced relaxation was investigated. Two protocols were adopted for these experiments: firstly, L-arginine or L-NMMA was added 10 min before addition of L-NOARG to determine if blockade could be prevented; and secondly, once blockade by L-NOARG had been established, the tissues were washed and re-equilibrated with L-NOARG, and L-arginine or L-NMMA was then added for 10 min to determine if blockade could be reversed.

Drugs

Acetylcholine chloride, adenosine 5′-triphosphate sodium salt, L-arginine hydrochloride, N^G-nitro-L-arginine (L-NOARG), 5-hydroxytryptamine creatinine sulphate and phenylephrine hydrochloride were obtained from Sigma (Poole, Dorset) and N^G-monomethyl-L-arginine (L-NMMA) was a generous gift from Dr D.D. Rees, Wellcome Laboratories (Beckenham, Kent). All drugs were dissolved and dilutions made in saline (0.9%).

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Phenylephrine-induced contraction

As previously reported (Martin *et al.*, 1986), endothelium-containing rings of rat aorta were less sensitive than endothelium-denuded rings to the contractile actions of

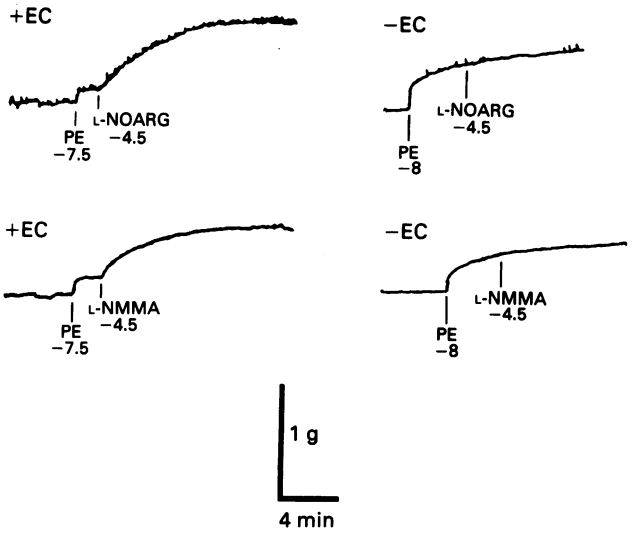

Figure 1 Individual experimental tracings showing the ability of N^G-nitro-L-arginine (L-NOARG, 30 μM) and N^G-monomethyl-L-arginine (L-NMMA, 30 μM) to augment phenylephrine (PE)-induced tone on endothelium-containing (+EC) but not endothelium-denuded (−EC) rings of rat aorta. Molar concentrations are given in log units.

phenylephrine (0.1 nM–30 μM). Following induction of submaximal tone to phenylephrine, addition of L-NOARG (1–100 μM) or L-NMMA (3–300 μM) resulted in an augmentation of tone on endothelium-containing but not endothelium-denuded rings (Figure 1). L-NOARG (1–100 μM) or L-NMMA (3–300 μM) had no effect by themselves in the absence of tone in endothelium-containing or endothelium-denuded rings, but pretreatment with either potentiated, in a concentration-dependent manner, phenylephrine (0.1 nM–0.1 mM)-induced tone in endothelium-containing (Figure 2) but not endothelium-denuded rings (data not shown). The ability of L-NOARG (100 μM) or L-NMMA (300 μM) to augment phenylephrine-induced tone in endothelium-containing rings was completely prevented by pretreatment with L-arginine (10 mM; Figure 2). In addition, L-arginine (10 mM) completely reversed the augmentation of phenylephrine-induced tone observed following treatment with L-NMMA (300 μM) but only partially reversed that with L-NOARG (100 μM)(data not shown).

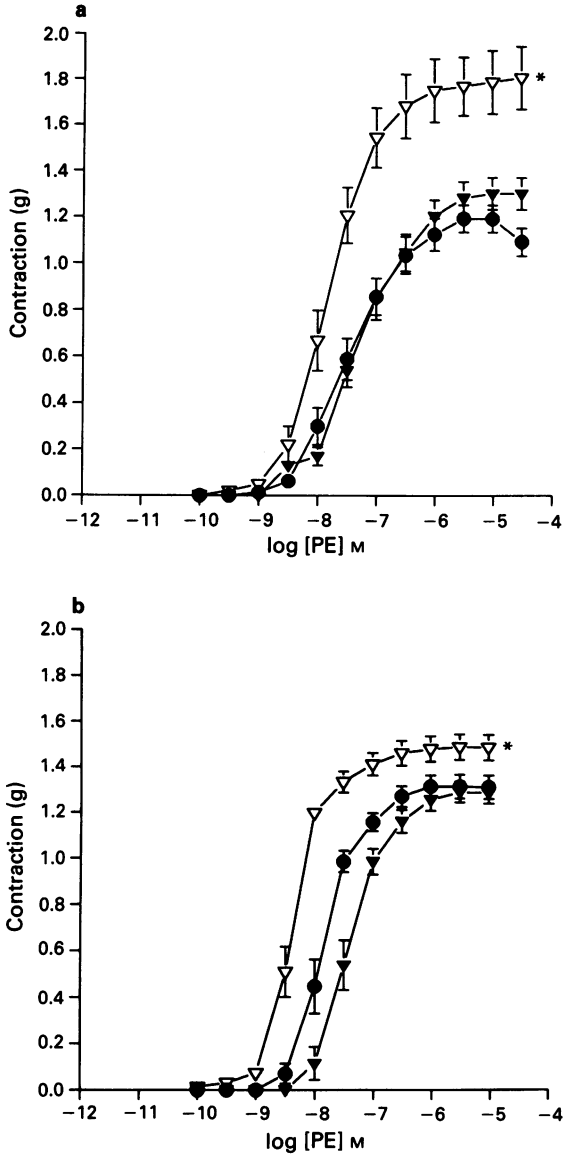

Figure 2 Concentration-response curves showing the contractile effects of phenylephrine (PE, ●) on endothelium-containing rings and the augmentation of contraction by (a) N^G-nitro-L-arginine (L-NOARG 100 μM, ▽) or (b) N^G-monomethyl-L-arginine (300 μM, ▽). The ability of L-arginine (10 mM) to protect against the actions of L-NOARG and L-NMMA is also shown (▼). Each point is the mean ± s.e.mean of 6–12 observations. *$P<0.05$, indicates a significant difference from untreated rings.

L-NOARG (100 μM) and L-NMMA (100 μM) also potentiated tone induced by 5-hydroxytryptamine (0.1–10 μM) on endothelium-containing, but not endothelium-denuded rings (data not shown).

Agonist-induced relaxation

Following induction of sub-maximal tone with phenylephrine, acetylcholine (1 nM–3 μM) induced a concentration-dependent relaxation in endothelium-containing but not endothelium-denuded rings (Figures 3, 4 and 6).

Treatment with L-NOARG (10–100 μM) resulted in a concentration-dependent inhibition of acetylcholine-induced relaxation (Figures 3 and 6). L-Arginine (10 μM–10 mM) had no effect by itself on acetylcholine-induced relaxation, but it concentration-dependently prevented (Figure 3) and also reversed the ability of L-NOARG (30 μM) to block relaxation.

In contrast to the effects of L-NOARG, treatment with L-NMMA (100–1000 μM) had no effect on acetylcholine-induced relaxation (Figure 4). It also failed to block acetylcholine-induced relaxation when 5-hydroxytryptamine (2 μM) was used as the contractile agent. L-NMMA (100–300 μM) was also without effect on the endothelium-dependent relaxation induced by ATP (0.1–100 μM), whereas L-NOARG (100 μM) produced substantial blockade (Figure 5).

Surprisingly, pretreatment with L-NMMA (30–300 μM) blocked, in a concentration-dependent manner, the ability of L-NOARG (30 μM) to inhibit acetylcholine-induced relaxation (Figure 6). L-NMMA (300 μM) also produced a partial

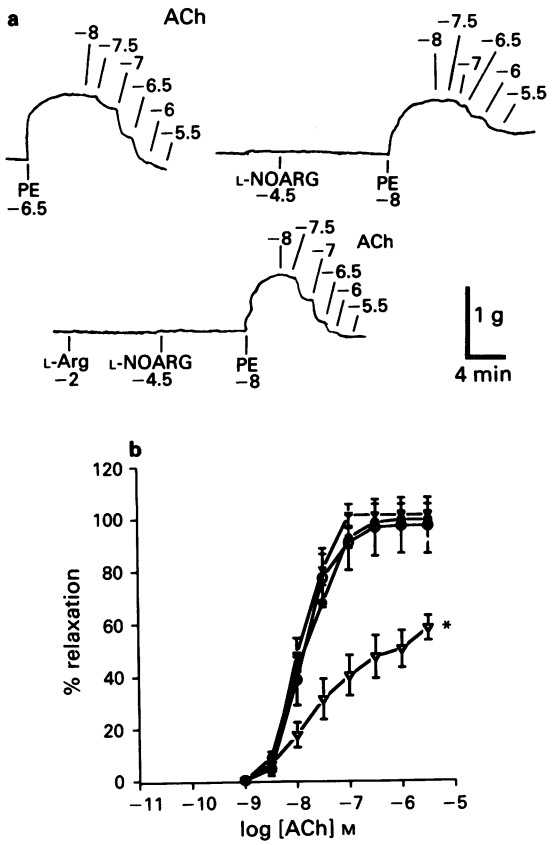

Figure 3 Individual experimental traces (a) and concentration-response curves (b) showing relaxation to acetylcholine (ACh, ●) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta, blockade of relaxation by N^G-nitro-L-arginine (L-NOARG, 30 μM, ▽) and protection against this blockade by L-arginine (L-Arg 10 mM, ▼). L-Arginine (10 mM, ○) itself had no effect on acetylcholine-induced relaxation. Each point is the mean ± s.e.mean of 6–8 observations. *$P<0.05$, indicates a significant difference from untreated rings.

reversal of the block of acetylcholine-induced relaxation obtained following treatment with L-NOARG (30 μM) (data not shown).

Discussion

Endothelium-containing rings of rat aorta are less sensitive to a wide range of vasoconstrictor stimuli than endothelium-denuded rings (Allan *et al.*, 1983; Eglème *et al.*, 1984) and this is satisfactorily explained by high basal production of nitric oxide by the endothelium in this tissue (Martin *et al.*, 1986; Martin, 1988). We found that two inhibitors of nitric oxide synthase, L-NOARG and L-NMMA (Rees *et al.*, 1989a; Moore *et al.*, 1990), augmented in an L-arginine-reversible manner phenylephrine-induced tone in endothelium-containing but not endothelium-denuded rings of rat aorta. This augmentation is likely to have occured as a consequence of inhibition of basal production of nitric oxide.

In keeping with the expected actions of an inhibitor of nitric oxide synthase (Rees *et al.*, 1989a; Moore *et al.*, 1990), we found that L-NOARG powerfully blocked, in an L-arginine-reversible manner, the endothelium-dependent relaxant actions of acetylcholine and ATP. Surprisingly, however, we found that despite blocking basal production of nitric oxide, L-NMMA in concentrations up to 1 mM, failed to block the endothelium-dependent relaxation induced by acetylcholine or ATP. A previous paper has described differential blockade of endothelium-dependent relaxation by an inhibitor of nitric oxide synthase (Martin *et al.*, 1992), and attributes this to differences in the efficacy of the relaxant. Our finding may represent an extreme example of the high efficacy of acetylcholine and ATP preventing inhibition of relaxation by L-NMMA, but other explanations are possible. Our data suggest that L-NMMA produces a selective inhibition of basal, but not agonist-stimulated production of nitric oxide in rat aorta. Since its development, L-NMMA has been shown to block agonist-stimulated endothelium-dependent relaxation in a wide range of tissues including rabbit aorta (Rees *et al.*, 1989a; Moore *et al.*, 1990) and coronary vascular bed (Amezcua *et al.*, 1989), dog coronary artery (Crack & Cocks, 1992) and bovine penile artery (Liu *et al.*, 1991). Why L-NMMA

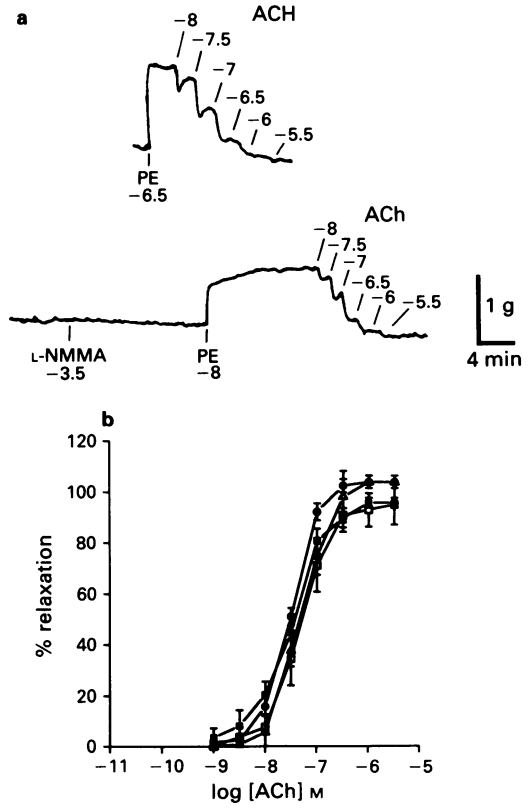

Figure 4 Individual experimental traces (a) and concentration-response curves (b) showing relaxation to acetylcholine (ACh, ●) in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta and the inability of N^G-monomethyl-L-arginine (L-NMMA) at 100 μM (■), 300 μM (□) and 1000 μM (△) to block this relaxation. Each point is the mean ± s.e.mean of 6 observations.

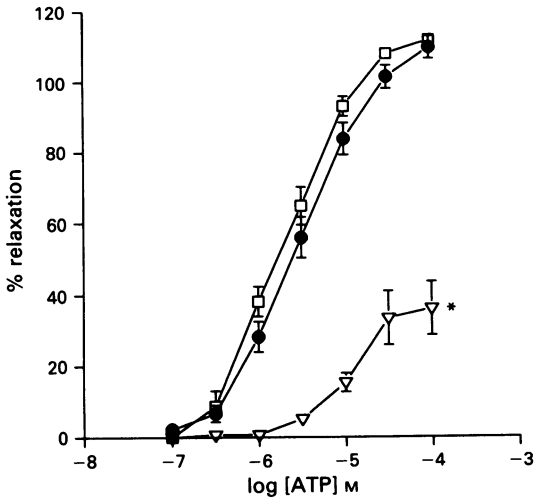

Figure 5 Concentration-response curves showing the relaxant actions of adenosine 5'-triphosphate (ATP, ●) in phenylephrine-contracted, endothelium-containing rings of rat aorta and blockade of this relaxation by N^G-nitro-L-arginine (100 μM, ▽) but not N^G-monomethyl-L-arginine (100 μM, □). Each point is the mean ± s.e.mean of 6 observations. *$P<0.05$, indicates a significant difference from untreated rings.

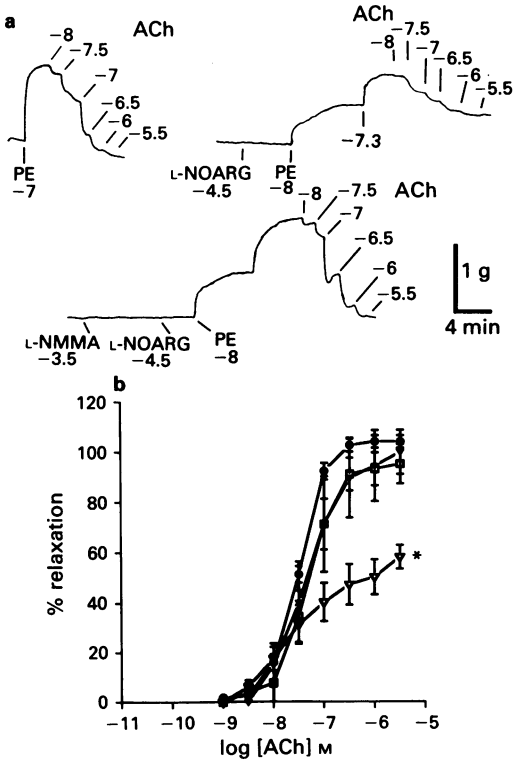

Figure 6 Individual experimental traces (a) and concentration-response curves showing relaxation to acetylcholine (ACh, ●) in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta, blockade of relaxation by N^G-nitro-L-arginine (L-NOARG, 30 μM, ▽) and protection against this blockade by N^G-monomethyl-L-arginine (L-NMMA, 300 μM, ▼). N^G-monomethyl-L-arginine (L-NMMA, 300 μM, □) itself had no effect on acetylcholine-induced relaxation. Each point is the mean ± s.e.mean of 6 observations. *$P<0.05$, indicates a significant difference from untreated rings.

does not block agonist-stimulated production of nitric oxide in rat aorta in unclear. It is unlikely to be due to a species differences, since L-NMMA inhibits acetylcholine-induced vasodilatation in the rat mesenteric bed (Moore *et al.*, 1990). It is equally unlikely that L-NMMA failed to gain access to, or was rapidly inactivated in, the endothelium of rat aorta, since basal production of nitric oxide was blocked effectively. Some workers have proposed that basal EDRF is free nitric oxide, while that released in response to agonists comes from a pre-formed store (Ignarro, 1991; Cocks & Angus, 1991) of a stable nitric oxide releasing molecule, such as an S-nitrosothiol (Myers *et al.*, 1990). This explanation too seems unlikely, since L-NOARG produced a rapid, powerful block of acetylcholine- and ATP-induced relaxation in our study. A more plausible explanation for the actions of L-NMMA is that it inhibits selectively the nitric oxide synthase isoenzyme responsible for basal but not agonist-stimulated production of nitric oxide. Previous reports have indicated that basal and agonist-stimulated production of EDRF require the presence of extracellular calcium (Long & Stone, 1985; Griffith *et al.*, 1986), suggesting that both occur through activation of the same calcium-dependent (Mülsch & Busse, 1991; Knowles *et al.*, 1989) constitutive form of nitric oxide synthase. However, more recent evidence indicates the presence in porcine freshly-harvested endothelial cells, of a calcium-insensitive form of nitric oxide synthase together with the expected calcium-sensitive form (Mülsch *et al.*, 1989). These could potentially account for basal and agonist-stimulated production of nitric oxide, respectively. Functional studies on isolated blood vessels also suggest differences between basal and stimulated production of nitric oxide. For example, while L-NAME inhibits basal as well as acetylcholine-stimulated production in the vascular bed of the rabbit ear, only in the former case is inhibition reversed by L-arginine (Randall & Griffith, 1991). Furthermore, thimerosal has no effect on basal production of nitric oxide yet powerfully blocks that stimulated by a wide range of agonists in dog coronary artery (Crack & Cocks, 1992).

The present demonstration that L-NMMA does not inhibit agonist-stimulated production of nitric oxide in rat aorta parallels our recent reports on non-adrenergic, non-cholinergic (NANC) nerves (Liu *et al.*, 1991; Martin *et al.*, 1993). NANC relaxation of the rat anococcygeus and bovine retractor penis (BRP) muscles clearly involves the L-arginine-nitric oxide pathway since it is rapidly abolished by L-NOARG (Hobbs & Gibson, 1990; Liu *et al.*, 1991). L-NMMA, however, blocks NANC relaxation in the rat anococcygeus by a maximum of about 50% (Gillespie *et al.*, 1989; Li & Rand, 1989) and, surprisingly, has no effect on that in the BRP (Liu *et al.*, 1991; Martin *et al.*, 1993). Another common finding between agonist-stimulated endothelium-dependent relaxation in rat aorta and NANC relaxation of the BRP (Martin *et al.*, 1993) is that pretreatment with L-NMMA protects against the blocking actions of L-NOARG. Thus L-NMMA behaves in a similar manner to the endogenous substrate, L-arginine, in competitively inhibiting the blockade of nitric oxide synthase. These findings suggest a complex interaction between L-arginine, L-NMMA and L-NOARG at the level of the nitric oxide synthase enzyme. This may be possible through the ability of L-NMMA to act as an alternative substrate (Olken & Marletta, 1992; Olken *et al.*, 1991) for, as well as an inhibitor of, nitric oxide synthase. Recent experiments using chemiluminescence detection have revealed that L-NMMA, but not L-NOARG, enhances nitric oxide production by rat aorta and pulmonary artery (Archer & Hampl, 1992), thus strengthening this view. Two possible explanations therefore emerge to explain the ability of L-NMMA to inhibit basal but not agonist-stimulated production of nitric oxide in rat aortic endothelium; either two separate forms of nitric oxide synthase are present, only one of which is inhibited by L-NMMA, or alternatively, only one form is present and activation of the enzyme so modifies the active site that L-NMMA behaves more like an alternative substrate than an inhibitor. A detailed study of the interactions of these agents with purified nitric oxide synthase from rat aortic endothelium will be required to establish which of these suggestions is correct.

In conclusion, we have shown that two inhibitors of nitric oxide synthase, L-NOARG and L-NMMA, each potentiate phenylephrine-induced tone in endothelium-containing rings of rat aorta by inhibiting basal production of nitric oxide. L-NOARG also blocked the production of nitric oxide stimulated by acetylcholine and ATP, but L-NMMA did not. In fact, L-NMMA, like the endogenous substrate L-arginine, inhibited competitively the ability of L-NOARG to block agonist-stimulated production of nitric oxide. Thus L-NMMA can discriminate between basal and agonist-stimulated production of nitric oxide by rat aortic endothelium.

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Transport of celiprolol across human intestinal epithelial (Caco-2) cells: mediation of secretion by multiple transporters including P-glycoprotein

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1 The transepithelial transport of the β-adrenoceptor blocking drug, celiprolol, was investigated in monolayers of the well differentiated human intestinal epithelial cell line, Caco-2.

2 The basal-to-apical transport (secretion) of [^{14}C]-celiprolol (50 μM) was 5 times higher than apical-to-basal transport (absorption). In the presence of an excess (5 mM) of unlabelled celiprolol the basal-to-apical transport was reduced by more than 80%, whereas the apical-to-basal transport remained unchanged.

3 Net celiprolol secretion obtained in the concentration range 0.01 to 5 mM displayed saturable kinetics with an apparent K_m of 1.00 ± 0.23 mM and V_{max} of 113 ± 11 pmol/10^6 cells min^{-1}. These results are consistent with saturable active secretion and provide an explanation for the dose-dependent bioavailability of celiprolol.

4 The secretion of celiprolol was sensitive to pH, and decreased in the absence of sodium and in the presence of ouabain, suggesting that transport was coupled to proton and sodium gradients.

5 The secretion of celiprolol was inhibited by substrates for P-glycoprotein (vinblastine, verapamil and nifedipine) and either inhibited or stimulated by typical substrates for the renal organic cation-H$^+$ exchanger (cimetidine, N^1-methylnicotinamide, tetraethylammonium and choline), suggesting that there are at least two distinct transport systems.

6 The secretion of celiprolol was also inhibited by other β-adrenoceptor blocking drugs (acebutolol, atenolol, metoprolol, pafenolol and propranolol) and by the diuretics, acetazolamide, chlorthalidone and hydrochlorothiazide, suggesting that the clinically observed effect of chlorthalidone on the bioavailability of celiprolol occurs at the level of the intestinal epithelium.

Keywords: Celiprolol; drug absorption; intestinal transport; epithelial transport; Caco-2; epithelial cell culture; intestinal epithelium; intestinal secretion; organic cation-proton exchanger; P-glycoprotein

Introduction

Celiprolol is a 'cardioselective' β-adrenoceptor blocking drug with intrinsic sympathomimetic activity and a weak vasodilator effect (reviewed by Riddel *et al.*, 1987). The drug exhibits dose-dependent bioavailability in rats and man after oral administration (Riddel *et al.*, 1987; Kuo *et al.*, 1993). In man, the bioavailability was 30% after an oral dose of 100 mg and 74% after a 400 mg dose (Gluth *et al.*, 1983; Hitzenberger *et al.*, 1983; Caruso *et al.*, 1985). The differences in bioavailability could not be explained by altered dissolution, first-pass metabolism or changes in excretion. We therefore investigated the possibility that celiprolol was actively transported across the intestinal epithelium (Kuo *et al.*, 1993). Microhistoautoradiography of frozen intestinal sections showed a time-dependent secretion of celiprolol from blood into the lumen of the rat intestine. Studies in rat isolated small intestinal cells showed that celiprolol was taken up by a time- and temperature-dependent mechanism, suggesting the presence of a carrier-mediated system. In addition, preliminary results using monolayers of the human intestinal epithelial cell line Caco-2 also suggested that celiprolol was actively secreted across the intestinal epithelium.

Celiprolol has intermediate lipophilic properties in comparison with other β-adrenoceptor blocking drugs, with an apparent partition coefficient in octanol-phosphate buffer of 0.2 at pH 7.4 and 37°C. It is a weak base with a pK_a-value of 9.7 and consequently it is present mainly as an organic cation at physiological pH. Intestinal secretion of organic cations including other β-blockers have been observed *in vivo* but so far the mechanisms behind the secretion have not been elucidated (e.g. George & Gruchy, 1979; Turnheim & Lauterbach, 1980; Lennernäs & Regårdh, 1993a, b). In contrast to the current understanding of organic cation transport in proximal tubular cells of the kidney (Weiner, 1985; McKinney, 1988), there is very little information on the handling of organic cations by the intestinal epithelium. Recently, an organic cation-H$^+$ exchanger for guanidine was characterized in rabbit intestinal brush border membrane vesicles (Miyamoto *et al.*, 1988). However, its substrate specificity was different from the extensively characterized organic cation-H$^+$ exchanger in the proximal tubular cells, since the typical substrates of the renal system such as tetraethylammonium, N^1-methylnicotinamide, cimetidine and choline failed to inhibit guanidine uptake in the intestine. To our knowledge, there is no information on the transport of organic cations in the human intestinal epithelium.

The brush border membrane of the intestinal epithelium contains an alternative secretory pathway for organic cations: the P-glycoprotein pathway. P-glycoprotein is overexpressed in multidrug-resistant tumour cells and is present in the apical plasma membrane of intestinal epithelial cells (Thiebaut *et al.*, 1987; Croop *et al.*, 1989). It has been demonstrated that it functions as an energy-dependent efflux pump for a variety of cytotoxic drugs and other hydrophobic compounds (reviewed by Gottesman & Pastan, 1988; Endicott & Ling, 1989; Ford & Hait, 1990). Thus, a major distinction

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between the function of the organic cation transport and P-glycoprotein is that the former mediates transcellular transport across the epithelium while the latter mediates active transport across the apical cell membrane.

In this paper, the transport of celiprolol was characterized in monolayers of the highly differentiated human intestinal epithelial cell line Caco-2 grown on permeable supports. This cell line forms confluent monolayers of well-differentiated enterocyte-like cells with the functional properties of transporting epithelia (reviewed by Neutra & Louvard, 1989; Artursson, 1991). Caco-2 cells have been used to study the transport of nutrients such as glucose (Blais *et al.*, 1987; Riley *et al.*, 1991), amino acids (e.g. Hidalgo & Borchardt, 1990; Hu & Borchardt, 1992; Nicklin *et al.*, 1992) and vitamin B-12 (Dix *et al.*, 1990), as well as a variety of drugs (e.g. Artursson, 1990; Hu & Borchardt, 1990; Wilson *et al.*, 1990; Conradi *et al.*, 1991; Inui *et al.*, 1992; Ranaldi *et al.*, 1992). A good correlation between drug absorption in Caco-2 monolayers in man has been established (Artursson & Karlsson, 1991).

The results of this study indicate active transport of celiprolol in the basal-to-apical direction (secretion) across the human intestinal epithelium that exceeds the level of transport in the apical-to-basal direction (absorption). These findings offer an explanation for the dose-dependent bioavailability of celiprolol. The basal-to-apical transport is inhibited by substrates for P-glycoprotein and either inhibited or stimulated by typical substrates for the renal organic cation-H^+ exchanger, suggesting that at least two distinct transport systems participate in the secretion of celiprolol.

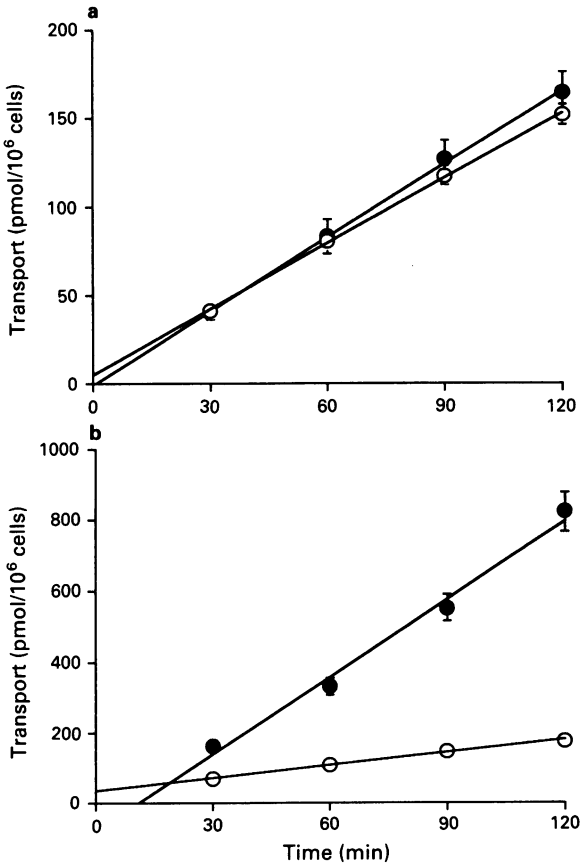

Figure 1 Apical-to-basal (a) and basal-to-apical transport (b) of 0.05 mM [^{14}C]-celiprolol across Caco-2 cell monolayers in the absence (●) and presence (○) of 5 mM unlabelled celiprolol as an inhibitor. Each point represents mean ± s.d.; $n = 4$.

Methods

Cell culture

The Caco-2 cell line was obtained from American Tissue Culture Collection (ATCC, Rockville, MD, U.S.A.) and was used between passage 90 and 105. The cells were cultivated according to previously published procedures (Artursson, 1990). For the transport studies, Caco-2 cells were seeded at a density of 4.2×10^5 cells cm^{-2} in polycarbonate filter cell culture inserts with a filter diameter of 12 mm or 24.5 mm and a mean pore diameter of 0.4 μm (Transwell; Costar, Badhoevedorp, The Netherlands). The cells were grown in Dulbecco's modified Eagle's medium (4500 mg l^{-1} glucose), supplemented with 1% non-essential amino acids, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% foetal calf serum in a humidified atmosphere of 10% CO_2:90% air at 37°C. The medium was changed every second day. All cell culture media and reagents were obtained from Gibco through Laboratory Design, Lidingö, Sweden. Transport studies were carried out with cell monolayers that were 20–35 days old.

Transport studies

The transport experiments were performed in Hanks' Balanced Salt Solution containing 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 25 mM HEPES (HBSS, pH 7.4). Initially, the monolayers were incubated for 15–30 min at 37°C in HBSS. Then, new prewarmed HBSS containing the radioactive compound, [^{14}C]-celiprolol, [^{3}H]-vinblastine or [^{14}C]-mannitol, was added to either the apical or the basal side of cell monolayer and drug-free HBSS was added to the opposite side. In studies on apical-to-basal transport, the cell culture insert was transferred to new basal chambers at regular intervals. The amount of radionuclide appearing after each sampling interval was determined and the cumulative transport was calculated. In studies on basal-to-apical transport, samples were taken from the apical side at regular intervals and were replaced with equal volumes of fresh HBSS. The cumulative transport was calculated after correction for dilution. Samples of the donor solutions were always taken prior to the experiment. The radioactivity of the samples was determined by liquid scintillation counting with a Tricarb 1900 CA scintillation counter (Packard Instrument, Downers Growe, IL, U.S.A.). Transport across the Caco-2 monolayers (apical-to-basal, J_{a-b}; basal-to-apical, J_{b-a}) are expressed as mol/10^6 cells min^{-1} or mol/10^6 cells 30 min^{-1}. The cell density of the Caco-2 monolayers was 0.9×10^6 cells cm^{-2} (Artursson, 1990). Net celiprolol transport (J_{net}) was defined as $J_{b-a} - J_{a-b}$.

Concentration-dependence of celiprolol transport

The transport of celiprolol (0.010 to 5.0 mM) from the apical-to-basal and basal-to-apical sides of the Caco-2 monolayers was followed for 120 min, with sampling every 30 min. To assess the effect of celiprolol concentration on the monolayer integrity, the transepithelial electrical resistance (TEER) and the permeability of the hydrophilic paracellular marker molecule [^{14}C]-mannitol were measured. TEER, expressed as Ω cm^2, was measured at the end of the transport experiments.

Sodium-dependence

The transport of 10 μM celiprolol in the apical-to-basal and basal-to-apical directions was determined after 30 min in the absence (control) or presence of the Na^+/K^+-ATPase inhibitor, ouabain (0.1 mM). Ouabain concentrations of 10 μM to 5 mM have earlier been used to determine the sodium-dependence of amino acid and dipeptide transport in Caco-2 cells (Dantzig & Bergin, 1990; Hidalgo & Borchardt, 1990; Hu & Borchardt, 1992; Nicklin *et al.*, 1992). The sodium-

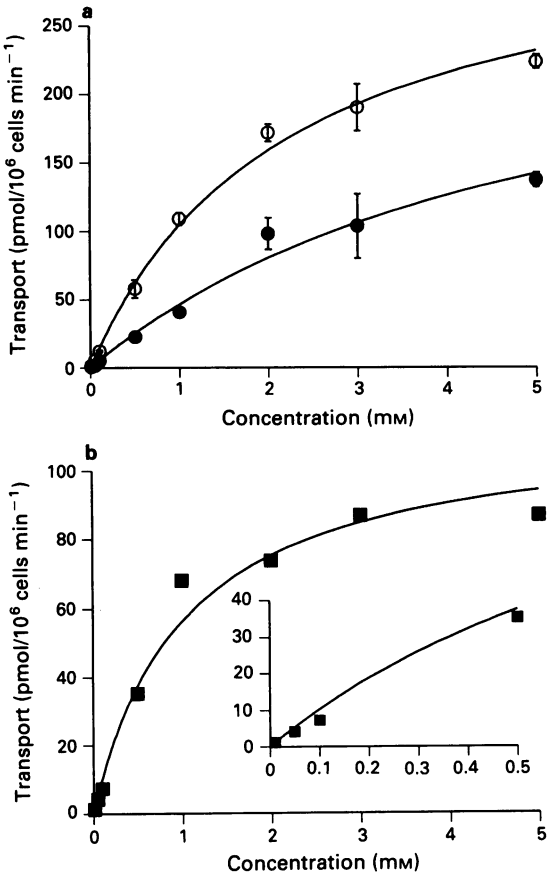

Figure 2 Concentration-dependence of celiprolol transport across Caco-2 cell monolayers. (a) Apical-to-basal (●) and basal-to-apical transport (○). Each point represents mean ± s.d.; $n = 4$. (b) Non-linear kinetics of celiprolol net secretion (■). The transport rates were calculated from the initial 30 min.

dependence of celiprolol transport was also investigated by measuring the transport in Na^+-free medium (HBSS that contained choline instead of Na^+). The monolayers were pre-incubated with ouabain and the Na^+-free medium for 30 min before the start of the transport experiments.

Proton-dependence and effect of amiloride

The transport of 10 μM celiprolol in the basal-to-apical direction was determined after 30 min and the effect of proton gradients was investigated by reducing the pH to 6.0 (HBSS buffered with MES) on either the apical or basal side of the monolayers. The influence of Na^+-H^+ exchange on the transport of celiprolol in the basal-to-apical direction was studied by addition of the Na^+-H^+ exchange inhibitor, amiloride (500 μM), to the apical or basal side. This concentration has previously been shown to inhibit effectively Na^+-H^+ exchange in Caco-2 cells (Watson *et al.*, 1991).

Inhibition of basal-to-apical transport of celiprolol

The transport of 10 μM [^{14}C]-celiprolol in the basal-to-apical direction was determined after 30 min in the absence (control) or in the presence of different inhibitors. In general, the inhibitors were added to either the apical or basal side at a concentration of 0.5 mM.

Chemicals

DL-[^{14}C]-celiprolol (37.3 μCi mg^{-1}) and unlabelled DL-celiprolol were obtained from Rhône-Poulenc Rorer Pharmaceutical Inc. (Collegeville, PA, U.S.A.). The radiochemical and chemical purity of [^{14}C]-celiprolol was 95%. The chemical purity of unlabelled celiprolol was greater than 98%. [^{14}C]-mannitol (49.3 mCi mmol^{-1}) was purchased from New England Nuclear, Boston, MA, U.S.A. [^{3}H]-vinblastine sulphate (8.3 Ci mmol^{-1}) was obtained from Amersham, Arlington Heights, IL, U.S.A. Pafenolol was a gift from Hässle Läkemedel AB, Mölndal, Sweden. Bambuterol was a gift from Draco Läkemedel AB, Lund, Sweden. 4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 4-morpholineethanesulphonic acid (MES), choline chloride, ouabain, amiloride, cimetidine, N^1-methylnicotinamide (NMN), tetraethylammonium (TEA), atenolol, acebutolol, metoprolol, propranolol, terbutaline, acetazolamide, chlorthalidone, hydrochlorothiazide, cephalexin, indomethacin, vinblastine, verapamil and nifedipine were purchased from Sigma, St. Louis, MO, U.S.A.

Kinetic and statistical analysis

Data are presented as means ± s.d. of n Caco-2 cell monolayers. Student's unpaired t test (two-tailed) was used to test the significance of the difference between two mean values. $P < 0.05$ was considered statistically significant. Kinetic constants for Michaelis-Menten kinetics were calculated by non-linear regression analysis using the computer software PCNONLIN (Statistical Consultants, Inc., Lexington, KY, U.S.A.). V_{max} and K_m are presented as parameter estimates ± s.e. (standard error of the estimate).

Results

The basal-to-apical transport at 50 μM [^{14}C]-celiprolol across Caco-2 monolayers was 7.35 ± 0.62 pmol/10^6 cells min^{-1} or 5 times higher than the apical-to-basal transport of 1.39 ± 0.10 pmol/10^6 cells min^{-1} (Figure 1a,b). Addition of 5 mM unlabelled celiprolol inhibited the basal-to-apical transport by more than 80%, whereas the apical-to-basal transport was unchanged (Figure 1a,b). These results are consistent with saturable active secretion and non-saturable passive absorption of celiprolol.

Figure 2a shows the transport rate of celiprolol over the initial 30 min as a function of the celiprolol concentration. Non-linear kinetics were observed for both basal-to-apical and apical-to-basal transport. However, the non-linear appearance of the apical-to-basal transport was shallow, indicating that the transport was mainly mediated by passive

Table 1 Effect of sodium depletion and ouabain on transport of celiprolol across Caco-2 cell monolayers

Condition	*Celiprolol transport* (pmol/10^6 cells 30 min^{-1}) *Apical-to-basal*		*Basal-to-apical*	
Control	10.8 ± 1.5	(100)	42.0 ± 3.3	(100)
Na^+-free medium	15.2 ± 0.8	(141)***	30.9 ± 3.2	(74)***
Ouabain	11.7 ± 1.9	(108)	23.6 ± 2.9	(56)***

Caco-2 monolayers were washed with HBSS (2 × 3 ml) and preincubated for 30 min with either HBSS alone (control), HBSS containing choline chloride instead of sodium chloride, or 100 μM ouabain. Subsequently, 10 μM [^{14}C]-celiprolol was added to the apical or basal side of the monolayers and the transport was determined after 30 min. The transport values are means ± s.d.; $n = 4$ ($n = 8$ for the control). The extent of transport expressed as % of control is given within parentheses. Statistical significance: ***$P < 0.001$.

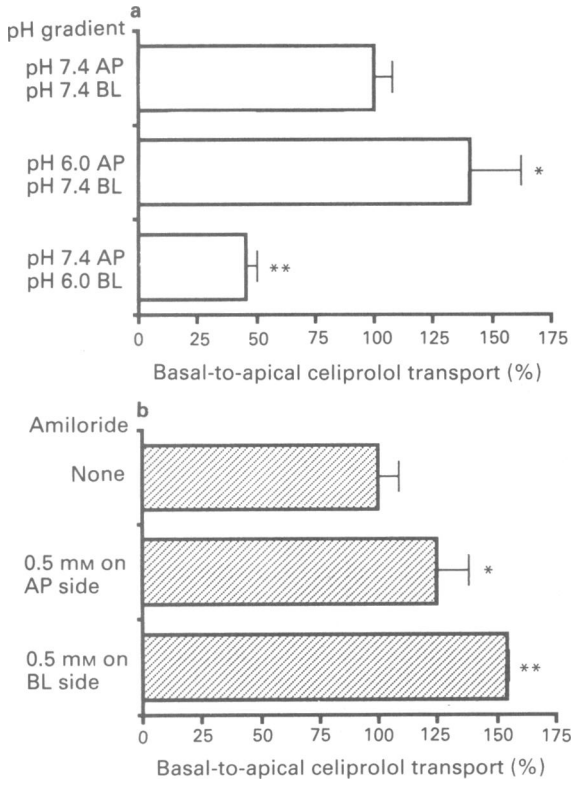

Figure 3 Effect of pH gradients (a) and amiloride (b) on the basal-to-apical transport of celiprolol across Caco-2 cell monolayers. The basal-to-apical transport of 10 μM [^{14}C]-celiprolol was determined after 30 min in the absence and presence of pH gradients (extracellular pH = 6.0) or 0.5 mM amiloride on the apical (AP) or basal (BL) side. Data are presented as % of control transport. Each bar represent means ± s.d.; $n = 4$. Significant differences: $^{*}P < 0.05$; $^{**}P < 0.01$.

diffusion. Subtraction of the apical-to-basal transport from the basal-to-apical transport gave a curve representing the net secretion of celiprolol (Figure 2b). The net secretion was evaluated kinetically by non-linear regression analysis giving an apparent K_m of 1.00 ± 0.23 mM and V_{max} of 113 ± 11 pmol/10^6 cells min^{-1} (102 ± 9 pmol cm^{-2} min^{-1}) as estimated from the Michaelis-Menten equation (Figure 2b).

Replacing sodium with choline in the transport buffer resulted in a significant decrease in the basal-to-apical transport (Table 1), and a significant increase in the apical-to-basal transport, providing further support for the suggestion that the secretion of celiprolol is influenced by active transport mechanisms. Inhibition of Na^+K^+ATPase with ouabain gave comparable results, suggesting that the basal-to-apical transport of celiprolol is coupled to the sodium gradient directly or indirectly (Table 1).

When the proton concentration was increased at the apical side of the cell monolayers (pH = 6.0), the basal-to-apical transport of celiprolol increased by 40% (Figure 3a). A corresponding increase in proton concentration at the basal side resulted in a 54% decrease in basal-to-apical transport, indicating that the transport of celiprolol is proton gradient dependent. Inhibition of the Na^+H^+exchanger with amiloride resulted in significant increases in secretion, independent of the side to which amiloride was added (Figure 3b).

Table 2 shows the varying effects of typical substrates for the organic cation H^+ exchanger on the basal-to-apical transport of celiprolol. When added to the apical side of the monolayers, choline, cimetidine and NMN significantly inhibited the basal-to-apical transport. However, when added to the basal side, no inhibition was observed and TEA and choline significantly stimulated the transport.

Celiprolol transport was also inhibited by apical addition of other β-blocking drugs of varying lipophilicity, and to a lesser degree by the β-adrenoceptor agonists, terbutaline and bambuterol, indicating that structurally related drugs interact with the same transporter(s) as celiprolol (Table 3). No correlation between the lipophilicity of the β-blocking drugs and the inhibition of the transport was observed. However, in contrast to the findings in Table 2, no stimulation of the transport of celiprolol was observed when the β-blocking drugs and β-adrenoceptor agonists were added to the basal side of the cell monolayers. Instead, an inhibition of the secretion was sometimes observed.

Since β-blocking drugs are often co-administered with diuretics and since the bioavailability of celiprolol is altered by concomitant administration of the diuretic, chlorthalidone (Walte, 1985), we also investigated the inhibitory effects of three diuretics on the basal-to-apical transport of celiprolol (Table 4). All diuretics, including chlorthalidone, inhibited the transport of celiprolol, suggesting that an interaction between celiprolol and diuretics may occur at the epithelial level.

The transport of celiprolol was not affected by cephalexin, a typical substrate for the dipeptide carrier (Inui *et al.*, 1992; Dantzig & Bergin, 1990), or indomethacin, which has recently been found to be an effective inhibitor of the ATP-dependent efflux of the anionic pH fluorochrome 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein (Collington *et al.*, 1991; 1992) (Table 4).

The transepithelial transport of a typical substrate for P-glycoprotein, [^{3}H]-vinblastine, was measured in the basal-to-apical and apical-to-basal directions (Figure 4). At a vinblastine concentration of 10 nM, the transport in the basal-to-apical direction (J_{b-a}, 13.1 ± 0.77 fmol/10^6 cells min^{-1}) was 15 times higher than that in the apical-to-basal direction (J_{a-b}, 0.87 ± 0.06 fmol/10^6 cells min^{-1}), consistent with recent results supporting the presence of a functional P-glycoprotein

Table 2 Effect of typical inhibitors of renal organic cation secretion on basal-to-apical transport (J_{b-a}) of celiprolol in Caco-2 cell monolayers

Organic cation (OC)[a]	*Celiprolol transport* J_{b-a} (pmol/10^6 cells 30 min^{-1}) *OC on apical side*		*OC on basal side*	
Control	60.7 ± 8.9	(100)	60.7 ± 8.9	(100)
Cimetidine	32.9 ± 4.1	(54)***	70.5 ± 5.7	(116)
NMN	36.0 ± 5.5	(59)***	52.3 ± 3.4	(86)
TEA	53.6 ± 8.1	(88)	85.3 ± 10.2	(141)***
Choline	49.9 ± 5.9	(82)*	73.5 ± 6.7	(121)*

The basal-to-apical transport of 10 μM [^{14}C]-celiprolol was determined after 30 min in the absence (control) or presence of 0.5 mM of each of the organic cations listed. The organic cations were added on either the apical or basal side of the monolayers. Transport values are means ± s.d.; $n = 4$ ($n = 15$ for the control). The extent of transport expressed as % of control is given within parentheses. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[a]Cimetidine has a log octanol/water partition coefficient of 0.4 (Craig, 1990). NMN (N^1-methylnicotinamide), TEA (tetraethylammonium) and choline are highly hydrophilic quarternary ammonium compounds.

Table 3 Effect of β-adrenoceptor agonist and antagonist drugs on basal-to-apical transport (J_{b-a}) of celiprolol in Caco-2 cell monolayers

Drug	log D[a]	Celiprolol transport J_{b-a} (pmol/10^6 cells 30 min^{-1}) Drug on apical side		Drug on basal side	
Control		63.9 ± 20.0	(100)	63.9 ± 20.0	(100)
Atenolol	− 2.14	37.1 ± 4.3	(58)**	69.4 ± 4.3	(109)
Celiprolol	− 0.70	24.0 ± 3.0	(38)***	42.0 ± 1.8	(66)*
Pafenolol	− 0.52	27.8 ± 6.6	(44)**	67.9 ± 7.1	(106)
Acebutolol	− 0.38	33.7 ± 5.8	(53)**	57.9 ± 8.1	(91)
Metoprolol	− 0.28	27.1 ± 4.2	(42)***	49.3 ± 4.2	(77)*
Propranolol	1.20	33.3 ± 4.6	(52)***	58.3 ± 4.2	(91)
Bambuterol	0.48	57.1 ± 2.8	(89)	53.0 ± 3.5	(83)*
Terbutalin	0.56	48.7 ± 12.3	(76)	51.1 ± 2.7	(80)*

The basal-to-apical transport of 10 μM [^{14}C]-celiprolol was determined after 30 min in the absence (control) or presence of 0.5 mM of the drugs (1.0 mM celiprolol and 0.2 mM propranolol). The drugs were added on either the apical or the basal side of the monolayers. Transport rates are means ± s.d., $n = 4$ ($n = 20$ for the control). The extent of transport expressed as % of control is given within parentheses.
Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[a]Log octanol/phosphate buffer (pH 7.4) partition coefficients (Lennernäs & Regårdh, 1993 and personal communications from Dr Kurt-Jörgen Hoffman, ASTRA Hässle AB, Göteborg, Sweden).

Table 4 Effect of diuretics and substrates for dipeptide and anionic transport, cephalexin and indomethacin, on basal-to-apical (J_{b-a}) transport of celiprolol in Caco-2 cell monolayers

Drug	log D[a]	Celiprolol transport J_{b-a} (pmol/10^6 cells 30 min^{-1}) Drug on apical side		Drug on basal side	
Control		61.5 ± 8.6	(100)	61.5 ± 8.6	(100)
Acetazolamide	− 0.26	33.8 ± 4.8	(55)***	57.8 ± 3.5	(94)
Chlorthalidone	0.24	46.4 ± 5.2	(75)**	66.8 ± 4.8	(109)
Hydrochlorothiazide	− 0.07	40.7 ± 12.6	(66)**	80.4 ± 9.0	(131)**
Cephalexin	0.65	57.2 ± 4.8	(93)	61.6 ± 4.3	(100)
Indomethacin	4.27	57.5 ± 5	(93)	67.2 ± 4.2	(109)

The basal-to-apical transport of 10 μM [^{14}C]-celiprolol was determined after 30 min in the absence (control) or presence of 0.5 mM of each of the drugs listed. The drugs were added on either the apical or basal side of the monolayers. Transport values are means ± s.d., $n = 4$ ($n = 20$ for the control). The extent of transport expressed as % of control is given within parentheses.
Statistical significance: **$P < 0.01$; ***$P < 0.001$.
[a]Log octanol/water partition coefficients (Craig, 1990).

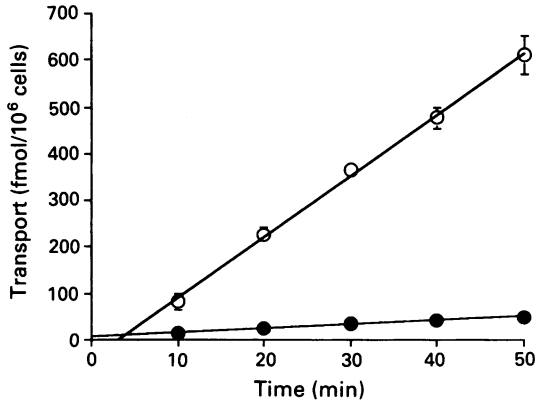

Figure 4 Transport of the P-glycoprotein substrate [^{3}H]-vinblastine (10 nM) across Caco-2 cell monolayers. Vinblastine transport in the apical-to-basal (●) and basal-to-apical (○) directions. Each point represents mean ± s.d.; $n = 4$–6.

transporter in Caco-2 cells (Hunter *et al.*, 1993). Table 5 shows the effect of typical substrates for P-glycoprotein on the basal-to-apical transport of [^{14}C]-celiprolol. Both vinblastine and its competitive inhibitor for the P-glycoprotein transporter, verapamil, inhibited the basal-to-apical transport of celiprolol, indicating that P-glycoprotein participates in the transport of celiprolol. Nifedipine was a less effective inhibitor. In addition, the basal-to-apical transport of [^{3}H]-vinblastine was inhibited by celiprolol (Figure 5).

Discussion

The results of this study show that celiprolol is actively transported across the human intestinal epithelium in the basal-to-apical direction. Saturation of the basal-to-apical transport of celiprolol in the intestine offers an explanation for the enhanced absorption of celiprolol at high doses and its non-linear absorption (Riddel *et al.*, 1987; Kuo *et al.*, 1993). A model composed of one saturable component, described by the Michaelis-Menten equation and a linear diffusional component failed to fit the data of the total apical-to-basal and basal-to-apical transport rates in Figure 2a. This analysis suggested that the observed transport of celiprolol was the sum of several undefined transport mechanisms. Therefore, the apparent kinetic parameters for the net transport rates were calculated. The resulting apparent V_{max} of 113 pmol/10^6 cells min^{-1} and K_m of 1.00 mM indicates that the Caco-2 monolayers have a large capacity for celiprolol secretion whereas the net affinity of the transporter(s) was relatively low.

P-glycoprotein has been identified in Caco-2 cells by monoclonal antibodies (Peters & Roelofs, 1992) and by recent functional studies on vinblastine transport showing that the drug is actively transported in the basal-to-apical direction and that this transport is completely inhibited by verapamil (Hunter *et al.*, 1993). In the present study, vinblastine as well as verapamil and nifedipine, which are two other typical P-glycoprotein substrates, clearly inhibited basal-to-apical transport of celiprolol. Furthermore, celiprolol inhibited the basal-to-apical transport of vinblastine. These results indicate that celiprolol was transported by the P-glycoprotein. The finding that the transport could be inhibited

Table 5 Effect of P-glycoprotein substrates on basal-to-apical transport (J_{b-a}) of celiprolol in Caco-2 cell monolayers

Drug	log D	Celiprolol transport J_{b-a} (pmol/10^6 cells 30 min^{-1}) Drug on apical side		Drug on basal side	
Control		66.3 ± 10.0	(100)	66.3 ± 10.0	(100)
Vinblastine	3.69[a]	23.4 ± 1.4	(35)***	23.5 ± 2.4	(35)***
Verapamil	4.8[a]	25.2 ± 2.0	(38)***	24.1 ± 1.2	(36)***
Nifedipine	2.4[b]	50.8 ± 7.6	(77)*	51.0 ± 3.4	(77)*

The basal-to-apical transport of 10 μM [^{14}C]-celiprolol was determined after 30 min in the absence (control) or presence of 0.5 mM of each of the P-glycoprotein substrates. The drugs were added on either the apical or basal side of the monolayers. Transport values are means ± s.d., $n = 4$ ($n = 8$ for the control). The extent of transport expressed as % of control is given within parentheses. Statistical significance: *$P < 0.05$; ***$P < 0.001$.
[a]log octanol/phosphate buffer (pH 7.4) partition coefficients (Zamora *et al.*, 1988; Selassie *et al.*, 1990)
[b]log octanol/water partition coefficient (Craig, 1990).

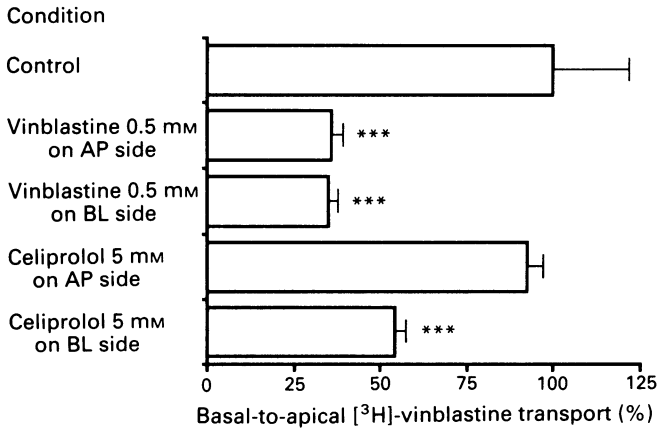

Figure 5 Effect of unlabelled celiprolol and vinblastine on basal-to-apical transport of [^{3}H]-vinblastine in Caco-2 cell monolayers. The basal-to-apical transport of 10 nM [^{3}H]-vinblastine was determined after 30 min in the presence of excess unlabelled celiprolol and vinblastine at the basal (BL) or apical (AP) side. Data are presented as % of control transport. Each bar represents mean ± s.d.; $n = 4$. Significant differences: ***$P < 0.001$.

from both the apical and basal sides can be explained by the high levels of lipophilicity of the inhibitors.

The complex inhibition/stimulation pattern obtained with typical substrates for the renal organic cation-H^+ exchanger suggest that multiple transport systems are involved in the transport of celiprolol. However, comparisons with previous studies on various organic cation-H^+ exchangers indicate that our results diverge somewhat from the expected inhibition pattern for an organic cation-H^+ exchanger (Sokol & McKinney, 1990; Saito *et al.*, 1992; Saitoh *et al.*, 1992). Moreover, it has recently been shown that the renal organic cation-H^+ exchanger and P-glycoprotein can be photoaffinity labelled by the same substrate (Holohan *et al.*, 1992). It is therefore fully possible that celiprolol is exclusively transported by the P-glycoprotein and not by a putative organic cation-H^+ exchanger in Caco-2 monolayers. However, there are several lines of evidence against this hypothesis. Firstly, photoaffinity labelling experiments show that NMN, a typical substrate for the organic cation-H^+ exchanger, is not a substrate for P-glycoprotein (Holohan *et al.*, 1990). Secondly, recent studies in cell lines expressing amplified multidrug resistance showed that a typical substrate for P-glycoprotein, quinidine, but not the organic cation-H^+ exchanger substrate cimetidine, reversed the resistance to adriamycin, indicating that cimetidine is not a substrate for P-glycoprotein (Dutt *et al.*, 1992). However, both NMN and cimetidine inhibited celiprolol secretion in the present study, supporting the hypothesis that a putative organic cation-H^+ exchanger or a similar transporter could be involved. Thirdly, the transport was proton-dependent and was inhibited by amiloride, which inhibits the basal Na^+-H^+ exchanger in Caco-2 cells (Watson *et al.*, 1991). This is in agreement with the hypothesis that organic cation-H^+ exchange is driven by a proton gradient established by the basal Na^+-H^+ exchanger. However, we note that amiloride itself is a substrate for the renal organic cation-H^+ exchanger and therefore additional inhibition of celiprolol transport by interaction with a putative intestinal organic cation-H^+ exchanger cannot be excluded (Wright & Wunz, 1989).

The finding that other β-blocking drugs inhibited the basal-to-apical transport of celiprolol is in conflict with our previous studies of the transport of β-blocking drugs in Caco-2 monolayers which showed that β-blockers such as atenolol, metoprolol and propranolol were transported across Caco-2 monolayers exclusively by passive diffusion (Artursson, 1990). There are several possible explanations for this discrepancy. Firstly, it is possible that only celiprolol is actively transported since it has a structure that is slightly different from that of the other β-blockers. However, this hypothesis is challenged by our own studies showing that propranolol is actively transported in rat isolated intestinal cells (Kuo *et al.*, 1993) and by studies indicating that propranolol reverses multi drug resistance, presumably by inhibition of P-glycoprotein (Zamora *et al.*, 1988; Hofsli & Nissen-Meyer, 1990). A more likely explanation is found in the differences in the experimental design between the two studies. In the earlier study, the concentration-dependence was investigated only up to a concentration of 0.1 mM and therefore, an active saturable transport mechanism may not have been detected. Moreover, the influence of the active transport mechanism on the overall transport of very lipophilic compounds such as propranolol and metoprolol is limited since these drugs easily pass the cells by passive diffusion (Kuo *et al.*, 1993).

Gastrointestinal secretion has been observed for β-blocking drugs other than celiprolol, including acebutolol, propranolol and pafenolol (George & Gruchy, 1979; Tai & Jackson, 1982; Kuo *et al.*, 1993; Lennernäs & Regårdh, 1993a,b). The β-blockers were found to be strong inhibitors of celiprolol transport when added to the apical side of the Caco-2 monolayers. These results support the hypothesis that various β-blocking drugs are actively secreted across the intestine. With the exception of celiprolol itself, none of the β-blocking drugs affected the basal-to-apical transport of celiprolol after basal addition. This suggests that their inhibitory effects were different from those of the typical substrates for the organic cation-H^+ exchanger and P-glycoprotein.

Diuretics such as chlorthalidone are often co-administered with β-blocking drugs. Since many diuretics are cations it is likely that these drugs would also interact with the transport of celiprolol. Indeed, the three diuretics chlorthalidone, acetazolamide and and hydrochlorothiazide all inhibited celiprolol secretion after apical addition. In addition, hydrochlorothiazide stimulated secretion after basal addition, suggesting that this drug may reduce the absorption of celiprolol. These

results may be of clinical significance since the absorption of celiprolol is altered by co-administration of chlorthalidone (Walte, 1985). Further studies on the effect of chlorthalidone on celiprolol transport in the apical-to-basal direction are needed to confirm this hypothesis.

In conclusion, the present study shows that (1) celiprolol is actively transported across the human intestinal epithelium and that the transport in the basal-to-apical direction (secretion) is larger than that in the apical-to-basal direction (absorption). These results offer an explanation for the enhanced absorption of celiprolol at high doses and its non-linear absorption; (2) the secretion of celiprolol is altered by typical substrates for P-glycoprotein and the organic cation-H^+ exchanger, suggesting that the secretion is dependent on multiple transporters and (3) the secretion of celiprolol is altered by diuretics, suggesting that the clinically observed effect of chlorthalidone on celiprolol transport occurs at the epithelial level.

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Antagonism of the stimulatory effects of efaroxan and glibenclamide in rat pancreatic islets by the imidazoline, RX801080

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1 The imidazoline α_2-adrenoceptor antagonist, efaroxan, stimulates insulin secretion from rat isolated islets and antagonizes the ability of diazoxide to inhibit glucose-induced insulin secretion. These effects result from closure of ATP-sensitive potassium channels although the mechanisms involved have not been elucidated.

2 In the present work, we have examined the effects of a close structural analogue of efaroxan, RX801080, in rat isolated islets of Langerhans. RX801080 was found to be ineffective as a stimulator of insulin secretion and did not prevent the inhibition of insulin secretion mediated by diazoxide.

3 RX801080 acted as an antagonist of the actions of several imidazolines (efaroxan, phentolamine and midaglizole) in rat islets. It dose-dependently inhibited the ability of efaroxan to antagonize the effects of diazoxide in islets and also completely inhibited the direct stimulation of insulin secretion mediated by efaroxan.

4 RX801080 also antagonized the effects of the non-imidazoline, ATP-sensitive potassium channel blocker, glibenclamide, in rat islets. It inhibited both the capacity of glibenclamide to stimulate insulin secretion and the ability of glibenclamide to overcome the inhibitory effects of diazoxide in rat islets.

5 Antagonism of glibenclamide responses by RX801080 was not due to inhibition of binding of the sulphonylurea to its receptor on the pancreatic β-cell.

6 The results suggest that imidazoline compounds and sulphonylureas interact with distinct binding sites on islet cells, but that these sites can interact functionally to control islet cell ATP-sensitive potassium channel activity and insulin secretion.

Keywords: Islets of Langerhans; insulin secretion; imidazoline receptors; potassium channel; efaroxan; glibenclamide; α_2-antagonist; sulphonylurea; RX801080; adrenoceptor

Introduction

The resting potential maintained across the plasma membrane of the pancreatic β-cell is determined primarily by a large efflux of potassium ions (Ozawa & Sand, 1986; Henquin, 1987; Dunne & Petersen, 1991; Ashcroft *et al.*, 1992; Boyd, 1992). This efflux is facilitated by a range of potassium channels in the β-cell plasma membrane, which remain open in the resting state. The most important of these is a channel the open state of which varies according to the ATP/ADP ratio within the cell (the K-ATP channel; Cook *et al.*, 1988; Dunne & Petersen, 1991; Ashcroft *et al.*, 1992; Boyd, 1992). Thus, the open state of this channel is determined by the metabolic status of the β-cell and, under conditions of increased glucose oxidation, the potassium permeability of the plasma membrane is reduced by closure of the channel (Cook & Hales, 1984; Ashcroft *et al.*, 1984; 1992; Dunne & Petersen, 1991; Ashcroft & Ashcroft, 1990; Boyd, 1992). Under these conditions the membrane depolarizes, allowing the gating of voltage-sensitive calcium channels and a rise in the rate of calcium influx. This, in turn, acts as the trigger for insulin secretion.

In addition to their sensitivity to the ATP/ADP ratio, β-cell K-ATP channels are also subject to modulation by a variety of pharmacological agents. These can either close (e.g. hypoglycaemic sulphonylureas; Sturgess *et al.*, 1985; Panten *et al.*, 1989; Ashcroft & Ashcroft, 1990; Nelson *et al.*, 1992) or open (e.g. diazoxide; Trube *et al.*, 1986; Dunne, 1989; Dunne *et al.*, 1990; Kozlowski & Ashford, 1992) the channels. It has been established recently that the activity of β-cell K-ATP channels can also be controlled by certain imidazoline compounds (Plant & Henquin, 1990; Chan *et al.*, 1991a; Jonas *et al.*, 1992). These were initially identified as a group of α_2-adrenoceptor antagonists (including efaroxan, phentolamine and midaglizole (DG-5128)) which share the unusual property of eliciting a direct increase in insulin secretion in the absence of any adrenoceptor agonist (Schultz & Hasselblatt, 1988; Smith & Furman, 1988; Chan & Morgan, 1990; Chan *et al.*, 1991a). It has now been established, however, that the primary determinant of this secretagogue activity is not α_2-antagonism *per se*, but rather the possession of an imidazoline ring within the molecule. Thus, certain other imidazolines which are not α_2-antagonists also have insulin secretagogue activity (Schulz & Hasselblatt 1989a); as does clonidine, which is an α_2-adrenoceptor agonist (Schulz & Hasselblatt, 1989b; Plant *et al.*, 1991).

Taken together, these data suggest that K-ATP channels in the pancreatic β-cell may be subject to regulation by a component which contains a binding site for imidazoline compounds. This site does not appear to belong to either of the recently designated I_1 or I_2 subclasses of 'imidazoline receptor' (although such sites may be present on islet cells (Remaury & Paris, 1992; Brown *et al.*, 1993)) since it appears to have very low affinity for idazoxan (Brown *et al.*, 1993). However, the putative 'imidazoline binding site' responsible for control of K-ATP channel activity in the β-cell shows stereoselectivity and the response is subject to down-regulation by agonists, suggesting that the binding site may represent a new type of imidazoline receptor (Chan *et al.*, 1993).

In order to characterize this islet imidazoline receptor fur-

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ther, and to study its role in the regulation of insulin secretion, we have attempted to identify compounds that may act as antagonists at this site. In the present paper, we describe the results of studies with RX801080, an imidazoline compound that is related in structure to efaroxan.

Methods

Isolation of islets of Langerhans

Islets of Langerhans were isolated by collagenase digestion (Montague & Taylor, 1968) from the pancreata of male Wistar rats (180–250 g body weight) allowed free access to food and water. The isolation medium was a bicarbonate buffered physiological saline solution equilibrated to pH 7.4 by gassing with O_2:CO_2 (95:5) (Gey & Gey, 1936). The buffer was supplemented with 4 mM glucose and 1 mM $CaCl_2$. Islets were selected individually under a binocular dissecting microscope and were used within 2 h of isolation.

Insulin secretion experiments

The method for islet incubation has been described in detail previously (Morgan & Montague, 1985). Briefly, groups of 3 islets were incubated in bicarbonate buffered medium, supplemented with bovine serum albumin (1 mg ml^{-1}) and appropriate test reagents. Islets were incubated for 1 h at 37°C and samples were then removed for measurement of insulin by radioimmunoassay.

Preparation of HIT-T15 cell membranes

Crude membranes were prepared from HIT-T15 cells cultured in RPMI-1640 containing 10% (v:v) foetal calf serum (Ashcroft *et al.*, 1986). The tissue culture medium was removed from the flasks, and the cells rinsed with phosphate buffered saline. Cells were then scraped from the flask surface and suspended in ice cold Tris (50 mM)/EDTA (1 mM)/$MgCl_2$ (10 mM) buffer (TEM) pH 7.5. The suspension was homogenized in a Teflon/glass Potter Elvjhem homogenizer (6 passes) and the homogenate centrifuged at 40000 *g* for 20 min (4°C). The membrane pellet was washed by resuspension in 40 ml TEM buffer and recentrifuged at 40000 *g* for 20 min (4°C). The final pellet was resuspended in TEM buffer and stored at − 80°C until required.

Radioligand binding experiments

[^{3}H]-glibenclamide (11 Ci $mmol^{-1}$) was used to label glibenclamide binding sites in HIT cell membranes (Niki & Ashcroft, 1991). Membranes (approx 400 μg protein), [^{3}H]-glibenclamide (2 nM) and competing agents were incubated in a final volume of 0.5 ml TEM buffer pH 7.5, for 1 h at 37°C. Non-specific binding was determined in the presence of 10 μM glibenclamide. Incubations were terminated by rapid vacuum filtration through Whatman GF/B filters. Filters were washed with 10 ml of ice cold Tris (50 mM)/EDTA (5 mM) buffer pH 7.4, and their radioactivity measured after addition of scintillant.

Protein determination

The protein content of HIT-T15 cell membrane preparations was measured by the bicinchoninic acid method of Smith *et al.* (1985).

Statistics

Results were analysed by Student's *t* test for unpaired data and differences were considered to be significant if the *t* value corresponded to a probability of 1:20 or less ($P<0.05$).

Materials

Efaroxan, RX801080 and RX831003 (Figure 1) were synthesized as described by Chapleo *et al.* (1984) and were provided by Reckitt & Colman Products. Diazoxide was obtained from Glaxo Pharmaceuticals, phentolamine from Pfizer and midaglizole from Reckitt & Colman Products. Glibenclamide was provided by SmithKline Beecham and [^{3}H]-glibenclamide was a gift from Hoechst Pharmaceuticals. Radioactive [^{125}I]-iodine (for radioimmunoassay) was from ICN Biomedicals, and anti-bovine insulin serum from Sigma. Culture medium RPMI-1640 and foetal calf serum were purchased from Gibco. Bicinchoninic acid reagents, for assay of protein, were purchased from Pierce. All other reagents were of analytical reagent quality.

Results

Screening of imidazoline compounds for activity in the pancreatic β-cell

In initial experiments two imidazoline compounds that are related in structure to efaroxan (Figure 1) were tested for activity in the pancreatic β-cell. In order to assess the effects of these compounds on islet function, their ability to reverse the inhibitory effect of 250 μM diazoxide on insulin secretion was studied. It has been shown previously that imidazoline compounds which can stimulate insulin secretion directly, are also able to overcome the inhibitory effects of diazoxide (Chan & Morgan, 1990; Chan *et al.*, 1991a,b; Plant & Henquin, 1990). Therefore the use of diazoxide provides a convenient assay system for determining the effectiveness of imidazolines to interact functionally with islet cells.

The results presented in Table 1 show that while efaroxan was able to reverse the inhibitory effects of diazoxide on insulin secretion, two close structural analogues, RX801080 and RX831003, were devoid of activity. We selected one of these (RX801080) for more detailed analysis and proceeded to investigate the effects of RX801080 on responses mediated by other imidazoline compounds in islets.

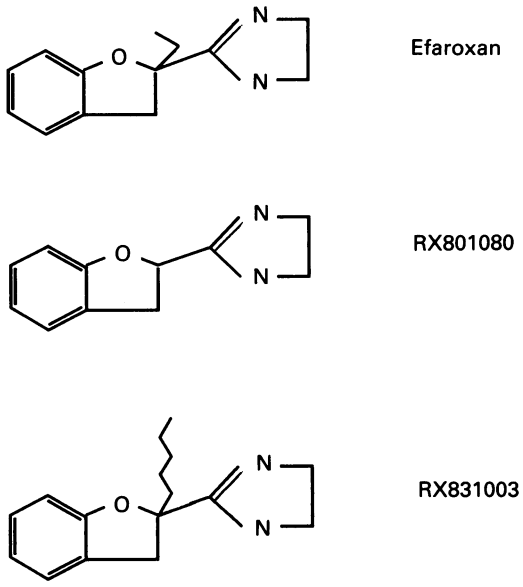

Figure 1 Structures of the imidazoline compounds tested for activity in pancreatic islets. The functional interactions of three imidazoline compounds with pancreatic islet cells were studied. The compounds employed were efaroxan, RX801080 and RX831003. Routes for synthesis of these compounds have been described by Chapleo *et al.* (1984).

Table 1 Effects of imidazolines on the inhibition of glucose-induced insulin secretion mediated by diazoxide

Imidazoline (100 μM)	*Diazoxide* (250 μM)	*Insulin secretion* (ng/islet h^{-1})
–	–	2.97 ± 0.22
–	+	0.52 ± 0.06
Efaroxan	+	2.80 ± 0.30*
RX801080	+	0.54 ± 0.09
RX831003	+	0.52 ± 0.06

Groups of 3 rat isolated islets were incubated for 1 h in the presence of 20 mM glucose. Diazoxide (250 μM) was included as shown, in the presence of each of three different imidazoline compounds (100 μM). Samples of medium were removed and insulin secretion measured by radioimmunoassay. Data are presented as mean rates of insulin secretion ± s.e.mean from 12 observations.
*$P<0.001$ relative to 20 mM glucose + diazoxide.

Table 2 Effects of RX801080 on direct stimulation of insulin secretion by efaroxan

Glucose (mM)	*Efaroxan* (100 μM)	*RX801080* (100 μM)	*Insulin secretion* (ng/islet h^{-1})
4	–	–	1.20 ± 0.15
20	–	–	4.85 ± 0.35*
4	+	–	3.65 ± 0.40*
4	+	+	1.45 ± 0.20**

Groups of 3 rat isolated islets were incubated for 1 h under the conditions shown. The incubation medium was then sampled and its insulin content measured by radioimmunoassay. Results are mean values ± s.e.mean from 10 observations.
*$P<0.001$ relative to 4 mM glucose alone; **$P<0.001$ relative to efaroxan alone.

Effects of RX801080 on responses mediated by imidazoline compounds in rat islets of Langerhans

Initially, it was established with several different islet preparations that RX801080 (up to 100 μM) did not modify insulin secretion induced by 20 mM glucose confirming that the compound lacks significant α_2-adrenoceptor agonist activity (Chapleo *et al.*, 1984) and does not exert other deleterious effects in the β-cell. When RX801080 was included in the incubation medium with efaroxan, it exerted a dose-dependent antagonistic effect, such that when present at an equimolar concentration with efaroxan, RX801080 completely prevented the reversal of diazoxide inhibition of secretion, mediated by efaroxan (Figure 2). The IC_{50} for this response was approximately 40 μM (Figure 2).

To examine this relationship further, we studied the effect of RX801080 on direct stimulation of insulin secretion induced by efaroxan (Table 2). Addition of 100 μM efaroxan to islets incubated in the presence of 4 mM glucose resulted in a 3 fold increase in insulin secretion, and introduction of 100 μM RX801080 abolished this response (Table 2). RX801080 did not alter basal insulin secretion in the absence of efaroxan (not shown).

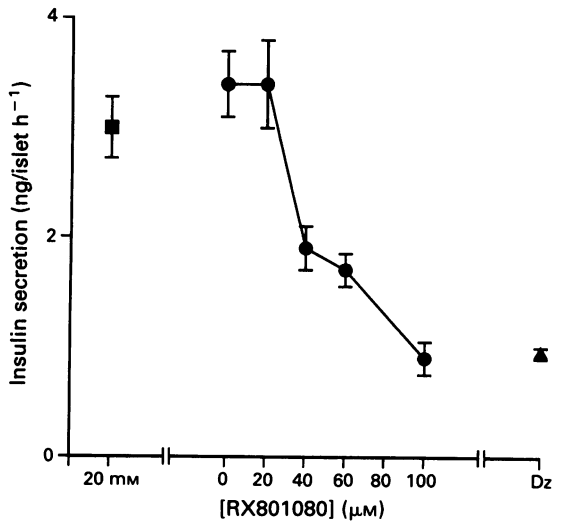

Figure 2 Antagonism of the effects of efaroxan in rat islets by RX801080. Rat isolated islets were incubated in groups of 3 for 1 h in the presence of 20 mM glucose, 250 μM diazoxide and 100 μM efaroxan (●). Increasing concentrations of RX801080 were included as shown. The rate of insulin secretion in response to 20 mM glucose alone (■) and 20 mM glucose plus diazoxide (▲) is also shown. Each value represents the mean rate of insulin secretion ± s.e.mean from a minimum of 10 observations.

The ability of RX801080 to antagonize stimulatory effects in islets was not restricted to responses mediated by efaroxan. Both phentolamine and midaglizole (100 μM) effectively reversed the inhibition of insulin secretion mediated by 250 μM diazoxide (Figure 3) and addition of 100 μM RX801080 antagonized the effect of each of these compounds. These results therefore suggest that RX801080 is an effective antagonist of responses mediated by a range of imidazoline compounds in the pancreatic β-cell.

Effects of RX801080 on responses mediated by glibenclamide in pancreatic islets

In view of the evidence that efaroxan may exert its effects on insulin secretion by inducing the closure of K-ATP channels, the effects of RX801080 on a non-imidazoline K-ATP channel blocker, glibenclamide, were studied.

As shown previously (Panten *et al.*, 1989) glibenclamide

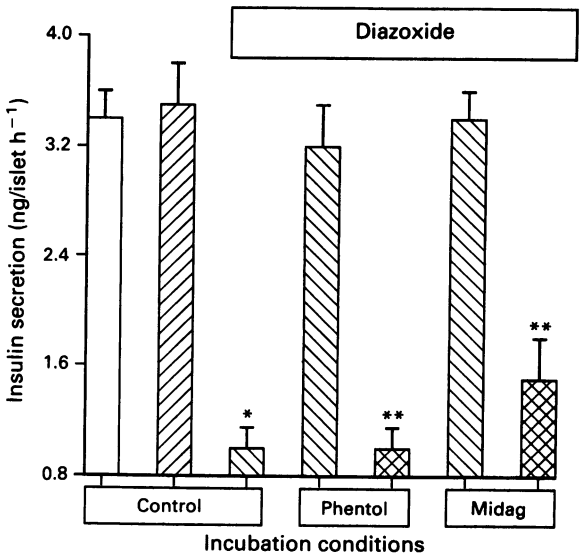

Figure 3 Antagonism of the effects of imidazoline compounds by RX801080. Groups of 3 rat islets were incubated at 37°C for 1 h in the presence of 20 mM glucose. Diazoxide (250 μM) was included as indicated and phentolamine (Phentol) or midaglizole (Midag) were each present at a final concentration of 100 μM. When present, RX801080 was included at a concentration of 100 μM (▨). Samples of the medium were removed and assayed for insulin content by radioimmunoassay. Each value represents the mean ± s.e.mean from at least 10 observations. *$P<0.001$ relative to 20 mM glucose alone (open column). **$P<0.001$ relative to test agent in the absence of RX801080 (▨).

was able to overcome significantly the inhibitory effect of diazoxide on glucose-induced insulin secretion from isolated islets (Table 3). Addition of RX801080 (100 μM) prevented this response (Table 3) suggesting that the compound was able to antagonize the effect of glibenclamide directly. In order to verify this conclusion, the effect of RX801080 on direct stimulation of insulin secretion by glibenclamide was studied (Table 4). Glibenclamide promoted a dose-dependent increase in insulin secretion from islets incubated in the presence of 4 mM glucose; this response was significantly inhibited in the presence of 100 μM RX801080 (Table 4).

Effects of RX801080 on binding of [^{3}H]-glibenclamide to HIT-T15 cell membranes

In order to examine the antagonistic effects of RX801080 on glibenclamide responses further, ligand binding studies were performed using [^{3}H]-glibenclamide to label sulphonylurea receptors on membranes from cultured β-cells. For these studies HIT-T15 cells were employed since they express abundant sulphonylurea receptors that have been well characterized pharmacologically (Boyd, 1988; Gaines *et al.*, 1988; Ashcroft & Ashcroft, 1990; Niki & Ashcroft, 1991; Schwanstecher *et al.*, 1992a,b).

The K_D for interaction of [^{3}H]-glibenclamide with its receptor in HIT cell membranes is in the sub-nanomolar range (Boyd, 1988; Gaines *et al.*, 1988) and concentrations up to 13 nM have been used successfully in displacement studies (Niki & Ashcroft, 1991). On the basis of preliminary experiments we found that a radioligand concentration of 2 nM was convenient for this purpose. Non-specific binding was determined in the presence of 10 μM unlabelled glibenclamide, and was found to represent approximately 20% of the total binding (not shown).

Table 3 Effects of RX801080 on the ability of glibenclamide to inhibit the action of diazoxide in glucose-stimulated islets

Glibenclamide (1 μM)	*Diazoxide* (250 μM)	*RX801080* (100 μM)	*Insulin secretion* (ng/islet h^{-1})
−	−	−	3.4 ± 0.20
−	+	−	1.2 ± 0.15*
+	+	−	2.6 ± 0.35**
+	+	+	0.8 ± 0.10***

Groups of 3 islets were incubated in the presence of 20 mM glucose for 1 h at 37°C. Glibenclamide, diazoxide and RX801080 were included as shown and the extent of insulin secretion measured. Results represent mean values ± s.e. mean from 12 observations.
*$P<0.001$ relative to 20 mM glucose alone; **$P<0.01$ relative to 20 mM glucose + diazoxide; ***$P<0.001$ relative to glibenclamide + diazoxide.

Table 4 Effects of RX801080 on stimulation of insulin secretion by glibenclamide in rat islets

Glucose (mM)	*Glibenclamide* (μM)	*RX801080* (100 μM)	*Insulin secretion* (ng/islet h^{-1})
4	−	−	0.27 ± 0.06
20	−	−	1.30 ± 0.19*
4	0.1	−	0.85 ± 0.12*
4	0.1	+	0.23 ± 0.09**
4	1	−	0.91 ± 0.13*
4	1	+	0.35 ± 0.10**

Groups of 3 islets were incubated with test reagents as shown for 1 h at 37°C. After this time samples of the incubation medium were removed and assayed for insulin content. Results represent mean values ± s.e.mean from 10 observations.
*$P<0.001$ relative to 4 mM glucose alone; **$P<0.001$ relative to glibenclamide in the absence of RX801080.

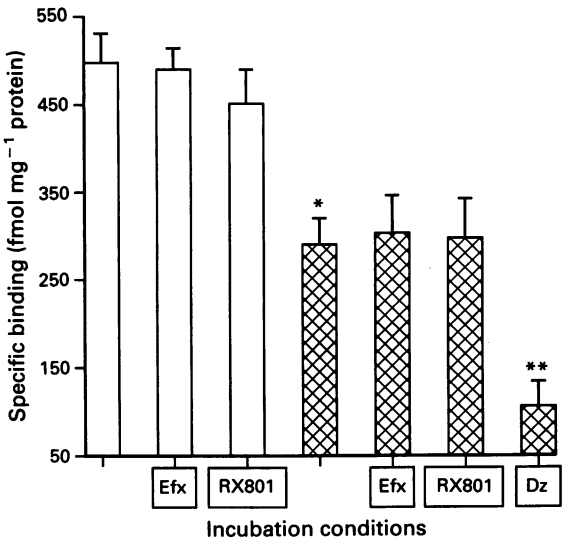

Figure 4 Effects of imidazoline compounds on the binding of [^{3}H]-glibenclamide to membranes from HIT-T15 cells. Crude membranes from HIT-T15 cells were incubated with 2 nM [^{3}H]-glibenclamide for 1 h at 25°C in the absence (open columns) or presence (cross hatched columns) of 1 mM Mg.ATP. Efaroxan (Efx, 300 μM), RX801080 (RX801, 300 μM) or diazoxide (Dz, 250 μM) were included as shown. Bound and free ligand were separated by vacuum filtration. Each value represents the mean binding ± s.e.mean, from at least 8 determinations. *$P<0.001$ relative to total specific binding in the absence of ATP. **$P<0.001$ relative to total specific binding in the presence of ATP.

Competition studies were performed to assess the ability of imidazolines to displace [^{3}H]-glibenclamide binding (Figure 4). These experiments revealed that neither efaroxan nor RX801080, at concentrations up to 300 μM, was able to displace [^{3}H]-glibenclamide from its binding site.

It has been shown previously that certain ligands require the presence of MgATP in order to displace [^{3}H]-glibenclamide binding effectively from β-cell membranes (Niki & Ashcroft, 1991; Schwanstecher *et al.*, 1991; 1992a,b). Therefore, we also examined the effect of ATP on [^{3}H]-glibenclamide binding. Addition of MgATP (1 mM) resulted in approximately 40% displacement of specific [^{3}H]-glibenclamide binding to HIT cell membranes (Figure 4). Under these conditions, diazoxide (250 μM) caused a further significant displacement of the radioligand (Figure 4). However efaroxan and RX801080 were still entirely ineffective in displacing [^{3}H]-glibenclamide binding, despite the presence of ATP.

Discussion

It has become evident from recent studies that the gating of K-ATP channels in the pancreatic β-cell can be regulated by a variety of pharmacological agents. The most potent of these are the sulphonyureas which are used widely in the management of Type II diabetes mellitus (reviewed by Ashcroft & Ashcroft, 1990; Nelson *et al.*, 1992). These agents control K-ATP channel activity by interacting with a specific receptor site located close to, or within, the channel (Sturgess *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987; Kramer *et al.*, 1988). However, the mechanisms by which occupation of this receptor results in channel closure have not been defined, and the receptor itself has eluded complete characterization.

By contrast with the sulphonylureas, certain other K-ATP channel blockers are less specific in their actions (e.g. tetraethylammonium, 9-aminoacridine; Henquin *et al.*, 1979; Karlsson & Ahren, 1992) and may not interact with specific receptors associated with the channels. Thus, both specific

and non-specific mechanisms have been implicated in K-ATP channel blockade.

In the present work we have extended our studies of the mechanisms involved in control of insulin secretion by imidazoline reagents. Previous results have shown that these compounds stimulate insulin release by inducing the closure of β-cell K-ATP channels (Chan *et al.*, 1991a; Plant & Henquin, 1990; Jonas *et al.*, 1992), but the nature of the interaction between imidazoline compounds and K-ATP channels has not been clarified. We have recently presented evidence that the ability of imidazolines to regulate insulin secretion is stereospecific and subject to agonist down-regulation (Chan *et al.*, 1993). These characteristics suggest that a functional receptor may be an important component of their site of interaction with the β-cell. The present results further support this hypothesis since they demonstrate that RX801080 can antagonize the stimulatory effects of other imidazoline compounds in islets.

The results described here also reveal that there are quite marked structural requirements determining efficacy at islet imidazoline receptors. For example, extension of the aliphatic chain on the chiral carbon of efaroxan (ethyl; Figure 1) by addition of 3 further carbon atoms (RX831033, Figure 1) resulted in loss of activity (Table 1). Presumably, this is due to the steric effect of an extended hydrocarbon chain reducing access of the molecule to the putative agonist binding site. RX801080 lacks substitution at the equivalent carbon atom of efaroxan (Figure 1) but is also inactive as an agonist. This lack of activity is unlikely to reflect reduced access to a binding site since any steric effects of substituents would be minimized in this molecule. Therefore, we investigated the possibility that RX801080 might interact with the efaroxan binding site in an antagonistic manner. The results obtained suggest that this may be the case, since RX801080 was found to antagonize the direct stimulation of insulin secretion mediated by efaroxan (Table 2) and also to prevent the effects of efaroxan and other imidazolines on responses induced by diazoxide in islets (Figures 2 and 3). Since the effects of efaroxan are attributable to changes in the open state of K-ATP channels, these results are consistent with the possibility that RX801080 acts antagonistically to other imidazolines at a regulatory site associated with K-ATP channels.

Confirmation of this hypothesis will require the development of a high affinity ligand that can be used to label the imidazoline binding sites in islets. In other tissues imidazoline sites can be labelled with [^{3}H]-idazoxan (Michel & Ernsberger, 1992; Kilpatrick *et al.*, 1992). However, although [^{3}H]-idazoxan will label non-adrenoceptor sites in rat islets (Brown *et al.*, 1993) and cultured β-cell lines (Remaury & Paris, 1992), it neither stimulates insulin secretion nor antagonizes the effects of other imidazolines in rat islets (Chan & Morgan, 1990; Brown *et al.*, 1993). Thus, it appears that the functionally important binding site for imidazolines in islets differs from those described previously in other tissues, and that [^{3}H]-idazoxan is not a suitable ligand with which to label this site.

Surprisingly, RX801080 not only antagonized the responses to imidazolines in islets but also inhibited the ability of glibenclamide to stimulate insulin secretion (Tables 3 and 4). This was an unexpected observation in that there is no obvious structural similarity between the sulphonylurea and any of the imidazolines tested. Indeed, neither RX801080 nor efaroxan was able to compete with [^{3}H]-glibenclamide for its binding site on β-cell membranes (Figure 4) even when present at concentrations more than 5 orders of magnitude greater than that of the radioligand. Thus, the inhibition of the effects of glibenclamide by RX801080 does not result from direct competition for the same binding site. Furthermore, RX801080 did not inhibit glucose-induced insulin secretion (Figure 3) emphasizing that RX801080 does not alter the regulation of islet K-ATP channels mediated by endogenous ATP.

Taken together, the results of the present study imply that sulphonylureas and imidazoline compounds may both interact with regulatory sites associated with K-ATP channels. These sites are clearly independent with respect to ligand binding but they appear to be related in a functional manner. The molecular basis of this interaction remains to be established but it may prove to be important in the context of diabetes therapy, since recent studies have demonstrated that administration of low doses of glibenclamide and efaroxan to rats *in vivo* leads to a synergistic increase in insulin secretion (Berridge *et al.*, 1992). Characterization of the islet imidazoline binding site may, therefore, yield important information about the regulation of K-ATP channels and also provide a new target for development of novel anti-hyperglycaemic agents.

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Characterization of the 5-HT_4 receptor mediating tachycardia in piglet isolated right atrium

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1 In order to explore whether 5-HT_4 receptor subtypes exist, we have characterized further the 5-HT_4 receptor that mediates tachycardia in the piglet isolated right atrium. All experiments were carried out in the presence of propranolol (400 nM) and cocaine (6 μM). We used tryptamine derivatives, substituted benzamides and benzimidazolone derivatives as pharmacological tools.

2 Tachycardia responses to 5-hydroxytryptamine (5-HT) were mimicked by other tryptamine derivatives with the following order of potency: 5-HT > 5-methoxytryptamine > α-methyl-5-HT = bufotenine > 5-carboxamidotryptamine = tryptamine (after treatment with pargyline) > 5-methoxy-*N*,*N*-dimethyltryptamine > 2-methyl-5-HT.

3 The substituted benzamides were all partial agonists relative to 5-HT except (−)-zacopride which was a full agonist. The stimulant potency order was renzapride > cisapride = (−)-zacopride > metoclopramide > (+)-zacopride.

4 The benzimidazolone derivatives had contrasting effects. 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) was a full agonist relative to 5-HT whilst BIMU 1 (endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) was a partial agonist with low intrinsic activity compared to 5-HT but had similar potency. We estimated a pK_B of 7.9 for BIMU 1 antagonism of 5-HT-induced tachycardia. DAU 6215 (N-endo-8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide, hydrochloride) had no chronotropic activity and was found to be a simple competitive antagonist with a pK_B of 7.1

5 SB 203186 (1-piperidinyl)ethyl 1H-indole 3-carboxylate) was a potent antagonist with a pK_B of 8.3. The affinity of SB 203186 was approximately 20 times higher than that of tropisetron (ICS 205-930; pK_B = 6.9) and DAU 6215 (pK_B = 7.0). GR113808 (([1-[2-[methylsulphonyl amino]ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate) and SDZ 205-557 ((2-diethylaminoethyl)2-methoxy-4-amino-5-chloro-benzoate) also antagonized 5-HT-induced tachycardia but not by simple competitive blockade.

6 The sinoatrial 5-HT_4 receptor in the piglet has a pharmacological profile that correlates well with 5-HT_4 receptors characterized in rat oesophagus, guinea-pig ileum and colon, mouse embryonic colliculi neurones and human atrium.

Keywords: 5-HT_4 receptors; piglet right atrium; tachycardia; tryptamines; benzamides; benzimidazolones; SB 203186; SDZ 205-557; DAU 6215; GR113808

Introduction

5-HT_4 receptors have been identified in a number of tissues including the brain, gastrointestinal tract and the heart (atrium and sinoatrial node). They were first described in primary cultures of mouse embryonic colliculi neurones (Dumuis *et al.*, 1988) and in guinea-pig hippocampal membranes (Shenker *et al.*, 1987; Dumuis *et al.*, 1988) where they mediate 5-hydroxytryptamine (5-HT)-induced stimulation of adenylyl cyclase activity.

Gastrointestinal 5-HT_4 receptors are present in guinea-pig stomach (Buchheit & Bertholet, 1992), ileum (Craig & Clarke, 1990; Eglen *et al.*, 1990) and colon (Elswood *et al.*, 1991; Wardle & Sanger, 1992) where they mediate contractile responses to 5-HT and/or increases in electrically-evoked twitch contractions induced by 5-HT. Stimulation of 5-HT_4 receptors in rat oesophagus causes relaxation (Baxter *et al.*, 1991; Reeves *et al.*, 1991) which occurs via adenylyl cyclase activation (Ford *et al.*, 1992).

5-HT_4 receptors in the heart mediate positive chronotropic effects of 5-HT in adult and newborn pigs (Villalon *et al.*, 1990; 1991; Kaumann, 1990) as well as mediating 5-HT-induced increases in contractile force, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content and cyclic AMP-dependent protein kinase stimulation in paced human right and left atrial preparations (Kaumann *et al.*, 1990; 1991a; Sanders & Kaumann, 1992). 5-HT_4 receptors also mediate positive inotropic effects and increases in cyclic AMP by 5-HT in piglet left atria (Kaumann *et al.*, 1991b).

Three families of compounds have agonist activity at some or all of the 5-HT_4 receptors described. 5-HT and other tryptamines including 5-methoxytryptamine (5-MeOT) often act as full agonists (Dumuis *et al.*, 1988; Craig & Clarke, 1990) whilst benzamide derivatives like cisapride and renzapride are sometimes full agonists (Dumuis *et al.*, 1989) but usually partial agonists at 5-HT_4 receptors (Kaumann, 1990; Kaumann *et al.*, 1991a).

Recently azabicycloalkyl benzimidazolone derivatives (Turconi *et al.*, 1991) have also been shown to be potent full or partial agonists at 5-HT_4 receptors (Dumuis *et al.*, 1991; Baxter & Clarke, 1992).

Tropisetron (ICS 205-930) has been extensively used as an antagonist of 5-HT_4 receptors despite its relatively low affinity and poor selectivity (reviewed by Bockaert *et al.*, 1992a). Several compounds have recently been reported to be more potent and selective 5-HT_4 antagonists than tropisetron, including SDZ 205-557 ((2-diethylaminoethyl)2-methoxy-4-amino-5-chloro-benzoate) (Buchheit *et al.*, 1991; 1992). DAU 6215 (N-(endo-8-methyl-8-azabicyclo[3.2.1]-oct-

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3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide, hydrochloride) (Turconi *et al.*, 1991; Dumuis *et al.*, 1991; Baxter & Clarke, 1992), SB 203186 ((1-piperidinyl)ethyl 1H-indole 3-carboxylate) (Kaumann *et al.*, 1992; Parker *et al.*, 1993) and GR113808 ([1-[2-[methylsulphonyl amino]ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate (Grossman *et al.*, 1993).

The present study was designed to characterize more fully the 5-HT_4 receptor described by Kaumann (1990) which mediates 5-HT-induced tachycardia in spontaneously beating right atria of newborn piglets. Using the various agonist and antagonists described above, we have compared the pharmacological properties of 5-HT_4 receptors in porcine sinoatrial node with those described for rat oesophagus (Baxter *et al.*, 1991; Baxter & Clarke, 1992), guinea-pig ileum and colon (Craig & Clarke, 1990; Eglen *et al.*, 1990; Wardle & Sanger, 1992), mouse embryonic colliculi neurones (Dumuis *et al.*, 1988; 1989; 1991) and human atrium (Kaumann *et al.*, 1990; 1991a; Sanders & Kaumann, 1992).

Methods

Male and female piglets (2–5 days old) were obtained from local farms and anaesthetized with halothane. The heart was excised and immediately washed free of blood with warm solution containing (mM): Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 34, HPO_4^{2-} 1, EDTA 0.04, and aerated with 95% O_2 and 5% CO_2. The right atrium was dissected in warm solution and mounted in a 50 ml organ bath using the apparatus described by Blinks (1965). The organ bath contained the solution described above at 37°C, supplemented with (mM): Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5, glucose 10 and continuously gassed with 95% O_2 and 5% CO_2 for the duration of the experiment. The water used throughout was deionised and twice distilled in glass.

Each spontaneously beating right atrium was suspended at a resting length just sufficient for measurable development of force. Care was taken not to overstretch the atrium since tachycardia could result (Blinks, 1956). The atrium was attached to a Swema SG4-45 strain gauge transducer by means of a stainless steel wire and force recorded on a Watanabe polygraph.

All experiments were carried out in the presence of 400 nM (±)-propranolol (to avoid possible indirect β-adrenoceptor-mediated effects due to noradrenaline; Kaumann, 1990), 6 μM cocaine (to block neuronal uptake; Kaumann *et al.*, 1990) and 200 μM ascorbate (to decrease oxidation of 5-HT). In some experiments atria were pretreated with pargyline (50 μM) for half an hour followed by washout to inhibit irreversibly monoamine oxidase (MAO).

A single cumulative concentration-effect curve was determined on each atrium by sequential addition of tryptamines, benzamides or benzimidazolones to the bath in amounts that increased the total concentration in steps of 0.5 log unit. Enough time was allowed for each effect to reach equilibrium. Control 5-HT curves were constructed in parallel tissues. In some experiments a concentration-effect curve for a partial agonist was followed by one for 5-HT in the presence of the highest concentration of the partial agonist. To assess the intrinsic activity of other agonists with respect to 5-HT, 600 μM 5-HT was administered after completion of a concentration-effect curve to an agonist (and in the presence of the latter).

To study the effects of antagonists separate atria were incubated with tropisetron, DAU 6215, SDZ 205-557, GR113808 or SB 203186 for at least 60 min before determination of the agonist concentration-effect curve. Control 5-HT curves were determined in other tissues in parallel.

All experiments were terminated by the addition of a saturating concentration of (−)-isoprenaline (200 μM) still in the presence of test agonist or antagonist, and 5-HT (600 μM). This (−)-isoprenaline concentration was calculated to surmount completely the antagonism by (±)-propranolol (Kaumann *et al.*, 1980).

The positive chronotropic effects of drugs were expressed as a percentage of the total increase in beating rate caused by agonist or agonist combination plus 200 μM (−)-isoprenaline (Δ HR % ΔIso). This procedure provided an independent way of estimating maximum increases in beating rates induced by 5-HT, other agonists, and partial agonists (Kaumann, 1990).

Agonist potency

The ability of agonists to induce tachycardia was expressed in absolute terms as pEC_{50} values relative to their individual maxima ($pEC_{50} = -\log EC_{50}$ concentration of agonist required to produce half-maximal effect).

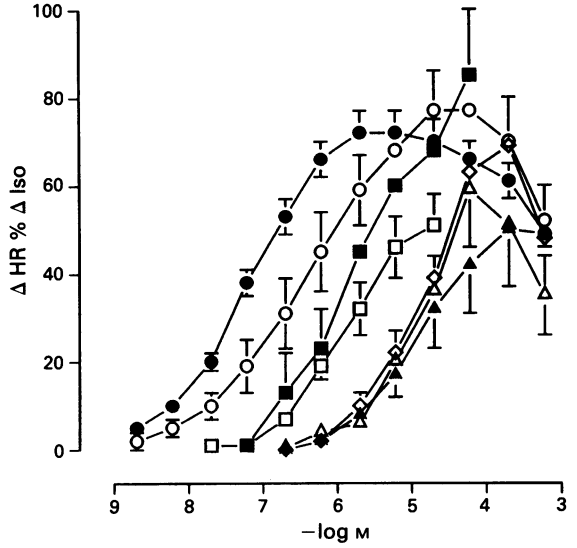

Figure 1 Concentration-effect curves to 5-HT (●, $n = 33$), 5-MeOT (○, $n = 7$), α-methyl-5-HT (■, $n = 7$), bufotenine (□, $n = 4$), tryptamine (◇, $n = 7$), 5-MeO-*N*,*N*-DMT (△, $n = 4$) and 5-CT (▲, $n = 4$) in piglet right atrium. Data are means ± s.e.mean. The chronotropic effects of agonists are expressed as a percentage of the increase in beating rate caused by agonist plus 200 μM isoprenaline (Δ HR % Δ Iso). 5-CT data from Kaumann (1990). For abbreviations, see text.

Table 1 Agonist potency and intrinsic activity in piglet right atrium

Agonist	*pEC_{50}*[a]	*Intrinsic activity*	n
5-HT	7.13 ± 0.1	1.0	33
5-MeOT	6.36 ± 0.3	1.07	7
α-Methyl-5-HT	5.90 ± 0.1	1.18	7
Bufotenine	5.95 ± 0.04	0.71	4
Tryptamine	4.87 ± 0.14	0.96	7
5-MeO-*N*,*N*-DMT	4.69 ± 0.21	0.82	4
5-CT	4.89 ± 0.13	0.69	4
Renzapride	6.70 ± 0.11*	0.69	4
Cisapride	6.44 ± 0.1*	0.39	4
(−)-Zacopride	6.41 ± 0.12	0.94	6
(+)-Zacopride	5.27 ± 0.24	0.33	4
Metoclopramide	5.73 ± 0.1	0.29	4
BIMU 8	6.70 ± 0.12	0.89	8
BIMU 1	7.13 ± 0.05	0.39	6

[a]$pE_{50} = -\log$ concentration of agonist required to produce 50% of the maximum increase in heart rate; mean ± s.e.mean.
*Data from Kaumann (1990). For abbreviations, see text.

Drug-receptor constants

The equilibrium dissociation constant K_B ($-\log K_B = pK_B$) for the antagonist-5-HT$_4$ receptor complex was calculated from

$$pK_B = \log (CR-1) - \log [B] \quad (1)$$

where CR is the concentration-ratio of agonist used in the presence and absence of antagonist (B). The assumption of simple competition (i.e. slope of unity) between antagonist and agonist for 5-HT$_4$ receptors was checked with a Schild plot (Arunlakshana & Schild, 1959).

The equilibrium dissociation constant Kp ($-\log K$p = pKp) was estimated by the method of Marano & Kaumann (1976), and Lemoine & Kaumann (1982). Kp was estimated from the slope of a weighted plot which relates equieffective concentrations of 5-HT in the absence (A_2) and presence (A_3) of a partial agonist P, $A_2 = i + mA_3$, where i is the ordinate intercept. The slope m of the regression equals $m = 1 - yp$, where the fractional 5-HT$_4$ receptor occupancy yp by the partial agonist P is given by [P]/([P] + Kp). pKp was calculated from

$$\log (1/m - 1) = \log [P] - \log K\text{p} \quad (2)$$

For simple competition between a 5-HT molecule and a partial agonist molecule for one 5-HT$_4$ receptor molecule, equation (2) has a slope of one, as in the Schild equation.

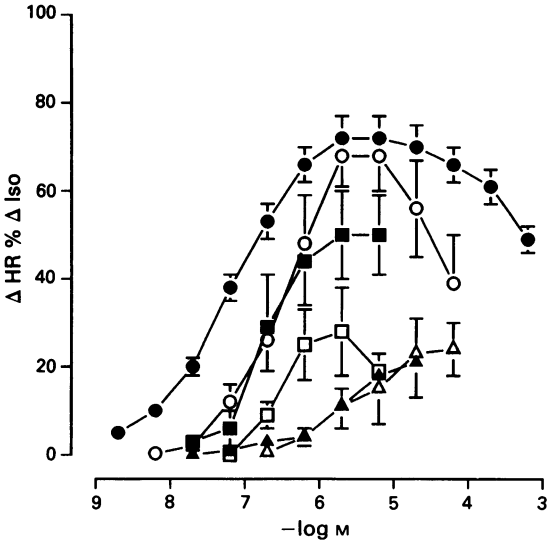

Figure 2 Concentration-effect curves to 5-HT (●, $n = 33$), (−)-zacopride (○, $n = 6$), renzapride (■, $n = 4$), cisapride (□, $n = 4$), (+)-zacopride (△, $n = 4$) and metoclopramide (▲, $n = 4$) in piglet right atrium. Data are means ± s.e.mean. The chronotropic effects are expressed as a percentage of the increase in beating rate caused by agonist plus 200 μM isoprenaline (Δ HR % Δ Iso). Renzapride and cisapride data from Kaumann (1990).

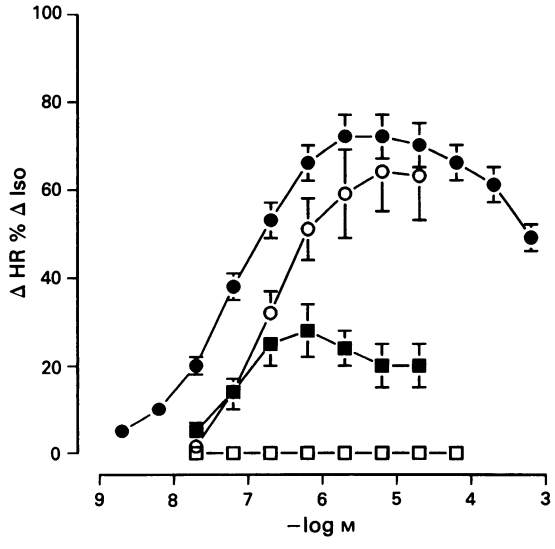

Figure 3 Concentration-effect curves to 5-HT (●, $n = 33$), BIMU 8 (○, $n = 8$), BIMU 1 (■, $n = 4$) and DAU 6215 (□, $n = 4$) in piglet right atrium. Data are means ± s.e.mean. The chronotropic effects are expressed as a percentage of the increase in beating rate caused by agonist plus 200 μM isoprenaline (Δ HR % Δ Iso). For abbreviations, see text.

Drugs

5-Hydroxytryptamine hydrochloride (5-HT), 5-methoxytryptamine hydrochloride (5-MeOT), 5-methoxy-N,N-dimethyltryptamine (5-MeO-N,N-DMT), (−)-isoprenaline hydrochloride, tryptamine hydrochloride, cocaine hydrochloride, bufotenine monoxalate and parglyine hydrochloride were all purchased from Sigma (UK).

α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT), 2-methyl-5-hydroxytryptamine maleate (2-methyl-5-HT) and tropisetron were obtained from RBI (Natick, MA, U.S.A.) and ascorbic acid from BDH (UK). The following drugs were gifts: (±)-propranolol hydrochloride from ICI Pharmaceuticals (Macclesfield, Cheshire), BIMU 1 (endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride), 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 6215 (N-(endo-8-methyl-8-azabicyclo [3.2.1]-oct-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1- carboxamide, hydrochloride) from Istituto de Angeli (Milan, Italy), cisapride from Janssen (Beerse, Belgium), and GR 113808 (([1-[2-[methylsulphonyl amino]ethyl]-4]piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate) from Glaxo Group Research (U.K.). (−)-and (+)-zacopride, renzapride hydrochloride, metoclopramide, SB 203186 ((1-piperidinyl)ethyl 1H-indole 3-carboxylate), SDZ 205-557 ((2-diethylamino-ethyl)2-methoxy-4-amino-5-chloro-benzoate) and 5-carboxamidotryptamine (5-CT) were synthesized in-house.

All stock solutions were made up in distilled water except tropisetron and cisapride (dimethylsulphoxide), 5-HT and (−)-isoprenaline (0.2 mM ascorbate).

Results

Agonist studies

Tryptamines The tryptamines 5-HT, 5-MeOT, α-methyl-5-HT, 5-CT, bufotenine, tryptamine (after pargyline pretreatment), and 5-MeO-N,N-DMT, were all chronotropic agonists in piglet right atrium. Concentration-effect curves to these agonists are shown in Figure 1 and pEC_{50} ($= -\log EC_{50}$) and intrinsic activity values are displayed in Table 1. The maximal effect of 5-HT was 72% of the effect of 200 μM isoprenaline. 5-MeOT, α-methyl-5-HT, 5-MeO-N,N-DMT and tryptamine were all full agonists relative to 5-HT whilst bufotenine and 5-CT were partial agonists. The 5-HT$_3$ receptor agonist, 2-methyl-5-HT, was a weak partial agonist with a pEC_{50} of ~3.9 (experiment not shown); its intrinsic activity could not be assessed since it caused depression of heart rate which could not be overcome by 5-HT or isoprenaline.

Concentration-effect curves to 5-HT, 5-CT, 5-MeOT and 5-MeO-*N*,*N*-DMT ($n = 3$) were unaffected by treatment of the right atria with the irreversible monoamine oxidase inhibitor, pargyline (not shown) and so pargyline pretreatment was not used in subsequent experiments. Pargyline was used routinely, however, in experiments with tryptamine since no response was obtained when pargyline was absent.

Substituted benzamides The 2-methoxy-4-amino-5-chloro-substituted benzamides renzapride, cisapride, (−)-zacopride,

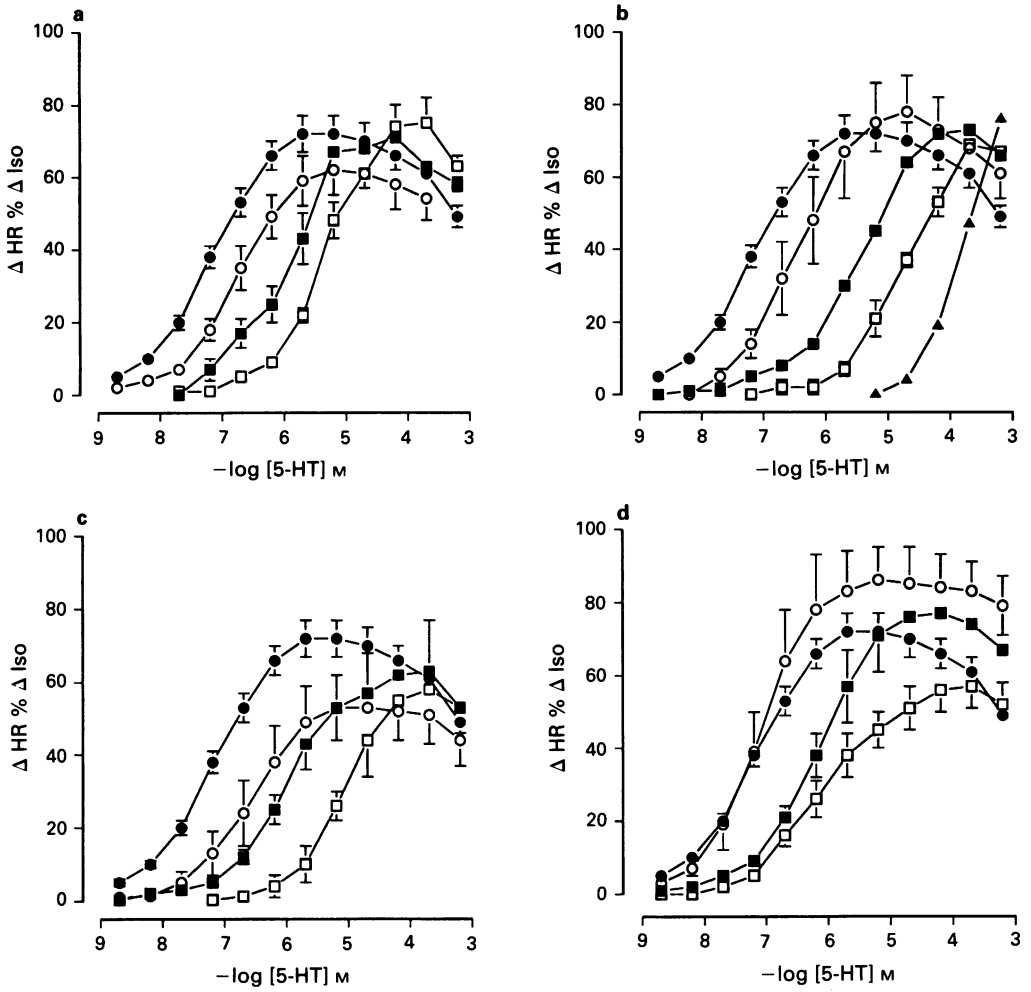

Figure 4 Concentration-effect curves to 5-HT in the absence (●, $n = 33$) and presence of (a) 0.1 (○, $n = 4$), 1.0 (■, $n = 6$) and 10 μM (□, $n = 5$) DAU 6215; (b) 0.02 (○, $n = 3$), 0.2 (■, $n = 2$), 2.0 (□, $n = 3$) and 10 μM (▲, $n = 2$) SB 203186; (c) 0.1 (○, $n = 5$), 1.0 (■, $n = 4$) and 10 μM (□, $n = 3$) SDZ 205-557; and (d) 0.002 (○, $n = 4$), 0.01 (■, $n = 4$) and 0.1 μM GR113808 (□, $n = 8$) in piglet right atrium. Data are means ± s.e.mean. The chronotropic effects of 5-HT are expressed as percentage of the increase in beating caused by agonist or agonist in the presence of antagonist, plus 200 μM (−)-isoprenaline (Δ HR % Δ Iso). For abbreviations, see text.

Table 2 Binding characteristics and affinity values of antagonists and partial agonists for 5-HT_4 receptors in piglet right atrium

Antagonists	pK_B *or* pKp[a]	*Slope*[d]	n
SB203186	8.26 ± 0.09	0.96 ± 0.09	10
DAU 6215	7.00 ± 0.10	0.85 ± 0.12	15
Tropisetron	6.89 ± 0.12[b]	1.09 ± 0.30	7
SDZ 205-557		0.58 ± 0.07	12
GR 113808		non-linear	16
Partial agonists			
BIMU 1	7.93 ± 0.11	1.08 ± 0.10	25
Renzapride	7.95 ± 0.13[c]	–	6
Metoclopramide	6.00 ± 0.5[c]	–	4

[a]pK_B or $pKp = -\log$ equilibrium dissociation constants; K_B, estimated using equation (1); Kp, estimated using equation (2).
[b]Data from Kaumann (1990).
[c]Estimated from single concentrations, 0.5 μM renzapride and 20 μM metoclopramide.
[d]Mean slope ± s.e.mean.

(+)-zacopride and metoclopramide were less potent agonists than 5-HT in causing tachycardia in piglet right atria (Figure 2). Only (−)-zacopride was a full agonist with respect to 5-HT. Renzapride was a potent partial agonist ($-\log EC_{50} = 6.7$) with an intrinsic activity of 0.7 (with respect to 5-HT), whilst (+)-zacopride, metoclopramide and cisapride were less potent partial agonists and had intrinsic activities of less than 0.4 (Table 1).

Renzapride and metoclopramide antagonized the effects of 5-HT so equilibrium dissociation constants for partial agonists (pKp) were calculated (see below). Above 60 μM, metoclopramide caused a dramatic decrease in heart rate.

Azabicycloalkyl benzimidazolone derivatives The azabicycloalkyl benzimidazolone derivative BIMU 8 was a potent full agonist relative to 5-HT in piglet right atrium (Figure 3). BIMU 1 was a chronotropic partial agonist of low intrinsic activity (Table 1, Figure 3). BIMU 1 also antagonized the effects of 5-HT, so that a pKp for partial agonist activity was calculated (see below).

DAU 6215 showed no chronotropic effects with concentrations of up to 60 μM (Figure 3) and caused some decrease in heart rate above this concentration. DAU 6215 was also tested for antagonist properties (see below).

Antagonist studies

Tropisetron (3 μM) antagonized the positive chronotropic effects of 5-MeOT ($n = 5$), α-methyl-5-HT ($n = 4$) and 5-MeO-N,N-DMT ($n = 3$), the log concentration-ratios being 1.28 ± 0.30; 1.33 ± 0.17 and 0.50 ± 0.30 respectively.

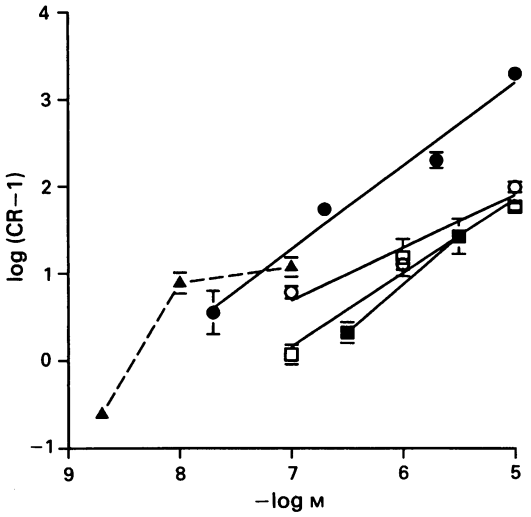

Figure 5 Schild plots from antagonism of 5-HT-induced tachycardia by DAU 6215 (□), SDZ 205-557 (○), SB 203186 (●), GR113808 (▲) and tropisetron (■) in piglet right atrium. Mean slopes ± s.e.mean: 0.96 ± 0.09 (SB 203186), 0.58 ± 0.07 (SDZ 205-557), 1.09 ± 0.3 (tropisetron) and 0.85 ± 0.12 (DAU 6215). A mean slope was not calculated for GR113808 because the plot was non-linear.

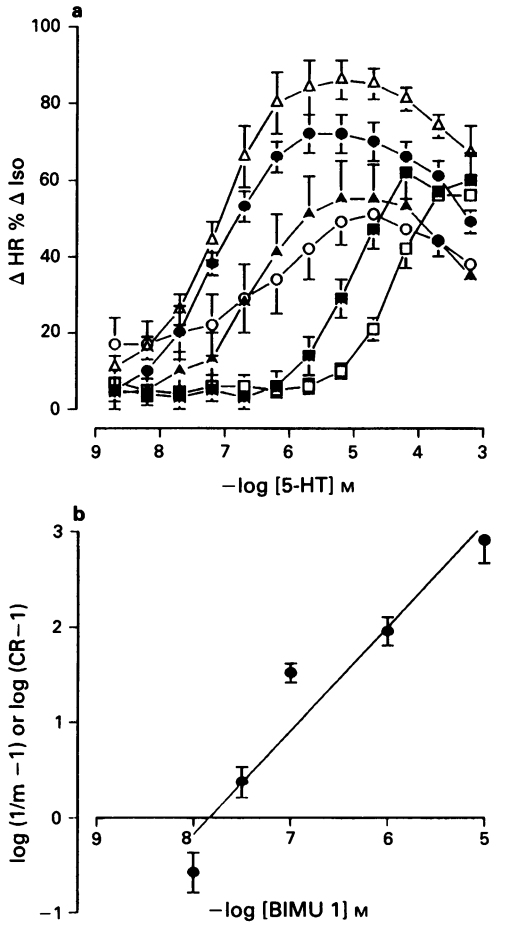

Figure 6 (a) Concentration-effect curves to 5-HT in the absence (●, $n = 33$) and presence of 0.01 (△, $n = 5$), 0.03 (▲, $n = 4$), 0.1 (○, $n = 5$), 1.0 (■, $n = 6$) and 10 μM (□, $n = 5$) BIMU 1 in piglet right atrium; data are means ± s.e.mean, chronotropic effects are expressed as a percentage of the increase in beating caused by agonist alone or in the presence of antagonist, plus 200 μM (−)-isoprenaline (Δ HR % Δ Iso). (b) Double log plot for simple competitive inhibition by BIMU 1; data from (a), calculated using equations (1) and (2); mean slope ± s.e.mean = 1.08 ± 0.10.

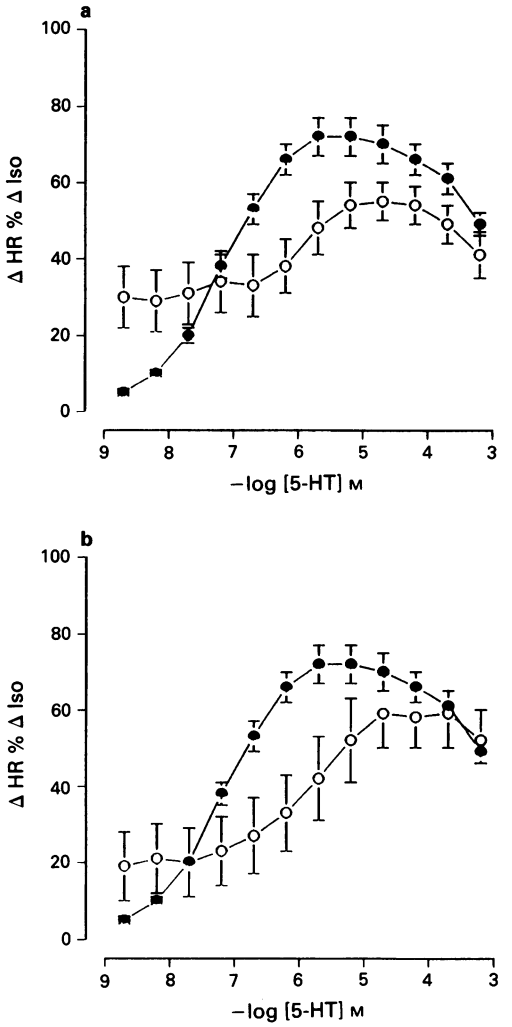

Figure 7 Concentration-effect curves to 5-HT in the absence (●, $n = 33$) and presence of (a) 0.5 μM renzapride (○, n = 6) and (b) 20 μM metoclopramide (○, $n = 4$) in piglet right atrium. Data are means ± s.e.mean. The chronotropic effects of 5-HT, renzapride and metoclopramide are expressed as a percentage of the increase in beating caused by agonist alone or in the presence of antagonist, plus 200 μM (−)-isoprenaline (Δ HR % Δ Iso).

Tropisetron (3 μM) also antagonized BIMU 8-induced increases in heart rate ($n = 4$) but the blockade was partially unsurmountable with up to 60 μM BIMU 8 (data not illustrated). Responses to tryptamine ($n = 4$) were completely abolished with 3 μM tropisetron (not shown).

DAU 6215 (0.1–10 μM) and SB 203186 (0.02–10 μM) caused parallel rightward shifts of concentration-effect curves to 5-HT (Figure 4a and b). The slopes of the Schild regression were not significantly different from unity and yielded estimated pK_B values of 7.0 and 8.3 respectively (Figure 5, Table 2). SDZ 205-557 (0.10–10 μM) also caused rightward displacement of the 5-HT curve (Figure 4c) but the slope of the Schild regression was only 0.58 (Figure 5) so a meaningful estimate of pK_B for SDZ 205-557 could not be made. Similarly, a meaningful pK_B estimate for GR113808 could not be made because although 0.01 and 0.1 μM shifted the 5-HT curve to the right, 0.1 μM also tended to reduce the maximum response to 5-HT and did not produce a significantly greater shift than 0.01 μM (Figure 4d). Since 0.002 μM GR113808 only caused marginal blockade and because of the lack of concentration-dependent antagonism with 0.01 and 0.1 μM, the resultant Schild plot was non-linear (Figure 5).

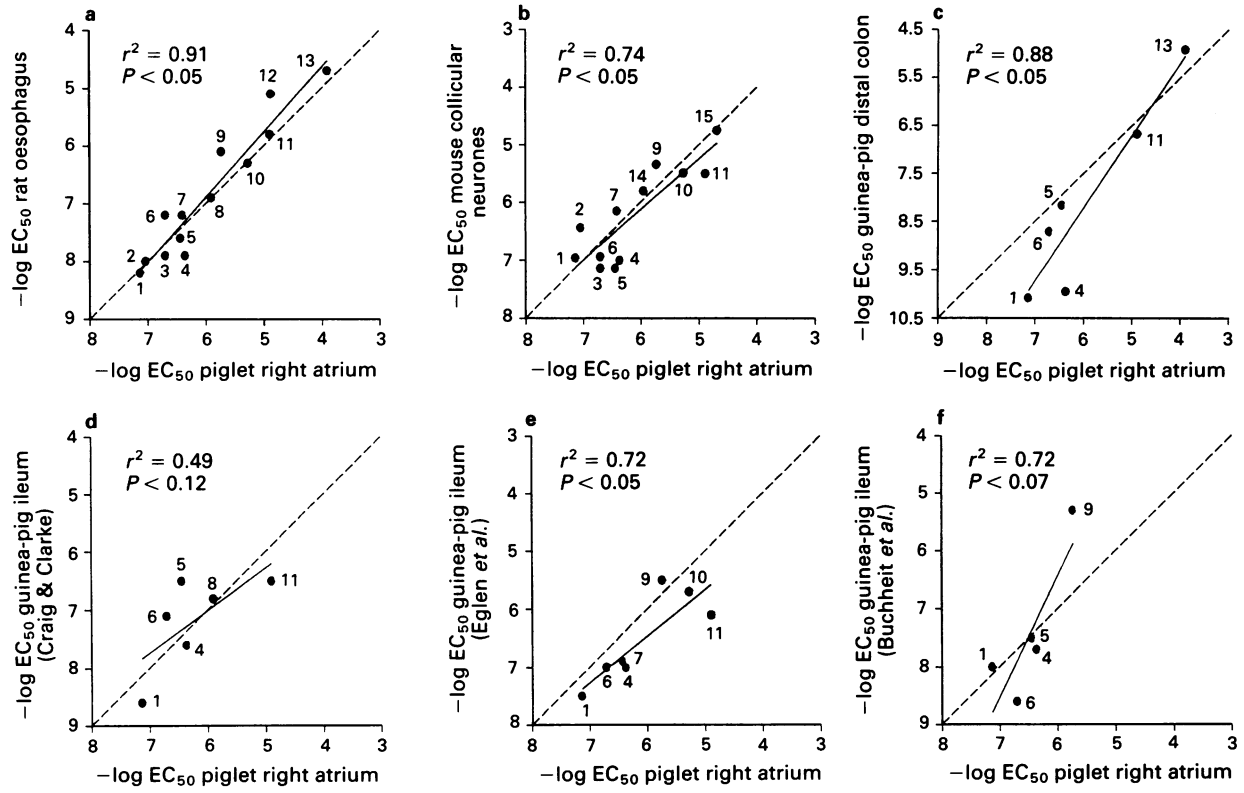

Figure 8 Squared correlation coefficients (r^2) and regression lines for 5-HT_4 receptor agonists in piglet right atrium and (a) rat oesophagus (Baxter *et al.*, 1991, Baxter & Clarke, 1992); (b) mouse embryonic colliculi neurones (Dumuis *et al.*, 1988; 1989; 1991); (c) guinea-pig distal colon (Wardle & Sanger, 1992); (d), (e) and (f) guinea-pig ileum (Craig & Clarke, 1990; Eglen *et al.*, 1990; Buchheit & Buhl, 1991, respectively); (1) 5-HT; (2) BIMU 1; (3) BIMU 8; (4) 5-MeOT; (5) cisapride; (6) renzapride; (7) (−)-zacopride, (8) α-methyl-5-HT; (9) metoclopramide; (10) (+)-zacopride; (11) 5-CT; (12) tryptamine; (13) 2-methyl-5-HT; (14) bufotenine, and (15), 5-MeO-*N*,*N*-DMT. For abbreviations, see text.

Partial agonist studies

BIMU 1 (Figure 6), renzapride (0.5 μM) and metoclopramide (20 μM) (Figure 7) also shifted 5-HT concentration-effect curves to the right. Since they had some agonist activity p*K*p values were estimated with equation (2) (Lemoine & Kaumann, 1982) although in some experiments, where they caused little or marginal stimulation, affinity values were estimated with equation (1) (Arunlakshana & Schild, 1959). The slope of the double log plot for BIMU 1 according to equations (1) and (2) was not significantly different from unity (Figure 6). The estimated p*K*p value for BIMU 1 was 7.9 (Table 2). p*K*p values were 7.9 for renzapride and 6.0 for metoclopramide as estimated from single concentrations (Table 2).

Discussion

The aim of our study was to compare piglet sinoatrial 5-HT_4 receptors (Kaumann, 1990) with 5-HT_4 receptors in other peripheral tissues and CNS. To exclude 5-HT_4 receptor-unrelated factors that determine agonist potency we used cocaine (which blocks tissue uptake of 5-HT thereby making more 5-HT available to the receptors, Kaumann *et al.*, 1990) and propranolol (which blocks β-adrenoceptors thereby preventing possible action of released noradrenaline, Kaumann, 1990). In some cases we also blocked monoamine oxidase (MAO).

The irreversible MAO inhibitor, pargyline, did not alter 5-HT-evoked responses which is consistent with the observations of Baxter *et al.* (1991). The responses to 5-MeOT, 5-CT and 5-MeO-N,N-DMT were also unaffected by pargyline. Conversely, no chronotropic responses were obtained with tryptamine unless pargyline was present, indicating a particular susceptibility of tryptamine to deamination by MAO. A similar effect of MAO inhibition was observed with tryptamine interacting with 5-HT_2 receptors of bovine large coronary artery (Frenken & Kaumann, 1988) indicating that the enzyme avidly oxidizes tryptamine in the neighbourhood of more than one class of 5-HT receptors.

pEC_{50} values were determined for tryptamine, benzamide and benzimidazolone derivatives for comparison with those obtained in rat oesophagus (Baxter *et al.*, 1991; Baxter & Clarke, 1992), guinea-pig ileum and colon (Craig & Clarke, 1990; Eglen *et al.*, 1990; Buchheit & Buhl, 1991; Wardle & Sanger, 1992) and mouse embryonic colliculi neurones (Dumuis *et al.*, 1988; 1989; 1991) (Figure 8). We assessed agonist potency in absolute terms as pEC_{50} values. This seemed the most relevant method of potency determination for this study, because the data we were using for comparison from other studies also used pEC_{50} values as a measure of agonist potency. Figure 8 shows there is excellent correlation between potency estimates for agonists in piglet atria and those in rat oesophagus and guinea-pig distal colon although the number of compounds compared is small for the latter. A weaker but still reasonable correlation is seen with piglet sinoatrial 5-HT_4 receptors and 5-HT_4 receptors in mouse embryonic colliculi neurones. There is borderline correlation between our data and the guinea-pig ileum data of Eglen *et al.* (1990) and Buchheit & Buhl (1991) whilst a poor correlation is seen with the data of Craig & Clarke (1990). This probably reflects a small sample size or that one drug is an outlier.

The relatively low stimulant potency of tryptamines and benzamides in piglet atria (Figure 8) suggests a relatively low 5-HT_4 receptor reserve or alternatively deficient coupling to biochemical effectors compared to guinea-pig ileum (Craig &

Clarke, 1990; Buchleit & Buhl, 1991) and distal colon (Wardle & Sanger, 1992) or rat oesophagus (Baxter *et al.*, 1991). The advent of a suitable 5-HT$_4$ receptor radioligand may reveal variations in 5-HT$_4$ receptor reserve in the different tissues. Indeed, preliminary autoradiographic studies with the novel 5-HT$_4$ receptor radioligand, [^{3}H]-GR113808, have revealed variations in specific binding to different brain regions of the rat and guinea-pig (Grossman *et al.*, 1993).

A number of tryptamine derivatives are full or partial agonists at 5-HT$_4$ receptors in the CNS (Dumuis *et al.*, 1988), gut (Craig & Clarke, 1990; Baxter *et al.*, 1991; Wardle & Sanger, 1992) and heart (Kaumann, 1990; Kaumann *et al.*, 1990; 1991a,b; Villalon *et al.*, 1990; 1991). We obtained the following order of potency for chronotropic activity: 5-HT > 5-MeOT > α-methyl-5-HT = bufotenine > 5-CT = tryptamine (after pargyline pretreatment) > 5-MeO-*N*,*N*-DMT > 2-methyl-5-HT. All tryptamines tested were full agonists compared to 5-HT except 5-CT, bufotenine and 2-methyl-5-HT.

Benzamides are key ligands for 5-HT$_4$ receptor characterization since they are more selective agonists than the tryptamines (although they can antagonize 5-HT$_3$ receptors). These compounds are usually partial agonists at 5-HT$_4$ receptors (Kaumann, 1990; Kaumann *et al.*, 1991a,b; Baxter *et al.*, 1991; Villalon *et al.*, 1990; 1991) although in mouse embryonic colliculi neurones their intrinsic activity often exceeds that of 5-HT (Dumuis *et al.*, 1989). Results from this study and of Kaumann (1990) in piglet right atria show that benzamides are partial agonists with low intrinsic activity except for (−)-zacopride which was a full agonist relative to 5-HT. The order of potency for these agents was renzapride > cisapride = (−)-zacopride > metoclopramide > (+)-zacopride. The similarity of the eudismic ratio for zacopride is consistent with similar steric configurations of the 5-HT$_4$ receptors in piglet, guinea-pig and rat. The stereoselectivity of the 5-HT$_4$ receptor to isomers of zacopride with a 10 fold potency separation between (−)-zacopride ($pEC_{50} = 6.41$) and (+)-zacopride ($pEC_{50} = 5.27$), is also a feature previously described in rat oesophagus (Baxter *et al.*, 1991) and guinea-pig ileum (Eglen *et al.*, 1990).

Because benzamides were partial agonists in piglet right atria, affinity estimates for renzapride and metoclopramide were obtained from experiments investigating antagonism of the chronotropic effects of 5-HT. The affinity estimate for renzapride ($pK_p = 7.9$) was greater than its stimulant potency estimate ($pEC_{50} = 6.7$) suggesting desensitization of 5-HT$_4$ receptors in addition to competition for the receptors. High renzapride concentrations have previously been shown to desensitize 5-HT$_4$ receptors (Dumuis *et al.*, 1989; Kaumann, 1990). In contrast, the affinity estimate for metoclopramide was similar to its potency estimate suggesting lack of desensitization and supporting the observations of Bockaert *et al.* (1992b) who showed desensitization with renzapride but not with metoclopramide. They proposed that 5-HT$_4$ receptor desensitization was proportional to agonist affinity.

Azabicycloalkyl benzimidazolones have also proved to be useful ligands for characterizing 5-HT$_4$ receptors (reviewed by Turconi *et al.*, 1991). BIMU 8 and BIMU 1 are potent full or partial agonists at the 5-HT$_4$ receptor in mouse colliculi (Dumuis *et al.*, 1991), rat oesophagus (Baxter & Clarke, 1992), and guinea-pig ileum (Rizzi *et al.*, 1992). Similarly, in the present study with piglet atria, BIMU 8 was a full agonist whilst BIMU 1 was a partial agonist relative to 5-HT. BIMU 1 was similar in potency to 5-HT but more potent than BIMU 8 as determined from the corresponding pEC_{50} values. We obtained an affinity estimate from experiments where the 5-HT concentration-effect curve was shifted to the right by BIMU 1. As with renzapride, desensitization seemed to be present since the affinity estimate ($pK_p \simeq 7.9$) for BIMU 1 was greater than the potency estimate ($pEC_{50} \simeq 7.1$), and at high concentrations fade of the BIMU 1-induced responses occurred.

Another benzimidazolone derivative, DAU 6215 was reported to be a weak partial agonist in mouse embryonic colliculi neurones (Dumuis *et al.*, 1991) but a competitive antagonist in rat oesophagus (Baxter & Clarke, 1992). In piglet right atria we found no chronotropic activity with DAU 6215 which at high concentrations even decreased the heart rate. DAU 6215 behaved as a simple competitive antagonist at sinoatrial 5-HT$_4$ receptors with a pK_B of 7.1 yielded from the Schild plot. This figure compared favourably with a pA_2 value of 6.7 in rat oesophagus (Baxter & Clarke, 1992), but was rather higher than the value of 5.5 estimated in mouse embryonic colliculi neurones by Dumuis *et al.* (1991).

Buchheit *et al.* (1992) recently introduced SDZ 205-557 as a 30 fold 5-HT$_4$ receptor selective (compared to 5-HT$_3$) antagonist of 5-HT-evoked contractions in guinea-pig ileum with an affinity of 7.4 whilst Eglen *et al.* (1991) showed that SDZ 205-557 had a pK_B of 7.1 in rat oesophagus. In piglet right atria SDZ 205-557 also antagonized 5-HT-induced tachycardia but this antagonism did not appear to be simple competitive. This may partially reflect a non-specific effect of this drug. We were unable to calculate a meaningful pK_B estimate since the slope of the Schild plot was 0.58.

GR113808 was recently presented as a novel 5-HT$_4$ receptor antagonist with a pA_2 of 9.2 and 9.5 in guinea-pig colon and rat oesophagus respectively (Grossman *et al.*, 1993). On piglet atrium, 2 nM GR113808 caused marginal blockade (Figures 4d and 5) and appeared to be less potent than on gut (Grossman *et al.*, 1993). In the present study we were unable to obtain a pK_B estimate since the Schild plot slope was non-linear, suggesting that the blockade was not simple competitive. There was a reduction in the maximum response to 5-HT with 0.1 μM GR113808 in our experiments, a phenomenon reported in the guinea-pig colon at the same concentration (Grossman *et al.*, 1993). This suggests a non-specific effect of GR113808 in these systems. The novel selective 5-HT$_4$ receptor blocker SB 203186 (Kaumann *et al.*, 1992) was the most potent competitive antagonist investigated in the present study. We obtained a pK_B of 8.3 for SB 203186, 20 fold or more potent than tropisetron and DAU 6215. Similar affinity estimates for SB 203186 have been obtained in piglet left atrium and human atrial strips (Parker *et al.*, 1993) where 5-HT$_4$ receptors have also been demonstrated (Kaumann *et al.*, 1990; 1991a,b).

In conclusion, we have shown that 5-HT$_4$ receptors in piglet right atria are sensitive to the three groups of agonists used to characterize this receptor, as well as being blocked by a number of antagonists reported to be selective for 5-HT$_4$ receptors in the literature. We suggest that piglet sinoatrial 5-HT$_4$ receptors are similar to 5-HT$_4$ receptors of rat oesophagus, guinea-pig ileum and colon, mouse embryonic colliculi neurones and human atrium.

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Cardiovascular effects of SCA40, a novel potassium channel opener, in rats

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1 Experiments have been performed to investigate the cardiovascular actions in the rat of SCA40, a novel potassium channel opener which is a potent relaxant of guinea-pig airway smooth muscle *in vivo* and *in vitro*.

2 SCA40 (0.01–30 μM) caused a complete and concentration-dependent relaxation of rat isolated thoracic aorta contracted with 20 mM KCl but failed to inhibit completely the spasmogenic effects of 80 mM KCl.

3 The ATP-sensitive K^+-channel blocker, glibenclamide (3 μM), failed to antagonize the relaxant action of SCA40 on 20 mM KCl-contracted rat isolated thoracic aorta.

4 SCA40 (0.001–100 μM) had dual effects on rat isolated atria. At low concentrations, SCA40 produced a concentration-dependent decrease in the rate and force of contractions. At higher concentrations (greater than 1 μM) SCA40 induced concentration-dependent increases of atrial rate and force.

5 *In vivo*, in normotensive Wistar rats, SCA40 elicited a dose-dependent (1–100 μg kg^{-1}) decrease in mean arterial pressure which was accompanied by a moderate dose-dependent increase in heart rate. SCA40 (100 μg kg^{-1}) had a slightly greater hypotensive effect than cromakalim (100 μg kg^{-1}) but the duration of the hypotension was longer with cromakalim than with SCA40.

6 The hypotensive effect of SCA40 was not reduced by propranolol, atropine, N^G-nitro-L-arginine methyl ester (L-NAME) or glibenclamide.

7 It is concluded that the mechanism by which SCA40 relaxes vascular smooth muscle *in vitro* and *in vivo* involves activation of K^+-channels distinct from glibenclamide-sensitive ATP-sensitive K^+-channels.

Keywords: Rat thoracic aorta; smooth muscle relaxation; SCA40; potassium channels; hypotensive activity

Introduction

SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carbonitrile) is a newly synthesized imidazopyrazine derivative possessing potent smooth muscle relaxant activity *in vitro* in guinea-pig isolated trachealis and potent anti-bronchospastic activity *in vivo*. Its weak cyclic AMP phosphodiesterase inhibitory activity only partially explains these relaxant properties (Bonnet *et al.*, 1992). Since SCA40 failed to inhibit completely the spasmogenic effects of 80 mM KCl in guinea-pig isolated trachealis, potassium channel opening properties have been proposed for it (Laurent *et al.*, 1993). SCA40 relaxant activity in guinea-pig isolated trachealis was not blocked by the ATP-sensitive K^+-channel blocker glibenclamide but was antagonized by charybdotoxin (ChTX), a purified peptide toxin present in *Leiurus quinquestriatus* venom, which has been found to block large-conductance Ca^{2+}-dependent K^+-channels in a variety of cells (Castle *et al.*, 1989). As opposed to potassium channel openers such as cromakalim, the relaxant activity of SCA40 does not involve ATP-sensitive K^+-channels, rather it appears to activate ChTX-sensitive K^+-channels such as large-conductance Ca^{2+}-activated K^+-channels.

ATP-sensitive K^+-channel openers, such as cromakalim, pinacidil and nicorandil have been shown to possess vascular smooth muscle relaxant and antihypertensive properties (Richer *et al.*, 1990). It has been proposed that potassium channel openers induce hyperpolarization of the smooth muscle cell membrane, which in turn reduces entry through voltage-sensitive channels of cytosolic calcium leading to vasorelaxation (Quast & Cook, 1989).

Recently evidence has been obtained for the involvement of Ca^{2+}-activated K^+-channels in the regulation of arterial tone. Small and large-conductance Ca^{2+}-activated K^+-channels have been identified in vascular smooth muscle cells from different species: bovine (Vazquez *et al.*, 1989); rabbit (Inoue *et al.*, 1986); guinea-pig (Benham *et al.*, 1986); and, rat (Van Renterghem & Lazdunski, 1992). Brayden & Nelson (1992) reported that TEA and ChTX were able to depolarize and constrict pressurized rabbit cerebral arteries. They concluded that the activation of Ca^{2+}-activated K^+-channels could lead to vasodilatation. Rusch *et al.* (1992) showed that a Ca^{2+}-activated K^+-current was enhanced in arterial membranes from genetic and experimental models of hypertensive rats. Asano *et al.* (1993) showed that ChTX-sensitive Ca^{2+}-activated K^+-channels were highly activated in arteries from spontaneously hypertensive rats (SHR) as compared to normotensive rats. All these findings suggest that activation of ChTX-sensitive Ca^{2+}-activated K^+-channels may be an important mechanism that regulates the myogenic tone, particularly in SHR arteries.

ATP-sensitive K^+-channel openers reduce the duration of the myocardial action potential in ventricular and atrial cells leading to negative inotropic activity (Shigenobu *et al.*, 1991). *In vivo*, ATP-sensitive K^+-channel openers lowered blood pressure and caused reflex tachycardia (Richer *et al.*, 1990). *In vitro*, ATP-sensitive K^+-channel openers have been shown to suppress spontaneous and oscillatory activities in isolated cardiac Purkinje fibres (Steinberg *et al.*, 1988) and to produce a negative chronotropic response in a dog heart preparation (Murakami *et al.*, 1992). On the other hand, an arrhythmogenic effect of ATP-sensitive K^+-channel openers has been postulated (Steinberg *et al.*, 1988).

The aim of the present study was to examine the effects of SCA40 *in vitro* in rat thoracic aorta and atria and to examine

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the cardiovascular properties of SCA40 in normotensive rats *in vivo*.

Methods

Effects of SCA40 against tone induced by KCl in rat thoracic aorta

Male Wistar rats (Iffa Credo, Lyon, France) weighing 300–350 g, were killed by a blow to the head and the thoracic aorta rapidly removed. Each aorta was cut into 4 rings, each 3–4 mm in length. Two stainless steel wire hooks were passed through the lumen of each ring. One wire was attached to the base of a 40 ml tissue bath and the other one to an isometric myograph transducer connected to a Physiograph Narco Bio-system. Tissues were suspended in a Chenoweth Koelle buffer. At the outset of each experiment, tissues were subjected to an applied tension of 0.5 g and allowed to equilibrate for 30 min during which time they were washed every 5 min. KCl (20 mM or 80 mM) induced contractions which reached stable maxima within 5 min. Cumulative log concentration-response curves to SCA40 were determined for aortic rings contracted with KCl (20 or 80 mM) taking the intensities of the initial contractions as 100%. Then, cumulative log concentration-response curves were determined for the relaxant action of SCA40 in aortic rings contracted with KCl (20 mM) in the absence (control) or in the presence of glibenclamide (3 μM). Relaxant responses were expressed as the percentage reduction in KCl-induced contraction. Relaxant potency was expressed as the negative log EC_{50}, where EC_{50} is the concentration producing 50% inhibition of the contraction. The EC_{50} values were calculated by linear regression analysis applied to the linear portion of each dose-response curve.

Rat isolated atria studies

Male Wistar rats were killed by a blow on the head and the heart was rapidly removed and placed in a beaker containing oxygenated Chenoweth-Koelle solution. Right and left atria were then removed and mounted in 40 ml organ baths filled with Chenoweth-Koelle solution. Changes in tension were measured isometrically with a myograph transducer connected to a Physiograph Narco Bio-system. The right atria were allowed to beat spontaneously, while left atria were paced at a frequency of 1.6 Hz (pulse duration of 5 ms and a voltage twice the threshold). After a 45 min equilibration period, the basal tension was adjusted to 1 g, right atria were used to measure the effects of drugs on rate, and left atria to measure the effects on tension. Cumulative concentration-response curves to SCA40 were determined. SCA40 effects were measured as differences in developed tension or rate from basal activity. Results are expressed as percentage variation from basal values.

Blood pressure studies in rats

Normotensive male Wistar rats weighing 300–350 g, fed with UAR A04 diet and fasted 18 h prior to the experiment, were used. Rats were anaesthetized with ethylurethane (1.2 g kg^{-1}, i.p.) and were maintained at a body temperature of 37°C. The left common artery and the tail vein were cannulated for the measurement of blood pressurre and the intravenous administration of drugs respectively. A Narco Bio-system pressure transducer was used to record the mean arterial pressure (MAP) and heart rate (HR) was derived from the arterial pulse signal. Following a stabilization period of 30 min, MAP and HR were recorded. SCA40 was injected intravenously in increasing doses (1, 3, 10, 30, 100 μg kg^{-1}). Blood pressure and heart rate were allowed to return to baseline between each SCA40 dose.

In a second set of experiments, the time-course of the blood pressure responses to SCA40 and cromakalim were evaluated after i.v. administration (doses of 100 μg kg^{-1} for each drug).

In another series of experiments, the effects of SCA40 (10 μg kg^{-1}) on rat MAP were evaluated before and after i.v. administration of specific drugs: propranolol (1 mg kg^{-1}); atropine (1 mg kg^{-1}); N^G-nitro-L-arginine methyl ester (L-NAME, 20 mg kg^{-1}); and, glibenclamide (20 mg kg^{-1}). These drugs were injected 15 min prior to the second administration of SCA40. β-Adrenoceptor and muscarinic cholinoceptor receptor blockade was assessed by i.v. administration of isoprenaline (1 μg kg^{-1}) and acetylcholine (2 μg kg^{-1}), respectively. ATP-sensitive potassium channel blockade was assessed by i.v. administration of cromakalim (75 μg kg^{-1}).

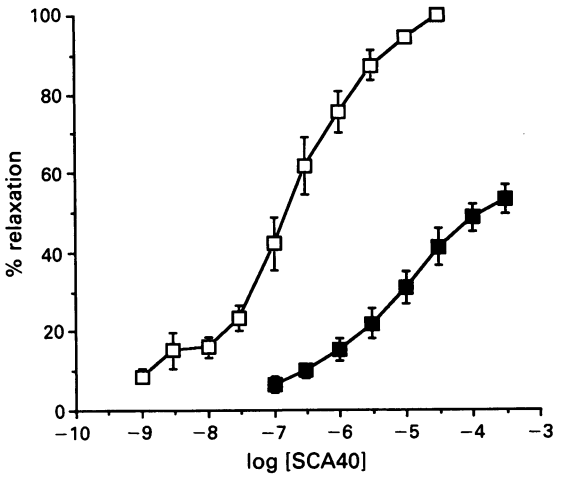

Figure 1 Rat isolated thoracic aorta: relaxant activity of SCA40 against established contraction to KCl 20 mM (□) and KCl 80 mM (■). Abscissa scale: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl. Each point is the mean ± s.e.mean derived from at least 6 experiments.

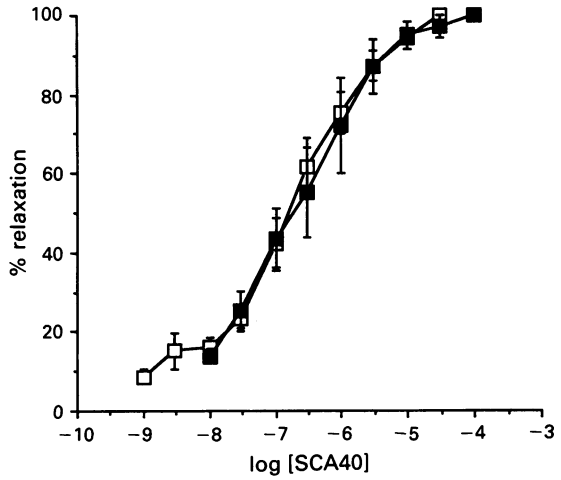

Figure 2 Rat isolated thoracic aorta: relaxant activity of SCA40 against established contraction to KCl 20 mM in absence (□) or in presence of glibenclamide 3 μM (■). Abscissa scale: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl 20 mM. Each point is the mean ± s.e.mean derived from at least 6 experiments.

Statistical evaluation of results

Statistical evaluation of the results was assessed by use of a two-tailed, unpaired *t* test. The null hypothesis was rejected when $P<0.05$.

Drugs and solutions

The substances used were obtained from the following sources: SCA40 was synthesized as already described (Bonnet *et al.*, 1992). (±)-Isoprenaline, propranolol, N^G-nitro-L-arginine methyl ester (L-NAME), acetylcholine, atropine and glibenclamide: (Sigma Chemicals (U.S.A.); cromakalim was a gift from Sanofi Laboratories (France); KCl, ethyl-carbamate (urethane) were from Prolabo (France).

For *in vivo* experiments, isoprenaline, L-NAME, propranolol, acetylcholine, atropine were dissolved in isotonic saline. SCA40, cromakalim and glibenclamide were dissolved in ethanol. Further dilutions of SCA40 and cromakalim were made in isotonic saline.

For *in vitro* experiments, 20 mM stock solution of SCA40 and glibenclamide were made up in ethanol. Further dilutions were made up in distilled water.

The Chenoweth-Koelle solution used in the tissue bath experiments had the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2$ 2.4, $MgCl_2$ 2.2, $NaHCO_3$ 15 and glucose 10. This solution was maintained at 37°C and gassed continuously with a mixture of 95% O_2, 5% CO_2.

Results

Rat thoracic aorta studies

Cumulative concentration-response curves to SCA40 on rat isolated thoracic aorta precontracted with 20 and 80 mM KCl are shown in Figure 1. SCA40 produced concentration-dependent inhibition of the response to 20 mM KCl, full relaxation of the KCl contraction being produced by 30 μM SCA40. When aortic preparations were contracted with 80 mM KCl, the maximum relaxation produced by SCA40 corresponded to approximately 50% of the maximum relaxation that could be achieved against 20 mM KCl-induced contraction. Moreover, the relaxation concentration-response curve to SCA40 against 80 mM KCl-induced contraction was shifted to the right approximately 1 000 fold compared with SCA40 relaxant activity against 20 mM KCl ($-\log EC_{50} = 6.86 \pm 0.10$ and 3.77 ± 0.09 respectively). In the presence of glibenclamide 3 μM (Figure 2), the relaxation concentration-response curve to SCA40 against 20 mM KCl-induced contraction was not modified with respect to the maximum response or location ($-\log EC_{50} = 6.86 \pm 0.10$ and 6.68 ± 0.12, in absence and presence of 3 μM glibenclamide respectively).

In vitro *chronotropic and inotropic activity of SCA40*

Cumulative concentration-responses curves on tension and rate of beating of rat isolated atria are shown in Figure 3. SCA40 produced dual effects in rat isolated atria. At low concentrations (0.001–1 μM), SCA40 exhibited a dose-dependent decrease in the rate of contraction (up to 28%). Similarly, SCA40, at low concentrations, produced a dose-dependent decrease in the force of contraction (up to 34%). At higher doses (>1 μM) SCA40 induced a dose-dependent increase of the beating frequency and contractile force such that 100 μM SCA40 exhibited positive chronotropic and inotropic activities.

Blood pressure studies in rats

Following the stabilization period of 30 min, the baseline mean arterial pressure (MAP) was between 95 and 120 mmHg and the baseline heart rate (HR) between 300 and 400 beats min^{-1}. The effects of SCA40 (1–100 μg kg^{-1}, i.v.) in anaesthetized normotensive rats are shown in Figure 4. SCA40 elicited a potent dose-dependent (1–100 μg kg^{-1}) decrease in MAP. The reduction in MAP was accompanied by a moderate dose-dependent increase in HR (less than 30 beats min^{-1} at 100 μg kg^{-1}). At lower doses (1 to 10 μg kg^{-1}), the reduction of MAP was maximal 10 s after administration of SCA40 and then returned to basal value within 1 min. At higher doses (30 and 100 μg kg^{-1}) MAP returned slowly, over 20 to 30 min, to the baseline level.

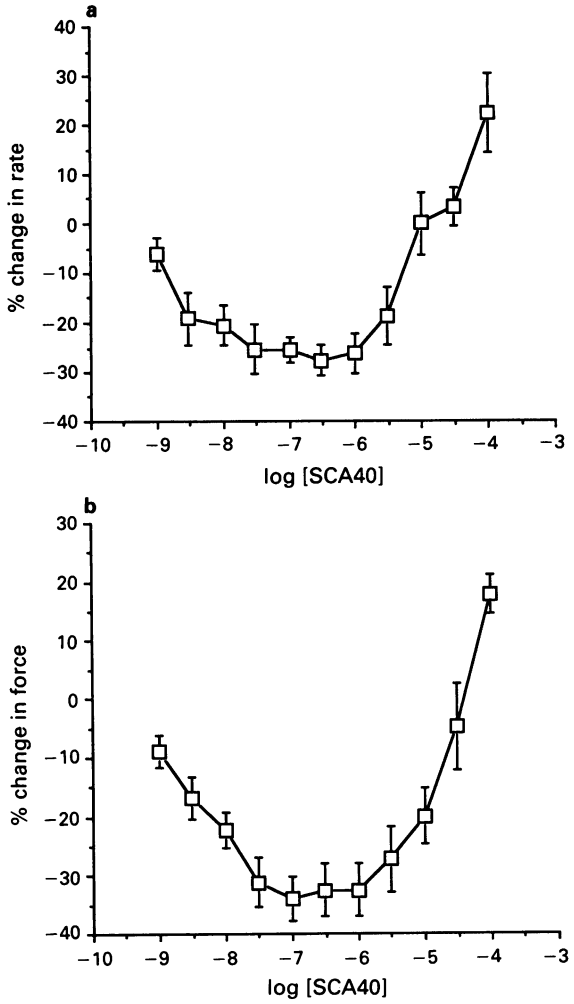

Figure 3 Concentration-response curves for the effects of SCA40 on: (a) rate and (b) contractile force of rat isolated atria. Abscissae scale: – log molar concentration of SCA40. Ordinate scales: change in rate and force expressed as a percentage of baseline values. Each point is the mean ± s.e.mean derived from 4 to 6 experiments.

Figure 5 shows the time course of the effects of i.v. administration of 100 μg kg^{-1} SCA40 and cromakalim on MAP. The fall in MAP produced by SCA40 was slightly greater than that induced by cromakalim but the hypotension lasted longer with cromakalim than with SCA40.

The effects of specific drugs on the decrease in blood pressure produced by SCA40 (10 μg kg^{-1}) are presented in Table 1. Intravenous injection of the β-adrenoceptor antagonist propranolol (1 mg kg^{-1}) had no significant effect on the SCA40 pressure response but caused a significant reduction of the isoprenaline-induced (1 μg kg^{-1}) blood pressure decrease. Atropine (1 mg kg^{-1}) also caused no attenuation of the SCA40 pressure response although muscarinic cholinoceptors were blocked as assessed by the significant decrease of the acetylcholine-evoked (2 μg kg^{-1}) pressor response. L-NAME (10 mg kg^{-1}) elicited an increase in MAP from 102.8 ± 10.4 to 153.5 ± 11.2 mmHg. After L-NAME the decreases in MAP induced by both acetylcholine (2 μg kg^{-1}) or SCA40 were enhanced. The fall in blood pressure due to SCA40 was not affected by glibenclamide (20 mg kg^{-1}) while the cromakalim-induced (75 mg kg^{-1}) decrease in blood pressure was significantly reduced by glibenclamide.

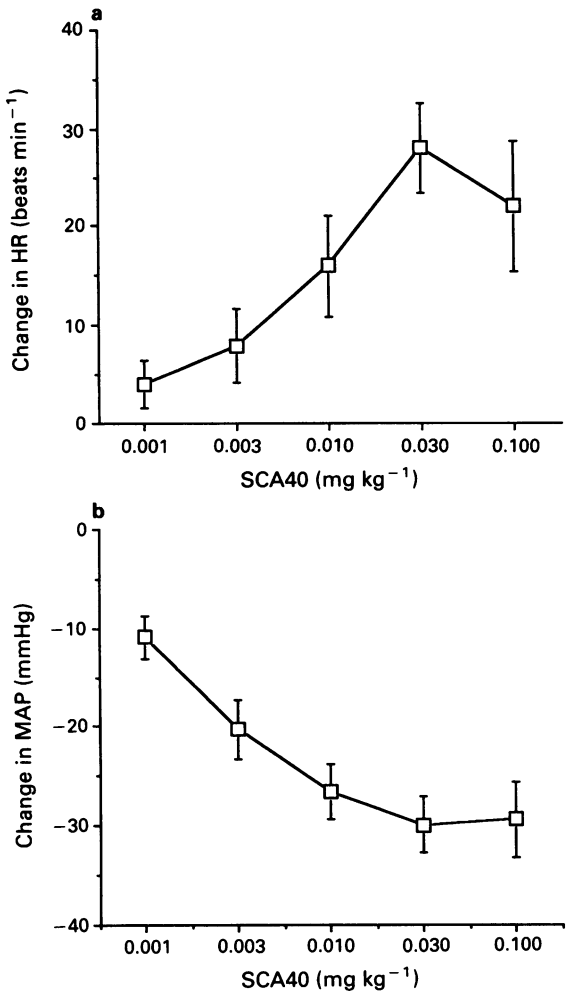

Figure 4 Dose-response curves for the effects of SCA40 on: (a) heart rate (HR) and (b) mean arterial pressure (MAP) in normotensive anaesthetized Wistar rats. Abscissa scale: i.v. doses of SCA40 (mg kg^{-1}, log scale). Ordinate scales: (a) change from baseline in HR (beats min^{-1}); (b) change from baseline in MAP (mmHg). Each point is the mean ± s.e.mean derived from 4 to 6 experiments.

Discussion

SCA40 is a newly synthesized imidazo[1,2-a]pyrazine derivative which exhibits potent smooth muscle relaxant properties *in vitro*, potent anti-bronchospasmic activity *in vivo* and moderate cyclic AMP phosphodiesterase inhibitory activity (Bonnet *et al.*, 1992). However, increased cyclic AMP formation due to the inhibition of cyclic AMP phosphodiesterase cannot totally explain the potent SCA40 smooth muscle relaxant activity (Bonnet *et al.*, 1992). In guinea-pig isolated trachea, SCA40 was able to inhibit completely the contractions induced by a low concentration of KCl (20 mM); in contrast, contractions induced by 80 mM KCl were only partially inhibited by SCA40 (Laurent *et al.*, 1993). Such a pharmacological profile has been described for K^+-channel openers (Hamilton *et al.*, 1986; Robertson & Steinberg, 1990). With high K^+ concentrations the potassium equilibrium potential is such that the hyperpolarization induced by K^+-channel openers is too weak to close voltage-operated Ca^{2+}-channels. The relaxant activity of SCA40 in guinea-pig

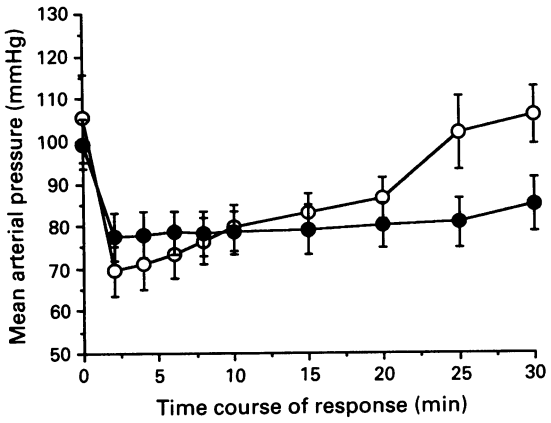

Figure 5 Time course of the effects of SCA40 100 μg kg^{-1} (○) and cromakalim 100 μg kg^{-1} (●) on mean arterial blood pressure (MAP) measured in anaesthetized normotensive Wistar rats after i.v. administration. Each point is the mean ± s.e.mean derived from at least 4 experiments.

Table 1 Effects of various antagonists (propranolol, 10 mg kg^{-1}; atropine, 1 mg kg^{-1}; N^G-nitro-L-arginine methyl ester (L-NAME) 10 mg kg^{-1}; glibenclamide, 20 mg kg^{-1}) on mean arterial pressor (MAP) responses evoked by i.v. administration of SCA40 (10 μg kg^{-1}), isoprenaline (1 μg kg^{-1}), acetylcholine (2 μg kg^{-1}) and cromakalim (75 μg kg^{-1}) in anaesthetized, normotensive rats

Pretreatment	*Agonist*	*Initial MAP* (mmHg)	*MAP change* (mmHg)	*% change*
Group 1				
Vehicle	SCA40	112.9 ± 7.0	− 30.1 ± 2.9	− 26.8 ± 2.6
Group 2				
Vehicle	Isoprenaline	92.8 ± 5.5	− 35.9 ± 3.8	− 38.7 ± 3.6
Propranolol	Isoprenaline	112.5 ± 6.3	− 8.2 ± 1.2[b]	− 7.3 ± 1.2[b]
	SCA40	95.8 ± 7.9	− 30.8 ± 5.9	− 31.5 ± 4.4
Group 3				
Vehicle	Acetylcholine	106.4 ± 10.5	− 34.7 ± 4.5	− 33.1 ± 1.8
Atropine	Acetylcholine	109.1 ± 16.6	− 10.7 ± 1.8[b]	− 9.8 ± 0.3[b]
	SCA40	100.7 ± 9.8	− 28.9 ± 5.4	− 28.1 ± 2.5
Group 4				
Vehicle	Acetylcholine	102.8 ± 10.4	− 37.7 ± 4.4	− 36.7 ± 1.8
L-NAME	Acetylcholine	153.4 ± 11.2	− 75.4 ± 6.4[b]	− 49.3 ± 3.3[b]
	SCA40	145.0 ± 13.3	− 62.2 ± 9.1[a]	− 42.1 ± 3.9[a]
Group 5				
Vehicle	Cromakalim	88.6 ± 7.9	− 25.6 ± 4.3	− 28.2 ± 2.7
Glibenclamide	Cromakalim	98.0 ± 8.4	− 8.7 ± 1.7[b]	− 8.7 ± 1.5[b]
	SCA40	109.6 ± 6.6	− 27.4 ± 2.8	− 25.2 ± 2.5

Rats were divided into 5 groups and each antagonist was injected i.v. to each rat 15 min before SCA40 injection. Acetylcholine, isoprenaline and cromakalim were injected 5 min before and 15 min after the antagonist administration. Each value represents the mean ± s.e.mean of four to six animals.

[a]Indicates a significant difference from the value in group 1, SCA40 alone (two-tailed unpaired *t* test); [b]indicates a significant difference from the corresponding values in the absence of each antagonist (two-tailed unpaired *t* test).

trachea was antagonized by charybdotoxin (ChTX) but not by glibenclamide, which suggested that the relaxant activity of SCA40 does not involve ATP-sensitive K^+-channels but rather large-conductance Ca^{2+}-activated K^+-channels or other ChTX-sensitive K^+-channels (Laurent *et al.*, 1993). In rat isolated thoracic aorta, SCA40 exhibited a similar profile. SCA40 was able to inhibit completely the contractions induced by low concentrations of KCl (20 mM) as opposed to high concentrations (80 mM) of KCl. As in guinea-pig isolated trachealis tissue, SCA40, at high concentrations (10–100 μM), retained some relaxant activity against the spasm induced by 80 mM KCl (50% of the maximum relaxation that could be achieved against 20 mM KCl-induced contraction). This relaxant activity of SCA40 at high concentration might be attributed to its cyclic AMP phosphodiesterase inhibitory properties. The relaxant activity of SCA40 in thoracic aorta was not antagonized by glibenclamide which suggests that the relaxant activity of SCA40 in vascular tissue, as in trachealis tissue, does not involve ATP-sensitive K^+-channels.

ATP-sensitive K^+-channel openers directly induce negative chronotropic and inotropic responses in heart preparations (Yanagisawa *et al.*, 1988; 1989; Murakami *et al.*, 1992) but little is known about the role of the large-conductance Ca^{2+}-activated K^+-channels or other ChTX-sensitive K^+-channels in heart, since no activators of these channels have yet been developed. In the present study, SCA40 produced dual effects on rat isolated atria. At low concentrations, SCA40 induced dose-dependent negative chronotropic and inotropic responses. ATP-sensitive K^+-channel openers have been shown to shorten the action potential in cardiac muscle and thereby produce negative inotropic responses (Shinegobu *et al.*, 1991). Due to its K^+-channel opener properties, SCA40 might increase outward potassium currents in cardiac cells, which might explain negative inotropic effects. In our experiments, SCA40 did not reduce the force of atrial contractions below 35% of the basal force. Thus, the maximal negative inotropic effects of SCA40 appeared to be less than those of ATP-sensitive K^+-channel openers since these compounds have been shown to reduce contractile force in cardiac muscle from guinea-pig and dog by 70 to 90% (Yanagisawa *et al.*, 1988; 1989; Shinegobu *et al.*, 1991; Murakami *et al.*, 1992). If the negative inotropic and chronotropic activities of SCA40 can be attributed to activation of ChTX-sensitive K^+-channels, these results suggest that ChTX-sensitive K^+-channels are present in sinoatrial pacemaker cells and in atrial cells and the channels might be involved in the pacemaker and contractile activities, but to a smaller extent than ATP-sensitive K^+-channels. At higher concentrations (> 1 μM) SCA40 induced a dose-dependent increase of the sinus rate and atrial contractility. The positive chronotropic and inotropic activities observed at high concentrations might be due to cyclic AMP phosphodiesterase inhibitory activity of SCA40.

In normotensive male Wistar rats, SCA40 displayed a dose-dependent (1–100 $\mu g\,kg^{-1}$) decrease in MAP after i.v. administration. The hypotensive action of SCA40 is consistent with the smooth muscle relaxant activity exhibited *in vitro* by this new potassium channel activator. The hypotensive effect of 100 $\mu g\,kg^{-1}$ SCA40 was slightly greater but shorter in duration than that of 100 $\mu g\,kg^{-1}$ cromakalim.

The hypotensive action of SCA40 was accompanied by a moderate dose-dependent increase in HR. The tachycardia induced by i.v. administration of SCA40 was abolished by prior administration of the β-adrenoceptor blocker, propranolol (data not shown) without affecting the hypotensive response, suggesting this to be a reflex effect rather than a direct action of SCA40 on the heart. Similar results have been reported for ATP-sensitive K^+-channel openers (Cook & Hof, 1988; Pacioreck *et al.*, 1990).

The hypotensive response induced by SCA40 (10 $\mu g\,kg^{-1}$) was not abolished by prior administration of the muscarinic cholinoceptor blocker, atropine (1 $mg\,kg^{-1}$) or the β-adrenoceptor antagonist, propranolol (1 $mg\,kg^{-1}$). These results suggest that the hypotensive response induced by SCA40 is not mediated by muscarinic cholinoceptor or β-adrenoceptor activation.

L-NAME (10 $mg\,kg^{-1}$) induced a large increase in MAP. Such a result has already been reported in rats (Van Gelderen *et al.*, 1991). Following administration of L-NAME, the fall in MAP induced by acetylcholine was increased. Van Gelderen *et al.* (1991) reported similar results in anaesthetized rats and concluded that the hypotensive response to acetylcholine in rat is largely independent of the arginine-NO pathway. The hypotensive response to SCA40 was also increased in the presence of L-NAME, indicating that the hypotensive response to SCA40 is also largely independent of the arginine-NO pathway. The hypotensive effects of SCA40 were not modified by prior i.v. administration of glibenclamide (20 $mg\,kg^{-1}$), whereas, in the same dose, glibenclamide significantly inhibited the fall in blood pressure induced by cromakalim. These results suggest that the hypotensive activity of SCA40 is not mediated by the same mechanisms as that of cromakalim and consequently, does not involve ATP-sensitive potassium channels.

The present study shows that SCA40, a novel potassium channel opener which has been shown to be a potent relaxant of guinea-pig airway smooth muscle *in vitro* and *in vivo*, is also a potent vascular smooth muscle relaxant *in vitro* and *in vivo*. As in tracheal tissue, the vascular smooth muscle relaxant activity of SCA40 does not involve ATP-sensitive K^+-channels.

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Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier

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1 Smooth muscle cells of the rat portal vein were dispersed by enzymatic treatment and recordings of whole-cell currents under calcium-free conditions were made by the voltage-clamp technique. The effects of the potassium (K)-channel opener, levcromakalim, on K-currents were compared with those of agents which modify protein phosphorylation.

2 Levcromakalim (1–10 μM) added to the extracellular (bath) fluid caused the development of a non-inactivating current ($I_{K(ATP)}$) and simultaneously inhibited the delayed rectifier current ($I_{K(V)}$) in a concentration-dependent manner. On prolonged exposure to levcromakalim (10 μM), $I_{K(ATP)}$ declined and $I_{K(V)}$ was further diminished.

3 Addition to the pipette (intracellular) solution of the selective inhibitor of protein kinase C, calphostin C, itself had no effect on K-currents and did not modify the induction of $I_{K(ATP)}$ or the simultaneous inhibition of $I_{K(V)}$ produced by 1 μM levcromakalim.

4 Addition of the protein kinase inhibitor (PKI(6-22)amide, 1 μM) to the pipette solution caused the production of a glibenclamide-sensitive, non-inactivating current and inhibited $I_{K(V)}$.

5 In an assay system, levcromakalim (10 μM) did not inhibit the activity of purified protein kinase A (Type 1 or Type 2).

6 Addition to the pipette solution of the phosphatase inhibitor, okadaic acid (1 μM), did not itself modify K-currents and had little effect on the simultaneous induction of $I_{K(ATP)}$ and inhibition of $I_{K(V)}$ by levcromakalim (1 μM).

7 When the pipette solution contained 1 mM MgATP (but was depleted of substrates for ATP production), a non-inactivating, glibenclamide-sensitive K-current developed spontaneously in 5 out of 11 cells with the simultaneous reduction of $I_{K(V)}$. In 3 of the 6 remaining cells, addition of the dephosphorylating agent, butanedione monoxime (5 mM) to the bath inhibited $I_{K(V)}$ and stimulated a glibenclamide-sensitive non-inactivating current.

8 Depletion of intracellular Mg^{2+} slightly enhanced $I_{K(V)}$. Under these conditions, levcromakalim (1 μM and 10 μM) did not significantly induce $I_{K(ATP)}$ or inhibit $I_{K(V)}$.

9 It is concluded that the effects of levcromakalim on K-currents can be mimicked by procedures designed to reduce channel phosphorylation. The results are consistent with the view that levcromakalim dephosphorylates the delayed rectifier channel, K_V, which becomes converted into a voltage-independent, non-inactivating form known as K_{ATP}. The possible mechanisms which underlie this interconversion are discussed.

Keywords: Levcromakalim; protein kinase C; protein kinase A; whole-cell voltage clamp; smooth muscle; butanedione monoxime; calphostin C; PKI(6-22)amide; delayed rectifier (K_V); ATP-sensitive K-channel (K_{ATP})

Introduction

K-channel openers such as levcromakalim, nicorandil, pinacidil and P1060 induce a voltage-independent, non-inactivating K-current in vascular smooth muscle. This current is thought to be carried by ATP-sensitive K-channels (K_{ATP}) (Clapp & Gurney, 1992; Noack *et al.*, 1992c; Russell *et al.*, 1992; Silberberg & van Breemen, 1992; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994) with an underlying unitary conductance in the range 10–30 pS (quasi-physiological conditions, Kajioka *et al.*, 1990; 1991; Noack *et al.*, 1992a; Bolton *et al.*, 1993; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Reducing the ability of the cell to synthesize ATP induces a K-current ($I_{K(ATP)}$; Noack *et al.*, 1992c; Silberberg & van Breemen, 1992) with characteristics identical to those of the current induced by the K-channel openers (Noack *et al.*, 1992c; Ibbotson *et al.*, 1993a). In smooth muscle, such results collectively suggest that these agents could compete with ATP for access to the inhibitory ATP binding site on an ATP-sensitive K-channel (K_{ATP}; see Edwards & Weston, 1993), a mechanism originally proposed to account for the actions of the K-channel opener RP49356 in cardiac myocytes (Thuringer & Escande, 1989). In support of this view is the recent finding in cultured fibroblasts that K-channel openers reduce the current through an ATP-sensitive chloride channel (Cl_{ATP}) which closes as ATP binding to the channel regulatory site *decreases* (Sheppard & Welsh, 1992).

In parallel with the induction of $I_{K(ATP)}$ by the K-channel openers in vascular smooth muscle is the simultaneous inhibition of the delayed rectifier current ($I_{K(V)}$; Beech & Bolton, 1989a; Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Furthermore, removal of substrates for ATP synthesis, and the presumed reduction in the intracellular ATP concentration ($[ATP]_i$), also inhibits $I_{K(V)}$ (Noack *et al.*, 1992c). Since in other tissues the channels (K_V) which carry $I_{K(V)}$ are sensitive to phosphorylation (Perozo & Bezanilla, 1990; Duchatelle-Gourdon *et al.*, 1991), the K-channel openers could reduce the phosphorylation of K_V by competing with ATP for access to sites on enzymes like protein kinases (see Scott, 1991).

The objective of the present study was to obtain more

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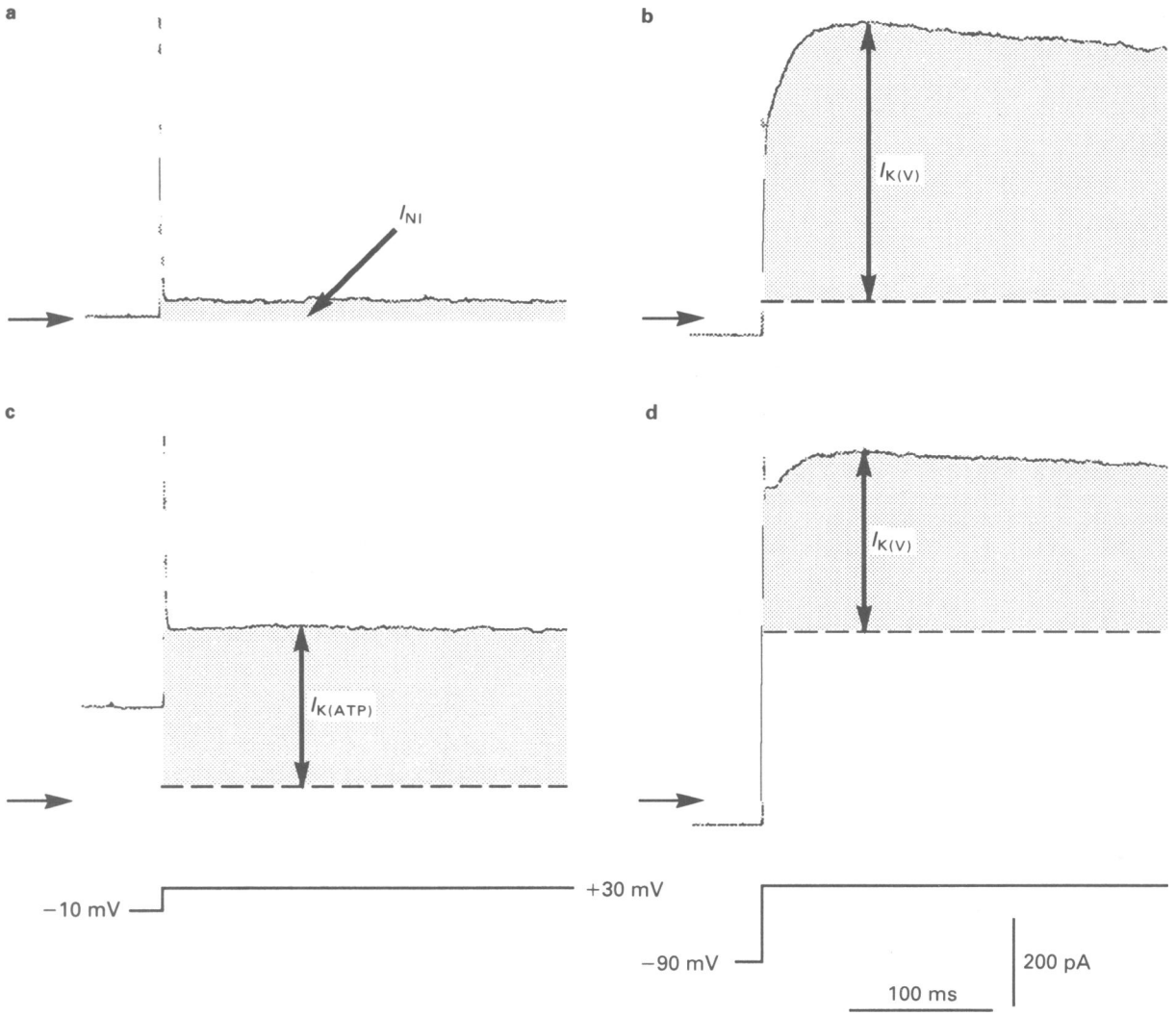

Figure 1 Currents obtained in isolated, single portal vein cells of the rat under calcium-free conditions in the absence (a, b) or presence (c, d) of levcromakalim. (a) On stepping to a test potential of + 30 mV from a holding potential of − 10 mV only a non-inactivating current (I_{NI}) was obtained (represented by shaded area above the zero current level). However, on stepping to the same test potential from a holding potential of − 90 mV (b) both I_{NI} and the rapidly activating and relatively slowly inactivating delayed rectifier K-current ($I_{K(V)}$) were observed. $I_{K(V)}$ (shaded area) is the portion of the total current remaining after subtraction of I_{NI} (magnitude indicated by dashed line). In the presence of 10 μM levcromakalim (c) a second non-inactivating current component was present on stepping from − 10 mV to + 30 mV. This component ($I_{K(ATP)}$, shaded area) is obtained by subtraction of I_{NI} (magnitude indicated by dashed line). On stepping to + 30 mV from a holding potential of − 90 mV in the presence of 10 μM levcromakalim, $I_{K(V)}$ was also present (d). Note that after subtraction of the non-inactivating currents (I_{NI} and $I_{K(ATP)}$; magnitude indicated by the dashed line), $I_{K(V)}$ (shaded area) was reduced (compare b and d). Each trace was derived by averaging the currents obtained by these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by the horizontal arrow.

information on the modulation of K-currents by levcromakalim in vascular smooth muscle using whole-cell voltage-clamp techniques. The effects of this K-channel opener were examined and compared with those of protein kinase inhibitors, a phosphatase inhibitor and a dephosphorylating agent. Using this approach, it was hoped to clarify how levcromakalim could induce $I_{K(ATP)}$ and simultaneously inhibit $I_{K(V)}$. A preliminary account of some of these findings has been given (Edwards *et al.*, 1993).

Methods

Protein kinase A assay

Unless otherwise indicated, protein kinase A activity was determined by incubating 2 μM adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (Type 1 or Type 2, derived from rabbit muscle; Sigma) with 100 μM ATP (magnesium salt), 10 μM cyclic AMP and substrate for protein kinase A, 50 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) in a 50 mM Tris HCl buffer solution containing 10 mM $MgCl_2$ and 0.25 mg ml^{-1} bovine serum albumin (pH 7.5) for 15 min at room temperature in the presence or absence of the potential modifying agents or of a protein kinase A inhibitor (PKI(2-22)amide). After addition of [γ-^{32}P]-ATP (ICN Biomedicals, 167 TBq $mmol^{-1}$; final concentration 55 nM) the tubes were incubated at 30°C for 6 min. The reaction was stopped by spotting 20 μl of the reaction mixture from each tube onto phosphocellulose paper discs which were washed firstly with 1% (v/v) phosphoric acid and then with water before being placed in scintillation vials containing 4 ml Ecoscint A (National Diagnostics). The radioactivity associated with the peptide-incorporated ^{32}P was determined by liquid scintillation counting.

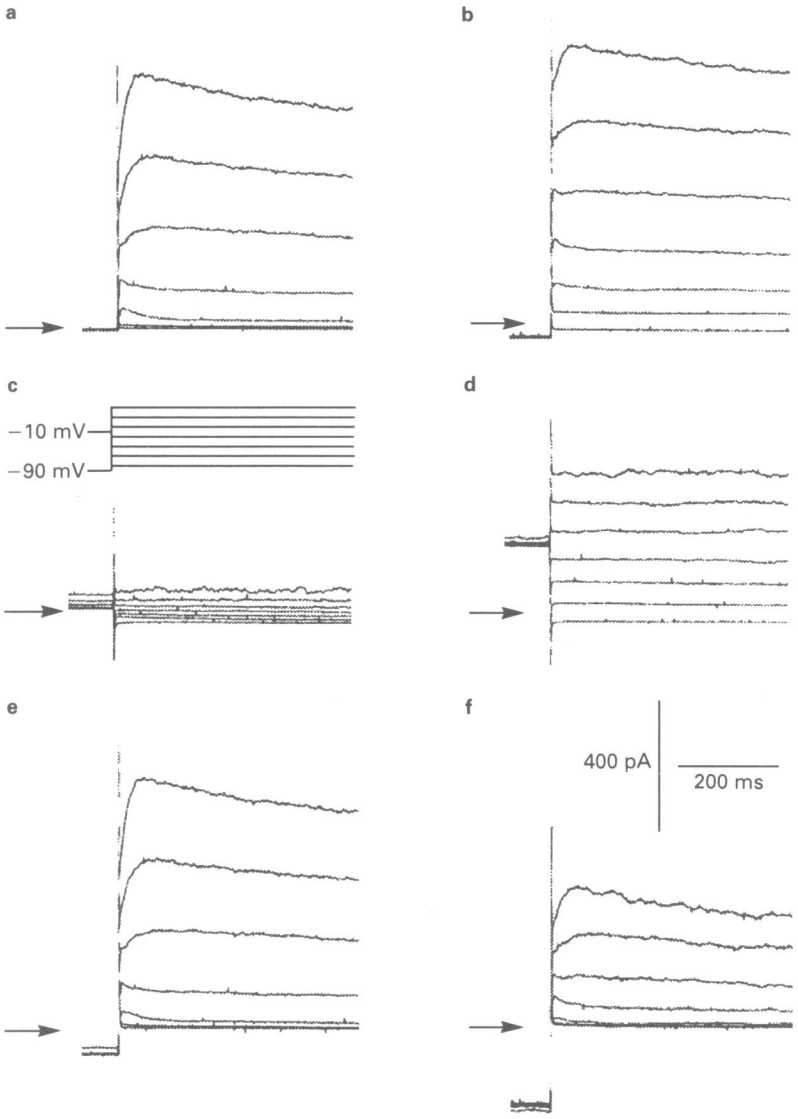

Figure 2 Families of currents obtained in isolated single portal vein cells of rat under calcium-free conditions in the absence (a, c, e) or presence (b, d, f) of levcromakalim. Cells were clamped at − 90 mV (a, b) or − 10 mV (c, d) and then stepped in 20 mV increments to a series of test potentials ranging from − 80 mV to + 40 mV (protocol illustrated in upper part of (c)). (a) Under control conditions, on stepping to the test potentials from a holding potential of − 90 mV, both I_{NI} and $I_{K(V)}$ were present. However, on stepping to the same test potentials from a holding potential of − 10 mV only I_{NI} was obtained (c). Digital subtraction of I_{NI} (c) from the peak total currents (a) for each test potential produced the trace shown in (e) which represents the component due to $I_{K(V)}$. In the presence of 10 μM levcromakalim (b) the total current at each test potential was slightly greater than in controls (a). However, the non-inactivating current component (which now comprises I_{NI} + the levcromakalim-induced $I_{K(ATP)}$) (d) was markedly enhanced (compare (d) and (c)). Subtraction of the non-inactivating currents from the total currents showed that $I_{K(V)}$ (f) was inhibited in the presence of 10 μM levcromakalim (compare (f) and (e)). Each trace was derived by averaging the currents obtained using these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by the horizontal arrow. See Figure 1 for further explanation of currents.

Production of isolated cells

All whole-cell voltage-clamp experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male Sprague-Dawley rats (100–125 g body weight), previously killed by stunning and bleeding. Each portal vein (about 15 mm length) was carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. For cell dispersal, intact portal veins were incubated in a low-Ca^{2+} physiological salt solution (PSS) containing collagenase and pronase (see Solutions) for 25 min. They were then cut into 4 segments and triturated in Kraftbrühe (Klöckner & Isenberg, 1985) in a wide bore, smooth-tipped pipette. The cells were used for experiments within 8 h of separation, during which time they were stored at 6°C in Kraftbrühe. All experiments were performed at 23°C.

Single-cell electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used in all experiments. Patch pipettes were pulled from Pyrex glass (H 15/10, Jencons, UK) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed as described by Noack *et al.* (1992b). For cell stimulation and for recording and analysing data the pCLAMP 5.5 programme was used (Axon Instruments, U.S.A.). Data acquisition and storage were as described by Ibbotson *et al.* (1993a). In each experiment the currents evoked by voltage steps from the stated holding potential were measured at their peak level.

We have previously demonstrated the ability of K-channel openers to simulate $I_{K(ATP)}$ under nominally calcium-free conditions (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a). On the

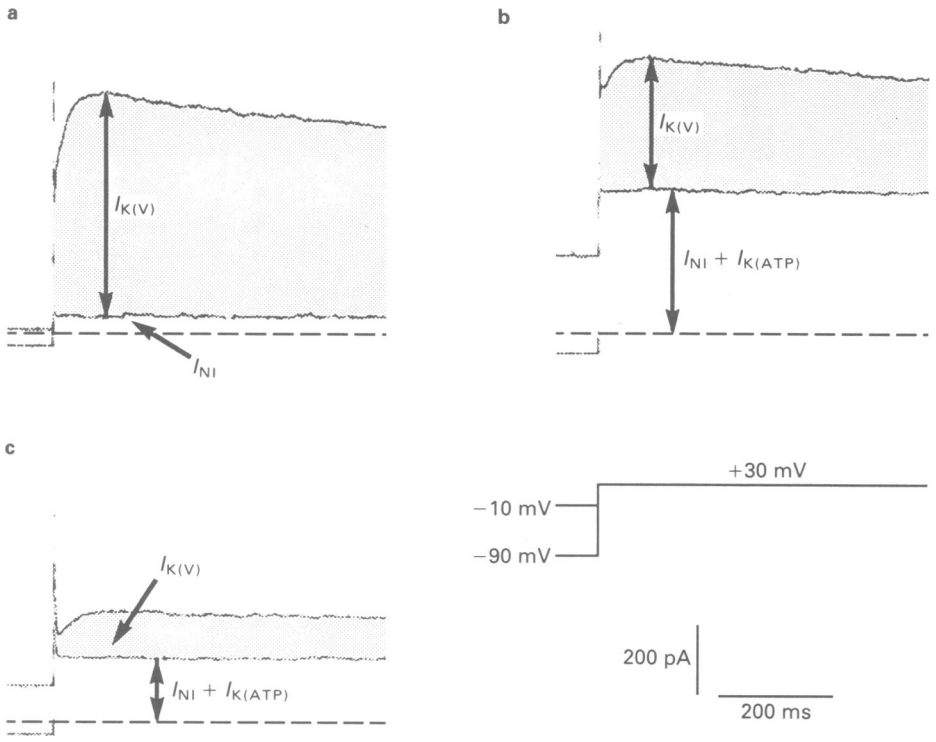

Figure 3 Currents obtained in isolated, portal vein cells of rat under calcium-free conditions in the absence (a) or presence (b, c) of levcromakalim. (a) On stepping to a test potential of + 30 mV from a holding potential of − 10 mV only a non-inactivating current (I_{NI}) was obtained. On stepping to the same test potential from a holding potential of − 90 mV both $I_{K(V)}$ (shaded area) and I_{NI} were observed. After 15 min superfusion with 10 μM levcromakalim (b) $I_{K(ATP)}$ and I_{NI} were present on stepping from − 10 mV to + 30 mV. On stepping to + 30 mV from − 90 mV, the total evoked current was slightly increased but $I_{K(V)}$ (shaded area) was markedly reduced (b). After exposure to levcromakalim for 30 min both $I_{K(V)}$ and $I_{K(ATP)}$, and thus the total current, were greatly reduced. Each trace was derived by averaging the currents obtained by these protocols in 4 separate cells from different animals. For each trace, the zero current level is indicated by a dashed line.

assumption that the bath and pipette solutions used in the present study had a contaminant calcium concentration of 10 μM (based on analysis by Petersen & Maruyama, 1983), the free calcium in these solutions was calculated (Fabiato, 1988) to be less than 1 nM due to the presence of EGTA. These conditions simplified the interpretation of data by effectively eliminating the involvement of K-currents carried by the large-conductance calcium-sensitive K-channel (BK_{Ca}). In whole portal veins, responses to levcromakalim are insensitive to selective inhibitors of BK_{Ca} (Winquist *et al.*, 1989; Wickenden *et al.*, 1991; Garcia & Kaczorowski, 1992).

The effects of the protein kinase A inhibitor, PKI(6-22)-amide, the protein kinase C inhibitor, calphostin C, and the phosphatase inhibitor, okadaic acid, were determined by inclusion of these agents in the pipette solution. The effects of butanedione monoxime (BDM), levcromakalim and glibenclamide were investigated by adding the appropriate amount(s) of these agents to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (volume: 1 ml) was continuously perfused (0.7 ml min^{-1}) with fresh external solution using a pump (Microperpex, Pharmacia LKB, Freiburg, Germany); a second identical pump was used to remove excess solution from the recording chamber.

Drugs and solutions

The low-Ca^{2+} PSS used for the cell separation comprised (mM): KCl 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (Type VIII, Sigma) 1 mg ml^{-1}, pronase (Calbiochem) 0.2 mg ml^{-1}, fatty acid free albumin 1 mg ml^{-1}, buffered with methanesulphonic acid to pH 7.4. Kraftbrühe comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid free albumin 1 mg ml^{-1}, pH adjusted to 7.2 with KOH. The PSS in the bath had the following composition (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, glucose 11, HEPES 10, EGTA (ethylene glycol-*bis* β-aminoethyl ether tetraacetic acid) 1.0, buffered with NaOH to pH 7.30; aerated with O_2. The pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, glucose 11, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered to pH 7.30 with KOH. Magnesium-free pipette solution was prepared similarly, but with exclusion of $MgCl_2$. The 1 mM MgATP pipette (internal) solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, MgATP 1. After the addition of MgATP, the pipette solution was buffered to pH 7.30 with KOH and used immediately.

Levcromakalim (Pfizer Central Research) and glibenclamide were first each dissolved in dimethyl sulphoxide (DMSO) to produce a concentrated stock solution (20 mM) from which dilutions were prepared with distilled water immediately before they were required. Calphostin C (Calbiochem) was dissolved in DMSO to give an 800 μM stock solution and diluted immediately before use. 2,3-Butanedione monoxime (BDM) and PKI(6-22)amide were dissolved directly in bath or pipette solutions, respectively, and were used immediately. Unless otherwise stated, all reagents and compounds were obtained from Sigma.

Data analysis

Treatment effects were analysed by 2-way within subject (repeated measures) ANOVA (Statistica v.3.0a:Statsoft). *P* values less than 0.05 were considered to be significant.

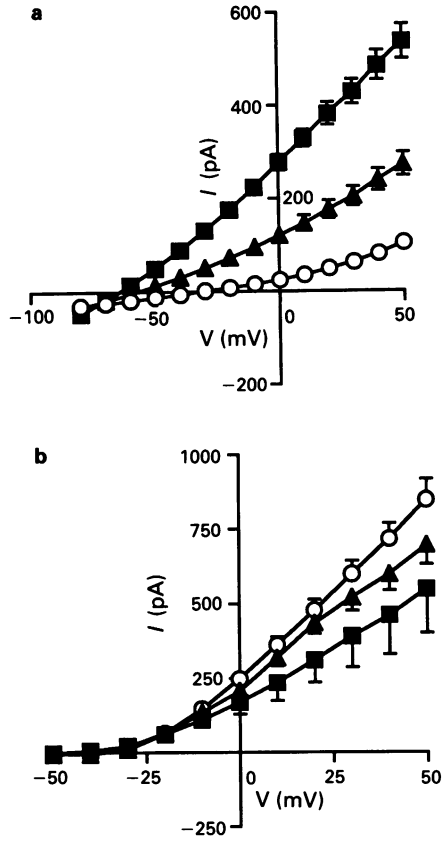

Figure 4 Effects of levcromakalim on current (I) – voltage (V) relationships determined in isolated single portal vein cells of rat under calcium-free conditions. Levcromakalim 1 μM (▲; $n = 10$–21) and 10 μM (■; $n = 4$–6) stimulated $I_{K(ATP)}$ ($P < 0.05$, a) and inhibited $I_{K(V)}$ ($P < 0.05$, b) in a concentration-dependent manner. (○) represents control non-inactivating (I_{NI}; a, $n = 26$) or inactivating ($I_{K(V)}$; b, $n = 14$) currents. Each point represents the mean ± s.e.mean value obtained.

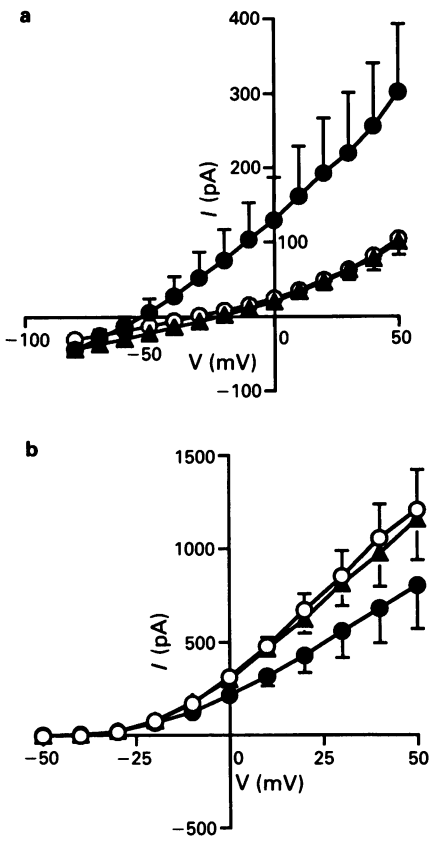

Figure 5 Effects of levcromakalim in the presence of a selective protein kinase C inhibitor, calphostin C (500 nM in the pipette solution) on (a) non-inactivating current and (b) the delayed rectifier K-current, $I_{K(V)}$: (○) represents control non-inactivating (I_{NI}; a) or inactivating ($I_{K(V)}$; b) current on breakthrough (i.e. immediately after obtaining the whole-cell recording configuration). Calphostin C (▲) had not modified either I_{NI} or $I_{K(V)}$ 18 ± 2 min after breakthrough. The induction of the non-inactivating current ($I_{K(ATP)}$) ($P < 0.05$) and the inhibition of $I_{K(V)}$ ($P < 0.05$) by 1 μM levcromakalim was similar in either the presence (●) or absence of calphostin C (see Figure 4). Each point represents the mean value obtained ± s.e.mean, $n = 4$.

Results

Effects of levcromakalim

In freshly-isolated rat portal vein cells under nominally calcium-free conditions, three types of current were evident. On stepping from a holding potential of − 90 mV to more positive test potentials, a rapidly-activating and -inactivating current developed (I_A). Although in some cells this could be observed over the full range of test potentials used (− 80 to + 50 mV), it was masked in most cells at potentials positive to approximately − 20 mV by the much larger delayed rectifier K-current, $I_{K(V)}$, which developed relatively slowly on stepping to potentials more positive than − 30 mV. $I_{K(V)}$ decayed fully over a period of several seconds leaving only the non-inactivating current (I_{NI}) which had a reversal potential of approximately − 30 mV. I_{NI} was thus not a pure K-current and was probably carried by cation channels.

To allow quantification of both the non-activating and inactivating currents, cells were clamped at a holding potential of either − 10 mV or − 90 mV (Figure 1). On holding at − 10 mV, the voltage-sensitive K-currents (I_A and $I_{K(V)}$) inactivated, and only the non-inactivating current component (I_{NI}) was present at each of the test potentials. $I_{K(V)}$ alone was thus determined by subtracting the non-inactivating current (holding potential − 10 mV) from the peak total current ($I_{NI} + I_{K(V)}$; holding potential − 90 mV) at each test potential (Noack *et al.*, 1992b).

Figure 2 shows the current obtained on stepping to a series of test potentials from a holding potential of − 90 mV (total currents) or a holding potential of − 10 mV (only non-inactivating currents available). Although the *total* current was slightly increased by 10 μM levcromakalim (Figures 2a and 2b), this agent produced a large increase in the non-inactivating current component by inducing $I_{K(ATP)}$ (Figures 2c and 2d). Under the conditions of the present study, subtraction of the non-inactivating current components ($I_{NI} + I_{K(ATP)}$) from the peak total current at each test potential revealed the current due to $I_{K(V)}$. It is evident from the traces shown in Figure 2 (e and f) that levcromakalim inhibited $I_{K(V)}$.

The non-inactivating K-current induced by levcromakalim ($I_{K(ATP)}$) is inhibited by glibenclamide and phentolamine and is thus easily distinguished from I_{NI} which is unaffected by these agents (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a). Note that $I_{K(ATP)}$ was never present under control, nominally calcium-free conditions. We have only observed this current in the presence of K-channel openers or under conditions in which protein dephosphorylation might occur (present study; Noack *et al.*, 1992c; Ibbotson *et al.*, 1993a).

Under control conditions $I_{K(V)}$ remained constant for at least 40 min (the duration of a typical experiment). However, in the presence of 10 μM levcromakalim (Figure 3) both $I_{K(V)}$ and $I_{K(ATP)}$ declined with time. Thus, 15 min after bath exposure to levcromakalim there was a large increase in the non-inactivating current component due to the induction of $I_{K(ATP)}$. However, $I_{K(V)}$ was simultaneously reduced, so that the total current in the presence of levcromakalim ($I_{K(V)} + I_{NI}$

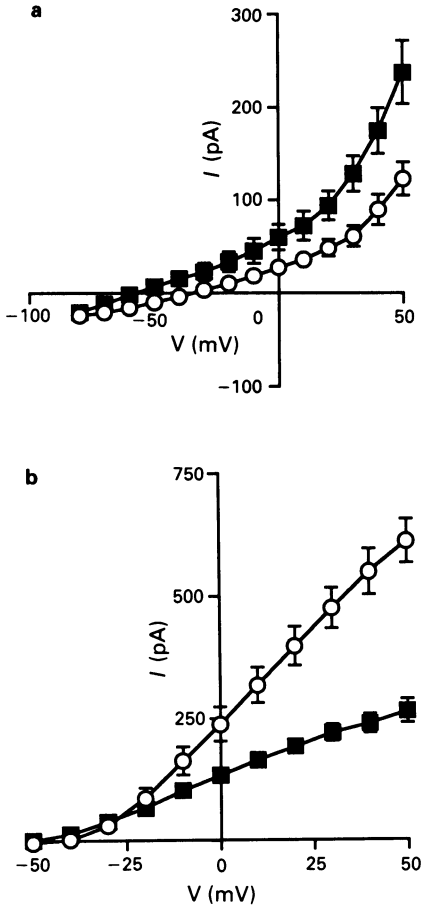

Figure 6 Induction of a non-inactivating K-current (a) and inhibition of the delayed rectifier K-current (b) by a protein kinase A inhibitor, PKI(6-22)amide (1 μM) in the pipette solution: (○) represents control non-inactivating (a) or inactivating ($I_{K(V)}$; b) currents on breakthrough (i.e. immediately after formation of the whole-cell recording configuration). (■) shows these currents at the peak of the development of the non-inactivating current (23 ± 2 min after breakthrough, $n = 5$). PKI(6-22)amide significantly enhanced the non-inactivating current and inhibited $I_{K(V)}$ ($P < 0.05$). Each point represents the mean ± s.e.mean value obtained, $n = 5$.

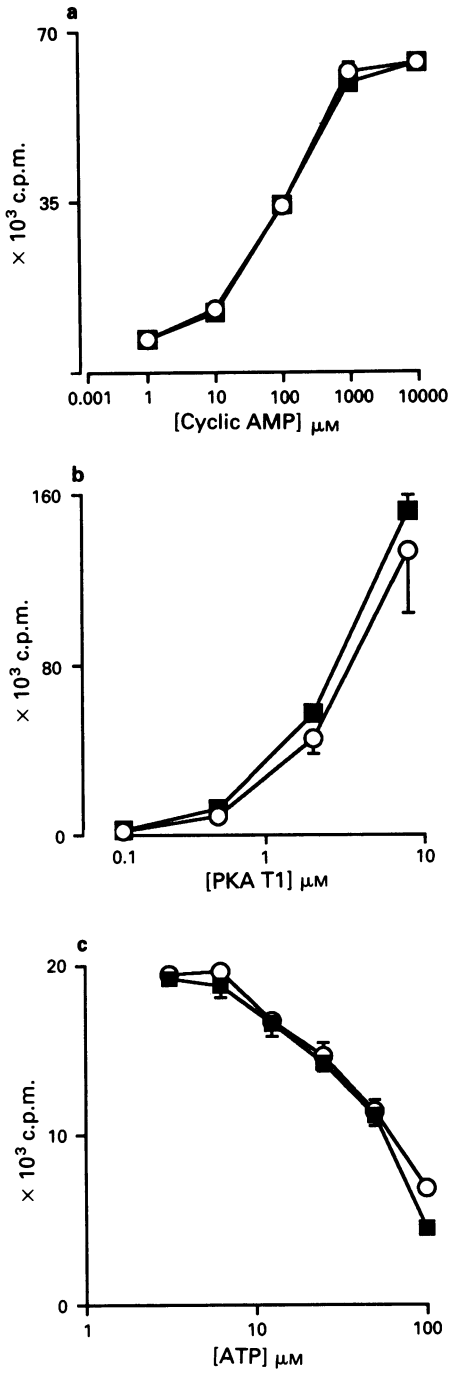

Figure 7 Effect of levcromakalim on Type 1 protein kinase A activity in the presence of varying concentrations of cyclic AMP (a) or type 1 protein kinase A (PKA T1; b). Incorporation of ^{32}P into the peptide substrate (counts per minute, c.p.m.) is an indication of the relative activity of protein kinase A in the presence of levcromakalim (■, 10 μM) or its vehicle (○, 0.1% dimethylsulphoxide). In (c), increasing the concentration of ATP reduced the incorporation of ^{32}P (○, vehicle control), an effect which was not modified by levcromakalim (■, 10 μM). Each point represents the mean value ± s.e.mean obtained ($n = 4$).

$+ I_{K(ATP)}$) was only slightly larger than that before superfusion with the drug ($I_{K(V)} + I_{NI}$). After 30 min exposure to levcromakalim the total current was markedly reduced and both $I_{K(V)}$ and $I_{K(ATP)}$ components had declined.

Figure 4a shows the concentration-dependent induction of $I_{K(ATP)}$ by levcromakalim. The current-voltage curves in the presence and absence of levcromakalim intersected between − 70 and − 80 mV, indicating that the induced current was K^+-selective. In every cell studied, the induction of $I_{K(ATP)}$ by levcromakalim was always accompanied by inhibition of $I_{K(V)}$ (Figure 4b). In the absence of K-channel openers the magnitude of both I_{NI} and $I_{K(V)}$ in the rat portal vein cells remained constant for up to 40 min under our control conditions.

Effects of protein kinase inhibitors

Addition of calphostin C (500 nM), a potent and highly selective inhibitor of protein kinase C (Kobayashi *et al.*, 1989), to the pipette solution had no effect on control currents and did not affect the induction of $I_{K(ATP)}$ or the inhibition of $I_{K(V)}$ by levcromakalim (1 μM) (Figure 5). In contrast, the inclusion of a potent inhibitor of protein kinase A, PKI(6-22)amide (1 μM; Glass *et al.*, 1989) in the pipette solution stimulated a non-inactivating K-current (Figure 6a). This current developed slowly, reaching a plateau at 23 ± 2 min ($n = 5$). Simultaneously, $I_{K(V)}$ was markedly reduced by this inhibitor in every cell tested ($P < 0.05$; Figure 6b). When the PKI(6-22)amide-induced K-current had reached its maximum it was inhibited by glibenclamide (1 μM; data not shown).

Several workers have concluded that K-channel openers might stimulate the opening of K_{ATP} by interfering with the binding of ATP to an inhibitory site on the channel (Thur-

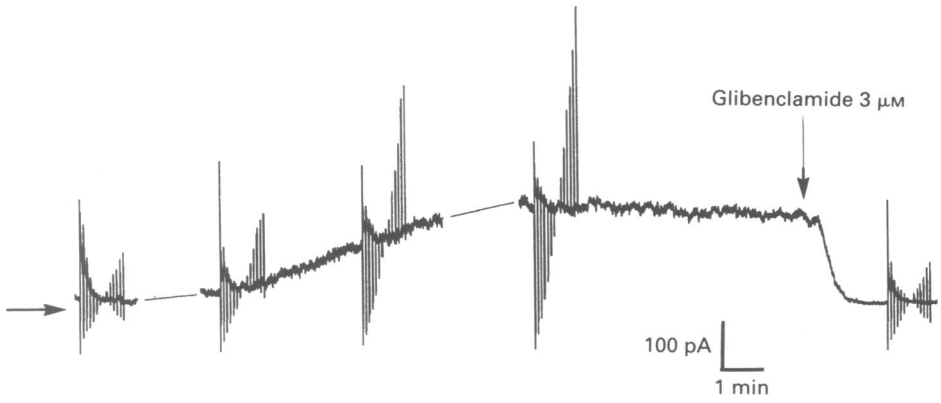

Figure 8 Slow development of a glibenclamide-sensitive outward current with time in a rat portal vein cell clamped at a holding potential of − 10 mV. The pipette solution contained 1 mM MgATP (see Drugs and Solutions). The trace commences immediately after breakthrough of the membrane within the pipette (i.e. formation of the whole-cell recording configuration), and zero current is indicated by the horizontal arrow. Deflections in the trace are the currents evoked on stepping from the holding potential to test potentials (− 80 to + 50 mV) in 10 mV increments. During gaps in the trace, the cell was clamped at − 90 mV to determine the changes in $I_{K(V)}$.

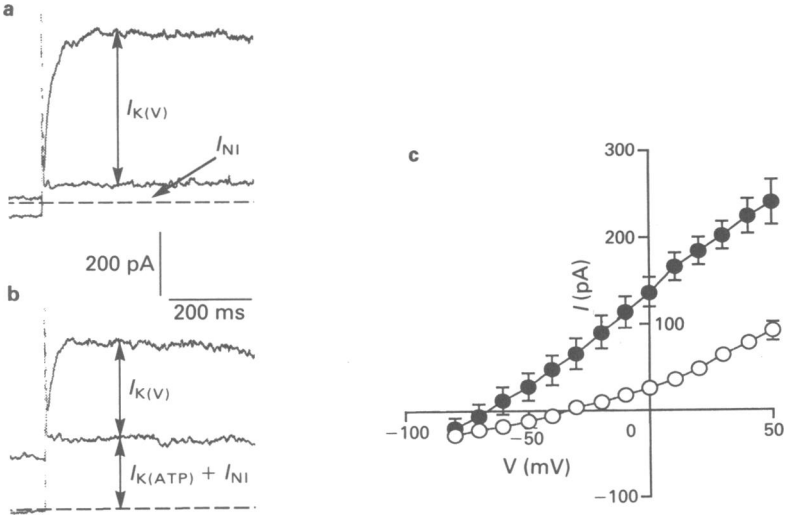

Figure 9 Induction of a non-inactivating current by butanedione monoxime (BDM). The trace in (a) shows the control non-inactivating current (I_{NI}) and the control delayed rectifier current ($I_{K(V)}$) obtained on stepping to a test potential of + 30 mV from holding potentials of − 10 mV or − 90 mV, respectively. (b) Shows the currents obtained in the same cell 6 min after superfusion with 5 mM BDM. Note that although the total current level (obtained on stepping from − 90 mV) was similar in the absence or presence of BDM, the non-inactivating current component ($I_{NI} + I_{K(ATP)}$, obtained on stepping from − 10 mV) was enhanced and the inactivating component ($I_{K(V)}$) was reduced in the presence of BDM (compare (a) and (b)). The graph in (c) represents the mean current (I) -voltage (V) relationship ($n = 3$) for the total non-inactivating currents ($I_{NI} + I_{K(ATP)}$) which were present at the peak of the response to bath application of BDM (●). Each point represents the mean values derived from those three (out of six) cells in which a non-inactivating current did not spontaneously develop when the pipette solution contained 1 mM MgATP (but was devoid of substrates for the tricarboxylic acid pathway) (see Drugs and Solutions). (○) represents the *I-V* relationship for the control non-inactivating current (I_{NI}) which was determined immediately before the addition of BDM. Each point represents the mean ± s.e.mean values obtained, ($n = 3$).

inger & Escande, 1989; Nakayama *et al.*, 1990). Inhibition by K-channel openers of the binding of ATP to a site on protein kinase A, and thus inhibition of protein kinase A activity, would be consistent with our finding that both the K-channel openers and the protein kinase A inhibitor stimulate a glibenclamide-sensitive, non-inactivating K-current and simultaneously inhibit $I_{K(V)}$. We therefore investigated the effect of a relatively high concentration of levcromakalim (10 μM) on protein kinase A activity in a biochemical assay. As shown in Figures 7a and 7b, levcromakalim had no effect on protein kinase A activity (measured as incorporation of ^{32}P into substrate, c.p.m.) over a range of concentrations of cycle AMP or of Type 1 protein kinase A (identical effects were produced with Type 2 protein kinase A, data not shown). Increasing the concentration of non-radiolabelled ATP reduced the transfer of the radiolabelled γ-phosphate by competing for binding sites on the kinase. Nevertheless, it is evident that levcromakalim had no effect on protein kinase A activity over a range of concentrations of ATP (Figure 7c).

Effects of butanedione monoxime

Channel phosphorylation is a dynamic process involving phosphorylation catalysed by kinases, and dephosphorylation stimulated by phosphatases. The dephosphorylating agent, butanedione monoxime (BDM), is known to inhibit $I_{K(V)}$ in human T-lymphocytes (Schlichter *et al.*, 1992). To avoid possible complications arising from effects of BDM on glycolysis and ATP production, we examined the effects of BDM on rat portal vein cells using a pipette solution con-

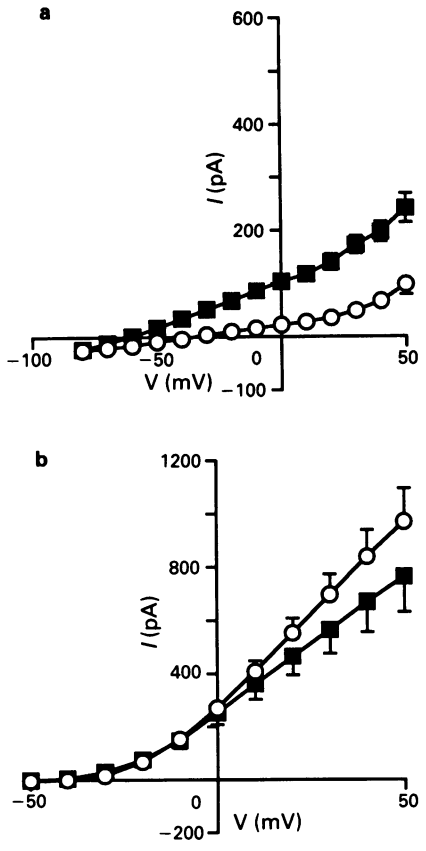

Figure 10 Effects of levcromakalim in the presence of a phosphatase inhibitor, okadaic acid (1 μM in the pipette solution). In the presence of okadaic acid, levcromakalim (1 μM) stimulated $I_{K(ATP)}$ ($P<0.05$; a) and inhibited the delayed rectifier K-current $I_{K(V)}$ ($P<0.05$; b): (○) represents control non-inactivating (I_{NI}; a) or inactivating ($I_{K(V)}$; b) currents before addition of levcromakalim and with 1 μM okadaic acid in the pipette; (■) shows the currents ($I_{NI} + I_{K(ATP)}$, a; $I_{K(V)}$, b) in the presence of 1 μM levcromakalim at the peak of the development of $I_{K(ATP)}$. Compare these effects with those produced by levcromakalim alone (Figure 4). Each point represents the mean ± s.e.mean value obtained, $n = 4$.

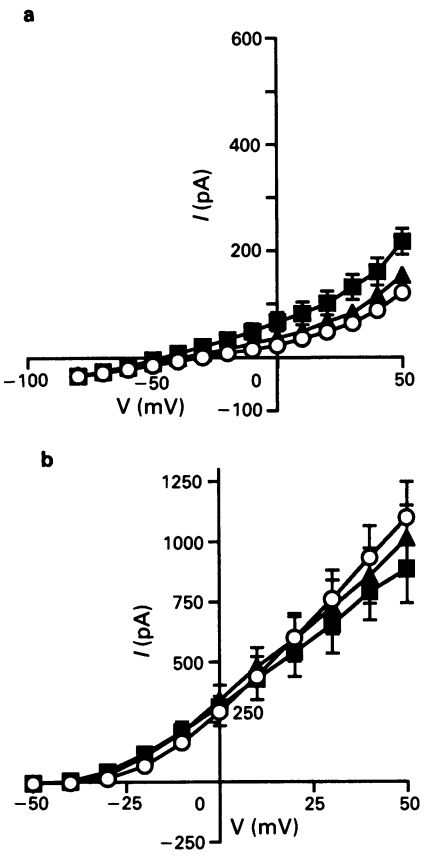

Figure 11 Failure of levcromakalim to induce $I_{K(ATP)}$ (a: $P>0.05$) and to inhibit $I_{K(V)}$ (b: $P>0.05$) when the pipette solution was essentially magnesium-free: (○) represents control non-inactivating (a) or inactivating ($I_{K(V)}$: b) currents on breakthrough ($n = 5$); (▲) and (■) show the currents after 15 min superfusion with 1 μM ($n = 4$) and subsequently 10 μM levcromakalim ($n = 3$), respectively. Compare this lack of effect with the changes produced by levcromakalim in the presence of magnesium (Figure 1). Each point represents the mean ± s.e.mean value obtained.

taining 1 mM MgATP (which was devoid of substrates for the tricarboxylic acid cycle, see *Drugs* and *Solutions*). Reducing the intracellular ATP concentration, by replacing the carboxylic acid substrates and glucose in the pipette solution with 1 mM MgATP and by omitting the extracellular glucose had an effect similar to the application of levcromakalim. In 5 out of 11 cells, $I_{K(V)}$ was reduced (data not shown) and a non-inactivating K-current was induced. Figure 8 shows the development of such a non-inactivating K-current with time and illustrates its glibenclamide-sensitivity. In 3 out of the 6 cells which did not spontaneously develop an outward current, bath application of BDM (5 mM) had no effect. However, in the remaining 3 cells, exposure to BDM immediately stimulated a non-inactivating, glibenclamide-sensitive K-current. Despite the induction of $I_{K(ATP)}$, the total whole-cell currents did not substantially increase (compare Figures 9a and 9b). Thus, as $I_{K(ATP)}$ appeared, $I_{K(V)}$ declined. The mean current-voltage relationship for the three cells in which BDM produced an effect is shown in Figure 9c.

Effects of phosphatase inhibition or magnesium depletion

One possibility emerging from our results was that the effects of the K-channel openers could be explained by stimulation of a phosphatase and thus induction of channel dephosphorylation. In an attempt to gain further insight into the mechanism of action of the K-channel openers we examined the effect of okadaic acid, an inhibitor of protein phosphatases 1 and 2A (Cohen, 1989), on the responses to levcromakalim. Figure 10 shows that inclusion of okadaic acid (1 μM) in the pipette solution had no effect on the non-inactivating currents but slightly enhanced $I_{K(V)}$ (compare with Figure 4). Furthermore, okadaic acid only slightly reduced the induction of $I_{K(ATP)}$ and the inhibition of $I_{K(V)}$ by levcromakalim. However, levcromakalim (1 μM and 10 μM) had no effect on $I_{K(V)}$ and did not induce $I_{K(ATP)}$ ($P>0.05$) when magnesium was omitted from the pipette solution (compare Figures 4 and 11). Depletion of intracellular magnesium alone enhanced $I_{K(V)}$ (compare Figures 4 and 11).

Discussion

The K-channel openers, levcromakalim, P1060 and aprikalim, simultaneously induce $I_{K(ATP)}$ and reduce $I_{K(V)}$ in venous and arterial smooth muscle (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). This modulation of K-currents could result from competition with intracellular ATP for access to the regulatory site on K_{ATP} (see

Edwards & Weston, 1993). Alternatively, or in addition, ATP binding to sites on protein kinases (Scott, 1991) could be modified by the K-channel openers resulting in inhibition of these enzymes. This would dephosphorylate channel proteins and modify channel gating properties (Perozo & Bezanilla, 1990; Duchatelle-Gourdon *et al.*, 1991). The primary objective of the present study was to assess the extent to which modification of channel phosphorylation could account for the actions of the K-channel openers.

Does levcromakalim inhibit a protein kinase?

If the changes in K-currents induced by levcromakalim resulted from channel dephosphorylation secondary to a reduction of kinase activity, then similar effects should be produced by protein kinase inhibitors. Under our essentially calcium-free conditions it seemed unlikely that K-channel openers could inhibit a calcium-dependent protein kinase C. However, the possibility existed that the divalent cation Mg^{2+} could substitute for Ca^{2+}, and in addition, calcium-independent forms of protein kinase C have been described (Andrea & Walsh, 1992). Thus the effects of calphostin C on whole cell K-currents in the presence and absence of levcromakalim were examined. Calphostin C, which is a potent, selective inhibitor of protein kinase C, was used since it inhibits the kinase by an effect on its regulatory subunits (Kobayashi *et al.*, 1989; Davis *et al.*, 1992). The use of less selective protein kinase C inhibitors such as staurosporine would have complicated interpretation of any resulting changes since these compounds inhibit several kinases by interacting with ATP binding sites (Davis *et al.*, 1992). Thus, any effects of staurosporine-like agents on K_{ATP} could have resulted from inhibition of the binding of ATP to its inhibitory site on this channel (site 2, see Edwards & Weston, 1993) rather than from inhibition of protein kinase C itself.

In the present study, calphostin C had virtually no effect in either the absence or presence of levcromakalim. It is nevertheless possible that inhibition of protein kinase C could contribute to the effects of the K-channel openers *in vivo* (i.e. in the presence of calcium). However, this is unlikely since the induction of $I_{K(ATP)}$ by these agents occurs in the absence of calcium (present study; Noack *et al.*, 1992a,b; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994). Furthermore, current-clamp studies (Noack *et al.*, 1992a) showed that under calcium-free conditions the associated membrane hyperpolarization was of similar magnitude to that observed with microelectrode recordings in whole tissues and in the presence of physiological concentrations of calcium (Hamilton *et al.*, 1986).

In contrast to the results with calphostin C, the inclusion in the pipette (intracellular) solution of a selective inhibitor of protein kinase A (PKI(6-22)amide; Glass *et al.*, 1989) did indeed reduce K_V and simultaneously stimulated a non-inactivating glibenclamide-sensitive K-current, suggesting that channel dephosphorylation following inhibition of this enzyme could be the mechanism which underlies the action of the K-channel openers. Although the unitary conductance of the channel underlying the induced non-inactivating current was not determined, the noise associated with the development of this current was typical of that associated with the current induced by the K-channel openers (Noack *et al.*, 1992a; Ibbotson *et al.*, 1993a; Criddle *et al.*, 1994) implying that the channel opened by the protein kinase A inhibitor had a small unitary conductance.

In the biochemical assays, however, a relatively high concentration of levcromakalim (10 μM) had no effect on protein kinase A activity irrespective of the concentration of protein kinase A, cyclic AMP or ATP employed. Thus, although the presumed channel dephosphorylation following inhibition of protein kinase A mimicked the effects of the K-channel openers, it seems unlikely that these agents exert their actions by inhibiting this enzyme.

Does levcromakalim stimulate a phosphatase?

Although the K-channel openers do not appear to dephosphorylate following *kinase* inhibition, the ability of PKI(6-22)amide to mimic the ability of the K-channel openers to inhibit $I_{K(V)}$ and to induce $I_{K(ATP)}$ suggested that dephosphorylation could underlie the effects of these agents. Thus the possibility that phosphatase stimulation was the basis of their action was tested.

Numerous types of protein phosphatase exist and these can be broadly characterized by the use of activators or inactivators (see Cohen (1989) and Shenolikar & Nairn (1991) for reviews). Of these, types 1 and 2A are sensitive to okadaic acid, with K_i values of 20 and 0.2 nM respectively (Bialojan & Takai, 1988; Cohen, 1989). In the present study, a relatively high concentration of okadaic acid (1 μM) in the pipette solution had only a small inhibitory effect on the actions of levcromakalim. This suggests that the magnesium-independent phosphatases 1 and 2A (Cohen, 1989) are not the site of action of the K-channel openers.

In the absence of magnesium the K-channel openers are unable to open K_{ATP} in insulinoma cells (Kozlowski *et al.*, 1989) or to induce $I_{K(ATP)}$ in smooth muscle (present study; Bolton *et al.*, 1993) a possible indication that a *magnesium-dependent* protein phosphatase is involved in the actions of the K-channel openers. Such an enzyme is the Type 2C protein phosphatase which is both magnesium-dependent (Cohen, 1989) and not inhibited by up to 10 μM okadaic acid (Bialojan & Takai, 1988). In the heart, such a magnesium-dependent phosphatase is thought to be involved in maintaining the normal degree of phosphorylation of K_V (Duchatelle-Gourdon *et al.*, 1991).

In the present study, removal of magnesium from the pipette had little effect on $I_{K(V)}$. However, under these Mg^{2+}-free conditions, levcromakalim was unable to induce $I_{K(ATP)}$ or to inhibit $I_{K(V)}$. Such results are consistent with the view that K-channel openers could exert a dephosphorylating action by stimulation of a Mg-dependent phosphatase. Furthermore, the so-called chemical phosphatase, butanedione monoxime (BDM), stimulated a non-inactivating, glibenclamide-sensitive K-current and inhibited $I_{K(V)}$, effects similar to those of the K-channel openers. The ability of this compound to inhibit K_V (in T-lymphocytes) has already been described (Schlichter *et al.*, 1992). Consistent with our findings that BDM enhanced a non-inactivating K-current in rat portal vein cells, this agent (0.1–3.75 mM) also stimulated K_{ATP} in insulinoma cells (under whole-cell recording conditions with the inclusion of 0.3 mM MgATP in the pipette solution; P.A. Smith, personal communication).

Collectively the results of the present study suggest that dephosphorylation via stimulation of a phosphatase is the mechanism which could underlie the effects of the K-channel openers on both $I_{K(V)}$ and $I_{K(ATP)}$. The involvement of such an action in ion channel modulation would not be novel since somatostatin is thought to enhance the opening of the large-conductance calcium-sensitive K-channel (BK_{Ca}) by stimulating a phosphatase (White *et al.*, 1991). Furthermore, somatostatin also activates K_{ATP} in insulinoma cells (de Weille *et al.*, 1989), and it is tempting to speculate that this could also involve phosphatase stimulation.

Protein phosphatases also seem to be involved in the regulation of calcium (Ca)-channels, since whole-cell Ca-currents are increased by okadaic acid (Hescheler *et al.*, 1988). Interestingly, inhibition of L-type Ca-channels, which was attributed to the stimulation of phosphatase 2A by atrial natriuretic peptide and somatostatin (White *et al.*, 1991; 1993), is also a feature of K-channel openers under certain conditions (Okabe *et al.*, 1990; Lodge *et al.*, 1992). The observations that these agents are capable of inhibiting L-type Ca-channels is consistent with the possibility that they reduce Ca-channel phosphorylation via phosphatase stimulation. In addition, the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, which is inhibited

by several K-channel openers (Sheppard & Welsh, 1992) is dephosphorylated and inactivated by protein phosphatase Type 2A (Berger *et al.*, 1993).

The K-channel openers convert $I_{K(V)}$ into $I_{K(ATP)}$ – a working hypothesis

In both the present and in previous studies, and using either phosphorylation modifiers or several chemically-distinct K-channel openers, we have never been able to induce $I_{K(ATP)}$ without simultaneously reducing $I_{K(V)}$ (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993a,b). In addition, numerous agents which inhibit $I_{K(V)}$ (alinidine, 4-aminopyridine, antazoline, cibenzoline, ciclazindol, clonidine, guanabenz, phencyclidine, phentolamine, quinidine, tedisamil) also inhibit the effects of K-channel openers in similar concentrations (Beech & Bolton, 1989a; Bray & Quast, 1992; Noack *et al.*, 1992b; Pfründer & Kreye, 1992; Ibbotson *et al.*, 1993a,b). Thus, with the exception of glibenclamide (see below), it is not possible pharmacologically to distinguish between $I_{K(V)}$ and $I_{K(ATP)}$. Furthermore, when the induction of $I_{K(ATP)}$ was prevented (by inclusion of MgATP in the pipette solution), $I_{K(V)}$ was not inhibited by the K-channel opener, P1060 (Ibbotson *et al.*, 1993a).

These data lead to the conclusion that the non-inactivating, voltage-independent K-channel currently designated K_{ATP} may not be a separate entity but may simply be a *partially-dephosphorylated* state of K_V. This state can be induced by agents such as the K-channel openers and by others which directly or indirectly influence the degree of channel phosphorylation. Although there was usually some increase in the total evoked current as $I_{K(ATP)}$ developed, such an observation does not conflict with our view that K_V and K_{ATP} are different states of the same channel. Indeed, some increase in current would be predicted if the property of inactivation were removed from a fraction of the population of K_V (see Hille, 1992).

The so-called ATP-sensitive K-channel has been well characterized in a variety of tissues (see Edwards & Weston, 1993 for review). In isolated patches, this channel rapidly runs down, i.e. enters a state from which channel opening is not possible. Since run-down can be slowed or reversed by the presence of MgATP (but not by non-hydrolysable analogues of ATP: see Ashcroft, 1988), it has been suggested that run-down itself results from dephosphorylation of site 1 on K_{ATP} (see Edwards & Weston, 1993). If the K-channel openers were to induce $I_{K(ATP)}$ by dephosphorylating K_V, then such channel dephosphorylation could only be partial, since full dephosphorylation would also inhibit $I_{K(ATP)}$. However, under conditions unfavourable to phosphorylation such as in the absence of MgATP, the K-channel openers *only* exert an *inhibitory* effect on K_{ATP} (Kozlowski *et al.*, 1989; Dunne, 1990). Such K-channel opener-induced inhibition thus probably results from run-down of K_{ATP} *via* further dephosphorylation. Furthermore, in the present study, relatively long exposure to a high concentration of levcromakalim induced marked inhibition of both $I_{K(ATP)}$ and $I_{K(V)}$ even with the presence of carboxylic acid substrates and glucose in the pipette.

Thus, under normal conditions of phosphorylation, we propose that the target channel of the K-channel openers is not K_{ATP} but is instead K_V, which these agents convert via dephosphorylation into K_{ATP}. This putative interconversion is summarised in the following scheme.

$$K_V \underset{\text{phosphorylation}}{\overset{\text{levcromakalim dephosphorylation}}{\rightleftharpoons}} K_{ATP} \underset{\text{phosphorylation}}{\overset{\text{levcromakalim dephosphorylation}}{\rightleftharpoons}} K_{ATP}(\text{inactive})$$

Further support for the view that K_{ATP} is a substate of K_V can be obtained by comparing the unitary conductances of these channels in smooth muscle. Analysis of the current induced by the K-channel openers suggests that the underlying mean single channel conductance is approximately 11 pS in quasi-physiological conditions (Noack *et al.*, 1992a; Bolton *et al.*, 1993; Ibbotson *et al.*, 1993a; Langton *et al.*, 1993; Criddle *et al.*, 1994). This is essentially identical to the mean value of 9 pS obtained for the smooth muscle K_V (Beech & Bolton, 1989b; Boyle *et al.*, 1992; Volk & Shibata, 1993).

The suggestion that dephosphorylation can convert $I_{K(V)}$ into a non-rectifying, voltage-sensitive current is not new. To our knowledge, such a change in the gating properties of the delayed rectifier current (induced by reducing $[ATP]_i$ in frog skeletal muscle) was first convincingly demonstrated by Fink & Wettwer (1978). In addition, numerous agents are known to reduce or abolish inactivation in sodium channels (see Hille, 1992). Channel conversion was also proposed by Beech & Bolton (1989a) to explain the reduction in $I_{K(V)}$ which occurred concurrently with the induction of a non-activating K-current by cromakalim in rabbit portal vein. This proposal did not gain favour, largely because of the inability of glibenclamide to inhibit K_V, despite the fact that it was an inhibitor of K_{ATP}.

To overcome this difficulty it is merely necessary to propose that glibenclamide binds selectively to the partially-dephosphorylated state of K_V (i.e. K_{ATP}). This proposal is entirely consistent with the finding of Schwanstecher and coworkers (1991) in pancreatic β-cells that [^{3}H]-glibenclamide binding is indeed enhanced under non-phosphorylating conditions. If K_{ATP} is not a separate entity but is rather a configuration of K_V, then it is possible that K_V in pancreatic β-cells is normally in the partially-dephosphorylated form (i.e. K_{ATP}). This would then explain both the sensitivity of this channel in the β-cell to glibenclamide and in addition its relative insensitivity to the K-channel openers (Garrino *et al.*, 1989). Under the present working hypothesis these agents do not open K_{ATP} *per se* but induce the K_{ATP} 'configuration' by dephosphorylation of K_V. Because the opening of K_{ATP} is voltage-independent (see Edwards & Weston, 1993), the change from K_V to K_{ATP} would automatically generate K-current flow by converting the channel from a state in which it is voltage-dependent and inactivates (i.e. K_V) to one in which it is voltage-independent and non-inactivating (i.e. K_{ATP}). Under prolonged dephosphorylating conditions, K_{ATP} runs down and becomes K_{ATP}(inactive), a process enhanced by the presence of K-channel openers (Kozlowski *et al.*, 1989; Dunne, 1990). Such run-down also occurs after exposure to high concentrations of levcromakalim (present study).

Conclusion

The results of the present study do not support the view (Thuringer & Escande, 1989) that the (so-called) K-channel openers compete with ATP for access to the inhibitory ATP binding site on K_{ATP} (site 2, see Edwards & Weston, 1993). Instead, all the effects of these agents on K-, Ca- and Cl-channels could be explained by a basic dephosphorylating action. Together with the results of previous studies it now seems likely that the target channel for the K-channel openers is the delayed rectifier, K_V, which loses its voltage-dependence and inactivation properties by conversion into a partially-dephosphorylated form, currently known as K_{ATP}. Dephosphorylation does not seem to be associated with inhibition of protein kinase A (associated with site 1 on K_{ATP}, see Edwards & Weston, 1993) but is more likely to be exerted via stimulation of a magnesium-dependent phosphatase. The exact mechanism by which the K-channel openers reduce channel phosphorylation forms the basis of an on-going study.

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Differential effects of K^+ channel blockers on antinociception induced by α_2-adrenoceptor, $GABA_B$ and κ-opioid receptor agonists

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1 The effects of several K^+ channel blockers (sulphonylureas, 4-aminopyridine and tetraethylammonium) on the antinociception induced by clonidine, baclofen and U50,488H were evaluated by use of a tail flick test in mice.

2 Clonidine (0.125–2 mg kg^{-1}, s.c.) induced a dose-dependent antinociceptive effect. The ATP-dependent K^+ (K_{ATP}) channel blocker gliquidone (4–8 μg/mouse, i.c.v.) produced a dose-dependent displacement to the right of the clonidine dose-response line, but neither 4-aminopyridine (4-AP) (25–250 ng/mouse, i.c.v.) nor tetraethylammonium (TEA) (10–20 μg/mouse, i.c.v.) significantly modified clonidine-induced antinociception.

3 The order of potency of sulphonylureas in antagonizing clonidine-induced antinociception was gliquidone > glipizide > glibenclamide > tolbutamide, which is the same order of potency as these drugs block K_{ATP} channels in neurones of the CNS.

4 Baclofen (2–16 mg kg^{-1}, s.c.) also induced a dose-dependent antinociceptive effect. Both 4-AP (2.5–25 ng/mouse, i.c.v.) and TEA (10–20 μg/mouse, i.c.v.) dose-dependently antagonized baclofen antinociception, producing a displacement to the right of the baclofen dose-response line. However, gliquidone (8–16 μg/mouse, i.c.v.) did not significantly modify the baclofen effect.

5 None of the K^+ channel blockers tested (gliquidone, 8–16 μg/mouse; 4-AP, 25–250 ng/mouse and TEA, 10–20 μg/mouse, i.c.v.), significantly modified the antinociception induced by U50,488H (8 mg kg^{-1}, s.c.).

6 These results suggest that the opening of K^+ channels is involved in the antinociceptive effect of α_2 and $GABA_B$, but not κ-opioid, receptor agonists. The K^+ channels opened by α_2-adrenoceptor agonists seem to be ATP-dependent channels, whereas those opened by $GABA_B$ receptor agonists are not.

Keywords: Clonidine; baclofen; U50,488H; antinociception; K^+ channels; sulphonylureas; 4-aminopyridine; tetraethylammonium

Introduction

Agonists of μ- and δ-opioid receptors open K^+ channels in neurones (North, 1989) and produce antinociception in experimental animals (Porreca *et al.*, 1984). The opening of K^+ channels seems to play a role in opioid-mediated antinociception, since the specific ATP-dependent K^+ (K_{ATP}) channel blocker, glibenclamide (an antidiabetic sulphonylurea) dose-dependently antagonizes the antinociceptive effect of morphine (Ocaña *et al.*, 1990; 1993; Wild *et al.*, 1991; Narita *et al.*, 1992), whereas the K^+ channel activator pinacidil produces opposite effects (Vergoni *et al.*, 1992). Moreover, the order of potency of different sulphonylureas in blocking K_{ATP} channels in CNS neurones (Amoroso *et al.*, 1990) and in antagonizing morphine antinociception is the same (Ocaña *et al.*, 1993), strongly suggesting that the opening of K_{ATP} channels underlies the morphine antinociceptive effect. This type of K^+ channel is also involved in δ-receptor-mediated antinociception, since glibenclamide antagonizes the antinociceptive activity of [D-Pen^2, D-Pen^5]-enkephalin (Wild *et al.*, 1991).

Agonists of α_2-adrenoceptors and $GABA_B$ receptors also open K^+ channels in neurones (Morita & North, 1981; Christie *et al.*, 1987; Christie & North, 1988; Lacey *et al.*, 1988) and promote antinociception (Sawynok, 1987; Fornai *et al.*, 1990). However, whether K^+ channel opening plays a role in the antinociceptive effect of agonists of these receptors has not been tested. Electrophysiological studies have shown that the K^+ channels opened by α_2-adrenoceptor and μ-opioid receptor agonists in neurones appear to be identical, and are insensitive to some K^+ channel blockers such as tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (North & Williams, 1985; Aghajanian & Wang, 1987). On the other hand, the K^+ conductances elicited by $GABA_B$ receptor agonists in neurones appear to be different, as they are antagonized by 4-AP and TEA (Inoue *et al.*, 1985; Stevens *et al.*, 1985).

In light of these facts it may be hypothesized that if K^+ channel opening underlies the antinociceptive effect of α_2-adrenoceptor and $GABA_B$ receptor agonists, K^+ channel blockers would be expected to antagonize this antinociception. Moreover, taking into account the results of the electrophysiological studies cited above, a differential sensitivity of α_2-adrenoceptor- and $GABA_B$ receptor-mediated antinociception to K^+ channel blockers would be expected. In the present study we evaluated the effect of the i.c.v. administration of sulphonylureas, TEA and 4-AP on the antinociception induced by clonidine, an α_2-adrenoceptor agonist, and baclofen, a $GABA_B$ receptor agonist.

Finally, to test the specificity of the effects of the K^+ channel blockers used, we evaluated whether these drugs antagonize the antinociception elicited by U50,488H, a κ-opioid receptor agonist (Von Voightlander *et al.*, 1983; Clark & Pasternak, 1988). Activation of κ receptors does not open K^+ channels but does close Ca^{2+} channels (Werz & MacDonald, 1984; Cherubini & North, 1985). Consequently, if the effects of sulphonylureas, TEA and 4-AP are due to K^+ channel blockade, they should not modify antinociception induced by U50,488H.

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Methods

Female CD-1 mice (Charles River, Spain) weighing 25–30 g were used for all experiments. The animals were housed in a temperature-controlled room (21 ± 1°C), with air exchange every 20 min and a standard 12 h light/dark cycle (lights on at 08 h 00 min and off at 20 h 00 min). The experiments were performed from 09 h 00 min to 15 h 00 min. Food and water were available *ad libitum* up to the beginning of the experiments. Naïve animals were used throughout. At all times the mice were handled in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

The tail flick test was run as previously described (Ocaña & Baeyens, 1991). Briefly, the animals were restrained in a plexiglass tube and placed on the tail flick apparatus (LI 7100, Letica, S.A., Spain). A noxious beam of light was focussed to the tail about 4 cm from the tip, and the latency to removal was recorded automatically to the nearest 0.1 s. The intensity of the radiant head source was adjusted to yield baseline latencies between 3 and 5 s; this intensity was never changed and any animal in which baseline latency was outside the pre-established limits was excluded from the experiments. The cut-off time was 10 s.

Two baseline tail flick latencies were recorded within 20 min before all injections. Then the solvent or drug was administered and tail flick latencies were measured 10, 20, 30, 45, 60, 90 and 120 min after treatment. The area under the curve of antinociception against time (AUC) was calculated for each animal according to the method of Tallarida & Murray (1987). The degree of antinociception in each animal was calculated according to the formula: % antinociception = $[(AUCt - AUCc)\ (AUCmax - AUCc)^{-1}] \times 100$, where AUCt and AUCc are the areas under the curve for treated and control animals respectively, and AUCmax is the area under the curve of maximum possible antinociception (10 s in each determination). Furthermore, to illustrate the time-course of the antinociceptive effect of the treatments, the degree of antinociception at each time was calculated according to the formula: % antinociception = $[(LTT\text{-}LTB)\ (CT\text{-}LTB)^{-1}] \times 100$, where LTT is the latency time in treated mice, LTB is the baseline latency time and CT is the cut-off time (10 s).

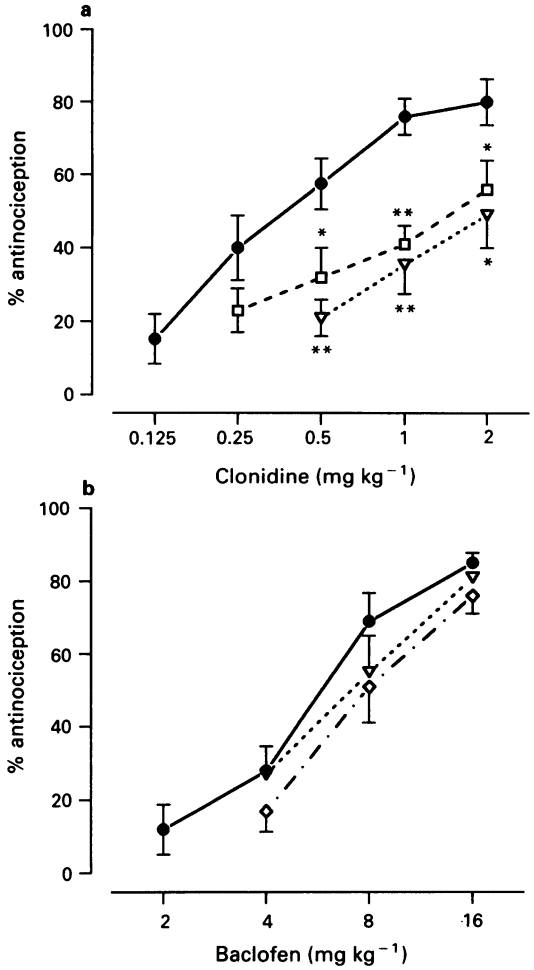

Figure 1 Effects of i.c.v. treatment with different doses of gliquidone on the antinociceptive effect of clonidine and baclofen (administered s.c.) in a tail flick test in mice. (a) Effects of clonidine + solvent (●), clonidine + gliquidone 4 μg/mouse (□) and clonidine + gliquidone 8 μg/mouse (▽). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. Statistically significant differences in comparison with clonidine + solvent: $^{*}P < 0.05$, $^{**}P < 0.01$ (Newman Keuls test). (b) Effects of baclofen + solvent (●), baclofen + gliquidone 8 μg/mouse (▽) and baclofen + gliquidone 16 μg/mouse (◇). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. No statistically significant differences in comparison with baclofen + solvent were found (Newman Keuls test).

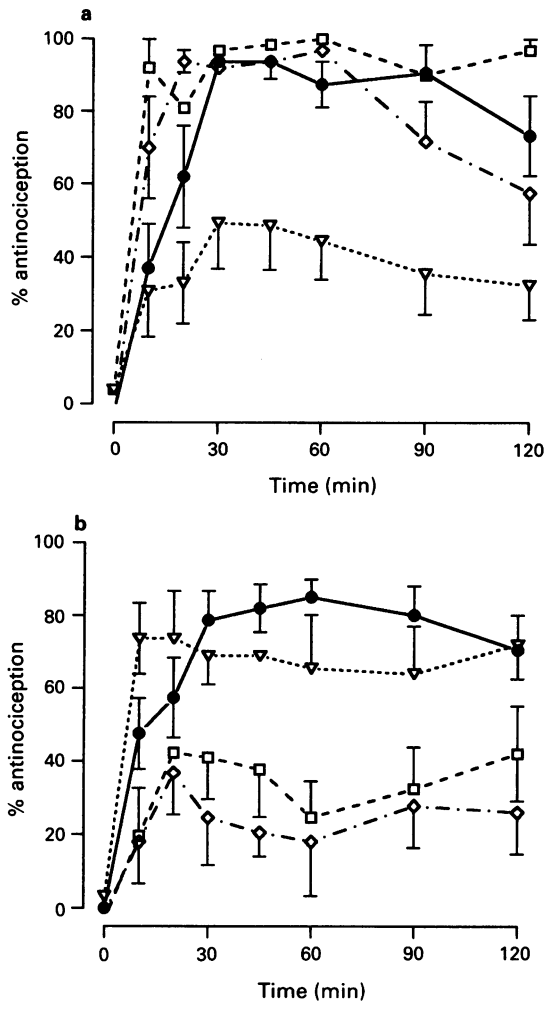

Figure 2 Time-course of the antinociceptive effect of clonidine (1 mg kg⁻¹, s.c.) and baclofen (8 mg kg⁻¹, s.c.) associated to different K^+ channel blockers in a tail flick test in mice. (a) Effects of clonidine (●), clonidine + gliquidone (8 μg/mouse, i.c.v.) (▽), clonidine + tetraethylammonium (TEA) (20 μg/mouse, i.c.v.) (□) and clonidine + 4-aminopyridine (4-AP) (25 ng/mouse, i.c.v.) (◇). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. (b) Effects of baclofen (●), baclofen + gliquidone (8 μg/mouse, i.c.v.) (▽), baclofen + TEA (20 μg/mouse, i.c.v.) (□) and baclofen + 4-AP (25 ng/mouse, i.c.v.) (◇). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals.

Table 1 Effects of different sulphonylureas on the antinociception induced by clonidine (1 mg kg^{-1}, s.c.) in a tail flick test in mice

Treatment[a]	*% antinociception*[b]
Clonidine + solvent	76.24 ± 5.09
Clonidine + gliquidone 2	45.09 ± 5.97**
Clonidine + gliquidone 4	41.25 ± 5.12**
Clonidine + glipizide 20	56.61 ± 6.25*
Clonidine + glipizide 40	52.06 ± 7.11*
Clonidine + glibenclamide 40	61.09 ± 7.11
Clonidine + glibenclamide 80	48.36 ± 7.39*
Clonidine + tolbutamide 80	62.09 ± 5.40
Clonidine + tolbutamide 160	48.93 ± 9.52*

[a]The numbers represent the dose (μg/mouse) of each sulphonylurea administered i.c.v.
[b]The values represent the mean ± s.e.mean of the results obtained in 8–12 animals.
Statistically significant differences in comparison to clonidine + solvent: *$P < 0.05$; **$P < 0.01$ (Newman Keuls test).

Once baseline latencies were obtained, the animals received a s.c. injection of clonidine, baclofen, U50,488H or their solvent, and an i.c.v. injection of one of the K^+ channel blockers or their solvents at time 0; the degree of antinociception was then measured during 2 h as described above. The s.c. injections were made in the interscapular region. The i.c.v. injections were administered to gently restrained unanaesthetized mice in Hamilton microlitre syringes, according to the method previously described (Robles *et al.*, 1992). After the antinociceptive test was finished, the trajectory of the i.c.v. injection was evaluated in each animal, and the results from those in which it was incorrect were discarded.

The drugs used and their suppliers were as follows: clonidine HCl (Sigma), baclofen (Sigma), *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pirrolidynyl]cyclohexyl) benzeneacetamide methanesulfonate salt (U50,488H) (Sigma), tetraethylammonium bromide (Sigma), 4-aminopyridine (Sigma) and the sulphonylureas glibenclamide, tolbutamide (both Sigma), gliquidone (Europharma, S.A.) and glipizide (Far-

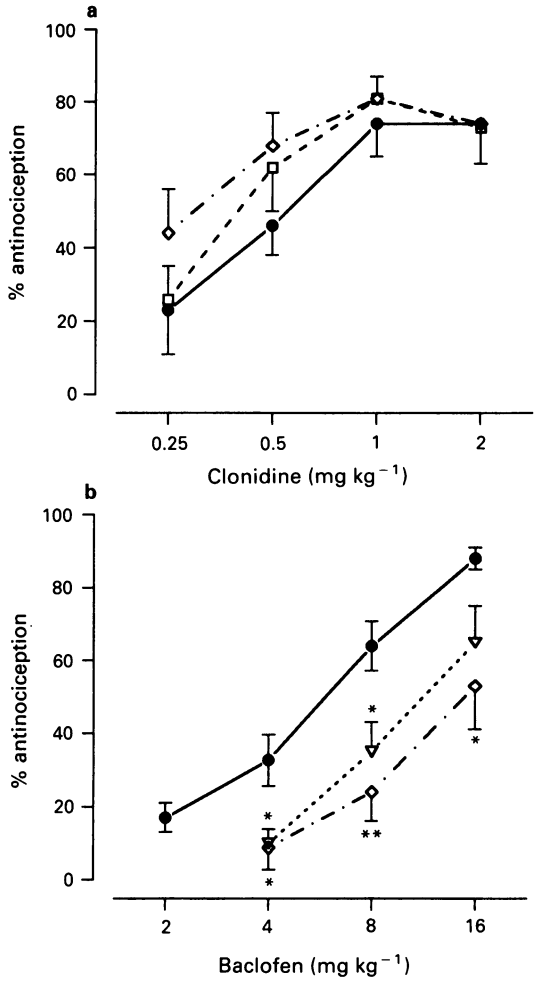

Figure 3 Effects of i.c.v. treatment with different doses of 4-aminopyridine (4-AP) on the antinociceptive effect of clonidine and baclofen (administered s.c.) in a tail flick test in mice. (a) Effects of clonidine + solvent (●), clonidine + 4-AP 25 ng/mouse (◇) and clonidine + 4-AP 250 ng/mouse (□). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. No statistically significant differences in comparison with clonidine + solvent were found (Newman Keuls test). (b) Effects of baclofen + solvent (●), baclofen + 4-AP 2.5 ng/mouse (▽) and baclofen + 4-AP 25 ng/mouse (◇). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. Statistically significant differences in comparison with baclofen + solvent: *$P < 0.05$; **$P < 0.01$ (Newman Keuls test).

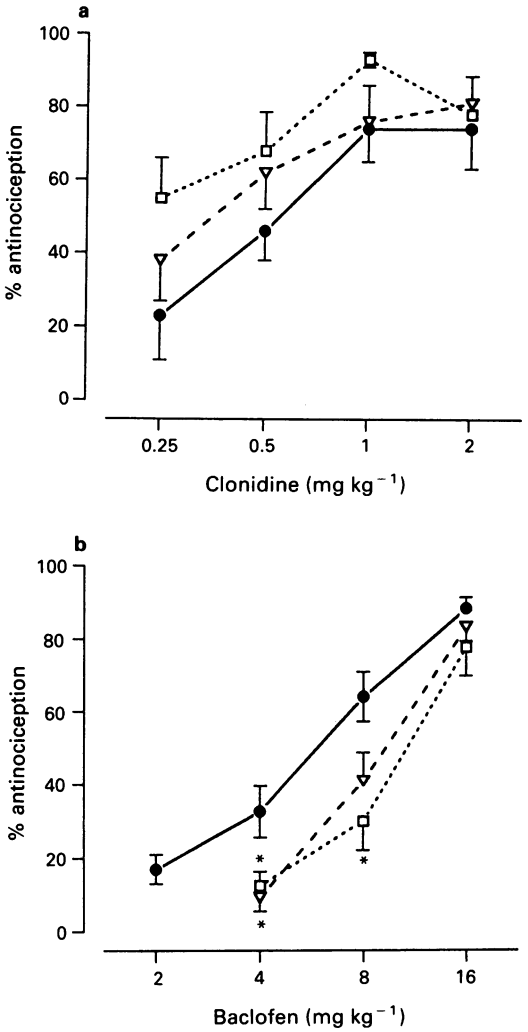

Figure 4 Effects of i.c.v. treatment with different doses of tetraethylammonium (TEA) on the antinociceptive effect of clonidine and baclofen (administered s.c.) in a tail flick test in mice. (a) Effects of clonidine + solvent (●), clonidine + TEA 10 μg/mouse (▽) and clonidine + TEA 20 μg/mouse (□). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. No statistically significant differences in comparison with clonidine + solvent were found (Newman Keuls test). (b) Effects of baclofen + solvent (●), baclofen + TEA 10 μg/mouse (▽) and baclofen + TEA 20 μg/mouse (□). Each point represents the mean ± s.e.mean of the values obtained in 8–12 animals. Statistically significant differences in comparison with baclofen + solvent: *$P < 0.05$ (Newman Keuls test).

mitalia Carlo Erba). Clonidine, baclofen and U50,488H were dissolved in demineralized water and injected subcutaneously in a volume of 5 ml kg^{-1}. All sulphonylureas were dissolved in 1% Tween 80 in demineralized water, whereas TEA and 4-AP were dissolved in demineralized water. All K^+ channel blockers were injected intracerebroventricularly in a volume of 5 µl per mouse. Control animals received the same volume of solvents.

Differences between the values in control and K^+ channel blocker-treated groups were analysed with analysis of variance (ANOVA) followed by a Newman Keuls test, and were considered significant when P was below 0.05.

Results

Effects of sulphonylureas on clonidine- and baclofen-induced antinociception

Both clonidine (0.125–2 mg kg^{-1}) and baclofen (2–16 mg kg^{-1}) induced a dose-dependent antinociception after subcutaneous administration to mice (Figures 1a and b). Gliquidone (4 and 8 µg/mouse, i.c.v.) significantly reduced clonidine antinociception, dose-dependently displacing to the right the clonidine dose-response line (Figure 1a). In contrast, gliquidone (8 and 16 µg/mouse, i.c.v.), i.e. at doses even greater than those used with clonidine, did not significantly modify baclofen-induced antinociception (Figure 1b). A representative example of the time-course of the antinociceptive effects of clonidine and baclofen plus gliquidone (8 µg/mouse, i.c.v.) is illustrated in Figure 2.

All sulphonylureas tested (gliquidone, glipizide, glibenclamide and tolbutamide) signficantly antagonized clonidine antinociceptive activity (Table 1). Considering the minimum dose of each sulphonylurea necessary to antagonize the clonidine effect, the order of potency was: gliquidone > glipizide > glibenclamide > tolbutamide (Table 1). None of the sulphonylureas significantly modified tail flick latency in control animals (data not shown), or induced any overt behavioural effect at the doses used.

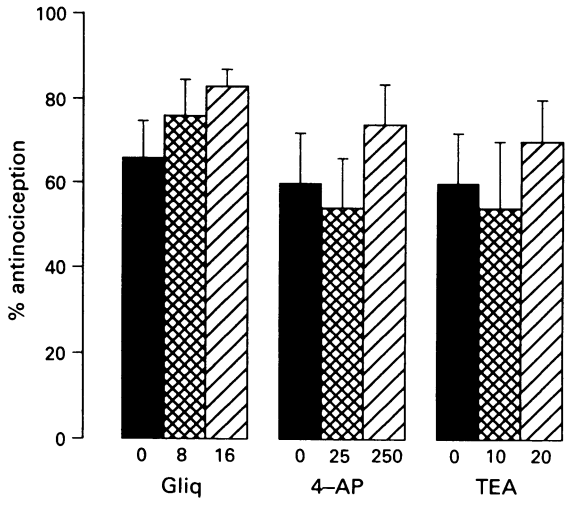

Figure 5 Effects of different K^+ channel blockers (Gliq = gliquidone; 4-AP = 4-aminopyridine; TEA = tetraethylammonium) on U50,488H-induced antinociception in a tail flick test in mice. The solid columns represent the effect of U50,488H (8 mg kg^{-1}, s.c.) + solvents. The doses of Gliq and TEA (µg/mouse), and those of 4-AP (ng/mouse) are shown below the columns. All the K^+ channel blockers were injected i.c.v. No statistically significant differences in comparison with U50,488H + solvent were found in any case (Newman Keuls test). Each column represents the mean ± s.e.mean of the values obtained in 8–12 animals.

Effects of 4-aminopyridine and tetraethylammonium on clonidine- and baclofen-induced antinociception

The i.c.v. administration of both 4-AP (2.5 and 25 ng/mouse) and TEA (10 and 20 µg/mouse) significantly antagonized the antinociceptive activity of baclofen, producing a displacement to the right of the baclofen dose-response line (Figures 3b and 4b). In contrast, neither 4-AP (25 and 250 ng/mouse, i.c.v.) nor TEA (10 and 20/µg mouse, i.c.v.) significantly modified the antinociceptive activity of clonidine (Figures 3a and 4a). An example of the time-course of the antinociception induced by clonidine and baclofen given with TEA (20 µg/mouse, i.c.v.) and 4-AP (25 ng/mouse, i.c.v.) is shown in Figure 2.

Neither TEA nor 4-AP significantly modified tail flick latency in control animals (data not shown), but the highest doses of both drugs produced signs of excitation in some animals (increased locomotor activity and number of explorations, groomings and rearings).

Effects of K^+ channel blockers on U50,488H-induced antinociception

As shown in Figure 5, i.c.v. administration of the K^+ channel blockers gliquidone (8 and 16 µg/mouse), 4-AP (25 and 250 ng/mouse) and TEA (10 and 20 µg/mouse) failed to modify significantly the antinociception induced by U50,488H (8 mg kg^{-1}, s.c.).

Discussion

Several kinds of K^+ channels with different electrophysiological characteristics and pharmacological sensitivities have been described in neurones (Halliwell, 1990; Aronson, 1992). Clonidine and other agonists of α_2-adrenoceptors open K^+ channels in neurones (Morita & North, 1981; Christie *et al.*, 1987; Tatsumi *et al.*, 1990) and produce antinociception (Fornai *et al.*, 1990). The possible relationship between these two effects was previously unknown, although it was suggested that noradrenaline, acting through α_2-adrenoceptors, may inhibit nociceptive input to the spinal cord by increasing potassium conductance in substantia gelatinosa neurones (North & Yoshimura, 1984). Our results show that clonidine-induced antinociception is antagonized by different sulphonylureas. All sulphonylureas tested to date specifically block ATP-dependent K^+ channels (Amoroso *et al.*, 1990; Cook & Quast, 1990; Schmid-Antomarchi *et al.*, 1990). Therefore, our results suggest that the opening of these K^+ channels is involved in clonidine antinociception. In support of this idea, it is interesting to note that the order of potency of different sulphonylureas in blocking ATP-dependent K^+ channels in CNS neurones – gliquidone > glipizide > glibenclamide > tolbutamide (Amoroso *et al.*, 1990; Schmid-Antomarchi *et al.*, 1990) – is the same order of potency as we found for blocking clonidine antinociception.

Neither TEA nor 4-AP antagonized clonidine-induced antinociception. That the lack of effect is not due to the use of low doses of the drugs, or to any methodological pitfall, is shown by the finding that the same or even lower doses of these K^+ channel blockers antagonize baclofen-induced antinociception, when administered by the same methods. Consequently, our results suggest that K^+ channels sensitive to TEA and 4-AP are not involved in the clonidine effect. These results were expected, as none of these K^+ channel blockers antagonizes the K^+ conductances induced by α_2-adrenoceptor agonists in neurones (North & Williams, 1985).

Various electrophysiological studies have suggested that the K^+ channels opened by α_2-adrenoceptor, μ- and δ-opioid receptor agonists are the same (Andrade & Aghajanian, 1985; North & Williams, 1985; Aghajanian & Wang, 1987; Tatsumi *et al.*, 1990). The present data confirm this idea, as sulphonylureas, but neither TEA nor 4-AP, antagonized cloni-

dine-induced antinociception, which is exactly the same pattern of activity as is shown by these K^+ channel blockers against morphine antinociception (Ocaña *et al.*, 1990; 1993; Wild *et al.*, 1991; Narita *et al.*, 1992) and the antinociception induced by [D-Pen^2, D-Pen^5]-enkephalin (Wild *et al.*, 1991), a proposed ∂_1-opioid receptor agonist (Mattia *et al.*, 1992). Taken together, these results suggest that α_2-adrenoceptors, μ- and ∂_1-opioid receptors are coupled to the same class of K^+ channels (probably K_{ATP} channels), and that the opening of these channels is involved in the antinociceptive effect of agonists of these receptors.

The antinociceptive effect of baclofen was antagonized by TEA and low doses of 4-AP. The degree of antagonism by TEA was lower than that caused by 4-AP, suggesting that 4-AP-sensitive K^+ channels play a more important role than TEA-sensitive channels in baclofen antinociception. These results are in agreement with the findings of electrophysiological studies showing that 4-AP markedly antagonized or even abolished the K^+ conductances induced by baclofen in neurones (Inoue *et al.*, 1985; Stevens *et al.*, 1985; Ogata *et al.*, 1987), whereas TEA did not antagonize (Inoue *et al.*, 1985) or only partially antagonized them (Stevens *et al.*, 1985; Lacey *et al.*, 1988). In addition, it has been shown that baclofen activates a 4-AP-sensitive K^+ channel in hippocampal neurones that mediates a voltage-dependent transient outward K^+ current with the characteristics of an A-current (Saint *et al.*, 1990). Bearing in mind these data it is tempting to assume that 4-AP antagonizes the baclofen antinociceptive effect by blocking this current. However, neither 4-AP nor TEA are specific blockers of a particular type of K^+ channel (Cook & Quast, 1990; Halliwell, 1990); consequently, considering only the sensitivity of baclofen-induced antinociception to these K^+ channel blockers, it is difficult to deduce what type of K^+ channel may underly this baclofen effect.

The antinociceptive effect of baclofen was not antagonized by gliquidone. The lack of effect of gliquidone cannot be an artifact, as under identical experimental conditions, even lower doses of gliquidone antagonized clonidine- (present study) and morphine-induced antinociception (Ocaña *et al.*, 1993). Consequently our results suggest that K_{ATP} channels are probably not involved in the antinociceptive effect of baclofen. Other drugs show a pattern of sensitivity to K^+ channel blockers similar to that of baclofen, the antinociception induced by [D-Ala^2]deltorphin II, a proposed ∂_2-opioid receptor agonist (Mattia *et al.*, 1992), being insensitive to glibenclamide but antagonized by TEA (Wild *et al.*, 1991).

It was previously shown that sulphonylureas did not antagonize U50,488H-induced antinociception (Narita *et al.*, 1992; Ocaña *et al.*, 1993); moreover, neither glibenclamide nor TEA antagonized the antinoception produced by U69,593 (Wild *et al.*, 1991), another κ-opioid receptor agonist. Our present results confirm, and extend these findings, since none of the K^+ channel blockers we tested antagonized U50,488H-induced antinociception. This lack of effect was expected, as U50,488H does not open K^+ channels in neurones, but does close Ca^{2+} channels (Cherubini & North, 1985; Christie & North, 1988; Xiang *et al.*, 1990). The lack of antagonism of the U50,488H effect by the different K^+ channel blockers is interesting, because it suggests that these drugs do not antagonize, in an unspecific and indiscriminate way, the antinociception induced by any drug. Instead, K^+ channel blockers seem to antagonize specifically only the antinociception due to drugs that activate receptors linked to K^+ channels.

In conclusion, our study shows that the antinociceptive effect of clonidine and baclofen are differentially antagonized by K^+ channel blockers. This suggests (1) that the opening of K^+ channels plays a role in antinociception, and (2) that different K^+ channels underlie the antinociceptive effect of α_2-adrenoceptor and $GABA_B$-receptor agonists.

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Characterization of purinoceptors mediating depolarization of rat isolated vagus nerve

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1 As part of a broader study to characterize neuronal purinoceptors, the effects of adenosine 5'-triphosphate (ATP) and a range of ATP analogues were investigated on the extracellularly recorded membrane potential of the rat isolated vagus nerve, using a 'grease-gap' technique.

2 ATP evoked depolarization of the rat vagus nerve. The concentration-effect curve to ATP was not monophasic: at the lower concentrations (1×10^{-5}–1×10^{-3} M) the curve was shallow (<50% of the near maximal response to 5-hydroxytryptamine (5-HT)) whilst at higher concentrations the relationship between concentration and amplitude of depolarization was steeper (>135% of the response to 5-HT at the highest concentration tested, 1×10^{-2} M). On washout of the high drug concentrations large after-hyperpolarizations were often observed.

3 α,β-methylene ATP (1×10^{-6}–3×10^{-4} M), β,γ-methylene ATP (1×10^{-6}–1×10^{-3} M), and 5'-adenylylimidodiphosphate (β,γ-imido ATP; 1×10^{-6}–1×10^{-3} M) were all more potent than ATP and produced large depolarizations of the rat vagus nerve at the highest concentrations tested (>150% of the response to 5-HT). The overall rank order of potency was α,β-methylene ATP > β,γ-methylene ATP = β,γ-imido ATP > ATP.

4 In contrast, 2-methylthio ATP (1×10^{-6}–1×10^{-3} M) produced relatively small depolarizations (<100% of the response to 5-HT). As was the case with low concentrations of ATP, the concentration-effect curve to 2-methylthio ATP was very shallow.

5 Adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine and adenosine 5'-O-(2-thiodiphosphate) (ADP-β-s; all 1×10^{-6}–1×10^{-3} M) evoked only small depolarizations of the vagus nerve, amounting to 47 ± 2.5%, 40.8 ± 7.8%, 33.7 ± 3.3% and 62.4 ± 12.7% of the response to 5-HT, respectively. Uridine 5'-triphosphate (UTP; 1×10^{-6}–1×10^{-3} M) was inactive.

6 The P_2 purinoceptor antagonist, suramin (1×10^{-5} M–1×10^{-4} M), antagonized responses to α,β-methylene ATP. The nature of this antagonism was not, however, consistent with simple competitive kinetics between agonist and antagonist. Depolarizations produced by β,γ-methylene ATP and β,γ-imido ATP were also attenuated by suramin (1×10^{-4} M), but in contrast, suramin had no effect on responses to ADP, 2-methylthio ATP, ADP-β-S or 5-HT.

7 In addition to its antagonist effects, suramin (10^{-4} M) markedly increased the maximum amplitude of the depolarization produced by ATP.

8 It is concluded that a heterogeneous receptor population mediates depolarization of the rat vagus nerve by purine nucleotides. Importantly, the large amplitude depolarizations to α,β-methylene ATP, β,γ-methylene ATP and β,γ-imido ATP are mediated via receptors that share many characteristics of the classical P_{2x} receptor. In contrast, the relatively small depolarizing effects of ADP, ADP-β-S and 2-methylthio ATP were suramin-resistant. Although it appears that other purinoceptors are present, these data suggest that the rat vagus nerve may serve as a useful preparation for studying the pharmacology of neuronal P_{2x} receptors.

Keywords: Rat vagus nerve; ATP; P_2 purinoceptors; suramin; depolarization

Introduction

It is widely accepted that adenosine 5'-triphosphate (ATP) acts as an excitatory co-transmitter of certain postganglionic parasympathetic, postganglionic sympathetic and sensory neurones (see Burnstock, 1990 for review). More recently direct evidence for a role for ATP in excitatory synaptic transmission has been provided (Evans *et al.*, 1992; Silinsky *et al.*, 1992). Neuromodulatory effects of purines in the central nervous system have also been described (Harms *et al.*, 1992; Sun *et al.*, 1992; Tschöpl *et al.*, 1992). As with other neurotransmitters, the neuronal effects of ATP are mediated through specific extracellular receptors. These receptors for ATP have been termed purinoceptors although they may belong to a larger family of nucleotide receptors (Burnstock & Kennedy, 1985; Cusack, 1993).

To date, relatively few studies have focused on the characterization of neuronal purinoceptors. Indeed, the framework for the present purinoceptor classification system (Burnstock, 1978; Burnstock & Kennedy, 1985) is founded almost exclusively on studies in non-neuronal, often multicellular preparations. From these studies purinoceptors have been divided into P_1 receptors, at which adenosine and AMP are more active than ADP and ATP, and P_2 receptors for which the reverse rank order of agonist potency occurs. Both the P_1 and P_2 receptor groups have been further subdivided on the basis of relative potencies of adenosine and ATP analogues and on the antagonist profiles of drugs such as the methylxanthines and suramin (see Fedan & Lamport, 1990, and Cussack, 1993, for reviews). At least four different subtypes of P_2 purinoceptors have been proposed (Burnstock & Kennedy, 1985; Gordon, 1986). The P_{2x} and P_{2y} receptor subtypes were first described on the basis of differential relative potencies of ATP analogues in vascular and visceral smooth muscles such that α,β-methylene ATP is considered to be most potent at P_{2x} receptors and 2-methylthio ATP is most potent at P_{2y} receptors (Burnstock & Kennedy, 1985). P_{2t} and P_{2z}

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receptors have been characterized in studies on platelets and mast cells, respectively (Gordon, 1986). Most recently a binding site resembling a P_2 purinoceptor in rat brain synaptosomes has been tentatively designated P_{2d} (Pintor *et al.*, 1993).

Electrophysiological experiments in single neurones and brain slices have provided some indication of the nature of purinoceptors mediating membrane depolarization (see Bean, 1992, and Illes & Nörenberg, 1993, for reviews). For example, structure-function relationships for purine nucleotide-evoked depolarization of rat cultured nodose ganglion neurones have been described (Krishtal *et al.*, 1988b) although no attempt was made to classify the receptor type(s) mediating this effect. In bullfrog dorsal root ganglion cells and rat and guinea-pig cardiac ganglia, membrane depolarization evoked by ATP is mediated via a P_2 purinoceptor which resembles the P_{2y} receptor (Allen & Burnstock, 1990; Tokimasa & Akasu, 1990; Fieber & Adams, 1991). In contrast, the purinoceptor that mediates excitation of neurones in the rat locus coeruleus paradoxically shares characteristics of both the P_{2x} and the P_{2y} receptor (Harms *et al.*, 1992; Tschöpl *et al.*, 1992; but see Illes & Nörenberg, 1993). However, the lack of simple quantitative neuronal assay systems for the measurement of relative agonist potencies and of antagonist affinities has undoubtedly hindered the characterization of neuronal purinoceptors.

We describe for the first time the depolarizing effect of ATP and ATP analogues on the extracellularly recorded membrane potential of the rat vagus nerve, measured by a 'grease-gap' technique. This preparation provides a technically convenient assay for the characterization of neuronal purinoceptors.

Methods

Extracellular recordings of agonist-induced depolarizations were made from segments of rat isolated cervical vagus nerve according to the method of Ireland & Tyers (1987). Briefly, male AHA Wistar rats (200–270 g) were stunned by a blow to the head, decapitated, and the cervical vagus nerves rapidly excised. Segments of nerve, approximately 15–20 mm long, were desheathed under a dissecting microscope, and transferred to heated (27°C) two-compartment Perspex baths such that approximately 50% of the nerve lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. The d.c. potential between the two compartments was measured with silver-silver chloride electrodes connected to the preparation through agar-saline/filter paper bridges. Signals were amplified, filtered (0.5 Hz) and displayed on a chart recorder (Lectromed Multitrace 8). Each compartment of the bath was perfused continuously with Krebs solution, preheated to 27°C and gassed with 95% O_2/5% CO_2, at a rate of 1–2 ml min^{-1}. Drugs were applied at known concentrations into the perfusate of the first compartment only.

Experimental protocols

After a 30 min equilibration period the viability of each preparation was assessed by exposure to a near maximal concentration of 5-HT (1×10^{-5} M) for 2 min. Preparations that depolarized by less than 250 μV were rejected (<15% of preparations). Repeated exposures to 5-HT (1×10^{-5} M) for 2 min at 30 min intervals were performed until reproducible depolarizations were obtained. After a further 30 min re-equilibration period, concentration-effect curves for agonist-induced depolarization were constructed non-cumulatively using serially increasing concentrations. Each concentration of agonist was applied for 2–2.5 min during which time a peak effect was reached. An interval of 45 min was left between agonist applications since in preliminary experiments depolarizing responses to purinoceptor drugs showed tachyphylaxis if shorter intervals were employed. Use of this protocol precluded construction of more than one concentration-effect curve in any one preparation. Relative agonist potencies and the effects of antagonists were thus determined by comparing with respective time- and vehicle-matched con-

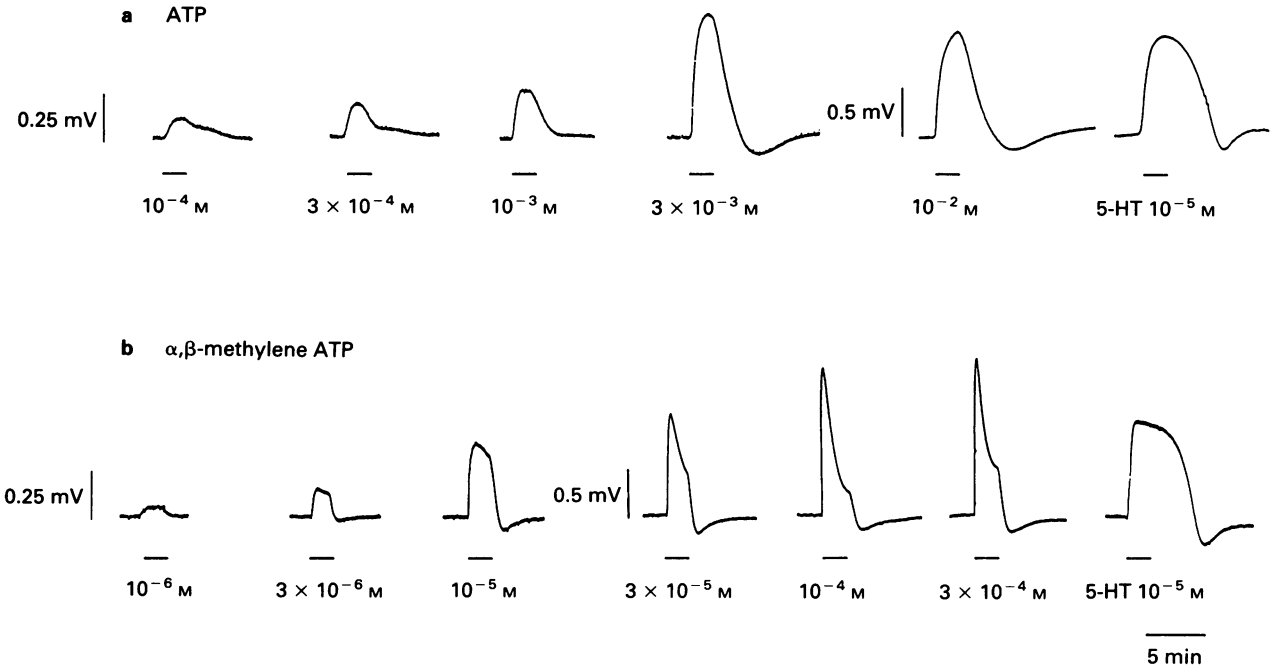

Figure 1 Discontinuous records of the effect of (a) adenosine 5'-triphosphate (ATP) and (b) α,β-methylene ATP on extracellularly recorded membrane potentials in the rat isolated vagus nerve. An upward deflection indicates depolarization of the nerve trunk, calibrated in mV. Each concentration of drug was perfused for the time period indicated by the solid horizontal bar; 45 min was left between agonist applications. For comparison the near maximal depolarization to 5-hydroxytryptamine (5-HT, 1×10^{-5} M) in the same preparation is also shown.

trol curves from other preparations. Antagonist contact times of 45 min were employed.

Analysis of results

The depolarization evoked by purinoceptor agonists was measured as the peak change (μV) in the d.c. potential between the two compartments, and expressed as a percentage of the response to 5-HT (1×10^{-5} M). Due to the limited availability of some agents it was not always possible to achieve the maximum of the agonist concentration-effect curve. Thus, to measure agonist potency, the concentration of agonist required to produce the equivalent of 100% of the response to 5-HT (1×10^{-5} M) was estimated from individual concentration-effect curves. This is referred to as EC_{100}. A response of this magnitude lay on the linear part of the concentration-effect curve for the purinoceptor agonists that evoked marked depolarizations. Antagonist effects were quantified, where possible, by measuring lateral displacements of the concentration-effect curves at a level of 50% of the maximum measured control response to give concentration-ratios between antagonist-treated and control, vehicle-treated preparations. In cases where the antagonist depressed the agonist response such that parallel displacements of the concentration-effect curve were not observed and lateral displacements could not be calculated, the results are expressed as percent inhibition of the agonist response at the highest concentration tested.

Data are expressed as arithmetic mean ± s.e.mean or geometric mean with 95% confidence limits where appropriate. Differences between groups were assessed by Student's unpaired t test and considered significant when $P < 0.05$.

Drugs and solutions

The composition of the Krebs solution was as follows (mM in de-ionised water): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, D-glucose 11.1, $CaCl_2.6H_2O$ 1.3. The following drugs were used: 5-hydroxytryptamine creatinine sulphate (5-HT), adenosine 5'-triphosphate disodium salt (ATP), α,β-methylene ATP lithium salt, uridine 5'triphosphate sodium salt (UTP), β,γ-methylene ATP, 5'-adenylylimidodiphosphate lithium salt (β,γ-imido ATP), adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate sodium salt (ADP), adenosine 5'-O-(2-thiodiphosphate) trilithium salt (ADP-β-s, all Sigma), 2-methylthio ATP tetra sodium salt (Research Biochemicals Incorporated), suramin (Bayer). All drugs were dissolved and diluted to the required concentration in Krebs solution, and stored on ice.

Results

Effect of ATP

Adenosine 5'-triphosphate evoked concentration-related depolarizations of rat isolated vagus nerve, amounting to 138.4 ± 22.0% ($n = 4$) of the amplitude of the response to 5-HT at the highest concentration tested (1×10^{-2} M; see Figure 1a and Figure 2). At the higher concentrations some preparations displayed marked after-hyperpolarizations on washout of the drug. The concentration-effect curve to ATP was not monophasic; at concentrations up to 1×10^{-3} M the relationship between concentration and depolarizing effect was very shallow whilst higher concentrations (1×10^{-3}–1×10^{-2} M) produced a much steeper concentration-effect curve.

Effect of analogues of ATP

A range of ATP analogues were tested for activity on the rat vagus nerve (Figures 2 and 3). Of these, α,β-methylene ATP

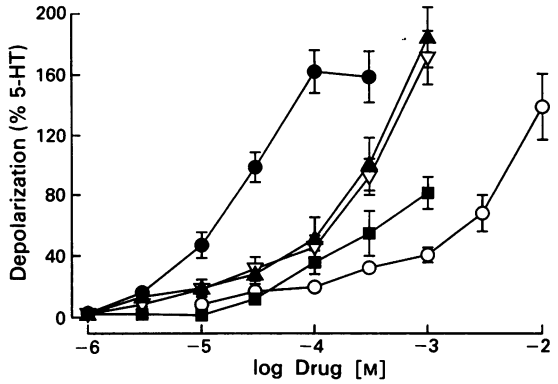

Figure 2 Comparison of the depolarizing effects of α,β-methylene ATP (●), β,γ-imido ATP (▽), β,γ-methylene ATP (▲), 2-methylthio ATP (■) and ATP (○) on rat isolated vagus nerve. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4$–8). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-hydroxytryptamine (5-HT, 1×10^{-5} M).

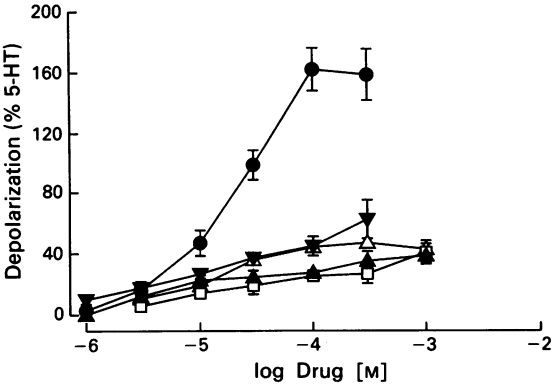

Figure 3 Depolarizing effects of ADP (△), AMP (□), adenosine (▲) and ADP-β-S (▼) on rat isolated vagus nerve. For comparison the effect of α,β-methylene ATP (●) is also shown. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4$–8). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-hydroxytryptamine (5-HT, 1×10^{-5} M).

was the most potent with a threshold concentration for depolarization of 1×10^{-6} M and an EC_{100} value of 1.75×10^{-5} M (95% confidence intervals 0.97–3.18×10^{-5} M, $n = 8$). The maximal effect of α,β-methylene ATP was 165.2 ± 15.0% of the response to 5-HT. Typically, depolarizations to α,β-methylene ATP reached the peak response more rapidly than responses to ATP and faded more rapidly in the continued presence of the drug (see Figure 1). Both β,γ-imido ATP and β,γ-methylene ATP were approximately 15 fold less potent than α,β-methylene ATP with respective EC_{100} values of 2.70×10^{-4} M (1.49–4.92×10^{-4} M, $n = 4$) and 3.48×10^{-4} M (2.30–5.27×10^{-4} M, $n = 4$) and evoked similar maximal depolarizations at the highest concentration tested (1×10^{-3} M; 184.3 ± 20.0% and 171.2 ± 17.5%, respectively). In general, depolarizations to β,γ-imido ATP and β,γ-methylene ATP also achieved the peak effect more rapidly than responses to ATP, but the marked fade observed with α,β-methylene ATP was less apparent. Marked after-hyperpolarizations were observed on washout of high concentrations of these methylene and imido analogues of ATP. The concentration-effect curve to 2-methylthio-ATP was shallow, and at the highest concentration tested (1×10^{-3} M) the amplitude of the depolarizing effect of this agent was less than 100% of the response to

5-HT. Consequently, an EC_{100} value could not be determined. ADP, AMP and adenosine (all 1×10^{-6} M–1×10^{-3} M) evoked relatively small depolarizations of the vagus nerve, the peak effects at 1×10^{-3} M being 47.0 ± 2.5% ($n = 4$), 40.8% ± 7.8 ($n = 4$) and 33.7 ± 3.3% ($n = 8$) of the response to 5-HT, respectively (see Figure 3). Similarly, depolarizations to ADP-β-S (1×10^{-6}–3×10^{-4} M) were small in comparison to the 5-HT response (62.4% ± 12.7, $n = 7$). Uridine 5'-triphosphate was inactive at concentrations up to 1×10^{-3} M.

Effect of suramin on depolarization to ATP analogues

Suramin (1×10^{-5}–1×10^{-4} M) had no effect on the extracellularly-recorded membrane potential, but produced rightward displacement of the concentration-effect curve to α,β-methylene ATP (see Figure 4a). At concentrations of 1×10^{-5} M and 3×10^{-5} M the agonist concentration-ratios in the presence of suramin did not appear to be concentration-related (8.8 ± 3.1, $n = 5$ and 3.8 ± 0.6, $n = 4$, respectively). The analysis of these data was complicated by an increase in the maximum response to α,β-methylene ATP in the presence of suramin. At the highest concentration of suramin tested (1×10^{-4} M) the displacement appeared parallel and the mean concentration-ratio was 15.5 ± 5.6 ($n = 5$). This corresponds to a pK_B value of 5.04 ± 0.15. Suramin (1×10^{-4} M) also antagonized responses to β,γ-imido ATP (see Figure 5a) and β,γ-methylene ATP; the percentage inhibition of depolarization at the highest concentration of agonist tested (1×10^{-3} M) was 67.1 ± 3.1% ($n = 4$) and 57.2 ± 10.7% ($n = 5$), respectively.

In contrast, depolarizations to 2-methylthio ATP (Figure 5b), ADP and ADP-β-S were resistant to antagonism by suramin (concentration-ratios of 0.93 ± 0.27, 2.34 ± 0.68 and 1.61 ± 0.57, all $n = 4$, respectively).

Effects of suramin on depolarizations to ATP

The maximum amplitude of the depolarization evoked by ATP was markedly enhanced by suramin (1×10^{-4} M; see Figure 5c).

Specificity of suramin

Concentration-effect curves to 5-HT (1×10^{-7}–3×10^{-5} M) were constructed in the presence of suramin or vehicle following the same protocol described for the ATP analogues. Maximal responses to 5-HT were achieved such that EC_{50} values could be calculated. 5-HT evoked concentration-related depolarizations of rat vagus nerve with a mean EC_{50} value of 4.79×10^{-7} M (3.04–7.51×10^{-7} M, $n = 4$). Suramin (1×10^{-5}, 3×10^{-5}, 1×10^{-4} M) had no significant effect on either the potency or maximum amplitude of depolarization produced by 5-HT (see Figure 4b).

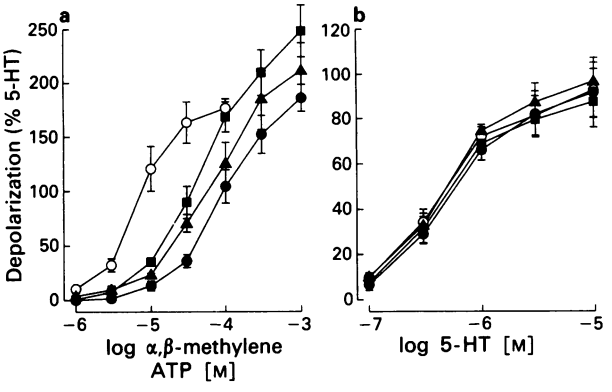

Figure 4 Effect of suramin on depolarizations of rat isolated vagus nerve evoked by (a) α,β-methylene ATP and (b) 5-hydroxytryptamine (5-HT). Symbols indicate responses to agonist in the presence of vehicle (○) or in the presence of 1×10^{-5} M (▲), 3×10^{-5} M (■) or 1×10^{-4} M suramin (●). Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4$–5). The abscissa scale shows the log molar concentration of drug and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-HT (1×10^{-5} M). Note the rightward displacement of the α,β-methylene ATP concentration-effect curve by suramin, and the increase in the maximum response to the agonist.

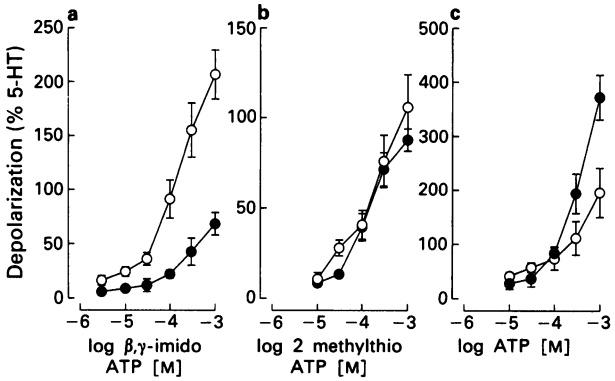

Figure 5 Effect of suramin on depolarizations of rat vagus nerve to (a) β,γ-imido ATP, (b) 2-methylthio ATP and (c) adenosine 5'-triphosphate (ATP). The responses to each agonist in the presence of vehicle or suramin (1×10^{-4} M) are represented by (○) and by (●), respectively. Each point represents the mean of single determinations and the vertical bars the s.e.mean ($n = 4$–5). In each panel the abscissa scale shows the log molar concentration of agonist and the ordinate scale the depolarizing response expressed as a percentage of the depolarization evoked by 5-HT (1×10^{-5} M). Note that suramin antagonizes responses to β,γ-imido ATP but not 2-methylthio ATP. Depolarizations to ATP were augmented in the presence of suramin.

Discussion

In the present study, using an extracellular recording system, we observed that ATP and other purine nucleotides depolarized the rat isolated vagus nerve. Although several other workers have shown that ATP excites neurones grown in culture from parasympathetic cardiac ganglia (Allen & Burnstock, 1990; Fieber & Adams, 1991) and nodose ganglia (Krishtal *et al.*, 1988a,b) this is, as far as we know, the first demonstration of a depolarizing effect of this agent on whole vagal nerve trunks.

The depolarizing action of ATP was marked but high concentrations were necessary. This, perhaps, reflects the rapid degradation of the nucleotide by ecto-nucleotidases to ADP, AMP and adenosine (see Welford *et al.*, 1987 and Hourani & Chown, 1989). In other neuronal preparations, adenosine evokes hyperpolarizing responses which offset the excitatory effects of ATP (e.g. Tschöpl *et al.*, 1992; Illes & Nörenberg, 1993). This would not, however, appear to be the case in the rat vagus nerve since adenosine itself evoked small depolarizations (< 50% of the response to 5-HT) rather than hyperpolarizations. Furthermore, in preliminary experiments responses to ATP were not modified by the adenosine antagonist 8-parasulphophenyltheophylline (3×10^{-5} M; data not shown). Another possibility is that ATP simultaneously activates an inhibitory purinoceptor on the vagus nerve. However, even though the marked depolarization evoked by high concentrations of ATP was often followed by a large after-hyperpolarization on washout of the drug it seems improbable that this reflects activation of an inhibitory receptor since there was no indication of an initial hyperpolarizing effect of any of the other purines tested. It seems more plausible that the after-hyperpolarization is due to activation of membrane Na^+/K^+ ATPases since similar, ouabain-sensitive after-hyperpolarizations have been reported with

other depolarizing agents (e.g. acetylcholine, Brown *et al.*, 1972). However, the ouabain-sensitivity of the after-hyperpolarization evoked by ATP has not been examined and so this possibility cannot be excluded.

The concentration-effect curve to ATP was clearly not monophasic; at low concentrations (1×10^{-5}–1×10^{-3} M) a very shallow relationship between concentration and the amplitude of depolarization was observed, whilst at higher concentrations ($> 1 \times 10^{-3}$ M) this relationship was much steeper. Importantly, the large amplitude depolarizations evoked by ATP could not be attributed to excitatory effects of either ADP, AMP or adenosine, since responses to these purines were relatively small. These data suggest that a P_2, rather than a P_1 purinoceptor mediates the large amplitude depolarizations to ATP (Burnstock & Kennedy, 1985).

The relative potencies and amplitudes of depolarization of other ATP analogues support this proposal. Of the purines tested only three other agonists (α,β-methylene ATP, β,γ-imido ATP, and β,γ-methylene ATP) evoked depolarizations whose peak amplitude was comparable with the effect of high concentrations of ATP. Although true maximal responses for β,γ-imido ATP and β,γ-methylene ATP could not be determined (due to limited availability of these compounds) the amplitude of the depolarizations to these agents at the highest concentration tested were similar to the maximal effect of α,β-methylene ATP. α,β-methylene ATP was the most potent being some 15–20 fold more potent than both β,γ-imido ATP and β,γ-methylene ATP, and 330 fold more potent that ATP itself. Similar rank orders of agonist potency for actions at P_{2x} receptors in the guinea-pig bladder (Cusack & Hourani, 1984) and the rabbit ear artery (O'Connor *et al.*, 1990) have been described. Indeed, this high potency of α,β-methylene ATP is generally considered indicative of an action at this receptor subtype. Moreover, 2-methylthio ATP and ADP-β-S, purines that show certain selectivity for the P_{2y} purinoceptor subtype (Burnstock & Kennedy, 1985; Cusack 1993) were relatively weak. Although it is prudent to be cautious when characterizing receptor subtypes on the basis of relative agonist potencies, taken together, these observations are consistent with the view that a P_{2x} purinoceptor mediates the large amplitude depolarizations of the rat vagus nerve to purine nucleotides.

In other preparations activation of P_{2x} purinoceptors by α,β-methylene ATP, and to a lesser degree ATP, evokes pronounced tachyphylaxis (Kasakov & Burnstock, 1983; see Fedan & Lamport, 1990). This phenomenon is not considered to occur with P_{2y} purinoceptors (Burnstock & Kennedy, 1985). Although tachyphylaxis was not specifically investigated in the present study, it was clear that the depolarization produced by high concentrations of α,β-methylene ATP faded rapidly in the continued presence of the drug. In addition, consecutive responses to this agent became progessively smaller if less than 45 min was permitted between drug applications. Interestingly, the fade of depolarizations produced by ATP and the β,γ-methylene and β,γ-imido analogues of ATP was less marked. It remains to be seen whether these superficial observations on fade of response correlate in any way with tachyphylaxis and receptor desensitization. Furthermore, more work is needed to determine whether desensitization is a characteristic of P_{2x} receptors in general or, alternatively, whether it is a specific property of the agonist α,β-methylene ATP itself.

The finding that the P_2 purinoceptor antagonist, suramin (Dunn & Blakeley, 1988; Leff *et al.*, 1990) inhibited depolarizations to α,β-methylene ATP, β,γ-imido ATP and β,γ-methylene ATP provides further evidence for the involvement of a P_2 receptor. Under our experimental conditions suramin did not, however, behave simply as a classical competitive antagonist, in that the magnitude of the displacement of the agonist concentration-effect curve was not directly related to the antagonist concentration. Interestingly, in rabbit ear artery suramin was shown to equilibrate only very slowly with the P_{2x} receptor and the nature of the antagonism was closely related to the incubation time (Leff *et al.*, 1990). Since in the present study the agonist concentration-effect curve took almost 5 h to construct it is possible that the complex nature of the antagonism is in some way related to the differing incubation times that the effects of each concentration of agonist were investigated at. The augmentation of responses to high concentrations of α,β-methylene ATP by suramin (1×10^{-5} M and 3×10^{-5} M, but not 1×10^{-4} M) is a further complicating factor. Indeed, somewhat surprisingly, suramin augmented rather than antagonized the responses to high concentrations of ATP. It seems unlikely that this reflects a non-specific membrane effect of suramin in that depolarizations to 5-HT were unaffected. A similar augmentation by suramin of contractions of the guinea-pig urinary bladder to α,β-methylene ATP, but not carbachol, has been reported (Hoyle *et al.*, 1990). It is plausible that low concentrations of suramin preferentially antagonize an inhibitory receptor activated by high concentrations of the purinoceptor agonist. Alternatively, suramin may bind to a subunit on the purinoceptor to facilitate allosterically agonist effects. Indeed, suramin has already been shown to modulate nicotinic receptor function in skeletal muscle by what seems to be an allosteric mechanism (Henning *et al.*, 1992). In the case of ATP, it is possible that suramin retards degradation via interaction with an ATPase, although this seems unlikely given the observation that very high concentrations of suramin (10 mM) are required to inhibit ecto ATPase in the guinea-pig urinary bladder (Hourani & Chown, 1989). No definitive evidence to support any of these hypotheses was obtained in the present study and therefore the mechanism(s) underlying this phenomenon remains unclear. Despite these complications, it is clear that suramin antagonizes the large amplitude depolarizations of the rat vagus nerve to the methylene and imido analogues of ATP thus supporting the proposal that the responses that they produce are mediated via P_2 purinoceptors.

In contrast, the small depolarizing responses to 2-methylthio ATP, ADP-β-S and ADP were resistant to suramin. These data imply that one or more purinoceptors, by definition distinct from the known classical suramin-sensitive P_2 purinoceptors, also mediate depolarization of the rat vagus nerve. Although the suramin-sensitivity of the P_{2u} receptor has not been reported it is unlikely that these responses are mediated via this receptor type since UTP was inactive as an agonist (Cusack, 1993). It is conceivable that these drugs activate a receptor that corresponds to the diadenosine polyphosphate binding site in rat brain synaptosomes, tentatively designated P_{2d} by Pintor and coworkers (1993). Another possibility is that these agents or their metabolites interact with adenosine (P_1) purinoceptors to evoke membrane depolarization; this supposition is at present the subject of investigation in our laboratory. Importantly, the suramin-resistance of these responses indicates that these agents are not partial agonists at the receptor activated by α,β-methylene ATP.

In summary, we have provided evidence that the rat cervical vagus nerve contains a heterogeneous purinoceptor population that mediates membrane depolarization. From the relative order of potencies of a series of ATP analogues, the predominant effect is mediated by a receptor which appears to be a P_{2x} receptor. Interestingly, from patch- and voltage-clamp studies on neurones cultured from parasympathetic intracardiac ganglia it appears that the excitatory effects of purines are mediated via a receptor that shares more similarities with the P_{2y} than the P_{2x} purinoceptor (Allen & Burnstock, 1990; Fieber & Adams, 1991). It remains to be established whether there is a genuine difference in the purine receptor population on the intracardiac neurones and on the axonal membranes of the cervical vagal nerve trunk or whether these contrasting findings can be attributed to the different methods used to study these receptors. For example, working with single cells may minimize some of the problems of purine nucleotide metabolism

associated with whole tissue experiments such that a more accurate index of the potency of the less stable purines (e.g. ATP and 2-methylthio ATP) is obtained. However, it is difficult to perform quantitative pharmacology using these methods and it is uncertain whether these neurones grown in culture express the same receptor types as the parent cells. The limitations of the available drug tools are another major problem, and the advent of more selective P_2 purinoceptor agonists and antagonists will undoubtedly aid in addressing these issues. The findings from this study indicate that the rat vagus nerve preparation may prove to be a useful preparation in which to study the activity of such novel agents on neuronal P_{2x} receptors.

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The effects of cholinoceptor agonists and antagonists on C-fibre evoked responses in the substantia gelatinosa of neonatal rat spinal cord slices

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1 The effects of cholinoceptor agonists and antagonists were studied on neurones in the substantia gelatinosa (SG) of an *in vitro* spinal cord slice and nerve preparation from neonatal rats.

2 Bath application of carbachol (1–50 μM) reduced, in a dose-related manner, the amplitude and duration of the excitatory postsynaptic potentials (e.p.s.ps) evoked in response to nerve stimulation.

3 The latencies and stimulation thresholds required to evoke these e.p.s.ps suggested that the majority were due to C-fibre activation.

4 The reduction in e.p.s.p. amplitude and duration produced by carbachol was reversed by the muscarinic antagonists, atropine (in 8 out of 11 cells), pirenzepine (in 7 out of 9 cells) and methoctramine (in 8 out of 9 cells) and by the nicotinic antagonist mecamylamine (in 3 out of 7 cells).

5 Injection of small hyperpolarizing or depolarizing pulses was associated with no change in conductance in 19 out of 26 (73%) of cells tested, suggesting that an action at a site presynaptic to the neurone studied could account for part of the effect of carbachol.

6 It is proposed that some of the cholinoceptors associated with the e.p.s.p. depression are located on C-fibres.

Keywords: Substantia gelatinosa; cholinoceptor; C-fibre; nociception; neonatal rat spinal cord

Introduction

Antinociceptive actions of cholinomimetics have been reported in a number of animal models of nociception (Yaksh *et al.*, 1985; Green & Kitchen, 1986; Smith *et al.*, 1989). A recent study (Bartolini *et al.*, 1992) has shown the muscarinic M_1 cholinoceptor agonists, MCN A-343 and AF102B, to be antinociceptive in models of mechanical, thermal and chemical nociception, whereas the M_2 agonist, arecaidine, produced hyperalgesia in mechanical and thermal tests.

A spinal mechanism of action of cholinoceptor antinociception is suggested by experiments in which cholinoceptor agonists were applied into the lumbar sub-arachnoid space (Yaksh *et al.*, 1985; Smith *et al.*, 1989). Antinociceptive effects in these experiments were reversed by the non-selective muscarinic antagonist, atropine but not by nicotinic antagonists. Antinociception was also produced with the acetylcholinesterase inhibitor neostigmine, suggesting the involvement of endogenous spinal cholinergic neurones in mediating antinociception. Depletion of acetylcholine by hemicholinium-3 also reduced the antinociceptive response to administration of neostigmine (Smith *et al.*, 1989).

There is evidence from binding studies for the presence of both muscarinic and nicotinic receptors in the spinal cord and dorsal root ganglia (DRG) in several species, including man (Seybold, 1985; Villiger & Faull, 1985; Gillberg *et al.*, 1988; Quirion *et al.*, 1989). Although the presence of a binding site does not necessarily indicate the presence of a functional receptor, it is at least suggestive of such a receptor. Using the muscarinic receptor antagonist [^{3}H]-quinuclidinyl benzilate (QNB), and specific M_1 and M_2 receptor antagonists, pirenzepine and 4-diphenylacetyl-*N*-methyl piperidine (4-DAMP), Bouchenafa & Livingston (1991), have shown that both M_1 and M_2 binding sites are present in the substantia gelatinosa (SG). Yamamura *et al.* (1983), have also shown the presence of binding sites in the SG using the M_1 receptor subtype antagonist [^{3}H]-pirenzepine. Muscarinic binding sites are found in the DRG and are transported in the sciatic nerve (Walmsley *et al.*, 1981); thus a presynaptic localization of receptors is likely. α-Bungarotoxin binding sites are also found in the superficial dorsal horn, being predominantely localized to laminae I and III. Binding sites are present in a population of large DRG neurones, transported in the sciatic nerve, and decreased in lamina III following dorsal rhizotomy, indicating their presence on large primary afferents (Ninkovic & Hunt, 1983). Neuronal bungarotoxin-sensitive nicotinic receptors have also been demonstrated in DRG in culture (Sucher *et al.*, 1990). Another study has shown the presence of α3, α4 and β2 nicotinic receptor subunit mRNA in the SG and α4 and β2 mRNA in lamina III (Wada *et al.*, 1989).

A number of studies have demonstrated the presence of cholinergic neurones and fibres in the dorsal horn (Barber *et al.*, 1984; Phelps *et al.*, 1984; Borges & Iversen, 1986; Ribeiro da Silva & Cuello, 1990). Borges & Iverson (1986) found cholinergic fibres to be most prominent in lamina III. Barber *et al.* (1984) showed the highest density of synaptic terminals containing choline acetyltransferase (ChAT) immunoreactivity to be in lamina III, whilst cell bodies were located in laminae III–IV but not in laminae I and II. A similar pattern was also seen by Ribeiro da Silva & Cuello (1990) who also showed cholinergic fibres in inner lamina II and to a lesser extent in outer lamina II and lamina I.

Effects of cholinoceptor agonists and antagonists have been reported on neurones in laminae III to V of rat spinal cord slices (Urban *et al.*, 1989); however little or no attention has been paid to the more superficial part of the dorsal horn.

Since the SG of the spinal cord is a major target for primary afferent fibres conducting in the C-fibre range, and is considered to be important in spinal nociceptive processing (Cervero & Iggo, 1980), we have examined the effects of cholinceptor agonists and antagonists on neurones in this region, especially with regard to the potentials evoked by primary afferent nerve stimulation. In this study we have focused on the role of muscarinic receptors in mediating the effects of carbachol on the e.p.s.ps evoked by afferent nerve stimulation.

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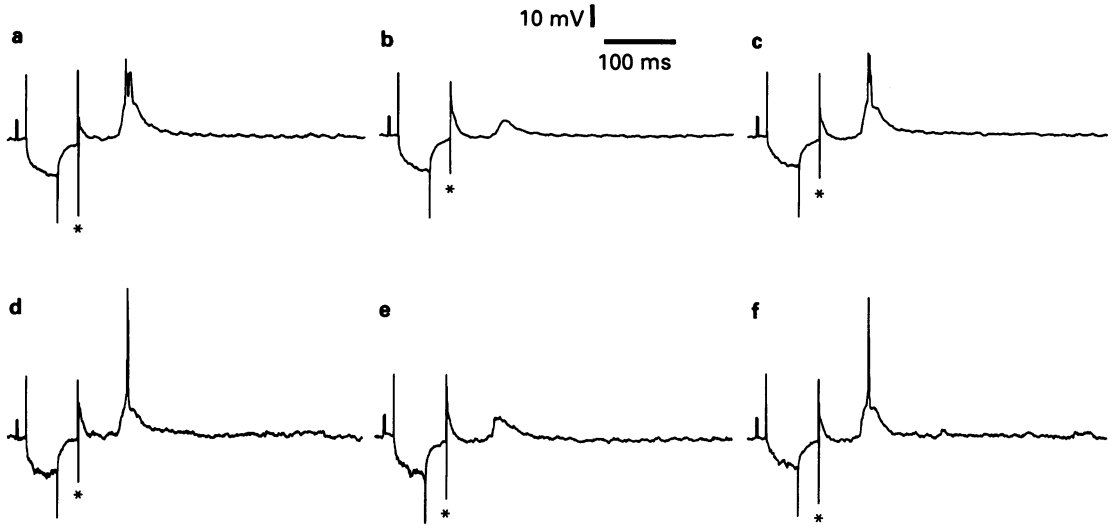

Figure 1 Effect of carbachol on an example of an e.p.s.p. evoked by nerve stimulation. (100 V, 0.5 ms, 0.2 Hz): (a), (b) and (c) show averages of 5 records; (d), (e) and (f) show representative single sweeps. (a) and (d) Show control responses before addition of carbachol (10 μM); (b) and (e) show responses obtained during carbachol administration, and (c) and (f) show recovery of the e.p.s.p. after washout with drug-free ACSF. In this figure and Figures 2, 3 and 4 the time of stimulus application can be seen by the presence of a stimulus artifact which is indicated by an asterisk. In a number of these records hyperpolarizing potentials are present just prior to the stimulus application. These were produced by hyperpolarizing current injection which was used to estimate conductance changes.

Methods

Neonatal rats (age 12–18 days) were anaesthetized with ether and the spinal cord was dissected, leaving the sciatic and femoral nerves attached on one side, in a bath continuously perfused with cold (4°C), gassed (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) at a flow rate of at least 5 ml min^{-1}. A 300–500 μm transverse lumbar spinal cord slice was cut with a modified vibrating microtome (Vibroslice, Camden Instruments). This microtome had been simply modified by turning the blade holder through 90° so that cuts could be made vertically as opposed to horizontally. The spinal cord slice, associated dorsal root ganglia (DRG) and nerve were placed in separate compartments of a three compartment bath (Morris, 1988). The spinal cord and DRG were completely submerged and perfused continuously at 3–4 ml min^{-1} with gassed ACSF maintained at 28 (± 1)°C. The composition of the ACSF was (in mM): NaCl 120, KCl 2.1, $KHPO_4$ 1.0, $MgSO_4$ 1.3, $NaHCO_3$ 25, glucose 10, $CaCl_2$ 2.4, Phenol red 0.014, pH 7.2 Many of the experiments were carried out in Mg^{2+}-free ACSF to permit the N-methyl-D-aspartate (NMDA) mediated components of the e.p.s.p. to be investigated. The nerve was placed on platinum wire stimulating electrodes and insulated with a mixture of liquid paraffin and petroleum jelly.

Intracellular recordings were made from neurones in the SG (lamina II) of the spinal cord. The slice compartment of the bath was transilluminated via its transparent sylgard base; this allowed direct targeting of the microelectrode on the SG, which was clearly visible as a more translucent band in the superficial dorsal horn. In addition, the position of a small number of neurones was verified by intracellular filling with lucifer yellow (2% lucifer yellow CH (Sigma) in 1 M lithium chloride). Recordings were made with 3 M potassium acetate-filled microelectrodes, made using a Flaming-Brown puller (Sutter Instruments), and having d.c. resistances of 120 to 240 MΩ. Signals were amplified using Axoprobe 1A (Axon Instruments) and recorded on video tape. Data were digitised using a CED 1401 interface (Cambridge Electronic Design) and analysed by computer.

Drugs were applied to the spinal cord by superfusing with a known concentration of the compound dissolved in ACSF. Compounds used were: carbamylcholine chloride (carbachol), atropine sulphate, methoctramine tetrahydrochloride, mecamylamine hydrochloride (all from Sigma), pirenzepine dihydrochloride, bicuculline methchloride (both from Research Biochemicals Inc.), 3-aminopropyl-diethoxymethyl-phosphonic acid, (CGP 35,348) (Malcangio *et al.*, 1991) (Ciba Geigy) and 3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) (Davies *et al.*, 1986) (Sandoz). Results shown for e.p.s.p. size were obtained by taking an average of 5–10 e.p.s.ps before addition of drug, 5–10 e.p.s.ps towards the end of the drug administration, and 5–10 e.p.s.ps after a period of wash with drug-free ACSF. Peak amplitude and total duration of the e.p.s.p. was measured for each average. For each response the averaged control values for e.p.s.p. amplitude and duration were expressed as 100%. The responses to drug application were then calculated as a percentage of this control value. This was done to scale the results according to their own controls. These percentages were used to calculate the average percentage decrease in e.p.s.p. size for groups of cells tested with a particular compound. All results are expressed as mean ± standard error of the mean (s.e.mean) for the number of cells tested (n). Statistical analysis was carried out on raw data values only.

Results

Intracellular recordings from 60 SG neurones were included in this study. Of these, 52 were synaptically activated by stimulation of the peripheral nerve, the others failed to respond to nerve stimulation at intensities up to 100 V and 0.5 ms duration. Only neurones with a resting membrane potential more negative than − 55 mV, and which produced action potentials in response to the injection of a depolarizing current pulse were included. The average resting membrane potential was − 72 ± 1.2 mV ($n = 60$). Stable recordings of individual cells were maintained for at least 30 min and often for several hours.

The sciatic or femoral nerve was stimulated at 0.05–0.5 Hz with stimulus intensities suprathreshold to that required for an e.p.s.p. to be evoked. Conduction velocity was calculated by measuring the distance between the cathode stimulating electrode and recording microelectrode (15–19 mm) and the latency to onset of the evoked potential. The average conduction velocity was 0.63 ± 0.1 ms^{-1} (mean ± s.e.mean) $n = 52$. The majority of these ($n = 45$) conducted more slowly than

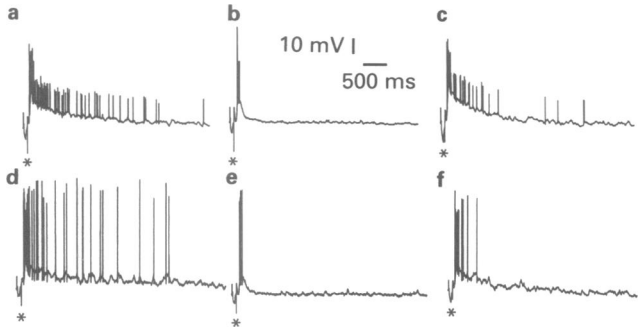

Figure 2 Effect of carbachol on an example of a long duration e.p.s.p. evoked by nerve stimulation. (100 V, 0.5 ms, 0.05 Hz): (a), (b) and (c) show averages of 5 records; (d), (e) and (f) show representative single sweeps. (a) and (d) Show control responses before addition of carbachol (10 μM); (b) and (e) show responses obtained during carbachol administration, and (c) and (f) show recovery of the e.p.s.p. after washout with drug-free ACSF.

1 ms^{-1} and correspond to the conduction velocity reported for C-fibres in rats of similar age (Fitzgerald & Gibson, 1984; Fitzgerald, 1985).

The location of a small number of neurones ($n = 5$) which were filled with lucifer yellow were located within the boundaries of the substantia gelatinosa, confirming that the microelectrodes were targeted correctly in this region of the spinal cord.

Effects of carbachol

Bath-application of carbachol to the spinal cord reduced, in a dose-related manner, the peak amplitude and duration of evoked e.p.s.ps in 83% of cells tested (43/52) (Figures 1, 2 and 4). For the results shown in Table 1, detailed measurements of the percentage of control e.p.s.p. size and duration were made on those cells in which carbachol was added and recovery obtained prior to application of antagonists. In many cells, antagonists were added immediately after application of carbachol, and before washout. On some cells, carbachol at 10 μM or 50 μM completely abolished the e.p.s.p. Washout with drug-free ACSF resulted in complete or near complete recovery of the e.p.s.p. to control values. There was no significant difference between the inhibitions produced by 10 μM and 50 μM carbachol ($P > 0.1$ using a pooled variance t test). Both long and short duration e.p.s.ps were reduced by carbachol. Alterations in e.p.s.p. duration, however, showed considerable variability, which would be expected in view of the probable contribution of polysynaptic pathways to these later e.p.s.p. components. The reduction of later components of the e.p.s.p., which in Mg^{2+}-free ACSF are blocked by the NMDA receptor antagonist CPP (2 μM), indicates that carbachol blocks both the non-NMDA and NMDA-mediated components of these primary afferent evoked e.p.s.ps.

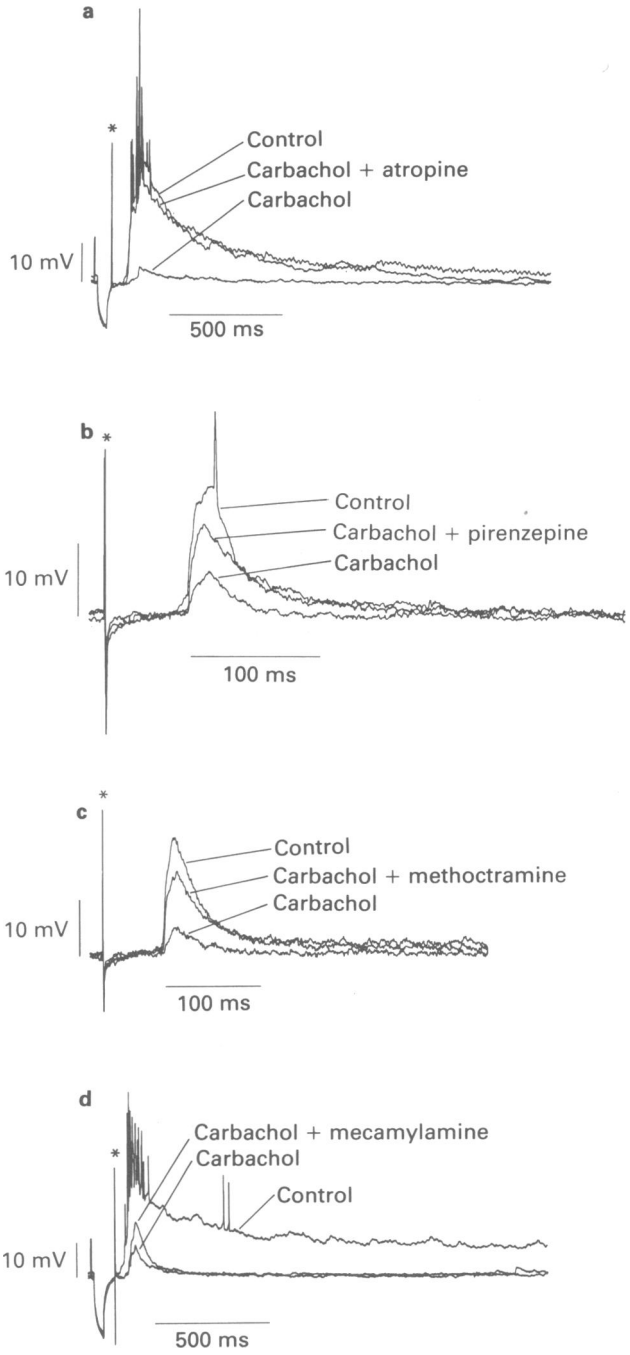

Figure 3 Reduction in e.p.s.p. size with 10 μM carbachol (a, b and c) or 50 μM carbachol (d) and reversal with the antagonists atropine (1 μM), pirenzepine (1 μM), methoctramine (1 μM) and mecamylamine (10 μM), (a, b, c and d, respectively). Each trace is the average of 5 sweeps.

Table 1 Effect of carbachol on e.p.s.p. size

		e.p.s.p. size		
Carbachol (μM)	*Units*	*Control*	*+ Carbachol*	*% of control*
1	mV	11.2 ± 1.3 (3)	10.8 ± 1.0 (3)	98.8 ± 12.1 (3)
1	ms	1713 ± 907 (3)	1733 ± 942 (3)	101 ± 7.4 (3)
10	mV	22.7 ± 2.2 (15)	10.7 ± 2.0 (15)**	55.7 ± 5.9 (15)
10	ms	1427 ± 107 (13)	415 ± 104 (13)**	35.9 ± 5.3 (13)
50	mV	14.6 ± 3.0 (7)	6.7 ± 2.3 (7)**	64.0 ± 11.5 (7)
50	ms	1053 ± 461 (7)	254 ± 92 (7)*	29.6 ± 9.6 (7)

E.p.s.p. peak amplitude (mV) and duration (ms), before (control), and in the presence of carbachol. The % of control figures given in both Tables 1 and 2 were calculated as described in the methods. Results are mean ± s.e.mean values for (n) cells tested. At 10 μM and 50 μM carbachol both the amplitude and duration of the e.p.s.ps are significantly reduced when compared to control values (asterisks in Tables 1 and 2, indicate ***$P < 0.01$; **$P < 0.02$; *$P < 0.1$, two-tailed paired t test).

Table 2 Effects of muscarinic receptor antagonists on reduction in e.p.s.p. size produced by carbachol

		e.p.s.p. size (mV)			*% of control response*	
Antagonist	*Carbachol* (μM)	*Control*	*Carbachol*	*Carbachol + antagonist*	*Carbachol*	*Carbachol + antagonist*
Atropine (1 μM)	10	26.6 ± 3.8 (4)	10.8 ± 4.2 (4)	23.4 ± 4.0* (4) ***	40.3 ± 11.6 (4)	88.0 ± 11.4 (4)
Atropine (1 μM)	50	17.0 ± 5.5 (4)	6.0 ± 2.9 (4)	14.9 ± 4.5* (4)	29.5 ± 14.9 (4)	90.0 ± 5.7 (4)
Pirenzepine (1 μM)	10	17.5 ± 1.5 (7)	6.5 ± 1.3 (7)	10.7 ± 1.6*** (5)	36.9 ± 5.7 (7)	60.4 ± 7.2 (5)
Methoctramine (1 μM)	10	17.4 ± 1.8 (8)	7.8 ± 1.1 (8)	12.9 ± 1.2** (6)	45.3 ± 5.3 (8)	76.4 ± 8.0 (6)

E.p.s.p. size before addition of carbachol (control), in the presence of carbachol, and with carbachol plus antagonist. Results are shown as absolute e.p.s.p. size (mV) and as % of control e.p.s.p. size before addition of carbachol. Results are mean ± s.e.mean values for (n) cells tested. The reversal of the effects of carbachol by atropine was highly significant ($P<0.01$) when the data from both concentrations of carbachol were pooled.

In most cells no change in membrane potential was seen; however, in some cells a hyperpolarization of 4–11 mV ($n = 3$), or a depolarization of 6–9 mV ($n = 3$) occurred.

No change in conductance during carbachol administration was seen in the majority of cells when constant hyperpolarizing or depolarizing current pulses of 0.1–0.3 nA, 50 ms were applied (19 out of 26 cells). In 7 cells, however, an increase in conductance of 3–23% ($n = 5$) or decrease in conductance of 8–14% ($n = 2$) was observed.

Cholinoceptor antagonists

On some cells, cholinoceptor antagonists were used to attempt to reverse the effects of carbachol. Reduction in e.p.s.p. size was first achieved with carbachol, followed immediately (without washout) by carbachol plus antagonist.

The reduction in e.p.s.p. size produced by carbachol was fully or partially reversed by 1 μM atropine in 8 out of 11 (73%) cells tested (Figure 3a, Table 2).

At 1 μM, pirenzepine, an antagonist with high affinity for muscarinic M_1 receptors (Hammer & Giachetti, 1982) partially reversed the effect of carbachol on evoked e.p.s.ps in 7 out of 9 (78%) cells tested (Figure 3b, Table 2). The M_2 receptor antagonist, methoctramine (Michel & Whiting, 1988), at 1 μM, was also effective in reversing the effect of carbachol in 8 out of 9 (89%) of cells tested (Figure 3c, Table 2). Although the overall trend was for antagonists to reverse the reduction in e.p.s.p. duration produced by carbachol, these were not statistically significant.

A nicotinic receptor antagonist, mecamylamine (Varanda *et al.*, 1985), at 1 μM, was also able to reverse partially the depression of the e.p.s.p. by carbachol in 3 out of 7 (43%) cells tested (Figure 3). The extent of reversal with this antagonist at the concentration used was small. Insufficient data are available at present for this antagonist to allow statistical analysis.

For all the antagonists tested, both the peak amplitude and duration of the e.p.s.p. were increased compared to their values during carbachol administration. The antagonists did not produce any marked effects on baseline membrane potential. Possible effects of the antagonists on e.p.s.p. size in the absence of carbachol have not been systematically explored and hence we have no data at present which would indicate whether the cholinergic inputs to these receptors are tonically active in this preparation. In no case did an antagonist result in a reversal of the carbachol produced inhibition of the e.p.s.p. amplitude to a value greater than control.

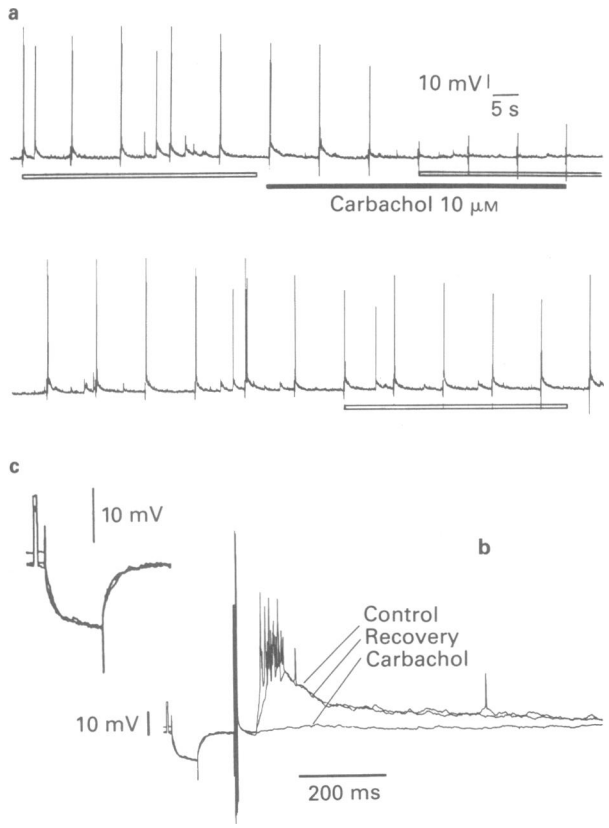

Figure 4 Example of a cell where carbachol reduced the e.p.s.p. size without affecting membrane potential or conductance. (a) Original trace showing effect of carbachol. The second part of the trace shows recovery after a period of washout using drug-free ACSF. (b) Average of 5 sweeps for control, carbachol and recovery, during the times shown as open bars in (a). (c) Conductance pulse (0.1 nA, 50 ms). (Nerve stimulation 100 V, 0.5 ms, 0.1 Hz.)

GABA receptor antagonists

Preliminary experiments have been carried out to examine the possibility that carbachol may be acting indirectly via interneurones which release inhibitory transmitters. These transmitters could then be responsible for the reduction in the size of the e.p.s.p. The $GABA_A$ receptor antagonist, bicuculline (10 μM, $n = 4$) and the $GABA_B$ receptor antagonist, CGP 35,348 (50 μM, $n = 6$) did not reduce the depressant effect of carbachol on the e.p.s.p.

Discussion

This study demonstrates a depressant action of a cholinoceptor agonist at synapses activated by slowly conducting primary afferent fibres in the SG of the spinal cord of the rat. Carbachol rapidly depressed and in many cases abolished,

the e.p.s.p. evoked by nerve stimulation, this effect being seen on the majority of cells tested. The depression in e.p.s.p. size was readily reproducible on the same cell with no obvious decline in response with repeated applications, suggesting that the receptors involved do not desensitize rapidly.

The lack of effect of carbachol on membrane conductance on most cells tested suggests an action of carbachol at a site presynaptic to the cell being recorded. Since the activation of SG neurones may be monosynaptic or polysynaptic, this presynaptic site may be at the primary afferent terminal or at interneurones between the primary afferent fibre and the cell studied. However, as the majority of C-fibres terminate in the SG it seems improbable that all the e.p.s.ps observed were mediated by polysynaptic pathways. The increase or decrease in conductance seen in a small number of cells in response to carbachol has not been investigated further but suggests an additional postsynaptic site of action.

The distribution of acetylcholine in the spinal cord has been studied using antibodies to the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT) which is considered to be a reliable marker for acetylcholine. ChAT immunoreactivity has been shown to be present from postnatal day 1 in the rat, reaching adult levels by day 21–28 (Phelps *et al.*, 1984). All cholinergic neurones in the dorsal spinal cord are thought to be intrinsic spinal neurones; no ChAT has been demonstrated in the DRG or dorsal roots, therefore a primary afferent origin of cholinergic fibres in the dorsal horn is unlikely. A supraspinal origin of cholinergic fibres is also improbable since spinal transection does not reduce ChAT activity caudal to a lesion (Barber *et al.*, 1984), and brainstem cholinergic neurones are not retrogradely labelled following spinal injections of tracer (Sherriff *et al.*, 1991). In the dorsal horn, acetylcholine-containing cell bodies are found in laminae III to V and some of these cells send dendrites towards lamina II (Ribeiro da Silva & Cuello, 1990), and lamina III (Barber *et al.*, 1984). These cell bodies are thought to contribute to the plexus of acetylcholine containing fibres and terminals in laminae II and III.

Ribeiro da Silva & Cuello (1990) have studied the distribution of ChAT immunoreactivity at the EM level in the dorsal horn and have shown the presence of ChAT-positive axonal and dendritic structures, particularly in inner lamina II and in lamina III. In these laminae, ChAT containing profiles were frequently seen to be part of both type I and type II synaptic glomeruli. ChAT containing dendritic profiles were often presynaptic to the axonal varicosity of the glomeruli, the varicosities originating from both myelinated (in type II glomeruli) and unmyelinated (in type I glomeruli) axons (Ribeiro da Silva & Coimbra, 1982; 1984; Ribeiro & Silva *et al.*, 1985). These studies provide anatomical evidence to support a presynaptic modulation of primary afferent fibres by cholinergic neurones; however, since ChAT-containing dendritic profiles presynaptic to the central axonal varicosity were found both in type II (presumed to be non-nociceptive) and type I (probably nociceptive) glomeruli, activation of cholinergic neurones could potentially inhibit both nociceptive and non-nociceptive transmission. Although these anatomical observations suggest a presynaptic site of action, acetylcholine released from cholinergic neurones in laminae III–V could act to stimulate transmitter release by inhibitory interneurones. Preliminary experiments suggest that the effect of carbachol is not mediated by GABA released from interneurones acting at $GABA_A$ or $GABA_B$ receptors, since neither the $GABA_A$ receptor antagonist, bicuculline, nor the $GABA_B$ receptor antagonist, CGP 35,348, prevented the depression of e.p.s.p. size caused by carbachol. It is possible however, that other inhibitory transmitters such as glycine may be involved.

Since a significant number of C-fibre afferents projecting to the SG are thought to be involved in mediating nociceptive input to the spinal cord, the suppression of the C-fibre evoked e.p.s.p. in our experiments may reflect suppression of nociceptive transmission by cholinergic spinal interneurones. A number of studies report only muscarinic receptors to be involved in mediating antinociception (e.g. Yaksh *et al.*, 1985; Smith *et al.*, 1989), however, there are also some reports of nicotinic antinociception (see Green & Kitchen, 1986). It is not clear from the present results at which receptor subtype(s) the muscarinic effect is mediated since the range of antagonist concentrations used was too limited to identify receptor subtypes. Full reversal of the reduction in e.p.s.p. size can be achieved by atropine; however, complete reversal of the effect of carbachol was not achieved with either of the more selective muscarinic antagonists at the concentrations used. Both M_1 and M_2 receptors may be involved since both are concentrated in the SG (Yamamura *et al.*, 1983; Bouchenafa & Livingston, 1991), however, their precise pre- or postsynaptic location in this region has not been demonstrated. Bartolini *et al.* (1992), have recently shown that selective M_1 agonists are capable of inducing antinociception in rats and mice, but a specific M_2 agonist caused mechanical and thermal hyperalgesia. They proposed that acetylcholine release was modulated by M_2 receptors located presynaptically, and that postsynaptic M_1 receptors mediated the observed antinociception. In our experiments the nicotinic component of the effect of carbachol appears to be small. At the concentration used, the nicotinic receptor antagonist mecamylamine reversed the carbachol induced depression of the e.p.s.p. by only a small amount. It is not yet clear which nicotinic receptor subtype is involved in this response.

From the preceding discussion of our results and those reported in the literature, we would conclude that some cholinergic neurones in laminae III–V give rise to axons which presynaptically inhibit the release of excitatory transmitter from C-fibres terminating in lamina II. This presynaptic inhibition appears to be largely mediated by muscarinic receptors. The question then arises as to how these inhibitory cholinergic spinal neurones are activated. There is anatomical evidence that they receive synaptic input from small myelinated and unmyelinated primary afferents (Ribeiro da Silva & Cuello, 1990), and thus may be involved in the interactions between sensory information conveyed by different types of afferent fibres. These cholinergic neurones may also be involved in mediating descending inhibition. Zhou & Gebhart (1991), using intrathecal administration of cholinoceptor antagonists have shown that a spinal cholinergic system (or systems) exerts a tonic inhibitory effect on noxious mechanical but not noxious thermal transmission, and that this effect is mediated by muscarinic but not nicotinic receptors. The same authors (Zhou & Gebhart, 1990) also reported spinal muscarinic cholinoceptor involvement in mediating descending inhibition from the nucleus reticularis gigantocellularis (NGC) and the nucleus gigantocellularis pars alpha (NGCα). They have also shown that bilateral transection of the spinal dorsolateral funiculi did not alter the antagonist effect of muscarinic antagonists on the inhibition of a cutaneous nociceptive reflex by a noxious visceral stimulus, supporting the involvement of cholinergic interneurones rather than a descending cholinergic pathway in this mechanism (Zhou & Gebhart, 1992).

In conclusion, we would suggest that intrinsic spinal cord cholinergic neurones inhibit synaptic transmission between C-fibres and substantia gelatinosa neurones and that this is partially due to presynaptic regulation of C-fibres via muscarinic receptors.

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Attenuation of reperfusion hyperalgesia in the rat by systemic administration of benzodiazepines

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1 An investigation into whether reperfusion hyperalgesia is modulated by prior systemic administration of two benzodiazepine agonists (diazepam and chlordiazepoxide), and an antagonist (flumazenil) was conducted.

2 Transient ischaemia was induced in conscious rats by applying an inflatable tourniquet to the base of the tail; when the rats exhibited a co-ordinated escape response, the tourniquet was deflated and reperfusion of the tail was allowed. Reperfusion hyperalgesia manifested as a decrease in tail flick latency, following tail immersion in 49°C water, after the release of the tourniquet.

3 Intraperitoneal administration of diazepam, chlordiazepoxide and flumazenil had no effect on the co-ordinated escape to the noxious ischaemic stimulus nor on tail flick latency after application of a sham tourniquet.

4 The hyperalgesia evident during reperfusion, was abolished by diazepam (1 and 5 mg kg^{-1}) and chlordiazepoxide (5 and 25 mg kg^{-1}). The antihyperalgesic effects of both diazepam (5 mg kg^{-1}) and chlordiazepoxide (25 mg kg^{-1}) were inhibited by flumazenil (1 mg kg^{-1}).

5 Rotarod performance was impaired in rats given diazepam and chlordiazepoxide at the same doses at which the benzodiazepines were antihyperalgesic. The impairment to motor function did not extend to the motor systems involved in the tail flick response.

6 In conclusion, benzodiazepines have antinociceptive properties during hyperalgesia.

Keywords: Hyperalgesia; ischaemia; tail flick test; benzodiazepines; diazepam; chlordiazepoxide; flumazenil; rotarod; motor function

Introduction

Hyperalgesia, an increased sensitivity to noxious stimulation, typically follows tissue injury, and depends on peripheral (Lynn, 1987) and central nervous system mechanisms (Woolf, 1983). Transient ischaemia acts as such a conditioning stimulus, in that hyperalgesia occurs to noxious thermal stimulation during reperfusion of previously ischaemic tissue (Gelgor *et al.*, 1986a). This reperfusion hyperalgesia can be attenuated by NMDA receptor antagonists (Sher *et al.*, 1992) as well as by inhibitors of prostaglandin synthesis (Gelgor *et al.*, 1992a). The antinociceptive action of these agents is confined to antihyperalgesia; they had no antinociceptive action against noxious stimulation in the absence of the conditioning stimulus. Hyperalgesia, therefore can be pharmacologically modulated by neuroactive agents which do not modulate the processing of noxious stimuli in the absence of hyperalgesia.

Benzodiazepine modulation of nociceptive processes is not well established in animal models. Procedural variations including route of administration, dosage, test of nociception (behavioural, electrophysiological) and nature of nociceptive stimulus have yielded different results. In models involving acute noxious stimulation such as the tail flick and hot plate assays, systemic administration of benzodiazepines produced no effect (Bragin *et al.*, 1989; Rosland & Hole, 1990; Carter, 1991), an antinociceptive effect (Rosland *et al.*, 1987; Zambotti *et al.*, 1991), and, in one report, even a hyperalgesic effect (Niv *et al.*, 1988). Intrathecal administration of benzodiazepines induces antinociception in behavioural tests such as the tail flick assay (Goodchild & Serrao, 1987; Pomeranz & Nguyen, 1987; Niv *et al.*, 1988; Serrao *et al.*, 1989) as well as a depression of electrophysiological responses in nociceptive pathways (Niv *et al.*, 1983).

There have been few studies in which benzodiazepines have been assessed for antinociceptive properties in animals displaying hyperalgesic behavioural responses. The effect of two prototype benzodiazepines, diazepam and chlordiazepoxide on behavioural responses to noxious ischaemia and to noxious thermal stimulation during reperfusion hyperalgesia was investigated. Naloxone was used to assess the interaction of diazepam with the opiate receptor system. Since benzodiazepines have motor side-effects, the effect of the same doses of diazepam and chlordiazepoxide on motor function in the rats was measured. Some of the results have been reported briefly to the Physiological Society of Southern Africa (Cartmell & Mitchell, 1990).

Methods

Animals

Male Sprague-Dawley rats weighing 230–260 g at the start of the experiment were housed in cages of five, at an ambient temperature of 21–23°C, on a 12 h light/12 h dark cycle with food and water *ad libitum*. All experiments were conducted between 09 h 00 min and 12 h 00 min. Groups of ten rats were used for each test.

Tests of nociception

The rats were habituated to perspex restrainers which allowed free movement of the tail and limited movement of the rest of the body, by placing them in the restrainers for 3 h on each of 2 days. At least 2 days elapsed between successive measurements on a single group of animals.

Responses to a noxious thermal stimulus were assessed by rapidly immersing the entire tail in hot water (49°C), and measuring tail flick latency as the time from immersion to the first coordinated motor response, measured on a stopwatch. The mean of three successive measurements made at 1 min

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intervals was recorded. In order to avoid thermally induced tissue damage the tails were routinely removed from the water if the rats had not responded by 20 s; in the current experiments all rats responded within the cutoff time. To obviate inter-obsever variability, a single observer measured all the tail flick latencies.

The potential effect of tail skin temperature on tail flick latency needs to be considered when tail flick latency is used as a test of nociception (Berge *et al.*, 1988). It has been shown recently by our laboratory that reperfusion hyperalgesia is not influenced by tail skin temperature changes induced by ischaemia (Gelgor *et al.*, 1992a). It is possible, however, that an agent under test may affect tail skin temperature changes, so, between measurements of tail flick latency, all but the proximal 20 mm of the rats' tails were immersed in a water bath maintained at 29°C. The time taken to move the tail from the 29°C to the 49°C bath was less than 1 s.

Ischaemia was induced by applying an inflatable tourniquet to the base of the tail; the tourniquet was connected to a sphygmomanometer and inflated to a pressure of 300 mmHg. The tourniquet was deflated when the animal exhibited a coordinated escape response such as jumping forward or turning around in the restrainer, or when the animal engaged in vigorous, sustained grooming. These responses were used as behavioural indicators of distress caused by the noxious stimulation arising from the ischaemia, and the time measured from inflation of the tourniquet to the first signs of such behaviour served as the escape latency. A transient escape response within the first 2 min was ignored. To reduce tissue damage, the ischaemia was relieved after 30 min if the animal had not responded. Control experiments were performed by applying an uninflated tourniquet (sham tourniquet) to the base of the tail for 12 min, the mean latency measured in previous experiments in our laboratory (Gelgor *et al.*, 1986a). Apart from providing a noxious stimulus, the tourniquet also was the conditioning stimulus used to induce hyperalgesia.

Tail flick latencies were measured immediately after cuff deflation and then following 30 and 60 min of reperfusion. The difference between tail flick latency during reperfusion and tail flick latency in the same animal before any treatment was employed as a measure of the hyperalgesia.

Motor function testing

A modification of the rotarod technique was used, the advantages of which have been detailed previously (Cartmell *et al.*, 1991). Briefly, 30 young rats were selected for their natural ability to perform on the rotarod, and then trained for 30 min a day over a 14 day period at increasing speeds of rotation.

To test potential effects of the agents on motor function, the rats were divided into groups of ten animals and, on each of 2 days, administered either with saline or the benzodiazepine in random order, each animal therefore serving as its own control. Diazepam, chlordiazepoxide or saline was administered intraperitoneally (i.p.) 30 min before placing the animals on the rotarod. The time from the onset of running until the rat fell off the rod served as each rat's performance time. The speed of the rod was 25 r.p.m. and the maximum running time limited to 60 min. As the distribution of performance times was skewed the response of each group of animals to the benzodiazepine was expressed as the percentage of animals failing to complete control rotarod performance times.

Drugs

The agents and doses used were: diazepam (Valium, Roche) 0.2, 1 and 5 mg kg^{-1}; chlordiazepoxide (Librium, Roche) 1, 5 and 25 mg kg^{-1}; flumazenil (Anexate, Roche) 0.01, 0.1 and 1 mg kg^{-1}; and naloxone (Narcan, Boots company) 1 mg kg^{-1}. All agents were administered intraperitoneally (i.p.) in 1 ml boluses except for flumazenil which was administered in a 2.5 ml bolus. All dilutions were made in normal saline. Diazepam and chlordiazepoxide were administered 30 min before application of the tourniquet (or sham tourniquet) and flumazenil and naloxone were administered 15 min before application of the tourniquet.

Statistical analyses

Data on nociception were analysed by two way and one way ANOVA's and significant differences identified by Student's *t* test with the Bonferroni correction for multiple comparisons. Rotarod data were compared by the Wilcoxon signed rank test.

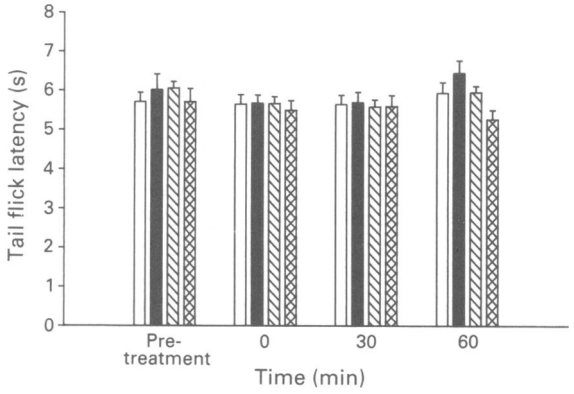

Figure 1 Tail flick latencies (mean ± s.e.mean) following i.p. administration of saline (open columns), diazepam (5 mg kg^{-1}, solid columns), chlordiazepoxide (25 mg kg^{-1}, hatched columns) and flumazenil (1.0 mg kg^{-1}, cross hatched columns) and application of a sham tourniquet. There was no significant difference between saline and any of the benzodiazepines in the absence of a conditioning stimulus.

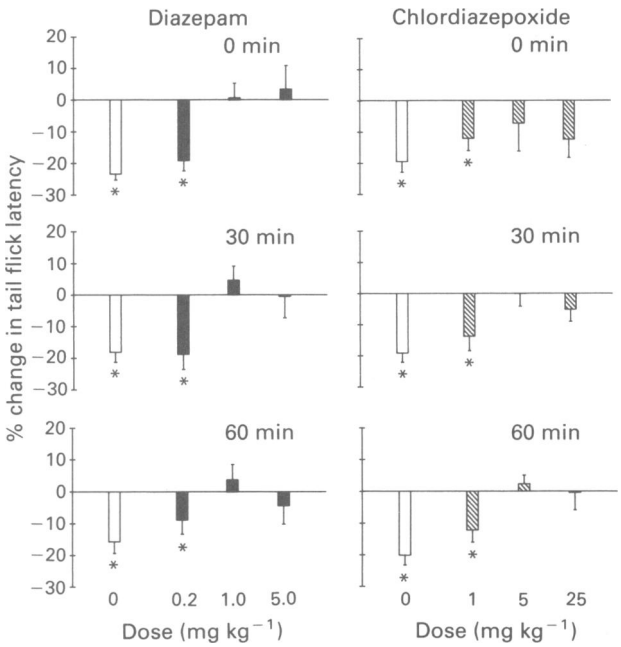

Figure 2 Percentage change in tail flick latency (mean ± s.e.mean) at three times during reperfusion of the tail in rats treated previously with saline (open column), diazepam (solid column, left panel) and chlordiazepoxide (hatched column, right panel). Reperfusion hyperalgesia is evident in rats given saline. At the lowest doses of diazepam (0.2 mg kg^{-1}) and chlordiazepoxide (1 mg kg^{-1}) reperfusion hyperalgesia was still evident but higher doses of diazepam (1 and 5 mg kg^{-1}) and chlordiazepoxide (5 and 25 mg kg^{-1}) abolished the reperfusion hyperalgesia evident in rats given saline. Asterisks indicate values significantly different from zero ($*P < 0.05$, $n = 10$ per group).

Ethical consideration

The experimental procedures were approved by the Animal Ethics Committee of the University of the Witwatersrand (Certificate Number 90/16/3) and complied with the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmerman, 1983).

Results

Administration of i.p. saline, diazepam, chlordiazepoxide and flumazenil, at the highest doses employed, did not change tail flick latency in sham tourniquet experiments, that is in the absence of ischaemia, at any of the time intervals tested (Figure 1, one way ANOVA $F_{3,156} = 1.43$, $P > 0.05$). These results imply that, at the doses tested, and in the absence of a conditioning stimulus, the benzodiazepines exert no antinociceptive effect against noxious thermal stimulation. Furthermore, at these same high doses, none of the agents had any effect on the escape latency to the noxious ischaemia. Following pretreatment with the highest dose of diazepam (5 mg kg^{-1}) the escape latency was 11.6 ± 1.7 min, and after chlordiazepoxide pretreatment (25 mg kg^{-1}) the escape latency was 10.6 ± 2 min. The mean escape latencies found in these experiments (11.8 ± 1.7 min) were no different from that found in our laboratory for other groups of rats (Gelgor *et al.*, 1986a).

The percentage decrease in tail flick latency following reperfusion of the previously ischaemic tail is shown in Figure 2. In rats given saline (open columns), significant decreases in tail flick latency from pre-ischaemic values, implying hyperalgesia, occurred at 0, 30 and 60 min during reperfusion ($P < 0.01$, $n = 10$ per group, paired t test with Bonferroni correction). The hyperalgesia was not a result of repeated noxious thermal stimulation (Fitzgerald & Lynn, 1977) because it was not evident in animals exposed to the same noxious thermal stimulation but not subjected to ischaemia (Figure 1, open columns). The hyperalgesia had the same characteristics as that described in our previous reports of post-ischaemic or reperfusion hyperalgesia (Gelgor *et al.*, 1986a, Sher *et al.*, 1992).

Diazepam abolished the reperfusion hyperalgesia evident in saline-treated rats at a dose of 1 mg kg^{-1} (one way ANOVA, $F_{3,36} = 0.12$, $P > 0.05$). Increasing the dose to 5 mg kg^{-1} had no further effect on tail flick latency (one way ANOVA, $F_{3,36} = 0.34$, $P > 0.05$). Reperfusion hyperalgesia also was inhibited by chlordiazepoxide, at a somewhat higher dose (5 mg kg^{-1}, one way ANOVA, $F_{3,36} = 1.11$, $P > 0.05$), and more effectively at 30 and 60 min of reperfusion than immediately after the release of the tourniquet. Again, increasing the dose of chlordiazepoxide five fold had no further effect (one way ANOVA, $F_{3,36} = 1.62$, $P > 0.05$). The antinociception produced by both diazepam and chlordiazepoxide was confined to antihyperalgesia, because, even at higher doses, the benzodiazepines did not increase tail flick latency beyond its pretreatment value.

Flumazenil at doses of 0.01, 0.1 and 1.0 mg kg^{-1} had no effect on responses to noxious thermal or ischaemic stimuli, nor on reperfusion hyperalgesia (Figure 3a). This observation is consistent with other reports that flumazenil itself, at least up to doses of 5 mg, has no antinociceptive properties

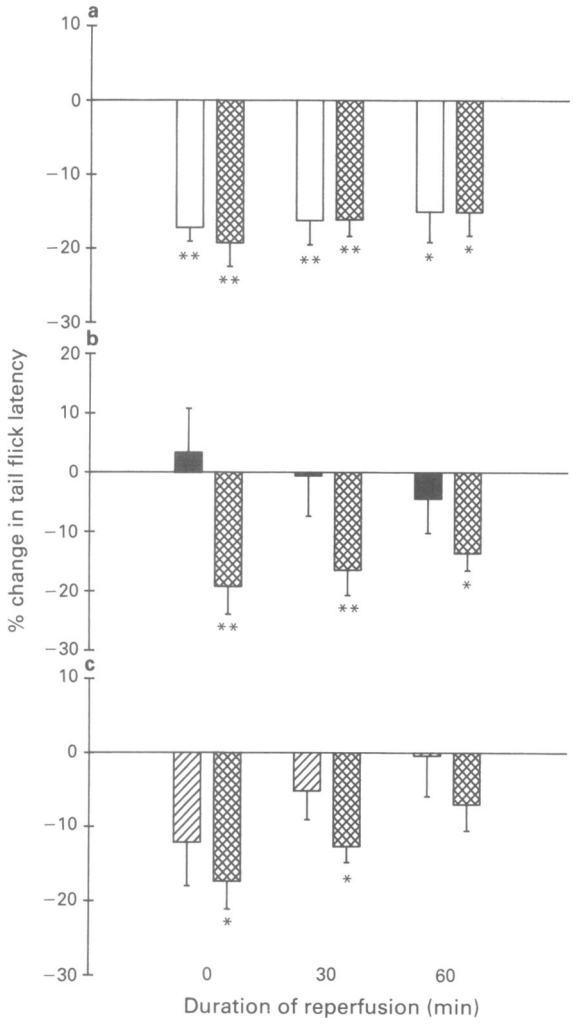

Figure 3 The effect of the competitive benzodiazepine antagonist, flumazenil, on the antihyperalgesia induced by 5 mg kg^{-1} diazepam and 25 mg kg^{-1} chlordiazepoxide. Flumazenil administered on its own (a) had no effect on the hyperalgesia during reperfusion. Flumazenil administered 15 min after diazepam (b) reversed the antihyperalgesia and returned tail flick latencies to values similar to those found in rats given saline. Flumazenil was less effective in attenuating the antihyperalgesia induced by chlordiazepoxide (c). (a) Left column – saline; right column, 1 mg kg^{-1} flumazenil; (b) left column, 5 mg kg^{-1} diazepam; right column, 5 mg kg^{-1} diazepam + 1 mg kg^{-1} flumazenil. (c) Left column, 25 mg kg^{-1} chlordiazepoxide; right column 25 mg kg^{-1} chlordiazepoxide + 1 mg kg^{-1} flumazenil. Asterisks indicate values significantly different from zero (**$P < 0.01$; *$P < 0.05$, $n = 10$ per group, paired t test).

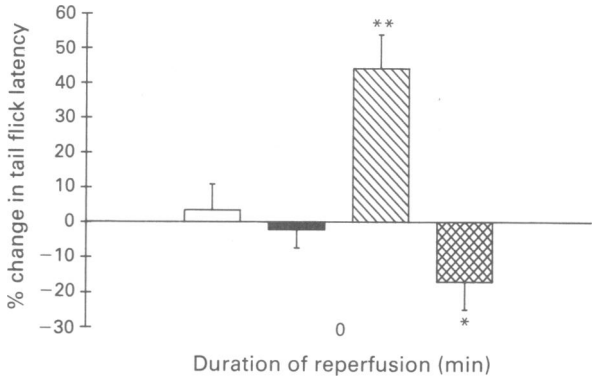

Figure 4 The effect of administering diazepam and diazepam + naloxone as well as morphine and morphine + naloxone on reperfusion hyperalgesia shown immediately after tourniquet deflation when hyperalgesia is maximal. Naloxone (1 mg kg^{-1}) administered 15 min after diazepam (5 mg kg^{-1}) had no effect, at any of the time intervals tested, on the antihyperalgesia found in rats treated with diazepam. The dose of naloxone is sufficient to antagonize the antinociceptive action of 5 mg kg^{-1} morphine. Open bar column, 5 mg kg^{-1} diazepam; solid column, 5 mg kg^{-1} diazepam + 1 mg kg^{-1} naloxone; hatched column, 5 mg kg^{-1} morphine; cross hatched column, 5 mg kg^{-1} morphine + 1 mg kg^{-1} naloxone. Morphine and morphine + naloxone data from Sher *et al.* (1992).

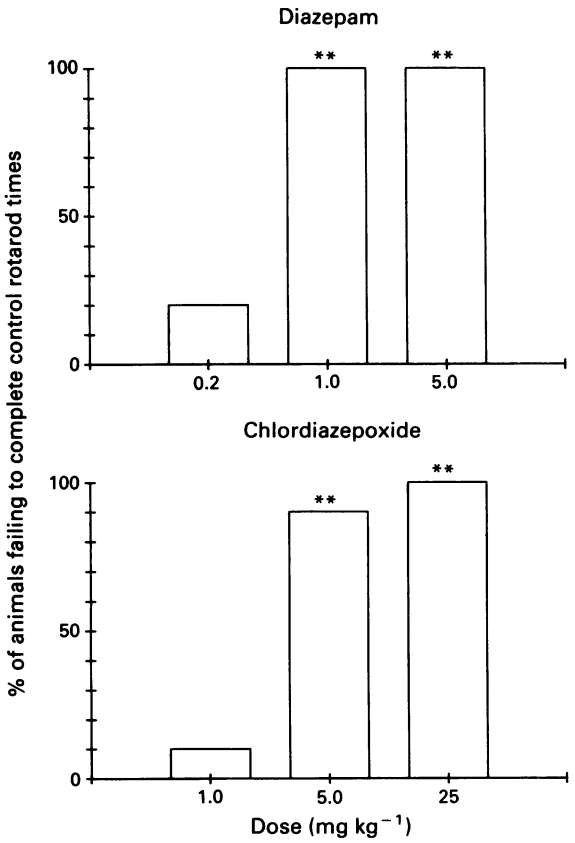

Figure 5 The percentage of animals failing to complete control rotarod performance times after administration of diazepam (top panel) and chlordiazepoxide (bottom panel). Significant decrements in rotarod performance were found for diazepam (1 and 5 mg kg^{-1}) and chlordiazepoxide (5 and 25 mg kg^{-1}) (**$P<0.01$, $n=10$ per group, Wilcoxon signed rank test).

(Goodchild & Serrao, 1987). The effect of administering 1 mg kg^{-1} flumazenil, 15 min after administering the highest doses of diazepam and chlordiazepoxide, on tail flick latency during reperfusion is shown in Figure 3. Flumazenil completely reversed the antihyperalgesic action of 5 mg kg^{-1} diazepam (two way ANOVA, $F_{treatment}=10.4$, $P<0.01$), and attenuated the antihyperalgesic action of 25 mg kg^{-1} chlordiazepoxide (two way ANOVA, $F_{treatment}=4.3$, $P<0.05$).

Naloxone (1 mg kg^{-1}, i.p.) had no effect on the antihyperalgesic action of 5 mg kg^{-1} diazepam (Figure 4). The dose of naloxone used is sufficient to antagonize the antinociception induced by 5 mg kg^{-1} of morphine (Sher *et al.*, 1992).

Diazepam and chlordiazepoxide induced a decrement in motor function, as measured by rotarod performance (Figure 5), at the same doses at which they inhibited reperfusion hyperalgesia. No rats were able to match their control performance times after being given the benzodiazepines ($P<0.01$, Wilcoxon signed rank test).

Discussion

The benzodiazepines, diazepam and chlordiazepoxide, abolished the reperfusion hyperalgesia which occurs in the rat's tail following a period of transient ischaemia. Their antihyperalgesic action was independent of changes in tail skin temperature (Rosland & Hole, 1990), and was attenuated by the benzodiazepine antagonist, flumazenil, implying that it was a specific effect of the benzodiazepines. In inflammatory models of nociception, the majority of which, such as the formalin test, rely on the chemogenic induction of hyperalgesia, Rosland *et al.* (1987) found that 2 mg kg^{-1} of diazepam induced an apparent antinociceptive effect which they suggested was an artefact resulting from motor deficits, while Abbott & Franklin (1986) found that 1 mg kg^{-1} diazepam had no effect. Using another chemogenic assay of nociception in mice, the phenylquinone writhing test, Fennesy & Sawynok (1973) found that chlordiazepoxide, but not diazepam, was antinociceptive at large oral doses. Diazepam reduces autotomy behaviour following peripheral deafferentation (Seltzer *et al.*, 1989), which apparently induces a hyperalgesic state. In our model, which relies on endogenous mediators released during ischaemia and reperfusion to induce temporary hyperalgesia, the benzodiazepines diazepam and chlordiazepoxide, were clearly antihyperalgesic.

System administration of benzodiazepines is accompanied by muscle relaxation (Bowman & Rand, 1980) with consequent deficits in motor function. We confirmed using the rotarod, that the benzodiazepines seriously compromised motor function at the doses at which they were apparently antihyperalgesic. The question whether the antihyperalgesia represented real antinociception, or was merely an artefact resulting from motor malfunction has to be addressed. If the antihyperalgesia found with systemic administration of diazepam and chlordiazepoxide was the result of an inability of the animal to respond mechanically to the noxious stimuli, we should have expected a failure to respond in the sham tourniquet experiments as well, and more particularly in the coordinated escape from the noxious ischaemia; neither of these responses was impaired. Consequently, the antihyperalgesia obtained with diazepam and chlordiazepoxide cannot be explained on the basis of motor deficits, although the ideal antinociceptive would produce antinociception without any deficit in motor function (Sher *et al.*, 1992).

The benzodiazepines were not analgesic. When morphine or pethidine were administered in the same test battery, they prolonged the escape latency to ischaemia and prolonged tail flick latency in the absence of a conditioning stimulus (Sher *et al.*, 1992). These properties, typical of narcotic antinociceptives, were not exhibited by the benzodiazepines. It is possible that both of these tests involve simple reflex pathways not associated with coordinated motor function and not vulnerable to agents affecting higher motor function. Such a proposition, however, would not explain the results we obtained in the case of the escape from the noxious ischaemia. The response observed which included attempting to turn or jump forward in the restrainer or sustained vigorous grooming, almost certainly is coordinated along the entire neuraxis. It seems as if the benzodiazepines, and perhaps other agents, can interfere with the motor function necessary to perform on a rotarod, without affecting even coordinated responses to noxious stimuli. The robustness of the pathways between afferent noxious input and efferent motor responses needs further systematic examination.

In man, diazepam has been reported to affect the emotional affective component of pain and not the sensory discriminative component (Chapman & Feather, 1973; Gracely *et al.*, 1978) allowing the hypothesis that any analgesic action is secondary to its anxiolytic mechanism. These human studies appear to have been conducted in healthy volunteers and, despite the extensive use of benzodiazepines in chronic pain management programmes (King & Strain, 1990), there are few systematic studies in which the efficacy of benzodiazepines in 'pathological' pain states has been evaluated. Serrao *et al.* (1992) have recently reported intrathecal midazolam to be an effective treatment for chronic mechanical lower back pain, reducing both sensory and affective components of pain. In animal models, it is difficult to distinguish the analogue of the emotional affective component of the antinociceptive response. Those studies which set out to do so usually employ exposure to novel stressors to create 'anxiety'. Stress in animals can produce either analgesia (Porro & Carli, 1988; Taukulis & Goggin, 1990) or hyperalgesia (Vidal & Jacob, 1982), both of which are attenuated by habituation. In our study, repeated exposure to the test battery resulted in

no change in the escape latency to noxious ischaemia nor in the tail flick latency following saline administration. Consequently, our rats did not show the characteristics of 'anxious' animals, so the antihyperalgesia we observed following systemic administration of benzodiazepines is likely to have resulted from modulation of sensory mechanisms involved in reperfusion hyperalgesia, rather than any anxiolytic action.

In principle, such modulation could occur in the periphery, that is at the nociceptors, or within the central nervous system. Benzodiazepines exert their action by binding to specific receptor sites co-located on the $GABA_A$-Cl^- complex and enhancing $GABA_A$-mediated inhibition (Schofield *et al.*, 1987). The major receptor sites for the benzodiazepines are in the central nervous system, and pharmacological actions of systemically administered benzodiazepines correlate well with their binding to specific central receptors (Mohler & Richards, 1988). A peripheral binding site has been shown to exist, but the structure-activity relationship as well as the correlations with function are distinct from those of the central type (Wang *et al.*, 1980). Although we have no way of localizing the antihyperalgesic activity of benzodiazepines which we observed, we assume tentatively that, like some other antihyperalgesic substances (Gelgor *et al.*, 1992b), they were acting within the central nervous system.

Whether benzodiazepine act centrally or peripherally, they could interact with opioid systems involved in antinociception. In clinical usage, benzodiazepines often are administered as adjuvants to opiates, and the combination has been considered to result in better pain relief (Kanto, 1981; Reves *et al.*, 1985). Studies in animals report that benzodiazepines may either attenuate (Mantegazza *et al.*, 1982; Daghero *et al.*, 1987; Rosland & Hole, 1990) or potentiate (Bradshaw *et al.*, 1973; Shannon *et al.*, 1976) the antinociceptive effect of morphine. We found that the antihyperalgesia induced by 5 mg kg^{-1} of diazepam is insensitive to naloxone, and similar insensitivity has been found by others when they have administered benzodiazepines intraperitoneally (Zambotti *et al.*, 1991) and intrathecally (Yanez *et al.*, 1990). Zambotti *et al.* (1991) have reported, however, that the antinociceptive effect of diazepam can be attenuated by the κ opioid receptor anagonist, MR2266. The interaction between benzodiazepines and opiates in antinociception therefore is complex.

With the discovery that benzodiazepines abolish reperfusion hyperalgesia, we bring to five the number of distinct classes of pharmacological agents that are known to abolish reperfusion hyperalgesia. In addition, the opiates (Sher *et al.*, 1992), antihistamines (Gelgor *et al.*, 1986b), cyclo-oxygenase inhibitors (Gelgor *et al.*, 1992a,b) and excitatory amino acid antagonists (Sher *et al.*, 1992) also abolish reperfusion hyperalgesia. The complexity of the interaction extends beyond that evident between opiates and benzodiazepines, and resolving the interaction between the mediators remains a major task for elucidating neurochemical mechanisms of hyperalgesia.

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Effects of ruthenium red and capsazepine on C-fibres in the rabbit iris

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1 We have investigated the effects of ruthenium red and capsazepine on a C-fibre-smooth muscle preparation (the rabbit isolated iris sphincter muscle).

2 Like capsaicin, ruthenium red and capsazepine were found to produce contractions in a concentration-dependent manner. C-fibre activation was held to be responsible since the contractions could be inhibited by tachykinin receptor blockade.

3 Both ruthenium red and capsazepine inhibited capsaicin-induced contractions concentration-dependently; the pIC_{50} values were 5.1 and 4.9, respectively. The contractions induced by bradykinin, which, like capsaicin, acts by releasing tachykinins from C-fibres, were also inhibited by ruthenium red and capsazepine in a concentration-dependent manner; the pIC_{50} values were 4.1 and 4.6, respectively.

4 Electrically evoked, tachykinin-mediated contractions were inhibited by ruthenium red and capsazepine in a concentration-dependent manner; the pIC_{50} values were 4.3 and 4.5, respectively.

5 The contractile response to neurokinin A (NKA) was inhibited by capsazepine (and by capsaicin), but not by ruthenium red, in a concentration-dependent manner; the pIC_{50} value was 4.3.

6 The results suggest that, besides their ability to antagonize capsaicin, ruthenium red and capsazepine possess a weak capsaicin-like effect. Conceivably, capsazepine interacts with binding sites for capsaicin, acting as a partial agonist/antagonist, while ruthenium red interacts with capsaicin-operated cation channels. The inhibition of electrically evoked- or bradykinin-induced responses by capsazepine and ruthenium red suggests that capsaicin/capsazepine binding sites and capsaicin-operated cation channels play a role in the process of transmitter release in response not only to capsaicin but also to other C-fibre stimuli. In addition, capsazepine (and capsaicin) may affect smooth muscle non-specifically since the response to NKA was also inhibited by this drug. The fact that ruthenium red did not affect the response to NKA provides further evidence that ruthenium red acts in a mode different from that of capsazepine.

Keywords: Capsaicin; ruthenium red; capsazepine; iris sphincter muscle (rabbit); neurokinin A

Introduction

Capsaicin activates sensory neurones (C-fibre neurones), causing release of transmitters, such as substance P, neurokinin A (NKA) and calcitonin gene-related peptide (for reviews, see Maggi & Meli, 1988; Bevan & Szolcsanyi, 1990; Holzer, 1991; Dray, 1992). Large doses of capsaicin bring about functional impairment of the C-fibres and depletion of neurotransmitters (Maggi & Meli, 1988; Bevan & Szolcsanyi, 1990; Holzer, 1991). It has been suggested that capsaicin acts on specific membrane receptors. This speculation is based on structure-activity studies that have identified specific structural requirements for capsaicin and its congeners (Szolcsanyi & Jancso-Gabor, 1975; 1976; Jhamandas *et al.*, 1984; Szallasi & Blumberg, 1989a; Dickenson *et al.*, 1990; Dray *et al.*, 1990; Maggi *et al.*, 1990). Moreover, capsaicin has been shown to displace competitively [^{3}H]-resiniferatoxin, a capsaicinoid (Szallasi & Blumberg, 1989a), from rat dorsal root ganglion membranes (Szallasi & Blumberg, 1989b).

Recently, ruthenium red has been reported to antagonize capsaicin-induced responses selectively (for reviews, see Amann & Maggi, 1991; Holzer, 1991). Ruthenium red inhibits a variety of sensory nerve-mediated responses to capsaicin and blocks the capsaicin-induced release of peptides from sensory nerve endings (Amann *et al.*, 1989; 1990; Maggi *et al.*, 1989; Franco-Cereceda *et al.*, 1989; 1990; Buck *et al.*, 1990; Jin *et al.*, 1990; Takaki *et al.*, 1992).

A recent advance of potential importance for understanding the mechanism of action of capsaicin was the development of capsazepine, a competitive antagonist to capsaicin (Dickenson & Dray, 1991; Dray *et al.*, 1991; Belvisi *et al.*, 1992; Bevan *et al.*, 1992). Capsazepine has been found to antagonize reversibly the capsaicin-evoked excitation of sensory nerve fibres (Dickenson & Dray, 1991; Belvisi *et al.*, 1992).

In the present study we have investigated the inhibitory effects of ruthenium red and capsazepine on capsaicin-induced contractions of the rabbit isolated iris sphincter muscle. During the course of this study, an attempt was made to investigate whether these compounds had effects of their own and whether they affected contractile responses evoked by stimuli other than capsaicin.

Methods

General

Albino rabbits of either sex, weighing 2.0–3.0 kg, were killed and exsanguinated. The eyes were immediately taken out. The iris was cut in half and the two halves were mounted in separate tissue baths (37°C), containing 8 ml of modified Krebs solution (Beding-Barnekow *et al.*, 1988). The solution was bubbled continuously with 93% O_2 and 7% CO_2, giving a pH of 7.4. The preparations were stretched gently with an initial tension of 1.5 mN and allowed to equilibrate for 90 min. The resting tension of the preparation was about 0.5 mN. Changes in tension produced by electrical stimulation or by the compounds added were measured and recorded by a Grass polygraph.

Effects of ruthenium red and capsazepine on contractile responses to capsaicin and to electrical stimulation

The experiments started with a standard electrical stimulation (square wave pulses, 20 Hz, 25 V, voltage drop 14–17 V over

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the electrodes, 0.3 ms duration, pulse train 10 s), applied by means of platinum electrodes connected to a Grass S4C stimulator in the presence of 10^{-6} M atropine and 5×10^{-6} M guanethidine. The two iris halves, mounted in separate baths, were stimulated in parallel, one preparation being exposed to a single concentration of ruthenium red or capsazepine, the other being exposed to vehicle (control preparation). After 30 min, the electrical stimulation was applied again and followed by application of 5×10^{-6} M capsaicin. This concentration of capsaicin was chosen to produce approximately 50% of the maximal response (Wang & Håkanson, 1992a). After the capsaicin-induced contraction had reached its maximum (usually within 10 min), the preparation was washed with drug-free Krebs solution and allowed to rest for 1 h. The preparation was then exposed to a buffer solution containing 137.7 mM KCl (but no NaCl) (Beding-Barnekow *et al.*, 1988); the resulting contraction was used as an internal standard. Each preparation was exposed to one concentration of ruthenium red or capsazepine only.

Effects of ruthenium red and capsazepine on contractile responses to bradykinin and NKA

In another series of experiments we investigated the effects of ruthenium red or capsazepine on the contractions induced by the subsequent application of 3×10^{-8} M bradykinin or 10^{-8} M NKA. The concentrations of bradykinin and NKA were chosen to produce approximately 50% of their maximal responses (Beding-Barnekow *et al.*, 1988; Wang & Håkanson, 1992a). These experiments were performed according to the same protocol as with capsaicin except that bradykinin or NKA were applied instead of capsaicin.

Analysis of results

In each experiment the contractile responses evoked by electrical stimulation, capsaicin or bradykinin in the drug-exposed preparations were compared with the responses in control preparations (set as 100%). The contraction induced by NKA in the presence of ruthenium red or capsazepine was expressed as % of that induced by NKA just before the exposure to ruthenium red or capsazepine; this was possible since repeated application of NKA is known to cause reproducible contractions in this preparation (Beding-Barnekow *et al.*, 1988). Concentration-response curves were constructed and the pIC_{50} values (i.e. the negative logarithm of the molar concentration of the antagonist that produces 50% inhibition of the contraction) were calculated.

Drugs

Capsaicin, bradykinin and ruthenium red were purchased from Sigma (St. Louis, MO, U.S.A.). NKA was from Peninsula Europe (St. Helens, UK). Capsazepine was generously donated by Dr S. Bevan, Sandoz Institute for Medical Research, London, UK. Spantide II, a tachykinin antagonist (Håkanson *et al.*, 1990; Maggi *et al.*, 1991), was synthesized at the Institute for Biomedical Research (Dr Karl Folkers, University of Texas at Austin, TX, U.S.A.). Capsaicin and capsazepine (10^{-2} M) were dissolved in pure ethanol. The stock solutions were diluted with 0.9% saline. Ruthenium red, bradykinin and NKA were dissolved in 0.9% saline. Spantide II (10^{-3} M) was dissolved in 0.5 N acetic acid.

Results

Contractile effects of capsaicin, ruthenium red and capsazepine

Capsaicin is known to contract the isolated iris sphincter concentration-dependently (Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985a; Håkanson *et al.*, 1987; Wang & Håkanson, 1992a). The contractile response reflects the release of tachykinins, since it can be blocked by tachykinin antagonists (Figure 1a) (Wahlestedt *et al.*, 1985a). Repeated applications of capsaicin cause tachyphylaxis (Figure 1e) (Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985a; Wang & Håkanson, 1992a). The contractions caused by the first and second application of 5×10^{6} M capsaicin were $113.1 \pm 5.4\%$ and $51.5 \pm 5.6\%$ of those evoked by 137.7 mM KCl buffer solution in control preparations ($n = 44$).

Ruthenium red produced concentration-dependent contractions (Figure 2) which usually reached maximum within 5–10 min and had subsided within 30 min after the application. Spantide II (10^{-5} M) inhibited the contractions by $91.3 \pm 4.4\%$ ($n = 4$), indicating that they were mediated by tachykinins (Figure 1b). Repeated application of 5×10^{-6} M ruthenium red three times with a time interval of 60 min (extensive washing) did not produce noticable tachyphylaxis (not shown). At higher concentrations, a second application of ruthenium red caused a reduced contraction, suggestive of tachyphylaxis (Figure 1f). The contractions caused by the first and second application of 10^{-4} M ruthemium red were $59.4 \pm 8.9\%$ and $10.7 \pm 5.1\%$, respectively, of those evoked by 137.7 mM KCl buffer solution ($n = 6$). There was no recovery of the response with time despite extensive washing.

Capsazepine also produced concentration-dependent contractions (Figure 2). The capsazepine-induced contractions usually reached maximum within 5 min and had subsided within 15 min after the application. As shown in Figure 1, the contractile effect of capsazepine was much weaker than that of ruthenium red. Spantide II (10^{-5} M) inhibited the contractions by $90 \pm 3.3\%$ ($n = 4$), indicating that they were mediated by tachykinins (Figure 1c). Repeated application of 10^{-5} M capsazepine failed to induce tachyphylaxis. At higher concentrations, a second application of capsazepine caused a reduced contraction, suggestive of tachyphylaxis (Figure 1g). The contractions caused by the first and second application of 10^{-4} M capsazepine were $27.4 \pm 4.6\%$ and $10.6 \pm 0.4\%$, respectively, of those evoked by 137.7 mM KCl buffer solution ($n = 6$). There was no recovery of the response with time, despite extensive washing.

Tetrodotoxin (TTX, 10^{-6} M) did not affect the contractions induced by capsaicin, ruthenium red and capsazepine (not shown).

Inhibitory effect of ruthenium red and capsazepine on capsaicin-induced contractions

Capsaicin was added to the bath 30 min after application of ruthenium red or capsazepine, at a time when the contractile responses evoked by the two drugs had subsided. Both ruthenium red and capsazepine inhibited the capsaicin-induced contractions concentration-dependently (Figures 3 and 4); the pIC_{50} values are given in Table 1. Ruthenium red was approximately equipotent with capsazepine in inhibiting the effect of capsaicin; at a concentration of 10^{-4} M, both ruthenium red and capsazepine nearly abolished the contraction induced by 5×10^{-6} M capsaicin.

Effects of ruthenium red and capsazepine on bradykinin-induced contractions

Bradykinin contracts the iris sphincter concentration-dependently, in a manner rather similar to that of capsaicin (Wahlestedt *et al.*, 1985a; Wang & Håkanson, 1992a). The contractions caused by bradykinin can be inhibited by tachykinin antagonists (Bynke *et al.*, 1983; Wahlestedt *et al.*, 1985a). Ruthenium red and capsazepine concentration-dependently inhibited the contractions induced by bradykinin (Figures 3 and 4). The pIC_{50} values are given in Table 1. At 10^{-4} M, ruthenium red suppressed the response by about 55%, while capsazepine abolished the response.

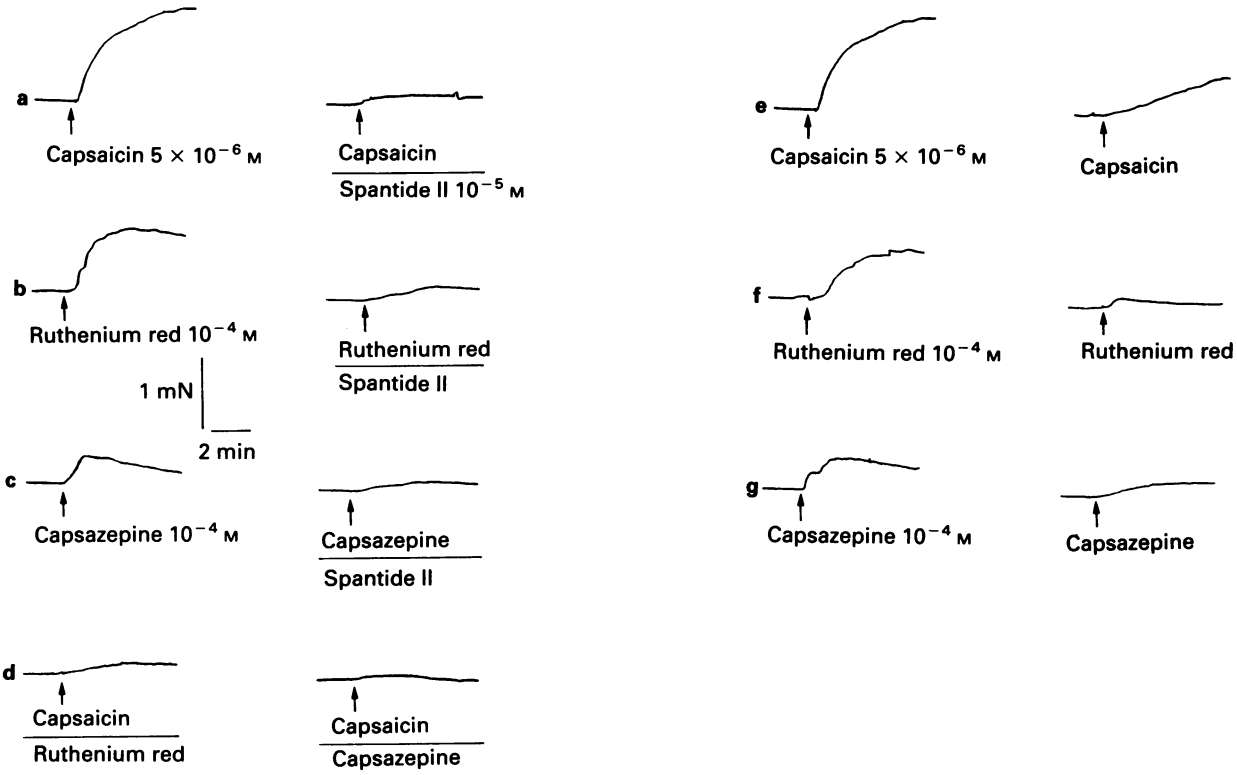

Figure 1 Original tracings illustrating the contractions of the rabbit iris sphincter induced by capsaicin (a), ruthenium red (b) and capsazepine (c). The contractions induced by all of the three drugs were inhibited by spantide II (parallel experiments) (a,b,c). The contractions induced by capsaicin were inhibited by either of ruthenium red or capsazepine (d). Consecutive application of either capsaicin (e), ruthenium red (f) or capsazepine (g) produced a progressively reduced response. Capsaicin: 5×10^{-6} M; ruthenium red 10^{-4} M; capsazepine 10^{-4} M; spantide II 10^{-5} M. Naive preparations were used in all experiments except in (e), (f) and (g).

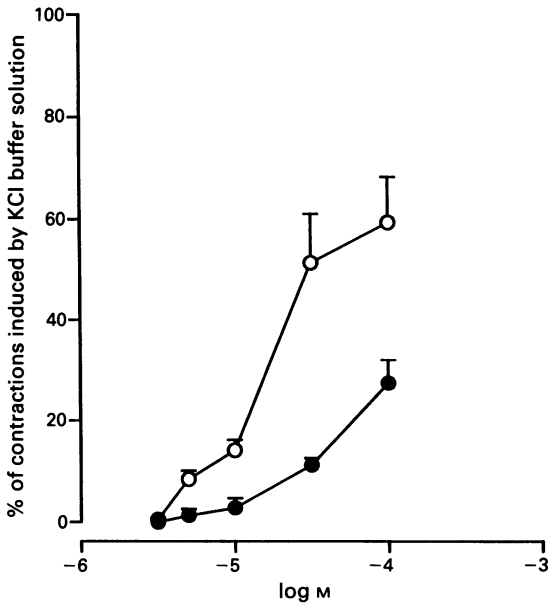

Figure 2 Ruthenium red (○) and capsazepine (●) contracted the iris sphincter in a concentration-dependent manner. Means ± s.e.mean of 8 experiments.

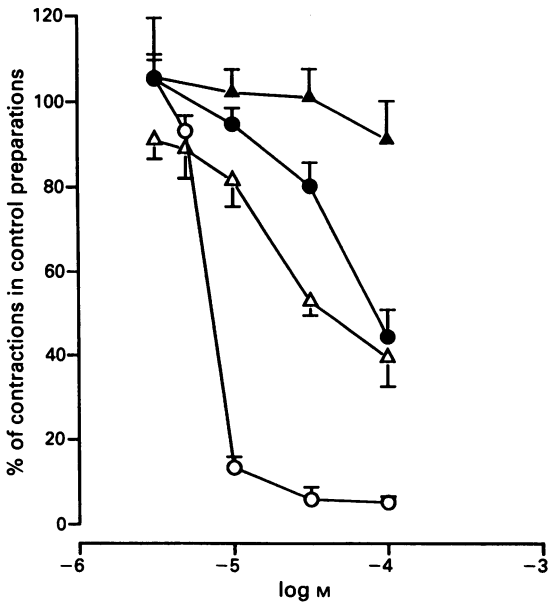

Figure 3 The contractile responses of the iris sphincter to 5×10^{-6} M capsaicin (○), 3×10^{-8} M bradykinin (●) and electrical stimulation (△) were inhibited concentration-dependently by ruthenium red. Ruthenium red was without effect on the contractions induced by 10^{-8} M neurokinin A (▲). Means ± s.e.mean of 6–8 experiments.

Inhibitory effects of ruthenium red and capsazepine on electrically evoked, non-cholinergic and non-adrenergic contractions

In the presence of atropine and guanethidine the contractile response of the iris sphincter to electrical stimulation is mediated by tachykinins (Wahlestedt *et al.*, 1985b; Wang & Håkanson, 1992b). Both ruthenium red and capsazepine concentration-dependently inhibited the contractions (Figures 3 and 4). The pIC_{50} values are given in Table 1. At 10^{-4} M, ruthenium red suppressed the response by about 60%, while capsazepine nearly abolished the response.

Table 1 Inhibitory effects of ruthenium red and capsazepine on contractile responses in rabbit iris sphincter muscle

Stimulus	Ruthenium red pIC_{50}	Ruthenium red MI (%)	Capsazepine pIC_{50}	Capsazepine MI (%)
Capsaicin	5.10	100	4.90	100
Electrical stimulation	4.32	60	4.50	89
Bradykinin	4.05	55	4.56	100
NKA	Inactive	<10	4.28	65

pIC_{50} value indicates the negative logarithm of the molar concentration of the antagonist that produces 50% inhibition of the contraction. MI (maximum inhibition) indicates the inhibition exerted by either of ruthenium red or capsazepine at a concentration of 10^{-4} M.

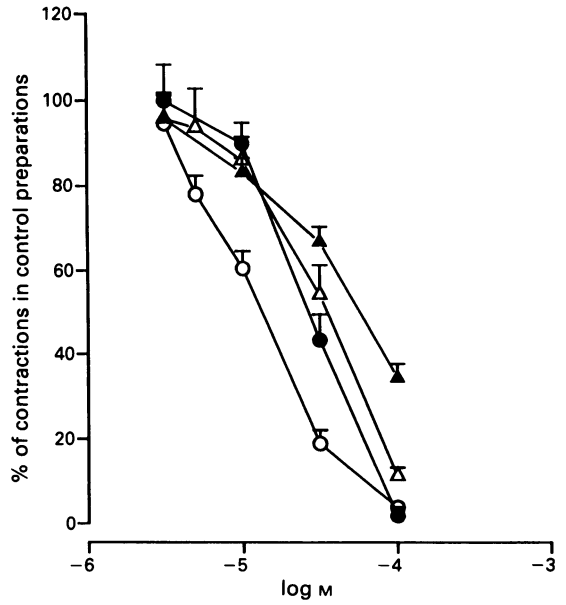

Figure 4 The contractile responses of the iris sphincter to 5×10^{-6} M capsaicin (○), 3×10^{-8} M bradykinin (●), electrical stimulation (△) and neurokinin A (▲) were inhibited concentration-dependently by capsazepine. Means ± s.e.mean of 6–8 experiments.

Effects of ruthenium red and capsazepine on NKA-induced contractions

Ruthenium red up to 10^{-4} M did not affect the contractions caused by NKA (Figure 3). However, capsazepine concentration-dependently inhibited the contractile response to NKA (Figure 4). The pIC_{50} value is given in Table 1. At 10^{-4} M, capsazepine suppressed the contraction by about 65%. Similar results have been obtained with capsaicin (Wang & Håkanson, 1992a).

Discussion

Capsaicin contracts the iris sphincter muscle of the rabbit, starting at a concentration of about 10^{-6} M (Wang & Håkanson, 1992a). The contraction reflects the excitation of sensory C-fibres and is mediated by tachykinins (Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985a; Håkanson *et al.*, 1987; Wang & Håkanson, 1992a). A typical feature of the capsaicin-induced stimulation of C-fibres is that the excitation soon subsides and that the fibres become progressively less responsive to repeated application of the drug (Maggi & Meli, 1988; Bevan & Szolcsanyi, 1990; Holzer, 1991; Dray, 1992). The refractoriness that develops to capsaicin upon repeated application reflects depletion of transmitters and/or blockade of nerve conduction (Gamse *et al.*, 1980; Petsche *et al.*, 1983; Pini *et al.*, 1983; Håkanson *et al.*, 1987; Maggi *et al.*, 1987; Marsh *et al.*, 1987; Amann & Lembeck, 1991; Docherty *et al.*, 1991; Holzer, 1991; Wang & Håkanson, 1992a). Recently, ruthenium red was shown to inhibit capsaicin-induced stimulation of sensory neurones and nerve fibres in various test systems (Amann & Maggi, 1991; Holzer, 1991); it is regarded as a 'functional' and non-competitive capsaicin antagonist (Amann & Maggi, 1991; Holzer, 1991; Maggi *et al.*, 1993). Capsazepine, on the other hand, is thought to be a competitive antagonist of capsaicin (Dickenson & Dray, 1991; Belvisi *et al.*, 1992; Bevan *et al.*, 1992; Maggi *et al.*, 1993).

Both ruthenium red and capsazepine were found to contract the iris sphincter concentration-dependently, in a manner resembling that of capsaicin. Thus, the contractile responses to ruthenium red and capsazepine were prevented by tachykinin receptor blockade, indicating an effect of the two drugs on C-fibres. Moreover, at concentrations of 10^{-4} M the two drugs displayed tachyphylaxis upon repeated administration. Capsazepine was much less effective in causing contraction than ruthenium red. At present, it cannot be unequivocally established whether ruthenium red and capsazepine stimulate the C-fibres of the iris sphincter by the same or by different mechanisms. Capsazepine is a capsaicin analogue and it is conceivable that it acts as a partial agonist, although this has not been demonstrated previously (Dray *et al.*, 1991; Bevan *et al.*, 1992). Recently, it was reported that injection of ruthenium red into the anterior chamber of the rabbit eye caused miosis (Andersson & Greves, 1991). The miosis could be inhibited not only by a tachykinin antagonist but also by the Ca^{2+} channel-blocking agent ω-conotoxin GVIA (Andersson & Greves, 1991). It was suggested that ruthenium red stimulates Ca^{2+} entry into the C-fibres, causing release of tachykinins and consequent miosis (Andersson & Greves, 1991). Our results are compatible with this interpretation.

Both ruthenium red and capsazepine concentration-dependently inhibited the capsaicin-induced contractile response of the rabbit iris sphincter. Since the effect of capsaicin on C-fibres is thought to depend on cation influx (Holzer, 1991), the inhibition by ruthenium red might reflect impaired influx of Ca^{2+} (Amann *et al.*, 1989; Chahl, 1989). Capsazepine on the other hand might inhibit the capsaicin-induced response by interacting with capsaicin binding sites (receptors?) to inhibit the capsaicin-induced contraction. Other mechanisms might also contribute. In the rabbit iris sphincter, the tachyphylaxis induced by repeated applications of capsaicin reflects the progressive depletion of the releasable pool of tachykinins (Håkanson *et al.*, 1987; Wang & Håkanson, 1992a). Thus, depletion of the tachykinin store by the two drugs may contribute to a reduced response to subsequent stimuli. Although ruthenium red and, in particular, capsazepine were much less effective than capsaicin in causing C-fibre excitation, the possibility cannot be excluded that the capsaicin-like agonism of ruthenium red and capsazepine could lead to inhibition of subsequent transmitter release.

Interestingly, ruthenium red and capsazepine inhibited not only the contractile response of the iris sphincter to capsaicin but also the response to bradykinin and to electrical stimulation. The concentrations needed to inhibit the contractile response to bradykinin and to electrical stimulation were higher than those needed to inhibit the response to capsaicin. Bradykinin contracts the rabbit iris by a mechanism similar to that of capsaicin (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985a). Thus, the contraction reflects the excitation of C-fibres and is mediated by tachykinins (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985a; Wang & Håkanson, 1992a). However, bradykinin acts on specific receptors (Regoli *et al.*, 1990; Lembeck *et al.*, 1991), distinct

from the capsaicin recognition sites, on the C-fibre plasma membrane. A specific bradykinin antagonist inhibits the response of the iris to bradykinin, but not to capsaicin (Griesbacher & Lembeck, 1987). Since Ca^{2+} entry is important for the release of neurotransmitters, the inhibition by ruthenium red of the response to bradykinin and to electrical stimulation could reflect impaired Ca^{2+} entry into the C-fibres. Whether capsazepine has a similar effect on Ca^{2+} transport is unknown. It cannot be excluded that while ruthenium red acts by blocking capsaicin-evoked Ca^{2+} entry, capsazepine interacts by blocking binding sites (receptors?) for capsaicin, acting as a partial agonist/antagonist. The inhibition of the responses to bradykinin and to electrical stimulation by ruthenium red and capsazepine suggests that capsaicin/capsazepine binding sites and capsaicin-operated cation channels may affect C-fibre transmitter release in general.

The finding that capsazepine, but not ruthenium red, inhibits the contractions induced by NKA was unexpected; the concentration of capsazepine needed was somewhat higher than that needed to inhibit the contraction induced by capsaicin. The finding is in line with our earlier observation that after incubation with 10^{-4} M capsaicin, the contractile response of the iris to a selective NK_1 agonist was reduced by 40% (Wang & Håkanson, 1992a). Thus, the observation is compatible with the notion that capsazepine acts as a partial capsaicin agonist/antagonist. NKA acts directly at tachykinin receptors on the smooth muscle cells to cause contraction and there is no evidence that it stimulates C-fibres. Since the NKA-induced contraction is much reduced in Ca^{2+}-free Krebs solution, Ca^{2+} entry into smooth muscle may play an important role in the contraction induced by NKA. Thus, the inhibition of NKA-induced contraction may suggest that capsazepine and capsaicin, unlike ruthenium red, can interfere with Ca^{2+} channels on smooth muscle. The observation also suggests that the inhibition of NKA-induced contraction may be at least partly responsible for the antagonism exerted by capsazepine to the contractile responses to capsaicin, bradykinin and electrical stimulation.

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The profiles of interaction of yohimbine with anxiolytic and putative anxiolytic agents to modify 5-HT release in the frontal cortex of freely-moving rats

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1 The interaction of yohimbine with anxiolytic and putative anxiolytic agents to modify 5-hydroxytryptamine (5-HT) release in the frontal cortex of the freely-moving rat was assessed using the microdialysis technique.

2 The α_2-adrenoceptor antagonist, yohimbine (5.0 mg kg^{-1}, i.p.) increased maximally the extracellular levels of 5-HT in the rat frontal cortex by approximately 230% of the basal levels.

3 The α_2-adrenoceptor agonist, clonidine (30–100 μg kg^{-1}, i.p.) decreased dose-dependently the extracellular levels of 5-HT in the rat frontal cortex by approximately 0–60% of the basal levels. A 5 min pretreatment with clonidine (50 μg kg^{-1}, i.p.) prevented the yohimbine-induced increase in the extracellular 5-HT levels.

4 The benzodiazepine receptor agonist, diazepam (2.5 mg kg^{-1}, i.p.) and the 5-HT$_3$ receptor antagonist, ondansetron (100 μg kg^{-1}, i.p.) (5 min pretreatment) completely prevented the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increases in the extracellular levels of 5-HT. The 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT (0.32 mg kg^{-1}, s.c.) partially antagonized the yohimbine response.

5 A 5 min pretreatment with the 5-HT$_3$/5-HT$_4$ receptor ligand **R**(+)-zacopride (10 μg kg^{-1}, i.p.) reversed the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increase in the extracellular levels of 5-HT to approximately 30% below the basal levels. A 5 min pretreatment with **S**(−)-zacopride (100 μg kg^{-1}, i.p.) failed to modify the response to yohimbine.

6 The present study provides evidence of the ability of the anxiogenic agent, yohimbine, to increase the activity of the central 5-hydroxytryptaminergic system and the ability of clonidine and various anxiolytic and putative anxiolytic agents to prevent the yohimbine response.

Keywords: 5-Hydroxytryptamine; *in vivo* microdialysis; α_2-adrenoceptor agonist and antagonist; diazepam; 5-HT$_{1A}$ receptor agonist; 5-HT$_3$ receptor antagonists; rat frontal cortex

Introduction

It is generally accepted that a reduction in the neuronal function of 5-hydroxytryptamine (5-HT) leads to an anxiolytic effect and that an increase in 5-HT function results in an anxiogenic profile (for reviews see Iversen, 1984; Chopin & Briley, 1987; Stein *et al.*, 1975; Thiebot, 1986). Thus, *p*-chlorophenylalanine (*p*-CPA), which reduces 5-HT neurotransmission by inhibition of tryptophan hydroxylase, showed anxiolytic-like effects in the rat social interaction test (File & Hyde, 1977; Barnes *et al.*, 1992b) and conflict procedures (Engel, 1986). These effects are reversed by the administration of 5-hydroxytryptophan (5-HTP) (Rochichaud & Sledge, 1969; Geller & Blum, 1970). Lesions produced by intraventricular or local stereotaxic administration of the 5-hydroxytryptaminergic neurotoxins, 5,6-dihydroxytryptamine (5,6-DHT) or 5,7-dihydroxytryptamine (5,7-DHT) result in anxiolytic-like effects in conflict tests (Stein *et al.*, 1975; Tye *et al.*, 1977) and in the rat social interaction tests (File *et al.*, 1979).

Anxiolytic benzodiazepine receptor agonists such as diazepam and chlordiazepoxide have also been demonstrated to inhibit 5-HT synthesis and metabolism in the rat or cat brain using *ex vivo* or *in vivo* measurements (Saner & Pletscher, 1979; Balfour, 1980; Collinge & Pycock, 1982). More recently, using the *in vivo* microdialysis technique, it has been reported that both the systemic and local administration of diazepam and flurazepam decreased the extracellular levels of 5-HT from the ventral hippocampus (Pei *et al.*, 1989) and frontal cortex (Barnes *et al.*, 1992a). It has also been demonstrated that exposure of rats to the elevated plus maze results in an increase in the levels of extracellular levels of 5-HT in the hippocampus, this effect being antagonized by the administration of diazepam and the 5-HT$_{1A}$ receptor agonist, ipsapirone (Wright *et al.*, 1992).

Some compounds which interact with 5-HT receptors such as the 5-HT$_{1A}$ receptor agonists buspirone, gepirone and ipsapirone, the 5-HT$_2$ receptor antagonists, ritanserin and ketanserin and the 5-HT$_3$ receptor antagonists, ondansetron, granisetron, tropisetron, MDL 72222 and zacopride, have been shown to have anxiolytic profiles in animal models of anxiety or in man (Cuelemans *et al.*, 1985; Goa & Ward, 1986; Newton *et al.*, 1986; Taylor, 1988; Lecrubier *et al.*, 1990; Briley & Chopin, 1991; Lader, 1991).

Yohimbine is a potent α_2-adrenoceptor antagonist (Goldberg & Robertson, 1983) and preclinical studies have indicated that yohimbine possesses anxiogenic-like effects in animal models of anxiety (Pellow *et al.*, 1985; 1987). In the clinical studies, it has been demonstrated that administration of yohimbine produces significant increases in subjective anxiety in healthy subjects (Charney *et al.*, 1983; 1992) and in patients with panic disorders (Albus *et al.*, 1992). The anxiogenic effects were antagonized by pretreatment with diazepam and clonidine (Charney *et al.*, 1983). The possible mechanism of such an effect may be the interaction of yohimbine at α_2-adrenoceptors resulting in an overactivity of the brain noradrenergic system which has been related to the production of some forms of anxiety (Charney *et al.*, 1983). There is also some evidence to indicate an interaction between α_2-adrenoceptors and 5-HT systems in the central nervous system (Fuxe, 1986; Maura *et al.*, 1982; Raiteri *et al.*, 1990) and the question arises as to whether 5-HT systems may play some role in the yohimbine-induced effects.

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The major aim of the present study was to investigate, by us of the *in vivo* microdialysis technique, the effect of systemic administration of yohimbine on the extracellular levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the frontal cortex of freely-moving rats. A further objective was to determine if the effect of pretreatment with anxiolytic and putative anxiolytic agents could influence the effect of yohimbine on the extracellular levels of 5-HT.

Methods

Animals

Male hooded-Lister rats (400–450 g) were housed in groups of 5 in a controlled environment (temperature 21 ± 1°C) under a 12 h light/dark cycle (lights on 07 h 00 min) and were given free access to food and water.

Stereotaxic implantation of chronic indwelling guide cannulae

Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and chloral hydrate (150 mg kg^{-1}, s.c.) before the insertion of 5 mm chronically indwelling guide cannulae (19 gauge stainless steel tubing; Coopers Needle Work Ltd) which were positioned above the frontal cortex (final probe tip location; right hemisphere, A +0.7, V −7.0, L −1.5, relative to bregma at an angle of 45°) and secured to the skull with screws and dental cement. The guide cannulae were kept patent with stylets.

Microdialysis probe construction, implantation and collection of dialysates

Microdialysis probes were constructed 'in house'. Briefly, stainless steel 'bodies' (11 mm, 23 gauge, Coopers Needle Work Ltd) and 'collars' (5 mm, 26 gauge, Coopers Needle Work Ltd) were fixed into a perspex holder. Dialysis membrane (external/internal diameter 310/220 μm, molecular weight cut off 40,000; AN69, Hospal Medical) was glued to the tip of the stainless steel 'body' and the end sealed with epoxy resin (Araldite) leaving 4 mm in total length exposed to the brain area. A silica glass fibre (external/internal diameter 140/74 μm, Scientific Glass Engineering PTY LTd) guided artificial cerebrospinal fluid (aCSF; mM: NaCl 126, $NaHCO_3$ 27.4, KCl 2.4, KH_2PO_4 0.5, $CaCl_2$ 1.1, $MgCl_2$ 1.3, Na_2HPO_4 0.49 and glucose 7.0; pH 7.4) to the tip of the microdialysis probe, which subsequently eluted from the probe (via coiled polypropylene tubing) following passage between the outer surface of the silica glass fibre and the inner surface of the dialysis membrane.

At least 14 days after stereotaxic location of the guide cannulae, rats were immobilized by a soft-cloth wrapping technique and the microdialysis probe was gently implanted into the frontal cortex and secured with cyanoacrylate adhesive (Permabond C2). The rat was placed in a single animal cage (with free access to food and water) for 12 h before being transferred to the test cage (42 × 24 × 26 cm; length, width, height) where the animal also had free access to food and water. Following a 30 min period for the rat to habituate to the test box, the microdialysis probe was connected, via polypropylene tubing, to a microdialysis pump (CMA 100, Carnegie) and was perfused at a constant rate of 2.0 μl min^{-1} with aCSF. The dialysate collected over the first 60 min was discarded and subsequent samples were collected every 20 min for a period of up to 6 h. All samples were analysed immediately for levels of 5-HT and 5-HIAA by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (e.c.d.).

H.p.l.c.-e.c.d. system for the quantification of 5-HT and 5-HIAA

For the simultaneous determination of 5-HT and 5-HIAA levels in dialysates, the h.p.l.c.-e.c.d. system comprised an isocratic pump (Waters 510 Solvent Delivery System) which was connected to an analytical column (Hypersil 5ODS; 150 × 4.6 mm; HPLC Technology) via an automatic injector (Waters Wisp). The analytical column was protected by a C_{18} guard column (Guard-Pak, Waters). The effluent from the

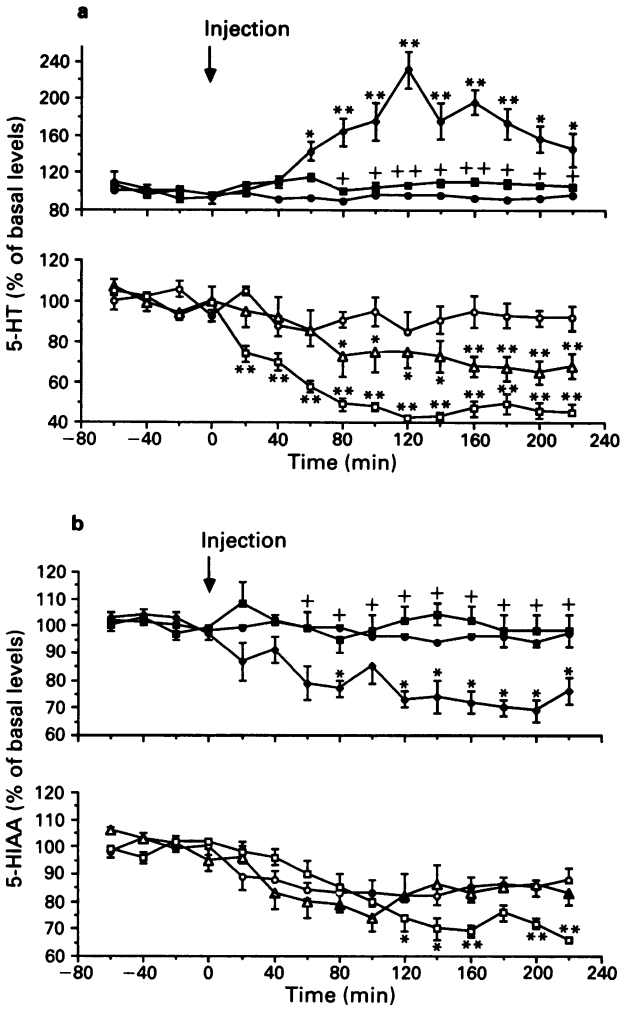

Figure 1 Effect of yohimbine (5.0 mg kg^{-1}, i.p.) (◆), clonidine (30 μg kg^{-1}, i.p. (○), 50 μg kg^{-1}, i.p. (△) and 100 μg kg^{-1}, i.p.) (□) and vehicle (saline, 1.0 ml kg^{-1}, i.p.) (●) and the ability of clonidine (50 μg kg^{-1}, i.p.) (■) to modify the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increase in (a) 5-hydroxytryptamine (5-HT) or decrease in (b) 5-hydroxyindoleacetic acid (5-HIAA) in the rat frontal cortex dialysates with measurements commencing 12 h after the probe implantation. Clonidine was administered 5 min prior to the injection of yohimbine when co-administered. 5-HT and 5-HIAA levels are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean of four to eight determinations. Significant increases or decreases compared to the basal levels and differences between the yohimbine-treated and clonidine + yohimbine-treated groups are indicated as $^{*}P<0.05$, $^{**}P<0.01$ (single factor ANOVA followed by Dunnett's *t* test). $^{+}P<0.05$, $^{++}P<0.01$ (two factor ANOVA followed by *t* test) respectively. Basal levels of 5-HT and 5-HIAA were – yohimbine: 54 ± 3.30 fmol/40 μl, 2.66 ± 0.05 pmol/40 μl; clonidine 30 μg kg^{-1}: 67 ± 4.90 fmol/40 μl, 4.6 ± 0.28 pmol/40 μl; clonidine 50 μg kg^{-1}: 69 ± 3.7 fmol/40 μl, 5.54 ± 0.11 pmol/40 μl; clonidine 100 μg kg^{-1}: 110 ± 5.60 fmol/40 μl, 5.82 ± 0.07 pmol/40 μl; yohimbine + clonidine: 95 ± 2.38 fmol/40 μl, 5.30 ± 0.45 pmol/40 μl; vehicle: 54 ± 2.50 fmol/40 μl, 2.41 ± 0.05 pmol/40 μl respectively.

analytical column was passed into an electrochemical detector (ESA Coulochem with model 5011 analytical cell, working electrode potential + 0.45 V versus a solid state reference electrode incorporated within the analytical cell). The output from the electrode was monitored with a recording/plotting integrator (Hewlett-Packard 3392A). A guard cell (ESA model 5020) was placed between the pump and injector and was set at + 0.50 V (relative to a solid state reference electrode incorporated within the cell) to reduce the background current originating from the mobile phase. The h.p.l.c.-e.c.d. system, with the exception of the integrator, was maintained at a constant temperature of 4°C inside a glass-fronted cool cabinet. The optimized mobile phase (methanol 11% v/v, disodium hydrogen orthophosphate 106 mM, citric acid 36 mM, tetraethylammonium bromide 2 mM, pH 6.2–6.3; slight adjustments to the pH and/or methanol concentration were made to overcome column to column variation) was delivered to the analytical column at a rate of 1.4 ml min^{-1}. Injections of external standards were made in order to identify and calibrate the peaks resulting from the injection of the dialysates.

Drugs

Yohimbine hydrochloride (Sigma), 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide, Research Biochemicals Inc.), ondansetron hydrochloride dihydrate (Glaxo), **R**(+)-zacopride and **S**(−)-zacopride hydrochloride ((±)4-amino-*N*-(1-azabicyclo[2,2,2]oct-3yl)-5-chloro-2-methoxy benzamide, A.H. Robins) and clonidine hydrochloride (Research Biochemicals Inc.) were dissolved in distilled water and diluted to volume in saline (0.9% w/v NaCl). Diazepam (Sigma) was prepared as a suspension in 10% polyethylene glycol 400 in saline. All drugs were used as received and were freshly prepared immediately before use.

Results

Effect of yohimbine and clonidine and their interaction to modify the extracellular levels of 5-HT and 5-HIAA

The administration of yohimbine (5.0 mg kg^{-1}, i.p.), caused marked increases in the extracellular levels of 5-HT in the rat frontal cortex (by approximately 230% of the basal levels), the maximal increases in the dialysate 5-HT levels were detected approximately 120 min following the injection, and then gradually decreased. At the end of the experiment (220 min), the levels of 5-HT were still significantly 40% higher than the basal levels (Figure 1). Yohimbine (5.0 mg kg^{-1}, i.p.) also caused a slight (25%) but significant decrease in the extracellular levels of 5-HIAA in the rat frontal cortex (Figure 1).

The administration of clonidine (30–100 μg kg^{-1}, i.p.) reduced in a dose-dependent manner the extracellular levels of 5-HT by some 0–60% of the basal levels in the rat frontal cortex. Such reductions began 20 min after the injection and reached a maximal effect in 120 min and remained at this level for the duration of the experiment (Figure 1). The high dose of clonidine (100 μg kg^{-1}, i.p.) (but not 30 and 50 μg kg^{-1}, i.p.) also slightly but significantly reduced the extracellular 5-HIAA levels from 98% ($t = 0$) to 70% (maximum reduction, Figure 1).

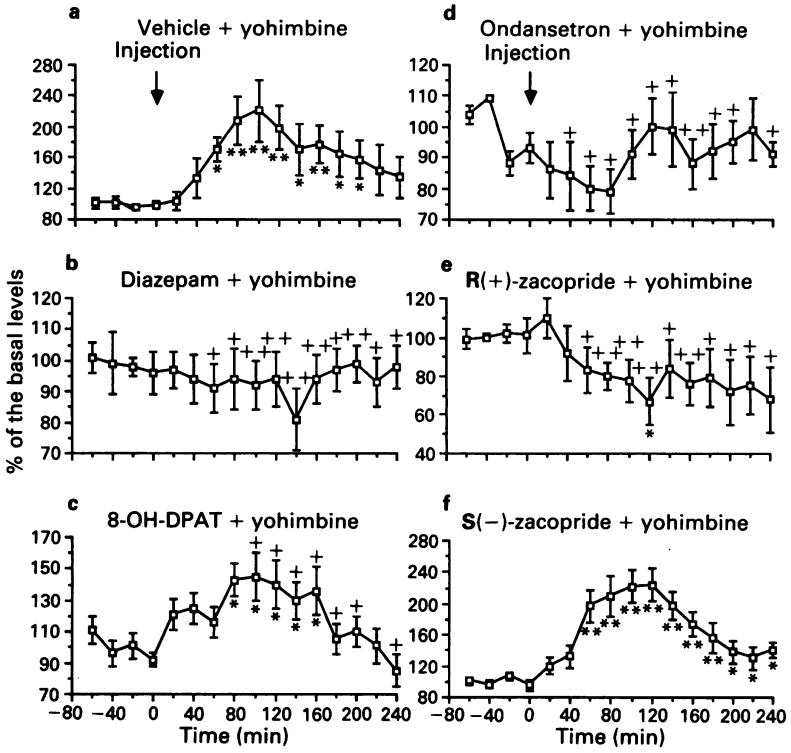

Figure 2 The ability of (a) vehicle (saline, 1.0 ml kg^{-1}, i.p.), (b) diazepam (2.5 mg kg^{-1}, i.p.), (c) 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 0.32 mg kg^{-1}, s.c.), (d) ondansetron (100 μg kg^{-1}, i.p.), (e) **R**(+)-zacopride (10 μg kg^{-1}, i.p.) and (f) **S**(−)-zacopride (100 μg kg^{-1}, i.p.) to modify the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increases or decreases in the extracellular levels of 5-HT in the rat frontal cortex dialysates with measurement commencing 12 h after the probe implantation. Diazepam, 8-OH-DPAT, ondansetron, **R**(+)-zacopride, **S**(−)-zacopride or vehicle were administered 5 min prior to the injection of yohimbine. 5-HT levels are expressed as the percentage of the meaned absolute amount in the 4 collections preceding the drug treatment. Data represent the mean ± s.e.mean of four to six determinations. Significant increases or decreases compared to the basal levels and differences between the vehicle + yohimbine treated and drugs + yohimbine treated groups are indicated as $^{*}P<0.05$, $^{**}P<0.01$ (single factor ANOVA followed by Dunnett's t test); $^{+}P<0.05$ and $^{++}P<0.01$ (two factor ANOVA followed by t test) respectively. Basal levels of 5-HT were – diazepam: 56 ± 2.10 fmol/40 μl, 8-OH-DPAT: 54 ± 2.39 fmol/40 μl; ondansetron: 38 ± 0.98 fmol/40 μl; **R**(+)-zacopride: 44 ± 2.83 fmol/40 μl; **S**(−)-zacopride: 47 ± 2.69 fmol/40 μl.

The administration of clonidine (50 μg kg^{-1}, i.p.) 5 min prior to the injection of yohimbine (5.0 mg kg^{-1}, i.p.) completely prevented the yohimbine-induced increases in the extracellular levels of 5-HT (Figure 1). The reduction in 5-HIAA levels induced by yohimbine were returned by clonidine to the vehicle-treated control levels (respective values of 73% and 102% at 120 min after drug administration, Figure 1).

Effect of diazepam, 8-OH-DPAT, ondansetron, R(+)-zacopride and S(−)-zacopride on the yohimbine-induced increases in the extracellular levels of 5-HT in the rat frontal cortex

The administration of diazepam (2.5 mg kg^{-1}, i.p.) and ondansetron (100 μg kg^{-1}, i.p.) 5 min prior to the injection of yohimbine (5.0 mg kg^{-1}, i.p.) completely prevented the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increases in the extracellular levels of 5-HT in the rat frontal cortex (Figure 2). The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.32 mg kg^{-1}, s.c.) partially prevented the effect of yohimbine, reducing the maximal yohimbine-induced increase in the 5-HT levels from 230% to 140% of the basal levels (t = 100 min, Figure 2). At the end of the experiment (220 min following the injection), the 5-HT levels had returned to the basal levels shown in the vehicle treated controls (Figure 2).

The administration of R(+)-zacopride (10 μg kg^{-1}, i.p.) 5 min before the injection of yohimbine (5.0 mg kg^{-1}, i.p.) reversed the yohimbine (5.0 mg kg^{-1}, i.p.)-induced increases in the extracellular 5-HT levels, the 5-HT levels significantly decreasing to approximately 30% below the basal levels (Figure 2). S(−)-zacopride (100 μg kg^{-1}, i.p.) administered 5 min before the injection of yohimbine (5.0 mg kg^{-1}, i.p.) failed to modify significantly the yohimbine-induced increases in the 5-HT levels (Figure 2).

Diazepam (2.5 mg kg^{-1}, i.p.), ondansetron (100 μg kg^{-1}, i.p.) R(+)-zacopride (10 μg kg^{-1}, i.p.), S(−)-zacopride (100 μg kg^{-1}, i.p.) and 8-OH-DPAT (0.32 mg kg, s.c.) failed to modify significantly ($P > 0.05$) yohimbine-induced decrease in 5-HIAA levels (data not shown).

Discussion

Using the *in vivo* microdialysis technique, the present study has demonstrated that the systemic administration of the α_2-adrenoceptor antagonist, yohimbine, having anxiogenic properties in animal models of anxiety and man (Charney *et al.*, 1983; 1992; Pellow *et al.*, 1987), significantly increased the extracellular levels of 5-HT in the frontal cortex of the freely-moving rat. Yohimbine also caused a slight but significant reduction in the frontal cortical dialysate 5-HIAA levels. Clonidine caused a dose-dependent reduction in the extracellular 5-HT levels, and the frontal cortical dialysate 5-HIAA levels were slightly but significantly reduced by the high dose of clonidine. Such findings might indicate that the anxiogenic-like effects induced by either an anxiogenic agent such as yohimbine or aversive situations (Wright *et al.*, 1992) result in increased 5-HT neuronal activity in the nerve terminal regions.

The influence of α_2-adrenoceptor ligands to modulate the activity of 5-HT neurones may occur either by an action on α_2-adrenoceptors on the 5-HT nerve terminals (Fuxe, 1965; Maura *et al.*, 1982; Raiteri *et al.*, 1990) or by an action on 5-HT neurones of the dorsal raphe nucleus (Fuxe, 1965). In the present study, therefore, the increases in the extracellular 5-HT levels induced by administration of yohimbine could be the result of blockade of an inhibitory noradrenergic innervation located on the 5-HT nerve terminals in the forebrain or 5-HT cells in the midbrain. This hypothesis is supported by data showing that the injection of the α_2-adrenoceptor antagonist, idazoxan into the dorsal raphe nucleus caused an elevation of the firing rate of 5-HT neurones in the dorsal raphe (Freeman, & Aghajanian, 1984), and systemic administration of idazoxan increased the extracellular levels of 5-HT and 5-HIAA in the rat frontal cortex (Garratt *et al.*, 1991). In the present study, the activation of α_2-adrenoceptors by clonidine, decreasing the extracellular levels of 5-HT when administered alone and preventing the yohimbine-induced increases in the 5-HT levels in the rat frontal cortex, are consistent with *in vitro* results showing that noradrenaline inhibits the depolarization-induced release of [^{3}H]-5-HT from rat hippocampal slices (Frankhuyzen & Mulder, 1980). In addition there are *in vivo* data indicating that clonidine inhibits depolarization-induced endogeneous 5-HT release in the hippocampus of freely-moving rats (Yoshioka *et al.*, 1992).

The present study also demonstrated that the yohimbine-induced increases of the extracellular levels of 5-HT were completely antagonized by pretreatment with diazepam, ondansetron and R(+)-zacopride, partially antagonized by 8-OH-DPAT and not affected by the administration of S(−)-zacopride. Diazepam, clonidine, ondansetron, R(+)-zacopride and 8-OH-DPAT have all been demonstrated to reduce, to different extents, the behavioural response to an aversive situation in the animal models of anxiety or possess antianxiety effects in man (Hoehn-Saric *et al.*, 1981; Engel *et al.*, 1984; Barnes *et al.*, 1990; Costall *et al.*, 1990; Young & Johnson, 1991). The doses chosen in the present study were based on the doses producing anxiolytic-like effects in the animal models of anxiety. The doses of diazepam, 8-OH-DPAT and R(+)-zacopride are also those we have previously demonstrated to decrease 5-HT release in the rat frontal cortex (Barnes *et al.*, 1992a) and for diazepam and 8-OH-DPAT in the rat ventral hippocampus (Pei *et al.*, 1989; Sharp *et al.*, 1989). However, this is not taken to imply that anxiolytic-like actions are necessarily related to a reduction in 5-HT release. Thus ondansetron failed to modify the extracellular levels of 5-HT when administered alone (Barnes *et al.*, 1992a) yet completely prevented the yohimbine-induced increase in the extracellular levels of 5-HT. But it remains possible that the actions of diazepam, 8-OH-DPAT and R(+)-zacopride in antagonizing the yohimbine-induced increases in the extracellular levels of 5-HT may reflect indirect and opposing actions. Yet there are additional possibilities of direct drug interactions on the 5-HT systems. Thus the ability of diazepam to decrease the depolarization-induced 5-HT release from slices of cortex (Collinge & Pycock, 1982) and hippocampus *in vitro* (Balfour, 1980), probably reflects the action of the benzodiazepine receptor agonists to enhance the action of GABA at GABA$_A$ receptors to reduce 5-hydroxytryptaminergic transmission in the brain (Stein *et al.*, 1977). Thus, in the present study, it is possible that diazepam potentiates the action of the GABA receptor to inhibit the synthesis (Saner & Pletscher, 1979) and metabolism of 5-HT (Koe, 1979) and to reduce 5-HT release in the terminal regions (Barnes *et al.*, 1992a) to attenuate the yohimbine-induced increases in the extracellular levels of 5-HT in the rat frontal cortex.

The ability of 8-OH-DPAT to attenuate significantly the yohimbine-induced increase in the 5-HT release may reflect the actions of a 5-HT$_{1A}$ receptor ligand with intrinsic activity to reduce the release of 5-HT in terminal regions following interaction with somato-dendritic 5-HT autoreceptors (Hutson *et al.*, 1989; Sharp *et al.*, 1989; Barnes *et al.*, 1992a). The partial blockade by 8-OH-DPAT may reflect the use of a modest dose. Thus, it has previously been demonstrated that idazoxan completely blocked the effects of 8-OH-DPAT in moderating 5-HT release and metabolism in the suprachiasmatic nucleus (SCN) of the chloral hydrate anaesthetized rat (Marsden & Martin, 1986).

The present results showing that R(+)-zacopride but not S(−)-zacopride significantly antagonized the effects of yohimbine in increasing the extracellular 5-HT levels are in agreement with the behavioural finding that R(+)-zacopride

possesses anxiolytic-like activity at low ($\mu g\,kg^{-1}$) dose levels whereas effective doses of S(−)-zacopride are at least 4 orders of magnitude higher than in the animal models of anxiety (Barnes *et al.*, 1990; Young & Johnson, 1991). Both R(+)-zacopride and S(−)-zacopride have potent antagonist effects at the 5-HT_3 receptors but their different potentials in modifying the effects of yohimbine adds to the differential activities of R(+)- and S(−)-zacopride to inhibit emesis and dopamine-induced hyperactivity, to reduce anxiety-related behaviours, to improve cognitive performance and to modify the extracellular levels of 5-HT in the rat frontal cortex (Barnes *et al.*, 1990; Costall *et al.*, 1990; Barnes *et al.*, 1992a). However, in addition to their 5-HT_3 receptor antagonism, S(−)-zacopride has a 5-HT_3 agonist potential and both R(+)- and S(−)-zacopride have been demonstrated to have agonist/partial agonist effects at the 5-HT_4 receptors in many tissues (see review by Eglen *et al.*, 1990; King, 1990; Bockaert *et al.*, 1992). In addition, R(+)-zacopride binds with much greater affinity than S(−)-zacopride to an unspecified recognition site (Barnes *et al.*, 1990; Kidd *et al.*, 1992). However, the relevance of such actions to their ability to modify behaviour in an aversive situation and neurotransmitter release is not known. It is reasonable to presume that the differential activities of R(+)- and S(−)-zacopride to modify the yohimbine-induced increases in the 5-HT release may reflect their interaction at 5-HT_3, 5-HT_4 and/or the R(+)-zacopride recognition site.

The extracellular levels of 5-HIAA were also slightly but significantly reduced by the administration of yohimbine and reductions caused by the yohimbine interaction with anxiolytic and putative anxiolytic agents were also significant. These results, however, are not in agreement with the previous finding that the α_2-adrenoceptor antagonist, idazoxan, increased 5-HIAA levels in the hypothalamus and dorsal raphe nucleus (Garratt *et al.*, 1991). The difference could be the result of a discrepancy between the different methods used. 5-HT could be deaminated before or after release and it would be difficult to conclude whether 5-HIAA accurately reflects 5-HT utilization or simply provides an index of monoamine oxidase activity. Thus, the changes in the extracellular levels of 5-HIAA are not necessarily an indication of corresponding changes in 5-HT release.

In summary, the present studies have demonstrated that the α_2-adrenoceptor antagonist, yohimbine, caused significant increases in the extracellular levels of 5-HT in the frontal cortex of freely-moving rats which are antagonized by pretreatment with the anxiolytic agent, diazepam, and the putative anxiolytic agents, clonidine, ondansetron, R(+)-zacopride and 8-OH-DPAT and not affected by S(−)-zacopride. The neurochemical data are interpreted in terms of drug action on 5-hydroxytryptaminergic systems to support a role of 5-HT in the behavioural response to drugs modifying behaviour to aversive situations in the animal models of anxiety.

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Adenosine receptor-induced second messenger production in adult guinea-pig cerebellum

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1 The effects of adenosine receptor agonists on cyclic nucleotides accumulation were investigated in adult guinea-pig cerebellar slices by use of radioactive precursors.

2 Adenosine elicited a rapid and maintained increase in cyclic AMP, that was fully reversed upon addition of adenosine deaminase. Adenosine analogues stimulated cyclic AMP generation up to 40 fold with the rank order of potency: 5'-N-ethylcarboxamidoadenosine (0.6 μM) > 2-chloroadenosine (6 μM) > adenosine (13 μM). CGS 21680 (10 μM) elicited only a small stimulation (1.2 fold).

3 The cyclic AMP response to NECA was reversed by the 1,3-dipropylxanthine-based adenosine receptor antagonists 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (XAC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and N-[2-(dimethylamino)ethyl]N-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide (PD 115,199) with estimated apparent inhibition constants of 15, 81 and 117 nM, respectively.

4 Pretreatment with adenosine also potentiated the cyclic GMP response to sodium nitroprusside, abolishing the decline in [^{3}H]-cyclic GMP observed with sodium nitroprusside alone, and allowing [^{3}H]-cyclic GMP levels to be maintained for at least an additional 10 min. This potentiation was fully reversed by adenosine deaminase.

5 Adenosine analogues potentiated the sodium nitroprusside-elicited cyclic GMP generation with the rank order of potency: 5'-N-ethylcarboxamidoadenosine (0.7 μM) > 2-chloroadenosine (6 μM) > adenosine (42 μM).

6 NECA potentiation of cyclic GMP formation was reversed by the antagonists XAC, DPCPX and PD 115,199 with apparent inhibition constants of 17, 102 and 242 nM, respectively.

7 The similar potencies of adenosine analogues and xanthine antagonists for stimulation of cyclic AMP and potentiation of cyclic GMP lead to the suggestion that these phenomena are mediated through the same adenosine receptor, the A_{2b} receptor. Furthermore, we suggest that potentiation of the sodium nitroprusside-induced cyclic GMP response may be mediated at the level of phosphodiesterase hydrolysis of the cyclic nucleotides.

Keywords: Cyclic GMP; cyclic AMP; A_{2b} adenosine receptor; guinea-pig cerebellum

Introduction

Adenosine appears to act as a general inhibitory modulator of neurotransmission throughout the CNS (Jacobson *et al.*, 1992; van Galen *et al.*, 1992). Although the physiological roles for adenosine in the cerebellum have yet to be fully clarified, a role for adenosine has been proposed in the regulation of long-term potentiation in hippocampus (de Mendonça & Ribeiro, 1990), a phenomenon similar to long-term depression in cerebellum, a proposed cellular mechanism for cerebellar motor learning. A neuromodulatory role for adenosine in the cerebellum has also been proposed (Ross *et al.*, 1990), whereby liberated adenosine (present at high levels in Purkinje cells, Braas *et al.*, 1986) might influence glutamate release from adjacent parallel fibres.

Adenosine receptors have been most closely studied in tissues of CNS origin, in which at least three classes have been described (Daly *et al.*, 1983). Thus, the A_1 class is linked to inhibition of adenylyl cyclase activity, and also appears coupled to a variety of other signal transduction mechanisms (for reviews see Jacobson *et al.*, 1992; van Galen *et al.*, 1992). In contrast, the A_2 class appears to be coupled exclusively to stimulation of adenylyl cyclase and adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation. Evidence for two subtypes of A_2 receptor was originally derived from the finding that although adenosine and its analogues could stimulate cyclic AMP generation in tissue slices from most brain regions (Daly, 1977) stimulation of adenylyl cyclase in particulate preparations from the CNS was limited to neostriatum, nucleus accumbens and olfactory tubercle (Fredholm, 1977). These subtypes were initially termed low and high affinity A_2 receptors based on the relative potency of adenosine analogues, and were later renamed A_{2b} and A_{2a} receptors, respectively (Bruns *et al.*, 1986). Further evidence for distinguishing these two subtypes has been gained through the A_{2a}-selective nature of the agonist CGS 21680 (Lupica *et al.*, 1990) and antagonist PD 115,199 (Bruns *et al.*, 1987b). Autoradiographic analysis of the binding of these two compounds labelled with ^{3}H underlines the discrete regional distribution of A_{2a} receptors (Bruns *et al.*, 1987b; Jarvis *et al.*, 1989). As yet, no selective agonist or antagonist, or radioligand binding assay is available for the A_{2b} receptor in the CNS.

High densities of A_1 receptors have been identified in the molecular layer of the cerebellum (Lewis *et al.*, 1981), the receptor class associated with inhibition of glutamate release in other brain regions (Fredholm & Dunwiddie, 1988). The use of mice with neurological deficits (Goodman *et al.*, 1983), as well as selective brain lesions (Wojcik & Neff, 1983), have implicated an association of A_1 receptors with cerebellar granule cells. These A_1 receptors inhibit adenylyl cyclase in *in vitro* assays (Wojcik & Neff, 1983). A_1 receptor radioligand

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binding has also been observed in cerebellum from rat, rabbit, guinea-pig and man (Murphy & Snyder, 1982). In the rodent neostriatum, the adenosine receptor agonists N^6-phenylisopropyladenosine exhibits a biphasic action on particulate adenylyl cyclase activity, with an inhibition at low concentrations (mediated through an A_1 receptor) and a stimulation at higher concentrations (via the A_{2a} receptor). In contrast, high concentrations of N^6-phenylisopropyladenosine failed to activate the particulate adenylyl cyclase in membranes from cerebellum. It was proposed, therefore, that A_2 receptors do not exist in mouse cerebellum (Wojcik & Neff, 1983). In cerebellar slices from the guinea-pig, adenosine had been shown to increase the generation of cyclic AMP, and also to increase levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in a Ca^{2+}-dependent manner (Ohga & Daly, 1977; Saito, 1977). In mouse cerebellum, there are contradictory reports of both increases and decreases in cyclic GMP formation (Ferrendelli *et al.*, 1973; Saito, 1977).

In the present study we have evaluated the role that different adenosine receptors play in the control of cyclic AMP levels in adult guinea-pig cerebellar slices using a number of adenosine analogues and antagonists. Furthermore, since we have recently observed a potentiation of sodium nitroprusside-stimulated cyclic GMP levels by forskolin (Hernández, Alexander & Kendall, unpublished observations), we also tested whether adenosine regulates cyclic GMP levels in adult guinea-pig cerebellar slices.

Methods

Tissue preparation and second messenger accumulation

Preparation and incubation of slices were essentially the same as described by Donaldson *et al.* (1990). Cross-chopped cerebellar slices (350 × 350 μm) were prepared from guinea-pigs (Dunkin-Hartley, either sex, weighing 200–300 g) with a McIlwain tissue chopper. They were then incubated in a shaking water bath for 60 min at 37°C in several changes of Krebs-bicarbonate buffer which contained (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, $CaCl_2$ 1.2m equilibrated with 95% O_2:5% CO_2. The slices were next suspended in fresh Krebs buffer and [^{3}H]-adenine or [^{3}H]-guanine was added to a final concentration of 74 kBq ml^{-1}. After an additional 60 min of incubation, the slices were washed and aliquots (25 μl) transferred into flat-bottomed plastic vials containing Krebs buffer (to a final volume of 300 μl). Slices were allowed to equilibrate for 15 min prior to addition of agents. The tubes were resealed under 95% O_2:5%CO_2 after each addition. Incubations were terminated by the addition of 200 μl of HCl (1 M), followed by 750 μl of ice-cold water. [^{3}H]-cyclic GMP and [^{3}H]-cyclic AMP were subsequently resolved by the double-column method of Salomon *et al.* (1974) using [^{14}C]-cyclic AMP and [^{14}C]-GMP as recovery markers. Typical basal levels of [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP were 2452 ± 108 and 1863 ± 103 d.p.m., respectively.

Slices used for determination of phosphoinositide turnover were pre-equilibrated with Krebs-bicarbonate buffer for 60 min and distributed as 25 μl aliquots into flat-bottomed vials in the presence of [^{3}H]-inositol (c. 40 kBq ml^{-1}) and LiCl (5 mM) to give a final volume of 300 μl as previously described (Alexander *et al.*, 1989). After 40 min, adenosine was added, followed by histamine. Following an incubation period of 45 min, the reaction was halted by the addition of 7.5% perchloric acid. After neutralization, [^{3}H]-inositol phosphates were then resolved by chromatography on Dowex-1 (chloride form) columns.

Binding of cyclopentyl-[^{3}H]-1,3-dipropylxanthine

Binding of the A_1-selective antagonist 8-cyclopentyl-[^{3}H]-1,3-dipropylxanthine ([^{3}H]-DPCPX) was carried out on a 20,000 *g* particulate preparation from guinea-pig cerebellum at 22°C for 90 min. The medium for radioligand binding was composed of 50 mM Tris, pH 7.4 containing 1 mM EDTA, 0.01% Triton X-100 and 1.25 u ml^{-1} adenosine deaminase. Saturation isotherms were constructed with six concentrations of [^{3}H]-DPCPX over the range 0.3–13 nM, defining non-displaceable binding in the presence of 2.5 mM theophylline. Incubation was halted by rapid filtration through Whatman GF/B filters using a cell harvester (Brandel Scientific/SEMAT, Herts, U.K.), washing the filters with 3 × 3 ml ice-cold 50 mM Tris, pH 7.4 containing 1 mM EDTA.

Calculations and statistical analysis

The computer programme GraphPad (GraphPad, California, U.S.A.) was used to generate parameters from radioligand binding data, and agonist and antagonist concentration-response curves. Antagonist dissociation constants (K_I) 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 exceeded the EC_{50} value. 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 a logistic equation as described 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_I was then determined from the relationship:

$$C/C' = (IC_{50}/K_I) + 1$$

In the text, values represent mean ± s.e.mean (except where indicated) of *n* independent experiments conducted in triplicate. Statistical analysis was performed with Student's unpaired *t* test.

Chemicals

[8-^{3}H]-guanine (324 GBq mmol^{-1}) and [8-^{14}C]-guanosine 3',5'-cyclic monophosphate (1.9 GBq mmol^{-1}) were purchased from Rotem Industries Ltd, Beer-Sheva, Israel and Moravek Biochemicals, California, U.S.A., respectively. [8-^{3}H]-adenine (962 GBq mmol^{-1}) was from Amersham International, Bucks, U.K. [Adenine-U-^{14}C]-adenosine 3',5'-cyclic monophosphate (11.4 GBq mmol^{-1}), [^{3}H]-inositol (455.1 GBq mmol^{-1} and [^{3}H]-DPCPX (4007.1 GBq mmol^{-1}) were purchased from DuPont NEN, U.K., respectively. Adenosine, 2-chloroadenosine, sodium nitroprusside and N^G-nitro-L-arginine were from Sigma Chemicals, Poole, Dorset, U.K. Adenosine deaminase was obtained from Boehringer Mannheim. 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), CGS 21680, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (XAC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from RBI SEMAT, Herts, U.K. PD 115,199 (N-[2-(dimethylamino) ethyl] N-methyl-4-(1,3-dipropylxanthine) benzene sulphonamide) was a generous gift from Warner Lambert, Ann Arbor, U.S.A. All other compounds were from standard commercial sources.

Results

Effects of adenosine on cyclic AMP accumulation

Incubation of [^{3}H]-adenine-prelabelled adult guinea-pig cerebellar slices in the presence of adenosine (1 mM) led to an increase in [^{3}H]-cyclic AMP levels. The response to adenosine was maximal by about 10 min (15 ± 1.7 fold over basal levels, $n = 6$) and stable for up to 30 min. This activation was fully reversible: addition of adenosine deaminase (1 u ml^{-1})

led to a rapid return of [^{3}H]-cyclic AMP to basal levels within a few minutes (Figure 1a).

The adenosine-induced increase of [^{3}H]-cyclic AMP levels was further characterized with a number of adenosine receptor agonists. Concentration-response curves for 5'-N-ethylcarboxamidoadenosine (NECA, EC_{50} value 0.62 ± 0.15 μM, $n = 3$), 2-chloroadenosine (2CA, 6 ± 1.3 μM, $n = 3$) and adenosine (12.8 ± 0.9 μM, $n = 3$) are shown in Figure 2a. Adenosine exhibited a reduced maximal response compared to NECA and 2CA (1 mM adenosine gave a response 56% of the response to 10 μM NECA, $P < 0.001$, $n = 6$). The class of adenosine receptor involved was further examined by the use of adenosine receptor antagonists. Increasing concentrations of antagonists were pre-incubated with slices for 10 min prior to a 10 min incubation period in the presence of a constant NECA concentration (10 μM). The inhibition curves for XAC (IC_{50} value 0.24 ± 0.1 μM, $n = 4$), DPCPX (1.27 ± 0.05 μM, $n = 3$) and PD 115,199 (1.83 ± 0.21 μM, $n = 3$) are shown in Figure 3a. The apparent inhibition constants were calculated (see Methods) and were 15, 81 and 117 nM for XAC, DPCPX and PD 115,199, respectively.

The potential role of the A_1 receptor subtype was further analysed with forskolin as stimulus of cyclic AMP generation and CPA as a selective agonist of this class of receptor. As can be seen in Figure 4a, forskolin (1 μM) increased [^{3}H]-cyclic AMP in [^{3}H]-adenine-prelabelled adult guinea-pig cerebellar slices. However, a 10 min preincubation with CPA failed to inhibit the forskolin response in the range of concentrations effective in cerebral cortex (10 nM–1 μM, Alexander *et al.*, 1992). A similar pattern was observed with 10 μM forskolin (data not shown). CPA increased [^{3}H]-cyclic AMP at concentrations of 300 nM or greater, suggesting a

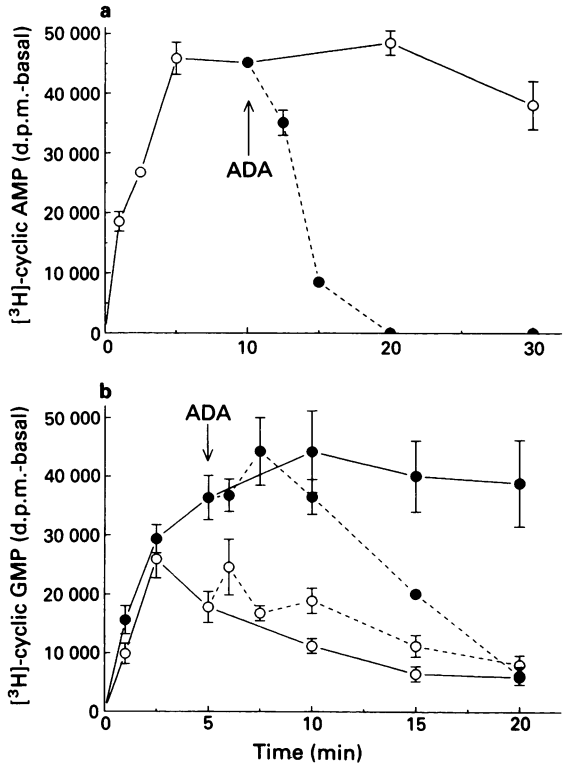

Figure 1 Time course profiles for adenosine-stimulated (a) [^{3}H]-cyclic AMP or (b) [^{3}H]-cyclic GMP accumulations. In (b), slices were incubated in the absence (○) or presence (●) of 1 mM adenosine for 10 min prior to addition of sodium nitroprusside. At the timepoint indicated by the arrow, 1 u ml^{-1} adenosine deaminase was added to some slices (dashed lines), which were then incubated further. Data are from a single experiment repeated on two additional occasions with essentially similar results. Bars show s.e.mean of triplicate determinations.

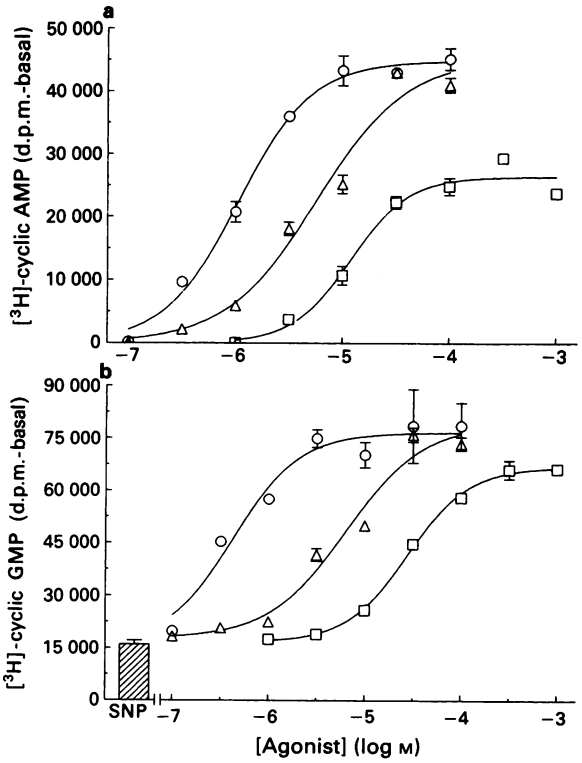

Figure 2 Concentration-response curves for the stimulation of (a) [^{3}H]-cyclic AMP or (b) sodium nitroprusside (SNP)-induced [^{3}H]-cyclic GMP accumulation in the presence of NECA (○), 2CA (△) or adenosine (□). The responses to NECA and 2CA were carried out, in both (a) and (b), after 10 min preincubation with 1 u ml^{-1} adenosine deaminase. Data are from single experiments representative of 3/4 separate experiments. Bars show s.e.mean of triplicate determinations. For abbreviations, see text.

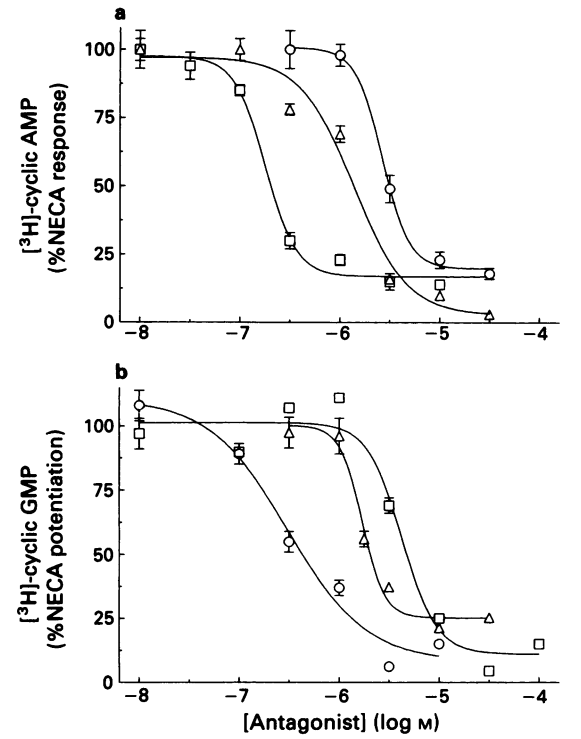

Figure 3 Inhibition of (a) NECA-stimulated [^{3}H]-cyclic AMP or (b) NECA-augmented [^{3}H]-cyclic GMP. Antagonists (a) XAC (□), DPCPX (△) or PD 115,199 (○) and (b) XAC (○), DPCPX (△) or PD 115,199 (□) were added 10 min prior to addition of 10 μM NECA. Data are from single experiments representative of 3/5 separate experiments. Bars show s.e.mean of triplicate determinations. For abbreviations, see text.

stimulation of A_{2b} receptors at such concentrations. Interestingly, at such concentrations CPA also enhanced the response to forskolin, suggesting that forskolin may potentiate receptor-mediated increases in cerebellar cyclic AMP. This hypothesis was further analysed by studying the response to forskolin in the presence and absence of adenosine deaminase (1 u ml^{-1}). As can be seen in Figure 5, adenosine deaminase shifted the concentration-response curve to forskolin to the right, suggesting a role for endogenous adenosine in the stimulation of cyclic AMP generation by low concentrations of forskolin in cerebellum.

Effects of adenosine on cyclic GMP accumulation

Pretreatment of cerebellar slices with adenosine (1 mM, 10 min) substantially changed the response to 1 mM sodium nitroprusside (SNP, Figure 1b). Adenosine pretreatment abolished the decline in [^{3}H]-cyclic GMP observed with SNP alone (10 min, 2.7 ± 0.2 fold over SNP alone, $n = 5$). The same pattern was obtained with 10 μM NECA (10 min, 3.36 ± 0.8 fold over SNP alone, $n = 10$). This effect of adenosine was fully reversible: addition of adenosine deaminase (1 u ml^{-1}) returned [^{3}H]-cyclic GMP content to basal levels within a few minutes (Figure 1b); 10 min treatment with 1 mM adenosine alone increased the basal levels of [^{3}H]-cyclic GMP (3.2 ± 0.3 fold, mean ± range, $P < 0.05$, $n = 2$). The same effect was observed with 10 μM NECA (10 min, 2.07 ± 0.25 fold over basal levels, $P < 0.001$, $n = 6$). In the presence of N^G-nitro-L-arginine (100 μM, 10 min preincubation), the response to 10 μM NECA alone was 63 ± 11% ($P < 0.01$, $n = 3$) of the response obtained in the absence of inhibitor. The potentiation of SNP-induced [^{3}H]-cyclic GMP levels produced by adenosine was analysed further with a number of adenosine receptor agonists. Concentration-response curves for NECA (EC_{50} value 0.67 ± 0.21 μM, $n = 3$), 2CA (5.85 ± 1.1 μM, $n = 4$) and adenosine (42 ± 16 μM, $n = 3$) are shown in Figure 2b. In this case, adenosine showed a similar maximal response compared to NECA (1 mM adenosine gave 81% of the response observed with 10 μM NECA, $P < 0.2$). The class of adenosine receptor involved was further examined by the use of adenosine receptor antagonists. Increasing concentrations of antagonists were pre-incubated with slices for 10 min prior to a 10 min incubation period in the presence of a constant NECA concentration (10 μM). The inhibition curves for XAC (IC_{50} value 0.25 ± 0.02 μM, $n = 4$), DPCPX (1.44 ± 0.1 μM, $n = 5$) and PD 115,199 (3.42 ± 0.36 μM, $n = 3$) are shown in Figure 3b, permitting calculation of the apparent inhibition constants as before (17, 102 and 242 nM for XAC, DPCPX and PD 115,199 respectively).

The role of A_{2a} and A_1 receptors in enhancing cyclic GMP levels was analysed with the specific agonists CGS 21680 and CPA, 10 min preincubation with 10 μM CGS 21680 potentiated only slightly the levels of [^{3}H]-cyclic GMP induced by 1 mM SNP (10 min, 1.2 ± 0.06 fold, $P < 0.1$, $n = 3$). Using concentrations in the range of A_1 receptor selectivity (up to 1 μM), CPA did not change either the response to SNP or basal levels of [^{3}H]-cyclic GMP (Figure 4b). However, in concentrations greater than 1 μM, the response to 1 mM SNP was potentiated, presumably through activation of A_{2b} receptors at these concentrations.

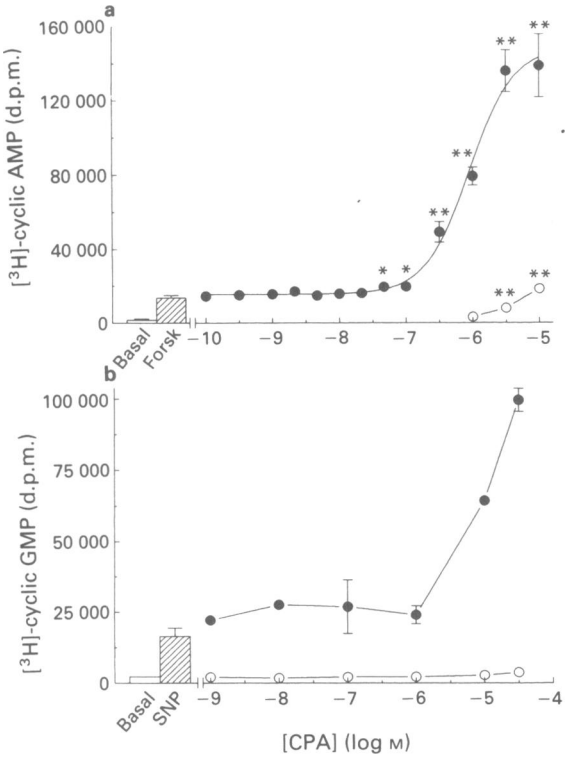

Figure 4 Concentration-response curves for CPA effects on (a) [^{3}H]-cyclic AMP or (b) [^{3}H]-cyclic GMP in the absence (○) or presence (●) of (a) forskolin (Forsk) or (b) sodium nitroprusside (SNP). Data are from single experiments representative of 3/4 separate experiments. Bars show s.e.mean of triplicate determinations. $*P < 0.05$, $**P < 0.01$ absence vs. presence of CPA. For abbreviations, see text.

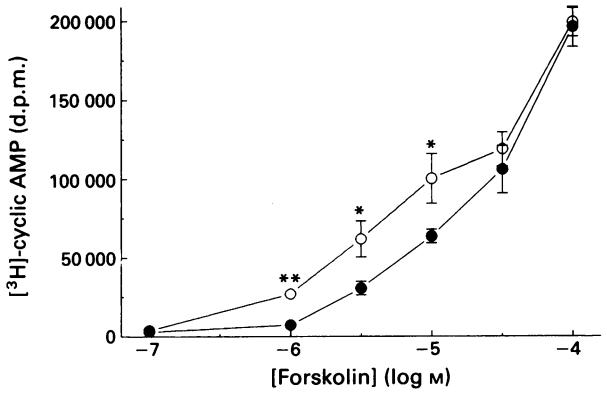

Figure 5 Concentration-response curves for forskolin-stimulated [^{3}H]-cyclic AMP accumulation in the absence (○) or presence (●) of of 1 u ml^{-1} adenosine deaminase. Data are from a single experiment repeated on a further occasion with essentially identical results. Bars show s.e.mean of triplicate determinations. $*P < 0.05$, $**P < 0.01$ absence vs. presence of adenosine deaminase.

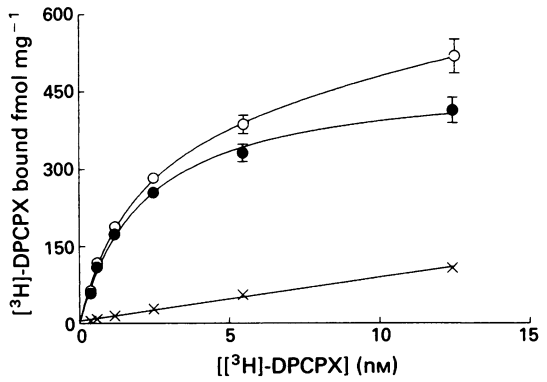

Figure 6 [^{3}H]-DPCPX binding to guinea-pig cerebellar membranes at the indicated concentrations in the absence (○) or presence of 5 mM theophylline (×). Displaceable [^{3}H]-DPCPX binding is indicated by (●). Data are means ± s.e.mean of triplicate determinations from three separate experiments.

Adenosine effects on phosphoinositide turnover in the guinea-pig cerebellum

Since adenosine has been observed to potentiate specifically H_1 histamine receptor-stimulated phosphoinositide turnover in guinea-pig cerebral cortical slices via an A_1-type adenosine receptor (Hill & Kendall, 1987), we investigated whether this phenomenon was apparent in the cerebellum. Basal accumulations of [^{3}H]-inositol phosphates (3004 ± 494 d.p.m., $n = 3$) were elevated in the presence of 100 μM histamine (470 ± 85% basal, $n = 3$). However, the presence of 100 μM adenosine failed to alter significantly the response to histamine (91 ± 15% histamine response, $n = 3$).

Binding of [^{3}H]-DPCPX to guinea-pig cerebellar membranes

Since we were unable to provide evidence from studies of second messenger responses for the presence of A_1 adenosine receptors in guinea-pig cerebellum, we investigated whether radioligand binding could demonstrate A_1 receptors in this tissue. Using a total particulate preparation from guinea-pig cerebellum, we observed saturable binding of the A_1-selective antagonist radioligand [^{3}H]-DPCPX, with a calculated K_D value of 2.1 ± 0.1 nM and a maximal binding capacity of 474 ± 32 fmol mg^{-1} protein ($n = 3$) (Figure 6).

Discussion

Adenosine receptor stimulation of cyclic AMP levels in guinea-pig cerebellum

Stimulation of cyclic AMP generation in guinea-pig cerebellum was enhanced by adenosine and its analogues with the rank order of potency; NECA > 2CA > adenosine. This rank order of potency is identical to that at the A_{2b} adenosine receptors of guinea-pig (Losinski *et al.*, 1993) and rat cerebral cortex (Bazil & Minneman, 1986), although the absolute potencies of the adenosine analogues appeared highest in guinea-pig cerebellum. The antagonists investigated showed an identical rank order of potency (XAC > DPCPX > PD 115,199) when compared with guinea-pig cerebral cortex (Losinski *et al.*, 1993), with, again, a slightly-enhanced affinity. DPCPX has been characterized as an A_1-selective antagonist (Lee & Reddington, 1986; Bruns *et al.*, 1987a; Lohse *et al.*, 1987), with low affinity at A_{2a} receptors. In contrast, PD 115,199 exhibits similar affinity at A_1 and A_{2a} receptors with low affinity at A_{2b} receptors (Bruns *et al.*, 1987b). XAC has been defined as an antagonist which exhibits high affinity at A_1 and A_2 receptors (Jacobson *et al.*, 1985; 1987). The rank order of potencies of both agonists and antagonists, therefore, identifies the adenosine receptor as an A_{2b} adenosine receptor.

Adenosine receptor stimulation of cyclic GMP levels in guinea-pig cerebellum

In the presence of 1 mM SNP, a transient elevation of [^{3}H]-cyclic GMP levels was observed which was enhanced and prolonged in the presence of adenosine or its analogues (Figure 1b). The rank order of potency of adenosine analogues for eliciting a potentiation of the SNP-induced [^{3}H]-cyclic GMP response was identical when compared to that observed for stimulation of cyclic AMP generation. Similarly, the antagonists elicited inhibition of NECA-enhanced [^{3}H]-cyclic GMP accumulation with the same rank order of potency as observed for NECA-stimulated [^{3}H]-cyclic AMP accumulation. These data strongly implicate A_{2b} adenosine receptors in both phenomena. This raises the possibility of a causative linkage between the two events, a suggestion which is further strengthened by our recent findings that forskolin and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, also exhibit a similar profile of enhancement of SNP-induced [^{3}H]-cyclic GMP accumulation in this tissue (Hernández, Alexander & Kendall, unpublished observations). Our present hypothesis to explain the enhancement of [^{3}H]-cyclic GMP levels is that competition at the phosphodiesterase level by either cyclic AMP (generated by forskolin or A_{2b} receptor stimulation) or 3-isobutyl-1-methylxanthine leads to an enhanced accumulation of [^{3}H]-cyclic GMP. We can provide a potential explanation for the increased maximal response to adenosine relative to NECA for augmenting cyclic GMP accumulation compared to the generation of cyclic AMP by suggesting that the phosphodiesterase responsible for degradation of [^{3}H]-cyclic GMP may become saturated by the cyclic AMP generated at ca. 70% of the maximal NECA response. Thus, since adenosine generates 56% of the cyclic AMP response to a maximally-active concentration of NECA, this will approach 80% of the cyclic GMP response.

In the absence of SNP, a small enhancement of [^{3}H]-cyclic GMP accumulation could be observed in the presence of adenosine. The reduction in basal [^{3}H]-cyclic GMP levels in the presence of oxyhaemoglobin and N^G-nitro-L-arginine suggests some endogenous production of nitric oxide in these slices (Hérnandez, Alexander & Kendall, unpublished observations), indicating the potential for adenosine receptor enhancement of endogenous nitric oxide-evoked cyclic GMP accumulation.

Other adenosine receptors in guinea-pig cerebellum

In guinea-pig cerebral cortical slices, it is possible to observe A_1 adenosine receptor-mediated inhibitions of cyclic AMP formation when forskolin is used as a stimulus (Alexander *et al.*, 1992). However, in the cerebellum, we were unable to provide evidence for inhibition of forskolin-stimulated cyclic AMP generation by concentrations of CPA active in the cortex. At relatively high concentrations of CPA, a direct stimulation of [^{3}H]-cyclic AMP generation was observed, together with an enhancement of the forskolin response, presumably through activation of A_{2b} receptors (DeLapp & Eckols, 1992). We were also unable to observe adenosine receptor potentiation of histamine-stimulated phosphoinositide turnover in the guinea-pig cerebellum, although this phenomenon is present in cerebral cortex (Hill & Kendall, 1987), hippocampus and neostriatum (R.M. Straw & D.A. Kendall, unpublished observation). Our investigations of [^{3}H]-DPCPX radioligand binding in particulate preparations from the guinea-pig cerebellum indicates relatively dense binding. This compares with our previously-reported investigation of [^{3}H]-DPCPX binding to guinea-pig cerebral cortical A_1 adenosine receptors (Alexander *et al.*, 1992) in which we observed a K_D value of 4.2 ± 0.4 nM with a maximal binding capacity of 1560 ± 278 fmol mg^{-1} protein. The signal transduction mechanism for the relatively abundant A_1 receptors in guinea-pig cerebellum therefore remains to be elucidated. The minor effect of CGS 21680 on [^{3}H]-cyclic GMP accumulation suggests an absence of A_{2a} adenosine receptors from guinea-pig cerebellum, a finding in agreement with radioligand binding studies in the rat (Bruns *et al.*, 1987b; Jarvis *et al.*, 1989) and mouse (Wojcik & Neff, 1983).

Conclusion

We are able to furnish evidence for signal transduction pathways for the A_{2b} adenosine receptor, but not A_1 or A_{2a} receptor classes, in adult guinea-pig cerebellum. The enhancement of cyclic GMP accumulation by A_{2b} adenosine receptor activation in guinea-pig cerebellum appears to be mediated through the stimulation of cyclic AMP generation. This phenomenon of cross-talk between second messengers could

well have important connotations for other systems where a stimulation of cyclic AMP is observed, the associated phenomena may be better explained as mediated through cyclic GMP.

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Effects of pyrimidines on the guinea-pig coronary vasculature

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1 The effects of the pyrimidines, uridine 5'-triphosphate (UTP), thymidine 5'-triphosphate (TTP) and cytidine 5'-triphosphate (CTP), were examined in the guinea-pig coronary bed, by use of a Langendorff technique. Comparisons were made with the actions of the purines adenosine 5'-triphosphate (ATP), inosine 5'-triphosphate (ITP) and guanosine 5'-triphosphate (GTP). The effect of, the nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME) and, the prostaglandin synthesis inhibitor, indomethacin on the vasodilator response to these purines and pyrimidines was examined. The effects of these inhibitors were assessed on their ability to inhibit both the amplitude and the area of the vasodilator response.

2 The relative order of potency of the purines and pyrimidines studied was ATP > UTP > ITP >> GTP, TTP, CTP.

3 The maximum amplitude and area of the vasodilator response to the pyrimidines, UTP (5×10^{-10}–5×10^{-7} mol), TTP (5×10^{-8}–5×10^{-7} mol) and CTP (5×10^{-7} mol), and purines, ITP (5×10^{-9}–5×10^{-7} mol) and GTP (5×10^{-8}–5×10^{-7} mol), were significantly reduced by L-NAME (3×10^{-5} and 10^{-4} M).

4 The inhibition of the response to ATP (5×10^{-8} mol), UTP (5×10^{-8} mol), ITP (5×10^{-8} mol), TTP (5×10^{-7} mol), CTP (5×10^{-7} mol) and GTP (5×10^{-7} mol) by L-NAME (3×10^{-5} M) was significantly reversed by L-arginine (1.5×10^{-3} M).

5 L-NAME (3×10^{-5} and 10^{-4} M) only inhibited the amplitude of the vasodilator response to a low dose of ATP (5×10^{-10} mol), although the area of vasodilator response to ATP (5×10^{-11}–5×10^{-7} mol) was significantly reduced by L-NAME (3×10^{-5} and 10^{-4} M).

6 The maximum amplitude of the vasodilator response to ATP (5×10^{-10}–5×10^{-7} mol) was significantly reduced by indomethacin (10^{-6} M), although the area of the vasodilator response to ATP was only significantly reduced at one intermediate dose (5×10^{-9} mol). Indomethacin (10^{-6} M) did not affect the maximum amplitude or area of the vasodilator responses to UTP (5×10^{-11}–5×10^{-7} mol), ITP (5×10^{-10}–5×10^{-7} mol), CTP (5×10^{-7} mol), TTP (5×10^{-8}–5×10^{-7} mol) and GTP (5×10^{-8}–5×10^{-7} mol).

7 It is concluded that in the guinea-pig coronary vasculature, the vasodilatation evoked by the pyrimidines, UTP, TTP and CTP, was mediated in large part via nitric oxide, as were the vasodilatations evoked by the purines ITP and GTP. The vasodilatations evoked by ATP, however, appear to involve prostanoids in addition to the release of nitric oxide.

Keywords: Nitric oxide synthase; L-N^G-nitroarginine methyl ester; prostanoids; indomethacin; coronary vasculature; relaxation of smooth muscle; purines; pyrimidines

Introduction

Adenosine 5'-triphosphate (ATP) produces powerful systemic effects; it influences many biological processes being released from nerve endings, platelets and endothelial cells in physiological and pathophysiological processes (Burnstock & Kennedy, 1986). In the cardiovascular system its ability to cause vasoconstriction or vasodilatation is mediated through activation of subtypes of P_2-purinoceptor (Burnstock & Kennedy, 1985; Hoyle, 1992). In the rat coronary vasculature, ATP causes vasoconstriction via P_{2X}-purinoceptors and vasodilatation via P_{2Y}-purinoceptors (Hopwood & Burnstock, 1987). The naturally occurring nucleotides, cytidine 5'-triphosphate (CTP), thymidine 5'-triphosphate (TTP) and uridine 5'-triphosphate (UTP) which are pyrimidines, and guanosine 5'-triphosphate (GTP), which is a purine, have also been shown to have effects on the vasculature. Of these, probably the most studied has been UTP which may be released from blood platelets (Goetz *et al.*, 1971; Urquilla, 1978). In many tissues, including the piglet aorta (Martin *et al.*, 1985), 2-methylthioATP (2-meSATP) is a potent agonist producing a similar maximum relaxant response to ATP. The relative order of agonist potencies of these two compounds is consistent with that conventionally described for P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985): 2-meSATP >> ATP = ADP >> α,β-methyleneATP (α,β-meATP), UTP. However there is also a variety of tissues in which ATP causes phospholipase C activation but to which the above agonist potency order does not apply; because of the common second messenger system in these tissues they have been loosely linked with the P_{2Y}-subtype (Kennedy, 1990; Boeynaems & Pearson, 1990). In these tissues 2-meSATP has little or no activity, for example, although ATP induces prostaglandin I_2 (PGI_2) production in bovine aortic smooth muscle cells, 2-meSATP does not (Demolle *et al.*, 1988). This indicates that there is a subpopulation of phospholipase C-linked P_2-purinoceptors that are insensitive to 2-meSATP. As such, these sites cannot correctly be classified as P_{2Y}-purinoceptors. This pattern is strengthened by the observation that UTP has similar agonist potency to ATP in many of the tissues that are unresponsive to 2-meSATP. Davidson and colleagues (1990) introduced the term 'nucleotide' receptor for the ATP/UTP-sensitive site on sheep pituitary cells. This convention was adopted and it was proposed that a nucleotide receptor may be characterized by the following agonist potency order: UTP = ATP > ADP > α,β-meATP, 2-meSATP (O'Connor *et al.*, 1991). Tissues like rat aorta (Dainty *et al.*, 1990) may contain a heterogeneous population of receptor types (possibly both P_{2Y} and 'nucleotide' recep-

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tors), activation of which results in the same functional response, in this case endothelium-dependent relaxation. To support this it has recently been demonstrated that two separate co-existing receptor populations (P_{2Y}-purinoceptors and nucleotide receptors) are located on bovine aortic endothelial cells (Motte *et al.*, 1993; Wilkinson *et al.*, 1993).

Endothelial cells play a key role in the control of vascular tone by virtue of their ability to synthesize and release endothelium-derived relaxing factors. Adenosine 5'-triphosphate has been shown to elicit vasodilatation in the coronary bed via an action at receptors located on endothelial cells, leading to release of these factors (Hopwood & Burnstock, 1987; Hopwood *et al.*, 1989). These factors include prostacyclin (Moncada & Vane, 1979) and endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980). Prostacyclin is a potent vasodilator (Moncada *et al.*, 1976) which can be released from endothelial cells by a variety of stimuli including UTP and ATP (Pearson *et al.*, 1983; Demolle *et al.*, 1988). In the guinea-pig coronary vasculature the release of EDRF, believed to be nitric oxide (NO, Ignarro *et al.*, 1986; Furchgott *et al.*, 1987) mediates relaxation evoked by ATP (Vials & Burnstock, 1992) and in the rat mesenteric arterial bed UTP also induces relaxation via NO (Ralevic & Burnstock, 1991).

This study investigates the relaxant effects of pyrimidine nucleoside triphosphates UTP, CTP and TTP and to compare them with the relaxant effects of the purine nucleoside triphosphates ATP, inosine 5'-triphosphate (ITP) and GTP, on the guinea-pig coronary vasculature. Inhibitors of the enzymatic synthesis of NO and prostaglandins are used, namely L-N^G-nitroarginine methyl ester (L-NAME) which is a competitive inhibitor of the synthesis of NO from L-arginine (Rees *et al.*, 1990) and is effective in inhibiting vasodilator responses to various agents including ATP in the guinea-pig coronary vasculature (Vials & Burnstock, 1992), and indomethacin which is a prostaglandin synthesis inhibitor (Vane, 1971).

Methods

Guinea-pigs (250–400 g) of either sex were injected with heparin (2 500 units i.p.) 15 min before being killed by cervical dislocation. The heart was removed and placed in cold Krebs solution (4°C) to arrest the beating. Extraneous fat and large vessels were removed, the heart was cannulated via the aorta, and the coronary circulation perfused by the method of Langendorff with a modified Krebs-Henseleit solution containing (mM): NaCl 115.3, KCl 4.6, $MgSO_4$. $7H_2O$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5 and glucose 11.1. Albumin (0.5 g l^{-1}) was also added to the solution to increase the oncotic pressure and reduce oedema. A water-filled silicone rubber balloon, connected to a pressure transducer (Viggo-Spectramed Bilthoven, model P23XL), was placed in the left ventricle for the measurement of left ventricular pressure. The left ventricular diastolic pressure did not exceed 10 mmHg. The average starting left ventricular systolic pressure was 41.92 ± 1.21 mmHg ($n = 57$). Perfusion pressure was monitored with a pressure transducer connected via a side arm to the cannula. A pair of platinum electrodes were placed in the right ventricle and the heart was paced at 4 Hz with electrical pulses of 5 ms duration at supramaximal voltage (usually around 20 V). The flow rate was gradually increased to obtain a starting perfusion pressure of 50–60 mmHg, using a Masterflex constant flow roller pump (Cole-Palmer Instruments Co., Chicago). The flow rate was determined by collecting the effluent, over a period of time, and the average rate was 13.26 ± 0.35 ml min^{-1}($n = 57$). The heart was left to equilibrate for at least 20 min before commencing the experiment.

When the perfusion had reached a steady state, the purines and pyrimidines were given as a bolus of 50 µl, injected over 3 s into the superfusing solution close to the heart. The duration of each individual experiment was no longer than 3 h. Due to this time restriction the effects of all the agonists could not be tested on the same heart. For this reason the agonists were chosen randomly and not more than four agonists were used on a particular heart. The order of exposure of the agonists to the heart was also random to minimize effects due to time-dependent changes and preparation variability. At least 5 min was left between the administration of each dose of agonist. When the effect of antagonists was examined, control dose-response relationships for the purines and pyrimidines were first obtained and L-NAME or indomethacin added to the perfusing solution and allowed to equilibrate for 20 min. The dose-responses were then repeated in the presence of the antagonist. After inhibition with L-NAME, L-arginine was also added to the perfusing solution to determine whether the inhibition could be reversed. The preparations were allowed to equilibrate for a further 20 min before a submaximal dose of agonist was repeated. For a given response, both its maximum amplitude and area were measured. The area of the vasodilator response was calculated using a measurement and analysis programme on an Apple II Computer. Student's t tests were used to assess statistical significance between responses obtained before and after antagonist, $P < 0.05$ being taken as significant. The rank order of potency of the purines and pyrimidines was determined empirically as maximum responses to these agents were not obtained and therefore pD_2 values could not be calculated. At the end of the experiment the heart was removed from the cannula, blotted and weighed. The mean wet weights were 1.46 ± 0.04 g ($n = 57$).

ATP, ITP, UTP, GTP, TTP, CTP, L-NAME, L-arginine and indomethacin were all obtained from Sigma Chemical Co. Ltd. Indomethacin was made up as a stock solution (10^{-2} M) in sodium carbonate solution (10^{-2} M). The remaining compounds were made up as stock solutions (10^{-1} M) in distilled water. A 50 µl bolus injection of distilled water caused no change in perfusion pressure other than a small injection artefact.

To test for the presence of ATP as a contaminant of GTP, TTP, CTP and ITP, solutions (10^{-2} M) of these agents were assayed for ATP with the luciferin-luciferase technique described by Stanley & Williams (1969). A negligible amount of ATP was found in any of these solutions.

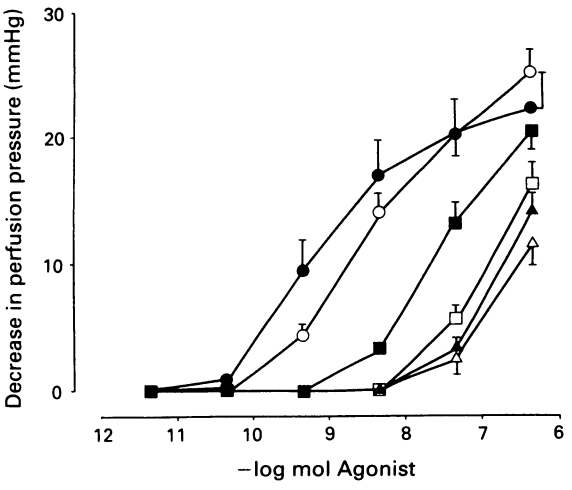

Figure 1 The amplitude of the vasodilatation evoked by adenosine 5'-triphosphate (●), uridine 5'-triphosphate (○), inosine 5'-triphosphate (■), thymidine 5'-triphosphate (□), guanosine 5'-trisphosphate (▲) and cytidine 5'-triphosphate (△) in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 8$.

Results

Dose-response to pyrimidines and purines

Bolus injections of ATP, UTP, ITP, GTP, CTP, and TTP produced dose-dependent vasodilatation in the guinea-pig coronary vasculature. Dose-response curves for the six agonists are illustrated in Figure 1. Due to the fact that maximum responses to these agents were not obtained, an arbitrary decrease in perfusion pressure of 10 mmHg was used to determine the relative order of potency. The potency order of these agonists was ATP > UTP >> ITP >> TTP, GTP, CTP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. There was a small but insignificant fall in the left ventricular systolic pressure on bolus administration of agonists at the high doses.

Effect of L-NAME and L-arginine

The effect of L-NAME on the maximum amplitude of the vasodilator response (Figure 2a–c), on perfusion pressure trace response (Figure 5) and on the area (Figure 3a–c) of the vasodilator response to ATP, UTP and ITP is demonstrated. The maximum amplitude and area of the vasodilator responses due to UTP (5×10^{-10}–5×10^{-7} mol) and ITP (5×10^{-9}–5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; Figures 2a,b, 3a,b and Figure 5a). L-NAME (3×10^{-5} and 10^{-4} M) only inhibited the amplitude of the vasodilator response to a low dose of ATP (5×10^{-10} mol; Figure 2c). In contrast, the area of the vasodilator response to ATP (5×10^{-11}–5×10^{-7} mol; Figures 3c and 5b) was significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M), reflecting an attenuation of the duration of the response. The maximum amplitude and area of

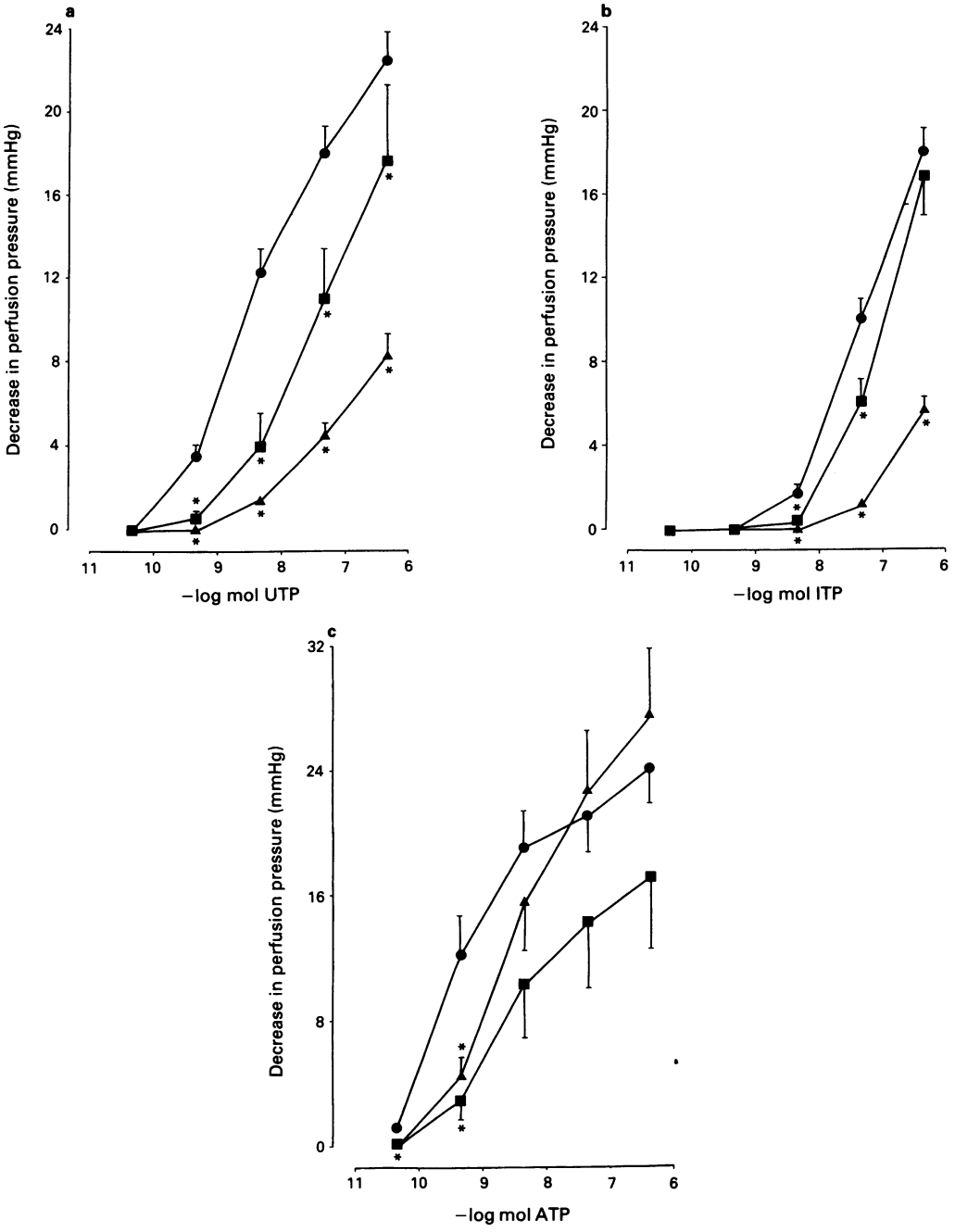

Figure 2 The amplitude of the vasodilatation obtained in response to (a) uridine 5'-triphosphate, (b) inosine 5'-triphosphate and (c) adenosine 5'-triphosphate, in the absence (●, mean of all controls) or presence of L-N^G-nitroarginine methyl ester (3×10^{-5} M (■) and 10^{-4} M (▲)), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences from control are $^*P < 0.05$.

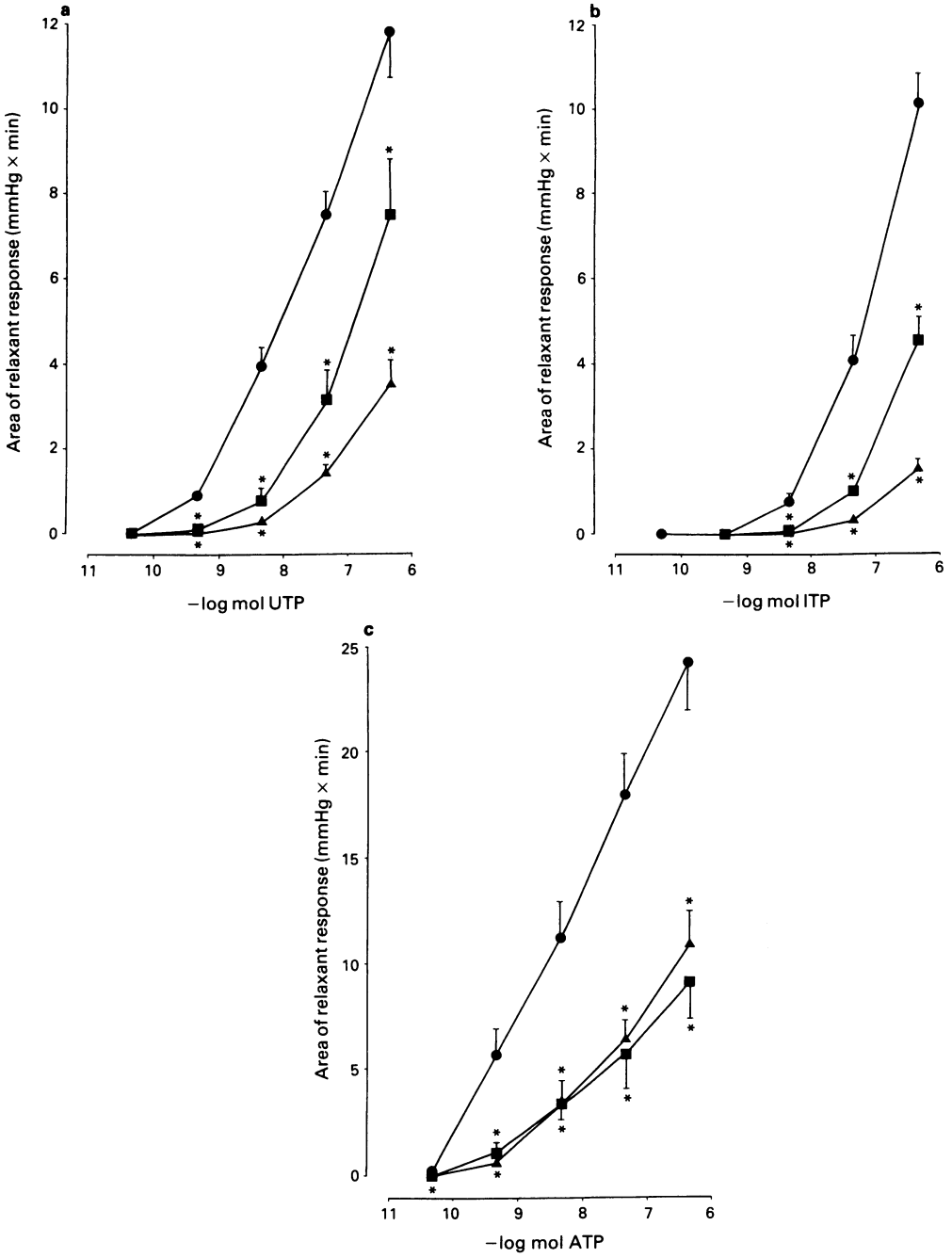

Figure 3 The area of the vasodilatation obtained in response to (a) uridine 5′-triphosphate, (b) inosine 5′-triphosphate and (c) adenosine 5′-triphosphate, in the absence (●, mean of all controls) or presence of L-N^G-nitroarginine methyl ester (3×10^{-5} M (■) and 10^{-4} M (▲)), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences from control are $^*P < 0.05$.

the vasodilator responses to GTP (5×10^{-8}–5×10^{-7} mol), CTP (5×10^{-7} mol) and TTP (5×10^{-8}–5×10^{-7} mol) were significantly inhibited by L-NAME (3×10^{-5} and 10^{-4} M; data not shown). The inhibition of the response to ATP (5×10^{-8} mol), UTP (5×10^{-8} mol), ITP (5×10^{-8} mol), TTP (5×10^{-7} mol), CTP (5×10^{-7} mol) and GTP (5×10^{-7} mol) by L-NAME (3×10^{-5} M) was significantly reversed by L-arginine (1.5×10^{-3} M; Table 1 and Figure 5a,b). L-NAME (3×10^{-5} and 10^{-4} M) and L-arginine (1.5×10^{-3} M) did not significantly affect the resting perfusion pressure or left ventricular pressure of the preparations.

Effect of indomethacin

Indomethacin (10^{-6} M) did not affect the maximum amplitude or area of the vasodilator responses to UTP (5×10^{-11}–5×10^{-7} mol; Figures 4a and 5a), ITP (5×10^{-10}–5×10^{-7} mol; Figure 4b), CTP (5×10^{-7} mol; data not shown), TTP (5×10^{-8}–5×10^{-7} mol; data not shown) and GTP (5×10^{-8}–5×10^{-7} mol; data not shown). In contrast, the maximum amplitude of the vasodilator response to ATP (5×10^{-10}–5×10^{-7} mol; Figures 4c and 5b) was significantly reduced by indomethacin (10^{-6} M). The area of the response to ATP was only significantly reduced at one intermediate dose (5×10^{-9} mol; data not shown). The resting perfusion pressure and left ventricular pressure of the preparations were unaffected by the addition of indomethacin (10^{-6} M).

Discussion

The results of this study revealed that the rank order of potency of the pyrimidines and purines in the guinea-pig

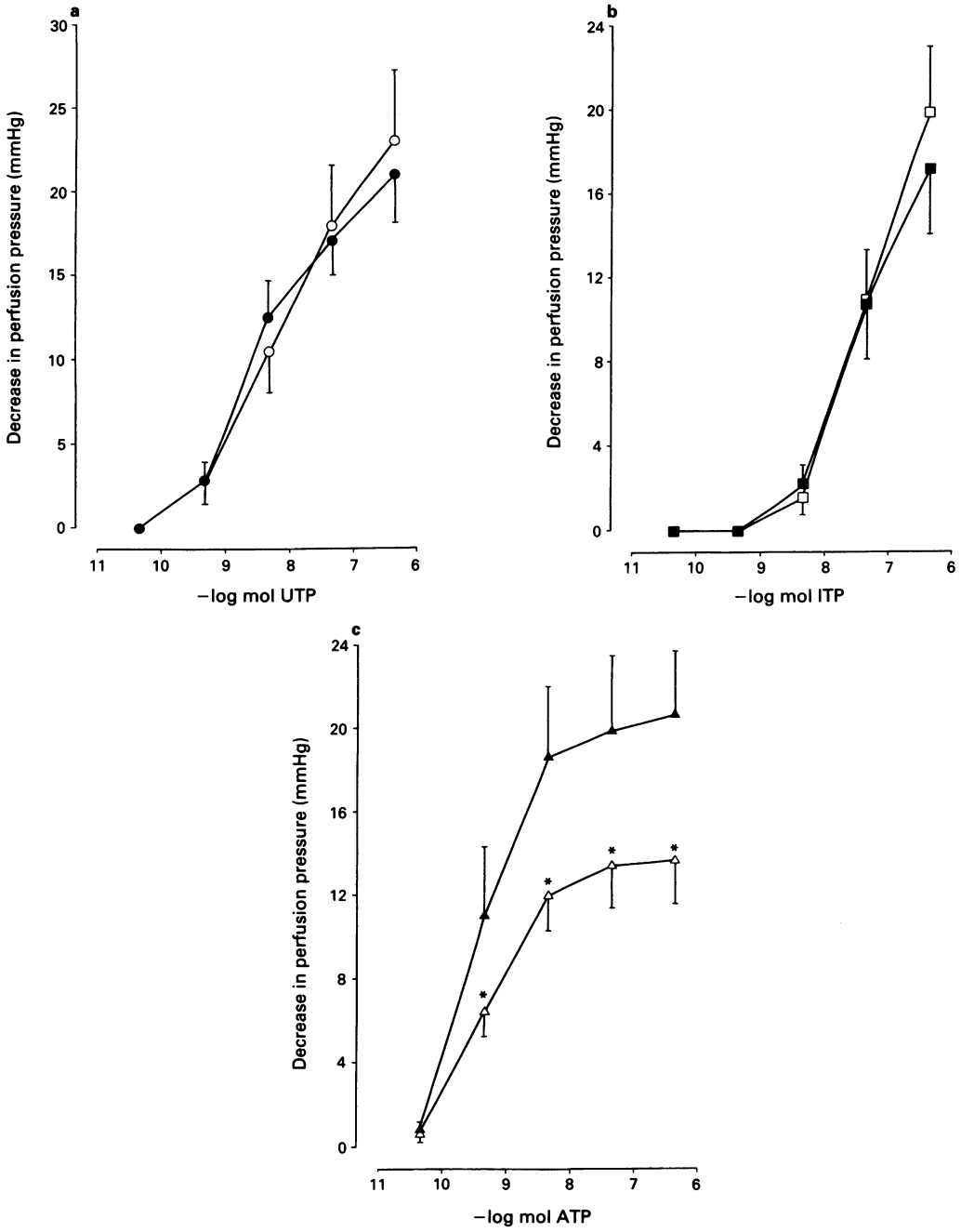

Figure 4 The amplitude of the vasodilator responses evoked by (a) uridine 5'-triphosphate, (b) inosine 5'-triphosphate and (c) adenosine 5'-triphosphate, in the absence (closed symbol) or presence (open symbol) of indomethacin (10^{-6} M), in the guinea-pig isolated perfused heart. The graph shows the mean ± s.e.mean, $n \geq 6$. The significant differences are $^{*}P < 0.05$.

coronary vasculature was ATP > UTP > ITP >> GTP, TTP, CTP. The pyrimidines UTP, CTP and TTP induce relaxation in a similar way to the purine compounds ITP and GTP in that the vasodilator responses to these pyrimidines and purines were dependent largely upon the synthesis of NO. In contrast, vasodilator responses evoked by ATP were only partially dependent upon the synthesis of NO. Prostanoids also play a role in the relaxation induced by ATP. TTP, GTP and CTP did not induce relaxation until they were used at relatively high doses. Contamination of these compounds could explain the responses obtained to these agents. Although minimal amounts of ATP were detected in solutions (10^{-2} M) of these compounds there is always the possibility that UTP is the contaminant.

It has been shown, in the rat mesenteric arterial bed, that relaxations induced by ATP, TTP, UTP and GTP are dependent upon an intact endothelium (Ralevic & Burnstock, 1991). In the pig aorta (Martin *et al.*, 1985) and human pial vessels (Hardebo *et al.*, 1987) relaxation to UTP is also endothelium-dependent. ATP can stimulate prostanoid production from perfused vascular beds and from endothelial cells in culture (Pearson & Gordon, 1985) and in the guinea-pig coronary vasculature it has been shown to induce release of NO (Kelm & Schrader, 1990). UTP has also been shown to stimulate prostacyclin production in endothelial cells (Forsberg *et al.*, 1987) and in the perfused rat liver (Haussinger *et al.*, 1988). In the rat mesenteric arterial bed, vasodilatation to UTP is in large part due to release of NO (Ralevic & Burnstock, 1991). We used this information to investigate and possibly to distinguish between the vascular mechanisms of pyrimidines and purines by using L-NAME, an inhibitor of the conversion of L-arginine to NO (Rees *et al.*, 1990), and indomethacin, a prostaglandin-synthesis inhibitor (Vane, 1971). A more direct approach to distinguish

Table 1 The area of the relaxation obtained in response to adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), inosine 5'-triphosphate (ITP), cytidine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP) and thymidine 5'-triphosphate (TTP) in the guinea-pig isolated perfused heart. The effect of L-N^G-nitroarginine methyl ester (L-NAME (3×10^{-5} M) in the perfusate) on the response to the agonists and the effect of L-arginine (L-Arg (1.5×10^{-3} M) in the perfusate along with L-NAME) on the inhibition by L-NAME is shown

		Area of relaxant response (mmHg × *min*)		
Agonist	*Dose (mol)*	*Control*	*After addition of: L-NAME*	*L-NAME + L-Arg*
ATP	5×10^{-8}	22.32 ± 3.40	10.53 ± 2.45*	20.85 ± 5.00**
UTP	5×10^{-8}	8.30 ± 1.13	3.14 ± 0.75*	5.63 ± 1.18**
ITP	5×10^{-8}	6.24 ± 1.27	1.00 ± 0.18*	4.10 ± 1.17**
CTP	5×10^{-7}	2.87 ± 0.78	0.03 ± 0.03*	1.74 ± 0.66**
GTP	5×10^{-7}	3.61 ± 0.40	0.83 ± 0.16*	3.58 ± 0.66**
TTP	5×10^{-7}	6.90 ± 0.98	1.49 ± 0.34*	3.37 ± 0.36**

The areas are expressed as the mean ± s.e.mean ($n \geq 6$). Significant differences from control are $*P < 0.05$. Significant differences from responses obtained in presence of L-NAME are $**P < 0.05$.

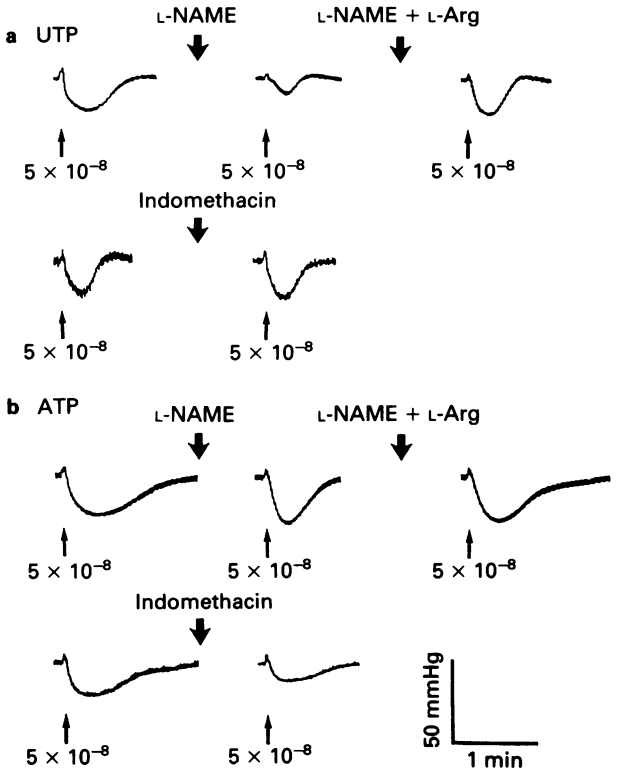

Figure 5 Typical perfusion pressure traces, obtained from guinea-pig isolated perfused hearts, showing the effects of (a) UTP and (b) ATP. The vasodilator response to these agents in the absence or presence (after ↓) of L-N^G-nitroarginine methyl ester (L-NAME, 3×10^{-5} M) and indomethacin (10^{-6} M) is demonstrated. Reversal of the inhibition of the vasodilator response to UTP and ATP, in the presence of L-NAME, by L-arginine (L-Arg, 1.5×10^{-3} M) is also demonstrated. The dose stated is the number of mol of vasodilator agonist that is injected into the perfusion system close to the heart.

between P_{2Y}-purinoceptor-mediated and 'nucleotide' receptor-mediated relaxations could not be adopted because of the absence of specific antagonists to the 'nucleotide' receptor and because the P_{2Y}-purinoceptor antagonist, reactive blue 2 (Burnstock & Warland, 1987), caused rapid deterioration of the tissue.

The maximum amplitude and area of the vasodilator responses to the pyrimidines, UTP, CTP and TTP, and the purines, ITP and GTP, were significantly reduced by L-NAME. These results suggest that the relaxant response to UTP, CTP, TTP, ITP and GTP take place largely through the formation and release of NO. In the rat mesenteric arterial bed, the vasodilator response to UTP is also largely dependent on the release of NO (Ralevic & Burnstock, 1991). The fact that L-arginine reversed the inhibition of the response to the pyrimidines and purines by L-NAME substantiates these claims in that it shows the L-NAME was selectively inhibiting the enzyme nitric oxide synthase. Nitric oxide synthase converts L-arginine into L-citrulline with the additional production of NO (Palmer *et al.*, 1988; Schmidt *et al.*, 1988; Mayer *et al.*, 1989; Palmer & Moncada, 1989). It therefore appears that the pyrimidines and purines studied induce relaxation in a similar manner with the exception of ATP. In the guinea-pig coronary vasculature it has been shown that ATP induces release of NO (Kelm & Schrader, 1990). However, as previously demonstrated (Vials & Burnstock, 1992), while L-NAME reduced the duration of the vasodilatation induced by ATP, it did not alter the peak response, suggesting that at least this part of the response is not due to the generation and release of NO.

Indomethacin, the prostaglandin synthesis inhibitor (Vane, 1971), significantly reduced the maximum amplitude of the vasodilator response to ATP. This suggests that prostanoids are also involved in part of the response to ATP. ATP has been shown to stimulate prostacyclin production from various beds and endothelial cells in culture (Needleman *et al.*, 1974; Boeynaems & Galand, 1983; Hellewell & Pearson, 1984). In the guinea-pig coronary vasculature, adenosine-induced relaxation was not mediated via prostanoids (Vials & Burnstock, 1993). Therefore the involvement of prostanoids in the relaxant response to ATP was not due to its breakdown to adenosine by highly active ectonucleotidases (Fleetwood *et al.*, 1989). The vasodilator responses to the pyrimidines UTP, TTP and CTP or the purines ITP and GTP were unaffected by the presence of indomethacin. Therefore prostanoids do not play a role in the vasodilatation produced in response to exposure to these pyrimidines and purines. In contrast UTP has been shown to induce prostacyclin production from bovine pulmonary artery endothelial cells (Lustig *et al.*, 1992).

In conclusion, we have demonstrated that the pyrimidines, UTP, TTP and CTP, and purines, ITP and GTP, induce relaxation in the guinea-pig coronary bed via formation and release of NO. ATP induces relaxation in the guinea-pig coronary vasculature via a combination of mechanisms involving both NO and prostanoids. Whether 'nucleotide' receptors are also present in the guinea-pig coronary vasculature is unclear. If they were present then action at these receptors induces relaxation via NO and not prostanoids. Selective antagonists will need to be established before a clear receptor profile in the guinea-pig coronary vasculature can be determined.

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Vasoconstrictor effects of various neuropeptide Y analogues on the rat tail artery in the presence of phenylephrine

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1 The increase in perfusion pressure induced by neuropeptide Y (NPY), peptide YY (PYY) and related peptides were compared in the perfused rat tail artery precontracted by a submaximal concentration (1 μM) of the vasoconstrictor, phenylephrine.

2 NPY, PYY, [Leu^{31},Pro^{34}]NPY, [Glu^{16}, Ser^{18}, Ala^{22}, Leu^{28,31}]NPY (ESALL-NPY) and the centrally truncated and stabilized analogues [D-Cys^{5}, 8-aminooctanoic acid$^{7-20}$, Cys^{24}]-NPY (D-Cys^{5}-NPY) and [D-Cys^{7}, 8-aminooctanoic acid$^{8-17}$, Cys^{20}]-NPY (D-Cys^{7}-NPY) produced a concentration-dependent enhancement of the vasoconstrictor response induced by 1 μM phenylephrine. PYY was two times more potent than NPY and [Leu^{31},Pro^{34}]NPY while ESALL-NPY, D-Cys^{7}-NPY and D-Cys^{5}-NPY were approximately 3, 5 and 16 times less potent than NPY respectively. NPY, D-Cys^{5}-NPY and D-Cys^{7}-NPY gave similar maximal responses whereas those observed for PYY, [Leu^{31},Pro^{34}]NPY and ESALL-NPY were much greater than that of NPY.

3 NPY 13–36 and [des-Ser^{3},Lys^{4}, Cys^{2}, 8-aminooctanoic acid$^{3-24}$, D-Cys^{27}]-NPY ([des-Ser^{3},Lys^{4}]Cys^{2}-NPY) were practically inactive at concentrations up to 3 μM, whereas [des-Ser^{3},Lys^{4}, D-Cys^{2}, 8-aminooctanoic acid$^{3-24}$, Cys^{27}]-NPY ([des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY), which differs from [des-Ser^{3},Lys^{4}]Cys^{2}-NPY in the disulphide bridge (a D-Cys in position 2 for [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY instead of an L-Cys for [des-Ser^{3},Lys^{4}]Cys^{2}-NPY) was a weak agonist the maximal effect of which could not be ascertained.

4 The contractile effects of [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY were additive with those of NPY and [Leu^{31}, Pro^{34}]NPY demonstrating that it is not a partial agonist but may simply not interact competitively with the receptor binding site for NPY. NPY and PYY interacted in a manner expected of agonists competing for the same binding site.

5 PYY, NPY and [Leu^{31},Pro^{34}]NPY were equipotent in displacing the I^{125}-labelled PYY from binding sites on membranes from Y_1-receptor expressing SK-N-MC cells, while the centrally truncated analogues were much less potent. The rank order of potencies for displacement of the I^{125}-PYY binding by these peptides in SK-N-MC cells correlated with their activity in enhancing the vasoconstrictor response of phenylephrine in the tail artery. For the [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY analogue, the displacement pattern was more complex in that the displacement analysis revealed the presence of two binding sites.

6 In conclusion, these data provide no evidence for other than postjunctional Y_1-receptors mediating the enhancement of the contractile response elicited by phenylephrine in the perfused rat tail artery. The effects of [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY indicate that the Y_1-receptor may possess an allosteric binding site.

Keywords: Neuropeptide Y (NPY); neuropeptide Y receptors; rat tail artery

Introduction

Neuropeptide Y (NPY) and peptide Y (PYY) are 36-amino acid peptides belonging to the pancreatic polypeptide family and were originally isolated from porcine brain (Tatemoto *et al.*, 1982) and gut (Tatemoto, 1982) respectively. NPY is widely distributed in the brain and in the peripheral sympathetic nervous system (Lundberg *et al.*, 1982; Allen *et al.*, 1983; Ekblad *et al.*, 1984) and is co-stored and co-released with catecholamines from sympathetic nerve terminals (Allen & Bloom, 1986; Kasakov *et al.*, 1988; Lundberg *et al.*, 1989).

A variety of functional roles in the cardiovascular system have been attributed to NPY (for review see Edvinsson *et al.*, 1987). Although NPY produces contractile responses by itself in some vascular preparations, it mainly enhances vasoconstriction elicited by various agonists and those evoked by electrical stimulation. Besides this potentiating effect, NPY also inhibits the release of noradrenaline evoked by electrical field stimulation of sympathetic nerves. These effects are mediated via two pharmacologically distinct receptors termed Y_1 and Y_2 (for review see Wahlestedt *et al.*, 1990), that are probably structurally distinct glycoproteins (Sheikh & Williams, 1990). Originally it was postulated that the Y_1-type receptor, which is involved in the direct and indirect effects on contractile activity, was located postjunctionally and required the whole NPY/PYY amino acid molecule for activation. The Y_2-type receptor seemed to be prejunctional and recognized not only NPY/PYY but also C-terminal fragments of both NPY and PYY (Wahlestedt *et al.*, 1986). However, this restricted functional localization has not been observed in recent studies. In the rat mesenteric arterial bed (McAuley & Westfall, 1992) and caval vein (Grundemar *et al.*, 1992) the presence of postjunctional Y_1 and Y_2 receptors was suggested and there is evidence for the existence of prejunctional Y_1 receptors (McAuley & Westfall, 1992). In addition to Y_1- and Y_2-receptors, there seems to exist an additional NPY receptor, that was recently designated Y_3 (Balasubramaniam & Sheriff, 1990; Michel, 1991). At Y_1 and Y_2 receptor subtypes, PYY is approximately equipotent with NPY while NPY is much more potent than PYY at Y_3 receptors. Recently the cloning and expression of cDNAs for a Y_1 receptor (Herzog *et al.*, 1992; Larhammar *et al.*, 1992) and most probably for a Y_3 receptor (Rimland *et al.*, 1991) were described.

NPY and PYY appear to be peptides with a folded structure (for review see Schwartz *et al.*, 1990). Recently, in order to examine the potential importance of the central amphi-

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pathic α-helical region of NPY in receptor binding, centrally truncated analogues were synthesized (Krstenansky *et al.*, 1989; McLean *et al.*, 1990). These peptides contain 8-amino-octanoic acid in place of selected residues of NPY and were stabilized with a disulphide bridge to restrict conformationally the NPY molecule to a folded helical structure.

The present study was performed to investigate how these centrally truncated and stabilized analogues of NPY may potentiate the phenylephrine-induced vasoconstriction in the rat tail artery. Their effects were compared to those obtained with NPY, PYY, NPY 13–36, the C-terminal fragment of NPY and [Leu^{31},Pro^{34}]NPY the Y_1-type receptor selective peptide (Fuhlendorff *et al.*, 1990). To elucidate further the identity of the receptors involved in the modulation of responses mediated by α-adrenoceptors the binding of all these peptides to the membranes of SK-N-MC cells, which expresses Y_1 receptors (Fuhlendorff *et al.*, 1990; Wahlestedt *et al.*, 1991), was also assessed. The results of this study suggest that, in the rat tail artery, the modulation of NPY of the contractile response elicited by α-adrenoceptors is mediated by Y_1-receptors.

Methods

Rat tail artery

Male Wistar rats (12 weeks old) were killed by cervical dislocation and exsanguinated. A segment of about 2–2.5 cm of the proximal part of the ventral tail artery was dissected out as previously described (Bucher *et al.*, 1987) and kept in oxygenated (95% O_2/5% CO_2) medium which contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25 and glucose 11. The arteries were cannulated at both ends and suspended vertically in an organ bath containing 4 ml of medium and perfused via their proximal ends with medium by means of a roller pump. The perfusion rate was gradually increased from 0 to 2.2 ml min^{-1} during the first 10 min after suspension of the arterial segment in the bath and kept constant thereafter. The intraluminal perfusion pressure was determined with a pressure transducer (Statham P23Db) and recorded on a pen recorder. Changes in the inflow perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. The arteries were allowed to equilibrate for 1 h before the contractile capacity was tested by exposing the arterial segment to a concentration of 3 μM phenylephrine before being contracted every 20 min by addition of 1 μM phenylephrine for about 10 min. Acetylcholine (10 μM) was added during one of these phenylephrine-induced contractions to verify the presence of a functional endothelium (Bucher *et al.*, 1992). The EC_{50} for phenylephrine in this vessel is about 2.5 μM. Once the artery had equilibrated and responded with 3 comparable phenylephrine-elicited contractions, concentration-response curves to NPY and related analogues were constructed non-cumulatively by the addition of a single concentration of the peptide to the arterial segment precontracted with 1 μM phenylephrine. When the contraction to NPY, or the analogues, had reached equilibrium, both phenylephrine and the peptide were washed out and the preparation left for 30 min before the addition of the same concentration of phenylephrine and a different concentration of peptide. All compounds were administered extraluminally to the bath fluid in a volume of either 10 or 30 μl. For calculation of the effects of NPY and of the different analogues, the contractile capacity of each artery was measured at the beginning of the experiment by adding 100 nM NPY to phenylephrine precontacted arterial segments; this contraction served as an internal reference and was set as 100%. Thereafter, all other contractions to either NPY or the analogues were expressed as a percentage of the 100 nM NPY-induced response. At the end of the experiment the same control contraction to 100 nM NPY was elicited; there was no significant difference between the first and last contractile responses.

Binding to SK-N-MC cell membranes

SK-N-MC human neuroblastoma cells (Biedler *et al.*, 1973) were obtained from the American Type Culture Collection. They were grown using MEM medium containing foetal calf serum (10%), L-glutamine (1%), non-essential amino acids (1%) and penicillin/streptomycin (100 i.u. and 100 μg ml^{-1} respectively). Confluent monolayer cultures were harvested by washing with medium and gently scraping with a rubber policeman. Cells were homogenized in 50 mM Tris HCl buffer (pH 7.4) with a Polytron homogenizer for 10 s at 4°C. The homogenate was then centrifuged at 1000 *g* for 15 min at 4°C. The pellet was discarded and the supernatant centrifuged at 18,000 *g* for 15 min, and the resulting pellet was resuspended in 50 mM Tris HCl buffer (pH 7.4) containing (mM): NaCl 115, KCl 15, $CaCl_2$ 5, $MgSO_4$ 1, KH_2PO_4 1.25, $NaHCO_3$ 25 and glucose 10; supplemented with 0.1% BSA and peptidase inhibitors (thiorphan 100 μM; bacitracin 4 mg ml^{-1}; leupeptin 0.4 mg ml^{-1}). The reaction was carried out in polypropylene tubes by adding 100 μg of cell membrane proteins to assay buffer containing [^{125}I]-PYY (specific activity 81.4 TBq mmol^{-1}; NEN, Les Ullis, France) at the indicated concentrations, with or without various concentrations of competiting non-labelled peptides, in a total volume of 500 μl. After incubation at room temperature for 120 min the incubation was terminated by washing the membranes with ice-cold 50 mM Tris HCl (pH 7.4) containing 0.1% BSA and filtration over Whatman GF/C glass fibre filters presoaked in 0.1% polyethyleneimine. The filters were quickly washed three times with 3 ml buffer. Filter-bound radioactivity was quantified in a gamma counter. Membrane protein content was determined according to a modified Lowry method (Lowry *et al.*, 1951) with bovine serum albumin used as a standard. All binding experiments were performed in duplicate. Specific binding was determined in the presence of 1 μM unlabelled PYY and was approximately 85% of total binding. Competition binding experiments were analyzed by fitting the experimental data to sigmoid function with a Hill slope of −1 using the InPlot program (GraphPad Software, San Diego, CA, U.S.A.) or by the LIGAND suite of programmes.

Drugs

The following drugs were used: (−)-phenylephrine hydrochloride (Sigma, L'Isle d'Abeau Chesnes, France); hNPY, hPYY, pNPY-(13–36), p[Leu^{31},Pro^{34}]NPY (Peninsula, Belmont, CA, U.S.A.). ESALL-NPY, [Glu^{16}, Ser^{18}, Ala^{22}, Leu$^{28, 31}$]

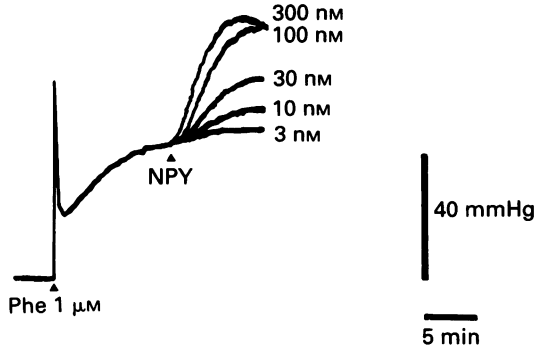

Figure 1 Composite of recordings from a typical experiment showing the enhancement of the perfusion pressure produced by different concentrations of neuropeptide Y (NPY) in the perfused rat tail artery precontracted with 1 μM phenylephrine (Phe).

NPY; [des-Ser^3,Lys^4]Cys^2-NPY, [des-Ser^3,Lys^4,Cys^5, 8-aminooctanoic acid$^{3-24}$, D-Cys^{27}]-NPY; [des-Ser^3,Lys^4]D-Cys^2-NPY, [des-Ser^3,Lys^4, D-Cys^2, 8-aminooctanoic acid$^{3-24}$, Cys^{27}]-NPY; D-Cys^5-NPY, [D-Cys^5, 8 aminooctanoic acid$^{7-20}$, Cys^{24}]-NPY; D-Cys^7-NPY, [D-Cys^7, 8 aminooctanoic acid$^{8-17}$, Cys^{20}]-NPY were from Marion Merrell Dow Research Institute (Strasbourg, France). Stock solutions of phenylephrine, NPY and the related analogues were prepared with Milli-Q water (Millipore) and subsequently stored frozen at − 20°C until use. The molecular extinction coefficient and the absorbance read at 280 nm were used to determine the concentration of the native peptides (NPY, PYY) and analogues using the formula:

$$A = \varepsilon.l.c$$

where A is the absorbance at 280 nm, ε the molecular extinction coefficient (specific for each peptide), l the path length, c the molar concentration.

Analysis of results

The relative potencies of the various analogues were derived from the comparison of their EC_{50} values, i.e., the concentration that produced 50% of the maximal effect, calculated by logit/log regression analysis. Results are expressed as mean ± s.e.mean of *n* experiments. The differences between the EC_{50} values were tested for significance by use of the non-parametric Mann-Whitney U-test. For multiple comparisons with the same group (in this case NPY), the limit of significance was divided by the number of comparisons according to Bonferroni (Wallenstein *et al.*, 1980). A probability level of 0.05 or less was considered significant.

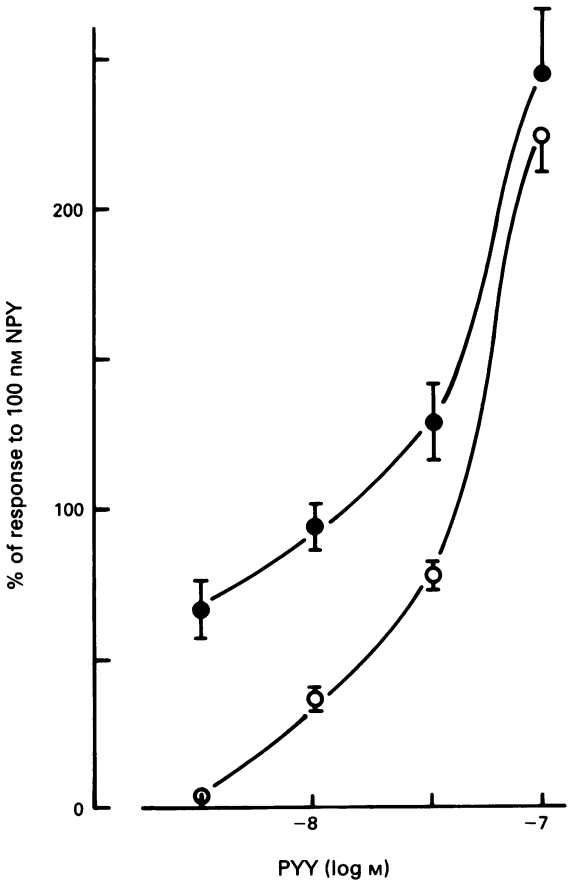

Figure 3 Effect of neuropeptide Y (NPY) on the concentration-response curve to peptide YY (PYY) in rat tail arteries precontracted with 1 μM phenylephrine: (○) shows the response obtained with PYY alone and (●) represent the responses obtained with PYY administered together with 50 nM NPY. Responses are expressed as in Figure 2. Each point is the mean ± s.e.mean of 6 arteries.

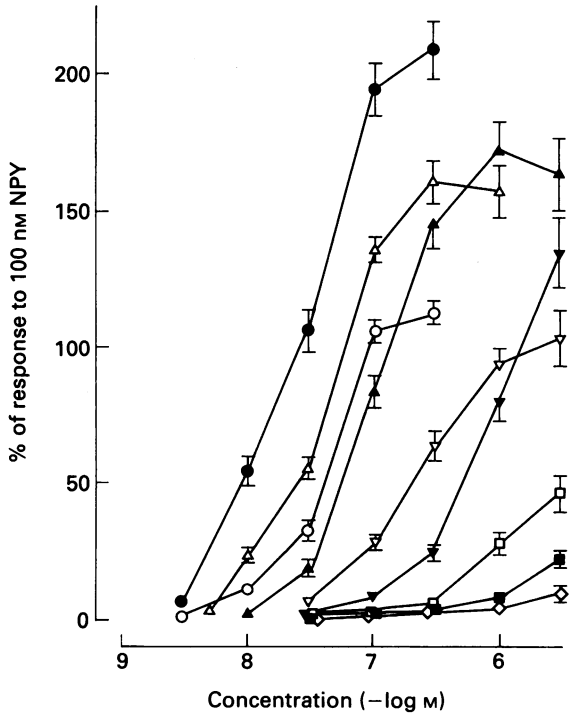

Figure 2 Concentration-dependent contractile responses produced by neuropeptide Y (NPY), PYY and NPY analogues in rat tail arteries precontracted with 1 μM phenylephrine. Responses are expressed as a percentage of the contractile response elicited by 100 nM NPY. Symbols indicate mean ± s.e.mean values: (○) NPY (12); (●) PYY (12); (◇) NPY13–36 (4); (△) [Leu^{31},Pro^{34}] NPY (12); (▲) ESALL-NPY (12); (▼) D-Cys^5-NPY (9); (▽) D-Cys^7-NPY (7); (□) [des-Ser^3,Lys^4]D-Cys^2-NPY (6); (■) [des-Ser^3,Lys^4]Cys^2-NPY (6).

Results

Contractile responses of the rat isolated tail artery

As illustrated in Figure 1, NPY elicited a concentration-dependent enhancement of the vasoconstrictions evoked by previous exposure of the rat tail artery to 1 μM phenylephrine. In the absence of phenylephrine, the vasoconstrictor effect of the highest concentration of NPY, PYY or the NPY analogues alone was less than 5% of that evoked with 100 nM NPY in phenylephrine precontracted arterial segments. In the presence of 1 μM phenylephrine, NPY, PYY and several of the NPY analogues enhanced vasoconstriction in a concentration-dependent manner (Figure 2). However, [des-Ser^3, Lys^4]D-Cys^2-NPY and [des-Ser^3, Lys^4]Cys^2-NPY were much less active than the other analogues and NPY 13–36 was almost inactive, high concentrations of the peptide (3 μM) causing only a slight increase in the phenylephrine-induced vasoconstriction (Figure 2; Table 1). There was a tendency for the centrally truncated peptides to have progressively lower EC_{50} values as the magnitude of the central amino acid deletion increased. The order of potency was PYY > [Leu^{31}, Pro^{34}]NPY ≥ NPY > [ESALL]NPY > D-Cys^7-NPY > D-Cys^5-NPY. The maximal effect elicited by PYY was about twice that elicited by NPY (Figure 2; Table 1). Although NPY and [Leu^{31},Pro^{34}]NPY were about equipotent, the maximal effect elicited by [Leu^{31},Pro^{34}]NPY was 1.5 fold that of NPY. Of the centrally truncated analogues with an 8-aminooctanoic acid residue, D-Cys^7-NPY was the most potent and D-Cys^5-NPY had an EC_{50} approximately 15 times

Table 1 Effects of neuropeptide Y (NPY), PYY and NPY analogues on rat isolated tail arteries precontracted with 1 μM phenylephrine

Compound	EC_{50} (nM)	E_{max}/NPY (100 nM)	n
NPY	47.2 ± 2.0	112.2 ± 3.7	12
PYY	19.7 ± 2.2**	208.0 ± 10.9**	12
[Leu^{31},Pro^{34}] NPY	42.2 ± 3.5	152.7 ± 9.4**	12
[ESALL] NPY	122.6 ± 12.4**	162.5 ± 13.4*	12
D-Cys^{7}-NPY	253.7 ± 23.1**	102.1 ± 10.8	7
D-Cys^{5}-NPY	765.2 ± 44.3**	134.3 ± 12.5	9
[des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY	>>1000	46.1 ± 6.6	6
[des-Ser^{3},Lys^{4}]Cys^{2}-NPY	>>1000	22.0 ± 1.5	6
NPY-(13–36)	>>1000	10.0 ± 1.8	4

Data are calculated from the experiments in Figure 2, $n = 4$ to 12 arteries per compound. Mean ± s.e.mean.
*$P < 0.05$, **$P < 0.01$ when compared to the value obtained with NPY.

less than that obtained for NPY (Table 1). However, these two analogues produced maximal effects identical to those of NPY. It should be noted that the maximal vasoconstrictor response (about 200 mmHg) produced by 10 μM phenylephrine was never attained by any combination of peptide with 1 μM phenylephrine. In the absence of 50 nM NPY the concentration-dependent effect of PYY was shifted to the left at low concentrations of PYY, but the maximal contractile effect was unchanged (Figure 3). The [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY analogue at 1 and 3 μM produced pressor responses 27% and 46% respectively of those obtained with 100 nM NPY (Figure 2). When 1 μM of [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY was co-administered with NPY, the potentiating effects of NPY were enhanced; the NPY concentration-effect curve was shifted to the left and the maximal response enhanced by about 28% (Figure 4). A 3 fold higher concentration of [des-Ser^{3}Lys^{4}]D-Cys^{2}-NPY shifted the NPY concentration-effect curve even further to the left and enhanced the maximal response by about 40% (Figure 4). Likewise, 1 μM [des-Ser^{3}, Lys^{4}]D-Cys^{2}-NPY augmented the vasoconstrictor responses of [Leu^{31},Pro^{34}]NPY on phenylephrine-precontracted arteries when the two peptides were co-administered and enhanced the maximal responses evoked by about 23% (Figure 5). The mean responses to each concentration of

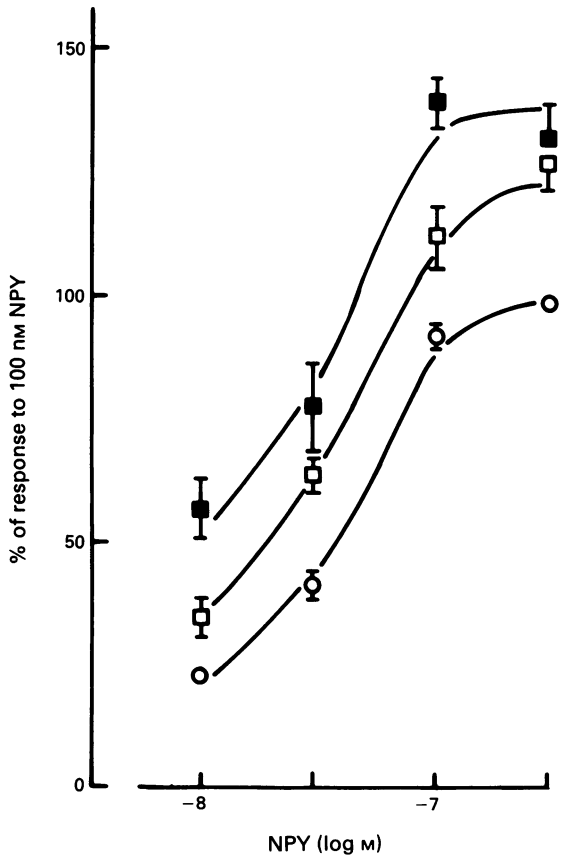

Figure 4 Effect of [des-Ser^{3},Lys^{4}, D-Cys^{2}, 8-aminooctanoic acid$^{3-24}$, Cys^{27}]-neuropeptide Y ([des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY) on the concentration-response curve to neuropeptide Y (NPY) in rat tail arteries precontracted with 1 μM phenylephrine: (○) shows the response obtained with NPY alone; (□) and (■) represent the responses obtained with NPY administered together with 1 μM and 3 μM [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY respectively. Responses are expressed as in Figure 2. Each point is the mean ± s.e.mean of 5–6 arteries.

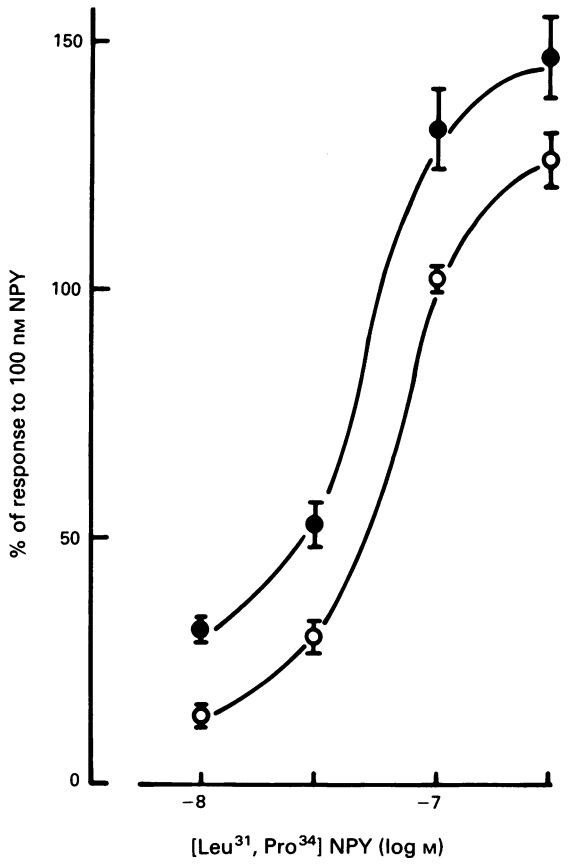

Figure 5 Effect of [des-Ser^{3},Lys^{4}, D-Cys^{2}, 8-aminooctanoic acid$^{3-24}$, Cys^{27}]-neuropeptide Y ([des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY) 1 μM on the concentration-response curve to [Leu^{31},Pro^{34}] NPY in rat tail arteries precontracted with 1 μM phenylephrine: (○) shows the response obtained with [Leu^{31},Pro^{34}] NPY alone; (●) represents the reponses obtained with [Leu^{31},Pro^{34}] NPY administered together with 1 μM [des-Ser^{3},Lys^{4}]D-Cys^{2}-NPY. Responses are expressed as in Figure 2. Each point is the mean ± s.e.mean of 6 arteries.

either NPY or [Leu^{31},Pro^{34}]NPY obtained in the absence of the [des-Ser^3,Lys^4]D-Cys^2-NPY analogue were significantly different ($P < 0.05$) from those obtained in the presence of either 1 or 3 μM of the [des-Ser^3,Lys^4]D-Cys^2-NPY analogue.

Characterization of [^{125}I]-PYY binding sites in SK-N-MC cells

Saturation binding isotherms demonstrated saturable high affinity [^{125}I]-PYY binding to SK-N-MC cell membranes (K_d 0.54 ± 0.04 nM, B_{max} 250 ± 40 fmol mg^{-1} protein; $n = 4$). Competition experiments with PYY, NPY and related analogues (Figure 6) produced a rank order of affinity of PYY (K_i 1.5 nM) ≥ [Leu^{31},Pro^{34}] NPY (K_i 1.8 nM) ≥ NPY (K_i 2.1 nM) > ESALL-NPY (K_i 7.7 nM) > D-Cys^7-NPY (K_i 13.2 nM) > D-Cys^5-NPY (K_i 74.9 nM) > NPY13–36 (K_i 147.9 nM). For [des-Ser^3,Lys^4]Cys^2-NPY and [des-Ser^3,Lys^4]D-Cys^2-NPY competition binding experiments produced Hill slopes significantly less than −1. In the particular case of [des-Ser^3, Lys^4]D-Cys^2-NPY, analysis of the total binding data (non-specific values were not subtracted) from six experiments using the LIGAND programme indicated that a two-site model was a better fit, than a single site model as indicated by the sums of squares. The F statistic yielded a P value < 0.001. Using this two-site model K_ds of 21.6 nM for the high affinity site and a K_d of 475 nM for the low affinity site were determined.

Discussion

The contractile responses to a variety of vasoactive agents are enhanced in the presence of NPY in arteries from different species (see Edvinsson *et al.*, 1987) and the present study shows that both NPY and PYY have a marked ability to enhance phenylephrine-mediated vasoconstrictions of rat isolated caudal arteries. Both the N- and C-terminal parts of NPY and PYY seem to be required for this postjunctional modulation of phenylephrine-evoked vasoconstrictions. Progressive N-terminal deletion results in severely reduced contractile activity in vascular preparations (Wahlestedt *et al.*, 1986) and the activity of NPY 13–36 was markedly reduced compared to that of NPY. The effect of NPY on the contractile responses to vasoactive agents is thought to be mediated via the Y_1-receptor because C-terminal fragments of NPY or PYY, selective for the Y_2 receptor, did not produce this effect (Wahlestedt *et al.*, 1990). [Leu^{31},Pro^{34}]NPY is regarded as a Y_1-receptor selective agonist (Fuhlendorff *et al.*, 1990) whereas NPY 13–36 is considered to be a selective Y_2-receptor agonist (Wahlestedt *et al.*, 1990). In the perfused rat tail artery, NPY and [Leu^{31},Pro^{34}]NPY are roughly equipotent whereas NPY 13–36 has only a slight effect at very high concentrations. These results are in agreement with earlier observations on other vascular preparations and indicate that, in the rat tail artery, the enhancement of the contractile response elicited by phenylephrine in the presence of NPY or PYY is mediated predominantly by Y_1 receptors.

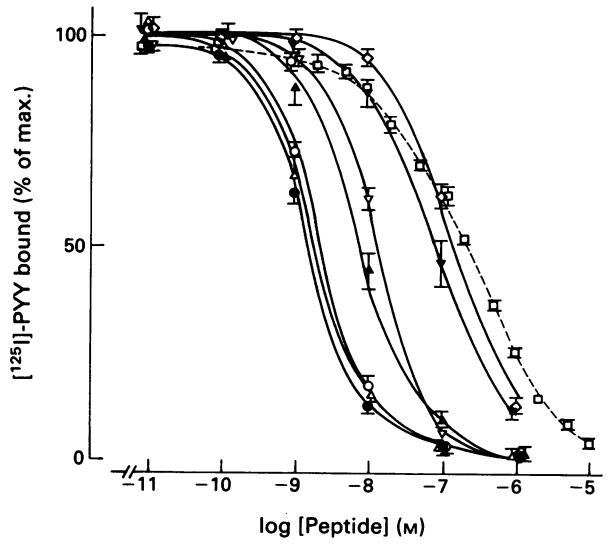

Figure 6 Competition of unlabelled peptides with [^{125}I]-PYY binding to SK-N-MC cell membranes. Membranes were incubated with 30 pM [^{125}I]-PYY in the presence of increasing concentrations of unlabelled peptides for 120 min at room temperature. The radiolabelled PYY was displaced by (●) PYY; (○) NPY; (◇) NPY 13–36; (△) [Leu^{31},Pro^{34}] NPY; (▲) ESALL-NPY; (▼) D-Cys^5-NPY; (▽) D-Cys^7-NPY; (□) [des-Ser^3,Lys^4]D-Cys^2-NPY. Data are the mean ± s.e.mean of 3–6 experiments.

The vasoconstrictor effects of various centrally truncated and stabilized NPY analogues were also examined. The D-Cys^7-NPY and D-Cys^5-NPY analogues concentration-dependently increased the perfusion pressure of phenylephrine precontracted arteries but were respectively about 5 and 16 times less potent than NPY. The [des-Ser^3,Lys^4]D-Cys^2-NPY and [des-Ser^3,Lys^4]Cys^2-NPY analogues were even less active and maximal effects could not be obtained (Figure 2). D-Cys^7-NPY has been shown to bind to mouse brain receptors and to pig spleen receptors with potency equivalent to that of NPY (McLean *et al.*, 1990). Moreover, the relative potency of D-Cys^7-NPY and [des-Ser^3,Lys^4]Cys^2-NPY observed in the present study is different from that observed in the rat jejunum mucosa (Cox & Krstenansky, 1991). Although D-Cys^7-NPY and D-Cys^5-NPY were less potent than NPY their maximal effects were similar to those of NPY. In the SK-N-MC human neuroblastoma cell line with a homogeneous population of Y_1 subtype receptors (Aakerlund *et al.*, 1990; Fuhlendorff *et al.*, 1990) it has been shown that the IC_{50} required to inhibit isoprenaline-stimulated cyclic AMP accumulation was 5 times higher for D-Cys^7-NPY than for NPY (Gordon *et al.*, 1990). Furthermore, it appears that a progressive central amino acid deletion as is the case for D-Cys^7-NPY, D-Cys^5-NPY, [des-Ser^3,Lys^4]D-Cys^2-NPY and [des-Ser^3,Lys^4]Cys^2-NPY, leads to a progressive loss of potency.

Increasing the potential for α-helicity as in the case of ESALL-NPY, designed as an amphipathic analogue (McLean *et al.*, 1990), produced a significantly different maximum response which was greater than that to the native peptide but not significantly different from that produced by [Leu^{31},Pro^{34}]NPY. The substitution of Ile for Leu in position 31 in the case of ESALL-NPY and [Leu^{31},Pro^{34}]NPY might then be of importance for the efficacy of the two peptides.

A significant substitution of the amphipathic α-helix and an L-Cys-D-Cys disulphide bridge as in the case of the [des-Ser^3,Lys^4]Cys^2-NPY, leads to an analogue with biological activity comparable to that of NPY 13–36. What is impressive, however, is the fact that the same deletion of the amphipathic α-helix but with a D-Cys-L-Cys disulphide bridge ([des-Ser^3,Lys^4]D-Cys^2-NPY) results in a more active analogue than that with an L-Cys-D-Cys disulphide bridge ([des-Ser^3,Lys^4]Cys^2-NPY).

In the present study, the low potency of the [des-Ser^3, Lys^4]D-Cys^2-NPY analogue prevented a direct determination of its efficacy in comparison with that of NPY. However, in the presence of either NPY or of the Y_1-receptor selective agonist [Leu^{31},Pro^{34}]NPY, the administration of different concentrations of [des-Ser^3,Lys^4]D-Cys^2-NPY did not show any antagonism of the vasoconstrictor effects of the peptides but a clear enhancement of the responses (Figures 3 and 4). The lack of inhibition demonstrates clearly that [des-Ser^3, Lys^4]D-Cys^2-NPY is not a partial agonist at these receptors. The interaction of different agonists, such as NPY and PYY at the same receptor should produce concentration-effect curves such as those shown in Figure 3 (Ariëns *et al.*, 1964). The additive responses observed with [des-Ser^3,Lys^4]D-Cys^2-NPY

or [Leu^{31},Pro^{34}]NPY (Figures 4 and 5) are harder to explain, but might indicate that [des-Ser^3,Lys^4]D-Cys^2-NPY interacts with different receptors from those activated by [Leu^{31},Pro^{34}]NPY and by NPY, or that [des-Ser^3,Lys^4]D-Cys^2-NPY interacts differently (allosterically) with the same receptors as NPY. A binding study of the interactions of NPY and [des-Ser^3,Lys^4]D-Cys^2-NPY was undertaken to try to elucidate these possibilities.

It was not possible to define specific [^{125}I]-PYY or [^{125}I]-NPY binding sites on arterial smooth muscle cell membranes from the tail artery so it was decided to characterize the binding properties of these analogues to well defined Y_1-receptors on SK-N-MC cells (Fuhlendorff *et al.*, 1990; Wahlestedt *et al.*, 1991). The curves illustrating the displacement of [^{125}I]-PYY from SK-N-MC cell membranes by PYY, NPY and [Leu^{31},Pro^{34}] NPY are consistent with a homogeneous population of NPY receptors of the Y_1 type as previously reported. Moreover, the rank order of potencies for the displacement of [^{125}I]-PYY binding in SK-N-MC cell membranes by NPY analogues appeared to fit with their rank order of potencies in enhancing the perfusion pressure in phenylephrine precontracted perfused tail arteries, tending to support their identification as Y_1 receptors. PYY and NPY exhibited similar affinities for [^{125}I]-PYY binding sites but their differing potencies *in vitro* (Table 1) indicates that they probably have differing efficiencies at Y_1 receptors. The biphasic nature of the interaction of [des-Ser^3,Lys^4]D-Cys^2-NPY with PYY receptors indicates a complex interaction of this compound with Y_1 receptors which could be purely allosteric, or a mixture of interactions at the NPY binding site and an allosteric site.

Several studies have suggested that at the postjunctional level the NPY receptor population is not homogeneous and that both Y_1 and Y_2 receptors may exist in the caval vein (Grundemar *et al.*, 1992) and mesenteric arterial bed (McAuley & Westfall, 1992) of the rat. In the nucleus tractus solitarius it has been proposed that NPY acts on receptors that cannot be characterized as either Y_1 or Y_2 (Grundemar *et al.*, 1991).

The results of the present study however, are compatible with the idea that the postjunctional effects of NPY are mediated via Y_1-receptors and no evidence for a mixture of receptors was obtained. The effects of [des-Ser^3,Lys^4]D-Cys^2-NPY, both in contractile experiments and in binding studies, indicate that this compound might interact with a different site on the Y_1-receptor from that recognized by NPY and PYY, but cannot exclude interaction with non-Y_1 receptors in the artery.

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Impaired sensory-motor nerve function in the isolated mesenteric arterial bed of streptozotocin-diabetic and ganglioside-treated streptozotocin-diabetic rats

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1 Adult male Wistar rats were treated with streptozotocin (65 mg kg^{-1}, i.p.) to induce diabetes. Subgroups of age-matched control and streptozotocin-treated rats were given daily injections of mixed brain bovine gangliosides (60 mg kg^{-1} body weight, i.p.). At eight weeks after treatment mesenteric arterial beds from rats in each of the four groups were isolated and perfused and the function of perivascular nerves (sympathetic and sensory-motor), endothelium and smooth muscle was assessed.

2 Values for basal tone of mesenteric beds from diabetic and diabetic-ganglioside rats were significantly lower than those of the control and control-ganglioside-treated rats. Perfusion pressures at basal tone were 25.55 ± 0.8 ($n = 11$), 22.58 ± 1.5 ($n = 12$), 28.42 ± 1.6 ($n = 12$) and 30.67 ± 1.9 ($n = 12$) mmHg for diabetic, diabetic-ganglioside, control and control-ganglioside-treated rats respectively.

3 There was no difference between the groups with respect to vasoconstrictor responses to sympathetic nerve stimulation, or to doses of noradrenaline. Vasoconstrictor responses to potassium chloride were also similar between the groups.

4 Perivascular nerve stimulation in the presence of the sympathetic blocker guanethidine (3 μM), with tone of the preparation raised with methoxamine (3–100 μM), elicited frequency-dependent vasodilatation of mesenteric arterial beds due to transmitter release from sensory-motor nerves. Sensory-motor nerve-induced vasodilator responses of mesenteric arterial beds from streptozotocin-diabetic and ganglioside-treated diabetic rats were significantly smaller than those of mesenteric beds from the controls (untreated and ganglioside-treated). Vasodilator responses to exogenously applied calcitonin gene-related peptide, the principal vasodilator transmitter released from these nerves, were not different between the groups. Vasodilator responses to the sensory neurotoxin capsaicin were also not different between the groups.

5 Endothelium-dependent vasodilator responses to acetylcholine were similar between the groups as were those to the endothelium-independent vasodilator sodium nitroprusside.

6 These results indicate that streptozotocin-induced diabetes produces marked impairment of sensory-motor nerve function in the rat mesenteric arterial bed. The significantly lower basal perfusion pressures of mesenteric beds from diabetic rats compared to controls may be a reflection of sympathetic dysfunction, but no differences were apparent from the vasoconstrictor responses produced when sympathetic nerves were electrically stimulated. There was no evidence for changes in endothelial vasodilator function, or smooth muscle vasodilator and vasoconstrictor function. Ganglioside treatment did not modify any aspect of vascular function of mesenteric beds from streptozotocin-diabetic or control rats.

Keywords: Sensory afferents; diabetes; gangliosides; mesenteric arterial bed; endothelium

Introduction

Diabetes is known to produce pathological changes in blood vessel structure and function involving both nerves (sensory, motor and autonomic) and endothelial cells. The pathogenesis of diabetic neuropathy has been extensively studied in the streptozotocin-treated rat, a model of insulin-dependent diabetes. In this model, sympathetic dysfunction is suggested by attenuated contractile responses due to stimulation of sympathetic nerves in the rat mesenteric bed (Takiguchi *et al.*, 1988) and tail artery (Hart *et al.*, 1988). Ultrastructural changes in sympathetic ganglia (Monckton & Pehowich, 1982) and neuronal deficits in tyrosine hydroxylase (the rate-limiting enzyme in the synthesis of noradrenaline) and 5-hydroxytryptamine (5-HT) specific to mesenteric perivascular sympathetic nerves (Webster *et al.*, 1991) have also been seen. The literature is conflicting with regard to the effect of diabetes on contractile activity to various agents including α-adrenoceptor agonists, 5-HT, K^+ and prostaglandins since increased vascular reactivity (Agrawal & McNeill, 1987; Abebe & MacLeod, 1990; Abebe *et al.*, 1990; White & Carrier, 1990) as well as no change (Furman & Sneddon, 1993) or diminished responsiveness (Longhurst & Head, 1985) to these same agents have been reported. Sensory-nerve conduction velocity has been reported to be decreased in diabetes (Moore *et al.*, 1980; Julu, 1988) and sensory neuropeptide-like immunoreactivity has been shown to be diminished in human diabetic skin (Levy *et al.*, 1989) and in *in vivo* corrected data in streptozotocin-diabetic rat mesenteric vessels (Webster *et al.*, 1991). Functional studies of perivascular sensory-motor nerves in diabetes, however, are relatively scarce, partly because of the few preparations in which motor responses to these nerves can be recorded. One such preparation is the isolated rat mesenteric arterial bed, in which electrical stimulation of capsaicin-sensitive primary sensory afferents during sympathetic blockade elicits vasodilatation which is mediated by the sensory neuropeptide, calcitonin gene-related peptide (CGRP) (Kawasaki *et al.*, 1988; Rubino *et al.*, 1992).

It is now known that endothelial cells play a crucial role in the control of blood vessel tone, particularly as mediators of

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vasodilatation to a number of vasoactive agents (Furchgott, 1983). Several studies have claimed that endothelial cells are adversely affected by diabetes, impaired endothelium-dependent relaxations having been demonstrated in clinical (De Tejada *et al.*, 1989) and experimental (Takiguchi *et al.*, 1988; Kamata *et al.*, 1989; Mayhan, 1992; Miyata *et al.*, 1992; Taylor *et al.*, 1992) diabetes. On the other hand, supersensitivity (Gebremedhin *et al.*, 1987; White & Carrier, 1990) and no change (Fortes *et al.*, 1983; Gebremedhin *et al.*, 1987; Andersson *et al.*, 1992; Furman & Sneddon, 1993) in endothelial responses in diabetes have also been described. The function of the underlying vascular smooth muscle in diabetes is generally unimpaired.

The aim of the current study was to assess the effects of streptozotocin-induced diabetes on peripheral nerve function in the rat mesenteric arterial bed. The functions of sympathetic and sensory-motor nerves were examined, as were postjunctional responses to the sympathetic neurotransmitter noradrenaline (NA), and the sensory neurotransmitter CGRP. The function of the vascular endothelium was assessed using the endothelium-dependent vasodilator, acetylcholine (ACh), and that of the underlying vascular smooth muscle was examined with the endothelium-independent vasodilator, sodium nitroprusside (SNP). Recently there has been considerable interest in the protective role of gangliosides (glycosphingolipids found in abundance in membranes from nervous tissue) in diabetes due to reports that these have improved some aspects of experimental diabetic neuropathy (Calcutt *et al.*, 1988; Ekstrom & Tomlinson, 1990; Soediono *et al.*, 1993). In view of this, we also examined the effects of ganglioside pretreatment on aspects of mesenteric arterial function in streptozotocin-induced diabetes.

Methods

Diabetes was induced in 23 adult male Wistar rats (weighing 400–450 g) by a single intraperitoneal injection (65 mg kg^{-1} body weight) of buffered streptozotocin (Belai *et al.*, 1988). Controls ($n = 24$) consisted of untreated animals of the same initial weight range. A week after the streptozotocin injection, subgroups of streptozotocin-treated ($n = 12$) and control (untreated) ($n = 12$) rats were given daily injections of a mixture of gangliosides (AFG_1) (60 mg kg^{-1} body weight per week; Fidia Abano, Terme, Italy). The onset of diabetes was established by the presence of rapid weight loss, polyuria and glycosuria. All groups were maintained under the same conditions, supplemented with food and water *ad libitum* until death, at 8 weeks. Previous studies in this laboratory showing no changes in a subgroup of streptozotocin-injected rats which failed to develop diabetes at any time during the 8 week period after streptozotocin injection are consistent with this model being one of streptozotocin-induced diabetes, rather than of the effects of streptozotocin *per se*.

Blood samples were taken from the posterior vena cava for blood glucose analysis under ether asphyxiation. Mesenteric arterial beds were isolated and set up for perfusion essentially as described previously (Ralevic & Burnstock, 1988). The abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was severed, the gut dissected away and the preparation mounted on a stainless steel grid (7 cm × 5 cm) in a humid chamber. The preparation was perfused at a constant flow rate of 5 ml min^{-1} by use of a peristaltic pump (Cole Parmer Instruments). Perfusion was with Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2:5% CO_2 and maintained at 37°C. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23, Gould) on a side arm of the perfusion cannula, and recorded on a polygraph (model 79D, Grass). The preparation was allowed to equilibrate for 30 min prior to experimentation.

Stimulation of perivascular nerves was achieved by passing a current between the cannulation needle and the wire grid on which the preparation rested. Sympathetic nerves were activated at basal tone by electrical field stimulation (90 V, 1 ms, 4–32 Hz for 30 s), and the resulting vasoconstriction could be abolished by guanethidine treatment. In separate preparations guanethidine (3 μM) was added to the perfusate at basal tone after 20 min equilibration and was present in the perfusate thereafter; the effectiveness of this treatment was confirmed after 10 min by establishing that vasoconstrictor responses to stimulation of sympathetic nerves were abolished. The tone of the preparation was then raised by the addition of methoxamine to the perfusate to a final concentration of 3–300 μM. Transmural nerve stimulation (60 V, 0.1 ms, 1–12 Hz, for 30 s) elicited vasodilator responses due to activation of primary sensory afferents and subsequent release of sensory transmitter. These vasodilator responses could be abolished by capsaicin confirming their sensory origin.

Drugs were administered as 50 μl bolus injections via an injection port proximal to the tissue. Vasoconstrictor responses to increasing doses of NA and ATP were established at basal tone at 3 min intervals or after tone had returned to baseline. Vasodilator responses to increasing doses of ACh, CGRP and SNP were established after the tone of the preparation had been raised with methoxamine. This was followed by a single dose of capsaicin (50 pmol). Intervals between doses were determined by the time it took for tone to return to its preconstricted level. Constrictor responses to potassium chloride (KCl, 0.15 mmol), at basal tone (following washout of methoxamine), were established at the end of each experiment as a measure of the contractile potential of the vascular smooth muscle.

Drugs

Acetylcholine chloride, sodium nitroprusside, noradrenaline bitartrate, methoxamine hydrochloride and capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) were obtained from Sigma, Poole, Dorset. Calcitonin gene-related peptide was from CRB Ltd., Cambridge. All drugs were made up in distilled water, except for noradrenaline, which was made up as a 10 mM stock solution in 0.1 mM ascorbic acid. Streptozotocin was donated by the Division of Cancer Treatment, National Institutes of Health, Bethesda, MD, U.S.A. Gangliosides were from Fidia Research Laboratories, Albano Terme, Italy.

Data analysis

All results were expressed as the mean ± s.e. Data analysis was done by analysis of variance, followed by Tukey's test to see where the differences lie. $P < 0.05$ was taken as significant.

Results

Diabetic model

The untreated controls gained weight during the 8 week period, reaching a final body weight of 566 ± 19.8 g ($n = 6$). Ganglioside-treated control rats weighed 611 ± 13.2 g ($n = 6$). Both the diabetic rats and the diabetic rats treated with gangliosides lost weight to a similar extent. Final body weight of the diabetic rats was 386.8 ± 27.1 g ($n = 6$) and of ganglioside-treated diabetic rats was 397.3 ± 16.3 g ($n = 6$). All diabetic rats used in the present study were severely hyperglycaemic with blood glucose levels of 43.4 ± 3.7 mmol l^{-1} (diabetics, $n = 6$) and 51.07 ± 2.9 mmol l^{-1} (ganglioside-treated diabetics, $n = 6$). Blood glucose levels of control rats

were 9.93 ± 0.7 mmol l^{-1} (controls, $n = 6$) and 12.17 ± 0.6 mmol l^{-1} (ganglioside-treated controls, $n = 6$). Distension of the large and small intestines and the presence of pale watery stools were observed at the time of death as previously described (Belai & Burnstock, 1990).

Basal tone

Diabetic and ganglioside-treated diabetic rats had significantly lower basal perfusion pressures than the control and ganglioside-treated control groups. Basal perfusion pressures were : 28.42 ± 1.6 ($n = 12$), 30.67 ± 1.9 ($n = 12$), 25.55 ± 0.8 ($n = 11$) and 22.58 ± 1.5 ($n = 12$) mmHg in mesenteric beds from control, ganglioside-control, diabetic and ganglioside-diabetic rats respectively. There was no significant difference between the groups with respect to vasoconstrictor responses to 0.15 mmol KCl. Values obtained were: controls, 55.75 ± 7.8 mmHg ($n = 12$); ganglioside-treated controls, 60.09 ± 7.9 ($n = 11$); diabetics, 53.91 ± 7.4 ($n = 11$); ganglioside-treated diabetics, 62 ± 6.3 ($n = 12$).

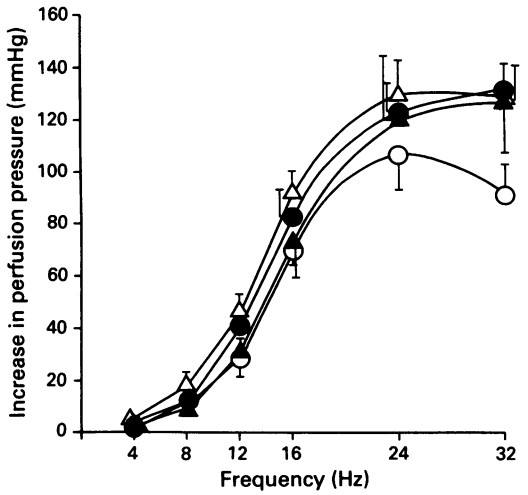

Figure 1 Frequency-response curves showing vasoconstrictor responses (increase in perfusion pressure, mmHg) of rat mesenteric arterial beds to electrical field stimulation of sympathetic nerves (4–32 Hz, supramaximal voltage, 1 ms, for 30 s): (●) control ($n = 6$); (▲) ganglioside-control ($n = 6$); (○) streptozotocin-diabetic ($n = 6$); (△) ganglioside-treated streptozotocin-diabetic ($n = 5$).

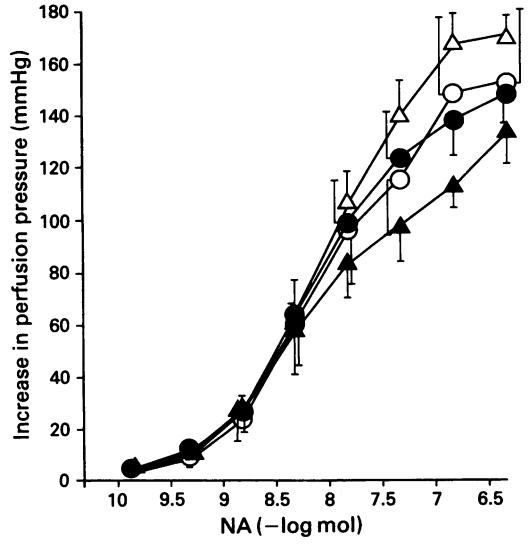

Figure 2 Dose-response curves showing vasoconstrictor responses (increase in perfusion pressure, mmHg) of rat mesenteric arterial beds to exogenous noradrenaline (NA): (●) control ($n = 6$); (▲) ganglioside-control ($n = 6$); (○) streptozotocin-diabetic ($n = 6$); (△) ganglioside-treated streptozotocin-diabetic ($n = 6$).

Sympathetic nerve stimulation

Stimulation of sympathetic nerves at basal tone elicited vasoconstrictor responses of the mesenteric vascular beds. There was no significant difference in vasoconstrictor responses between the groups (Figure 1).

Responses to noradrenaline

Mesenteric vasoconstrictor responses to NA were not different between the groups (Figure 2).

Raised-tone

There was no difference in the amount of tone produced by methoxamine in the raised-tone preparations, or in the concentration of methoxamine required to produce this degree of tone. Values for raised tone were: control, 65.65 ± 5.5 mmHg ($n = 12$); ganglioside-treated controls, 61.07 ± 6.7 mmHg ($n = 11$); diabetics, 55.4 ± 6.0 mmHg ($n = 11$); ganglioside-pretreated diabetics, 60.05 ± 5.8 mmHg ($n = 12$).

Sensory-motor nerve stimulation

Electrical field stimulation of mesenteric beds in the presence of guanethidine and methoxamine elicited frequency-dependent vasodilatation. Mesenteric beds from diabetic and ganglioside-treated diabetic rats produced significantly smaller vasodilator responses than did preparations from control and ganglioside-treated groups at 2, 4 and 8 Hz (Figures 3 and 4).

Responses to calcitonin gene-related peptide and capsaicin

There was no significant difference in vasodilator responses to CGRP or to capsaicin between the groups (Figure 5).

Responses to acetylcholine and sodium nitroprusside

There were no significant differences in vasodilator responses to ACh (Figure 6) or to SNP (Figure 7) between the groups.

Discussion

Diabetic neuropathy typically involves detrimental changes in autonomic, sensory and motor nerves. Defective axonal transport is believed to be the critical initiating factor in degenerative distal neuropathies. The present study provides a functional correlate for morphological and electrophysiological evidence for peripheral diabetic sensory neuropathy by showing that the vasodilator function of sensory-motor nerves in rat mesenteric arteries is severely impaired in streptozotocin-induced diabetes. Interestingly, we have shown in a previous study that chronic acrylamide treatment, suggested to produce similar peripheral neuropathies to diabetes, was also associated with impaired sensory-motor nerve function in the rat mesenteric arterial bed (Ralevic *et al.*, 1991).

The principal transmitter associated with the motor (efferent) function of sensory-motor nerves in the rat mesenteric arterial bed is CGRP (Kawasaki *et al.*, 1988). In the present study, while marked attenuation of sensory-motor nerve-mediated vasodilatation was observed, vasodilator responses to exogenous CGRP were not significantly different betweent the groups, indicating that streptozotocin-induced diabetes had caused peripheral sensory neuropathy and not a dysfunction of the postjunctional receptor for CGRP. There was no differences between the groups with respect to vasodilator responses to the sensory neurotoxin, capsaicin, used at a dose producing vasodilator responses of similar magnitude to those produced by electrical field stimulation at 8 Hz. Electrical stimulation of primary sensory afferents pro-

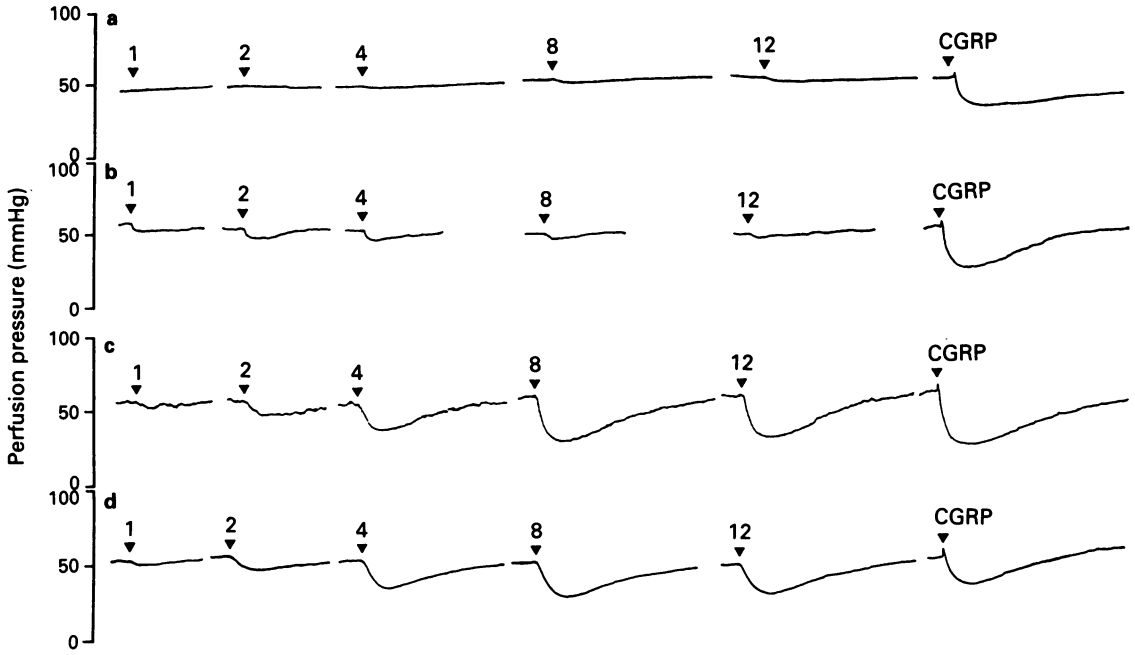

Figure 3 Traces showing frequency-dependent vasodilator responses of rat mesenteric arterial beds to electrical field stimulation of sensory-motor nerves (1–12 Hz, 60 V, 0.1 ms, for 30 s) in the presence of guanethidine (3 μM) and methoxamine. Similar vasodilator responses to a bolus dose of exogenous calcitonin gene-related peptide (CGRP, 50 pmol) were produced between the groups. Mesenteric beds from: (a) streptozotocin-diabetic; (b) ganglioside-treated streptozotocin-diabetic; (c) control; (d) ganglioside-treated control.

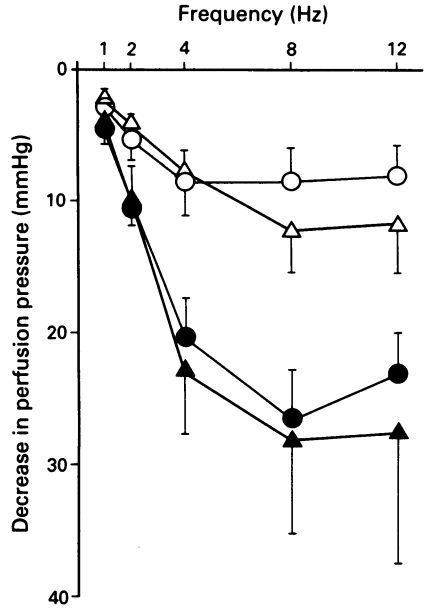

Figure 4 Frequency-response curves showing vasodilator responses of mesenteric arterial beds from control (●, $n = 6$); ganglioside-treated control (▲, $n = 5$); streptozotocin-diabetic (○, $n = 6$); ganglioside-treated streptozotocin-diabetic (△, $n = 5$) rats to sensory-motor nerve stimulation (1–12 Hz, 60 V, 0.1 ms, for 30 s) in the presence of guanethidine and methoxamine.

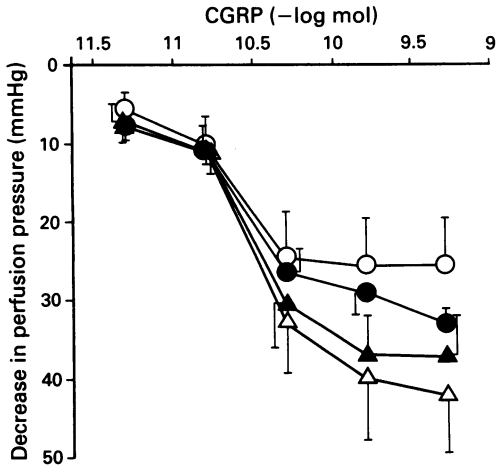

Figure 5 Dose-response curves showing vasodilator responses of rat mesenteric arterial beds to exogenous calcitonin gene-related peptide (CGRP). Control (●, $n = 6$); ganglioside-treated control (▲, $n = 5$); streptozotocin-diabetic (○, $n = 5$); ganglioside-treated streptozotocin-diabetic (△, $n = 6$).

duces antidromic invasion of sensory nerve terminals thus mimicking the axon reflex, while capsaicin stimulates the terminals of these nerves via specific receptors coupled to non-selective cation channels (Maggi & Meli, 1988). The different responses of mesenteric sensory-motor nerves to electrical stimulation, but similar responses to capsaicin which were evident between streptozotocin-diabetic and control rats indicates that the sensory defect produced by streptozotocin-diabetes is related to mechanisms which can be differentiated by physiological and non-physiological release processes.

There has been some interest in the neuroprotective role of gangliosides in diabetes following demonstrations that they are associated with prevention of the development of several neurological defects associated with short-term experimental diabetes, including axonal transport of 6-phosphofructokinase (Calcutt *et al.*, 1988) and acetylcholinesterase (Marini *et al.*, 1986), and in addition are involved in nerve regeneration (Triban *et al.*, 1989; Ekstrom & Tomlinson, 1990). On the other hand, ganglioside treatment was found not to improve impaired axonal transport of substance P in the rat sciatic nerve (Calcutt *et al.*, 1990). The mechanisms of action of gangliosides are not clearly understood but this latter study indicates that their effects are selectively beneficial

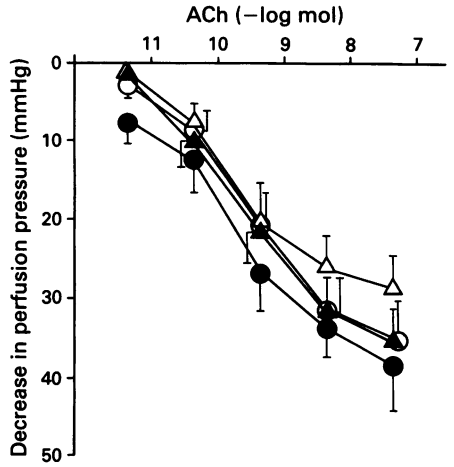

Figure 6 Dose-response curves showing vasodilator responses of rat mesenteric arterial beds to exogenous acetylcholine (ACh). Control (●, $n = 5$); ganglioside-treated control (▲, $n = 4$); streptozotocin-diabetic (○, $n = 6$); ganglioside-treated streptozotocin-diabetic (△, $n = 6$).

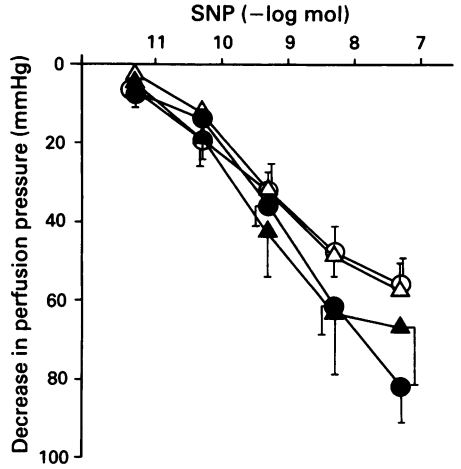

Figure 7 Dose-response curves showing vasodilator responses of rat mesenteric arterial beds to exogenous sodium nitroprusside (SNP). Control (●, $n = 6$); ganglioside-treated control (▲, $n = 6$); streptozotocin-diabetic (○, $n = 6$); ganglioside-treated streptozotocin-diabetic (△, $n = 6$).

and may explain why, in the present study, impaired mesenteric sensory-motor nerve-mediated vasodilator responses were not improved by ganglioside treatment.

The effects of experimental diabetes on sympathetic nerves, forming the principal vasoconstrictor nerve supply to mesenteric blood vessels, have been more extensively studied than have its effects on sensory-motor nerves. Other workers, also using the *in vitro* rat mesenteric arterial bed preparation, have shown that sympathetic neuropathy is a consequence of streptozotocin-induced diabetes (Longhurst & Head, 1985; Takiguchi *et al.*, 1988). In these and our study rats were used at a similar time after induction of diabetes; however, in the former studies diabetes was induced in rats weighing 200–275 g, while in our study they were 400–450 g. It is possible that the age of induction of diabetes is critically related to the susceptibility and hence to the severity of diabetes. An implication of this is that sensory-motor nerves are more susceptible to the effects of diabetes than are sympathetic nerves, thus sensory-motor dysfunction may represent an early stage in diabetic mesenteric neuropathy.

The significant decrease in basal tone of mesenteric beds from streptozotocin-induced diabetic rats seen in the present study may be a reflection of sympathetic neuropathy. However, we found that vasoconstrictor responses to electrical stimulation of sympathetic nerves were preserved. It is possible that stimulation at parameters necessary to elicit sympathetic vasoconstrictor responses may overcome subtle differences in the content and/or release of transmitter from sympathetic nerves. An alternative explanation for this apparent discrepancy involves the dynamic balance between vasoconstrictor sympathetic nerves and vasodilator sensory-motor nerves; transmural stimulation of the mesenteric bed produces responses which are the result of activation of both of these types of nerves. Hence, it is possible that sympathetic neuropathy was masked by a corresponding sensory neuropathy. On the other hand, the difference in basal tone may be unrelated to sympathetic neuropathy *per se*, but may reflect an adaptive increase in vessel calibre occurring as a consequence of the hyperphagia and marked increase in mesenteric blood flow characteristic of streptozotocin-induced diabetes. In this respect it has been reported that an enlargement of mesenteric nerves and arteries accompanies hypertrophy of the rat gut (Cracco & Gabella, 1991).

It is unlikely that the observed decrease in basal tone of the diabetic preparations seen in the present study is due to changes in the smooth muscle since vasoconstrictor responses to exogenous NA and KCl were unchanged. While this is in agreement with a recent study of the isolated mesenteric arterial bed by Furman & Sneddon (1993), the literature is variable regarding the effects of diabetes on rat mesenteric vascular responses to exogenous agents including NA and KCl, with increased (Agrawal & McNeil, 1987; Abebe & MacLeod, 1990; Abebe *et al.*, 1990; White & Carrier, 1990) or decreased (Longhurst & Head, 1985; Takiguchi *et al.*, 1988; Andersson *et al.*, 1992) contractile responses also having been shown. Some of the variability characteristic of the literature on the effects of diabetes on vascular function is likely to be due to differences in the duration of diabetes and in the different susceptibility of different vascular beds. However, it is difficult to understand why such discrepancies are also commonly reported between different groups using the same vessel and similar methods and time of induction of diabetes. The reason for these discrepancies remains unexplained.

In the present study there was no evidence for dysfunction of endothelial or smooth muscle vasodilator mechanisms. With respect to the effects of vasoconstrictor and vasodilator (endothelium-dependent and -independent) agents our results are similar to those of Furman & Sneddon (1993) who also found no evidence for changes in vascular responsiveness of the isolated rat mesenteric arterial bed after long-term (15–17 weeks) streptozotocin-induced diabetes. Furthermore, these results are compatible with those of a recent *in vivo* study by Kiff *et al.* (1991) who demonstrated raised mesenteric vascular conductance in streptozotocin-diabetic rats and no differences in endothelium-dependent vasodilatation to ACh. Attenuated relaxations to endothelium-dependent vasodilators, but generally unimpaired endothelium-independent relaxations have been demonstrated in clinical (De Tejada *et al.*, 1989) and a variety of experimental (Takiguchi *et al.*, 1988; Kamata *et al.*, 1989; Mayhan, 1992; Miyata *et al.*, 1992; Taylor *et al.*, 1992) diabetes, while endothelium-independent relaxations are generally unimpaired. It is possible that diabetes affects the different components of the vasculature with a different time course, with neuropathy occurring before the onset of endothelial changes. In this respect, Takiguchi and coworkers (1988) showed that sympathetic neuropathy in streptozotocin-induced diabetes (seen at 8 weeks after injection with streptozotocin) occurred before functional evidence for endothelial damage (occurring at 12 weeks).

In conclusion, the results of the present study provide evidence that sensory-motor nerve vasodilator function is impaired in mesenteric arteries of streptozotocin-diabetic rats. There was no evidence for a change in the postjunc-

tional receptor for CGRP. The significantly lower basal perfusion pressure shown by mesenteric beds from diabetic rats may be indicative of sympathetic neuropathy, but there was no evidence for differences in vasoconstrictor responses to electrical stimulation of sympathetic nerves. Chronic streptozotocin-induced diabetes did not modify vasoconstrictor responses to NA or KCl. Moreover, neither endothelium-dependent or endothelium-independent vasodilator responses were altered compared to age-matched controls. Ganglioside treatment did not modify any aspect of vascular function in our model. These results suggest that sensory-motor nerves may be more susceptible to diabetic neuropathy than sympathetic perivascular nerves, and these changes may precede other changes commonly reported in diabetes such as changes in the vascular endothelium.

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Effects of β-adrenoceptor agonists in human bronchial smooth muscle

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1 We have investigated the potency and duration of action of isoprenaline and a range of β-adrenoceptor agonists as relaxants of inherent tone in human superfused, isolated bronchial smooth muscle, a tissue reported to contain a homogeneous population of β_2-adrenoceptors.

2 All of the β-adrenoceptor agonists caused concentration-related inhibition of inherent tone, with isoprenaline having an EC_{50} of 27 nM. The rank order of agonist potency was: formoterol ⩾ salmeterol ⩾ clenbuterol > fenoterol = isoprenaline > terbutaline ⩾ salbutamol > quinprenaline.

3 Relaxant responses to salmeterol were fully reversed by the selective β_2-adrenoceptor blocking drug, ICI 118551, demonstrating the involvement of β_2-adrenoceptors.

4 Rt_{50}, i.e. the time taken for 50% recovery from the effects of an EC_{50} concentration of agonist, differed considerably between the different β_2-adrenoceptor agonists. Most agonists were short-acting, having Rt_{50} values less than 13 min. Quinprenaline was of moderate duration, with an Rt_{50} value of ⩾ 20 min. In contrast, salmeterol was extremely long-acting, with no sign of recovery within 4 h.

5 Estimates of relative potency and duration of action were similar to those previously determined for these agonists in the guinea-pig isolated trachea. These results suggest, therefore, that guinea-pig trachea is a suitable alternative to human bronchus for the evaluation of the actions of β-adrenoceptor agonists on airways smooth muscle.

Keywords: β-Adrenoceptor agonists; salmeterol; formoterol; human bronchus; inherent tone; relaxation; duration of action

Introduction

The guinea-pig isolated trachea has been widely used to evaluate the actions of β-adrenoceptor agonists on airways smooth muscle *in vitro* (Castillo & DeBeer, 1947; Foster, 1966; Coleman & Nials, 1986). We have previously used the electrically-stimulated superfused preparation to identify the potent and long-acting β_2-adrenoceptor agonist, salmeterol (Ball *et al.*, 1991) and subsequently, to investigate its mechanism of action (Nials *et al.*, 1993).

More recently, we have attempted to carry out similar studies with β-adrenoceptor agonists on electrically-stimulated human superfused bronchial smooth muscle (Coleman *et al.*, 1993), a tissue which has previously been shown to contain a homogeneous population of β_2-adrenoceptors which mediate relaxation (Goldie *et al.*, 1984). However, the presence of inherent tone in this tissue complicates the evaluation of the relaxant activity of β-adrenoceptor agonists. On the other hand, such inherent tone in human bronchial smooth muscle is well-maintained, resistant to the effects of indomethacin, atropine and phenoxybenzamine (Rabe *et al.*, 1992a; Coleman *et al.*, 1993) and stable for many hours. Inherent tone may, therefore, be suitable for the study of both the potency and the duration of action of spasmolytic agents.

We now report the potency and duration of action of a range of β-adrenoceptor agonists as relaxants of inherent tone in human bronchial smooth muscle.

Methods

Tissue preparation

Samples of human bronchus were obtained from patients undergoing surgical resection of the lung. No account was taken of previous drug therapy. Bronchial tissue was carefully dissected clear of lung parenchyma and vascular tissue and placed in a modified Krebs solution. The composition of the Krebs solutions was as described by Apperley *et al.* (1976). Bronchus of lumen diameter 2–4 mm was cut into rings of 2–3 mm width, which were then opened to form strips. The preparations were either used immediately or stored overnight in oxygenated (5% CO_2 in O_2) Krebs solution at 4°C.

The preparations were mounted in superfusion chambers by tying a cotton thread to one end for attachment to a strain gauge, and a cotton loop to the other end for anchoring the tissue within the chamber. The superfusion apparatus employed in these experiments has been described previously (Coleman *et al.*, 1986; Coleman & Nials, 1989). The preparations were mounted under a resting tension of 1 g, and superfused at a rate of 2 ml min^{-1} with oxygenated (5% CO_2 in O_2) modified Krebs solution maintained at 37°C and containing indomethacin (2.8 μM) to inhibit endogenous prostanoid synthesis. In order to demonstrate the presence of inherent tone, a single concentration of isoprenaline (100 nM) was infused on to each preparation. Experiments were carried out on preparations which underwent changes in tension of greater than 0.2 g in response to the isoprenaline infusion.

Determination of agonist potency

The relaxant activities of β-adrenoceptor agonists were measured against inherent tone. For each preparation, two cumulative concentration-effect curves to the standard β-adrenoceptor agonist, isoprenaline, were obtained by infusing increasing concentrations onto the tissue until the response to each concentration was maximal. Tissues were superfused with agonist-free Krebs solution for 30 min between curves. A cumulative concentration-effect curve to a test agonist was then constructed in an identical fashion. The magnitude of each response was measured and calculated as a percentage

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of the maximum isoprenaline response obtained in the final control curve.

Potency values for the β-adrenoceptor agonists were expressed in both absolute terms (concentration required to induce 50% of the maximum response to each agonist, EC_{50}) and relative to isoprenaline, as an equieffective concentration (EEC, i.e. EC_{50} for the test agonist/EC_{50} for isoprenaline). An EEC of less than unity indicates greater potency than isoprenaline, and an EEC greater than unity, a lower potency than isoprenaline.

Determination of the times for onset and offset of action

A single concentration-effect curve to isoprenaline was constructed by infusing increasing concentrations onto the tissue preparations in a sequential fashion. This was achieved by infusing each concentration until peak effect was attained, the infusion then being stopped and the tissues allowed to recover before the next concentration was administered. A curve to the test agonist was then constructed in a similar fashion. However, if no recovery was observed from the relaxant responses to the test agonist, the experimental protocol was altered. Thus, long-acting β_2-agonists (i.e. salmeterol and quinprenaline) were evaluated on paired preparations, in each case a sequential concentration-effect curve to isoprenaline first being constructed. Following this, a single concentration was added to each preparation. The concentration of test agonist chosen was that which caused a response approximately 20–40% of maximum on one preparation and 60–80% of maximum on the other. A composite, two point concentration-effect plot spanning the EC_{50} was then constructed.

Onset time (Ot_{50}) is defined as the time from administration of an EC_{50} of an agonist to attainment of 50% maximal response. Recovery (Rt_{50}) is defined as the time from stopping administration of the test agonist to attainment of 50% recovery from the EC_{50}. Ot_{50} and Rt_{50} values were determined by interpolation from a plot of % response against time to attainment of 50% of each response (for Ot_{50}), or of 50% recovery from the response (for Rt_{50}; Coleman & Nials, 1989).

Drugs used

The following compounds were used: ascorbic acid (BDH Chemicals, UK), atropine sulphate (Sigma, UK), clenbuterol (Glaxo Group Research, UK), fenoterol (Sigma, UK), formoterol (Glaxo Group Research, UK), ICI 118551 (erythro-DL-1 (7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, ICI Pharmaceuticals, UK), indomethacin (Sigma, UK), isoprenaline sulphate (Sigma, UK), mepyramine maleate (May & Baker, UK), papaverine (Sigma, UK), propranolol hydrochloride (Sigma, UK), quinprenaline (Pfizer, USA), salbutamol (Glaxo Group Research, UK), terbutaline (Glaxo Group Research, UK).

β-Adrenoceptor agonists were dissolved in 2 drops of glacial acetic acid and diluted to stock concentrations with phosphate buffer (pH 7.0). Dilutions of stock concentrations were made with 0.9% w/v saline. To prevent the autooxidation of isoprenaline and to maintain identical vehicle constituents, all solutions of β-adrenoceptor agonists contained ascorbic acid (11 μM).

Results

Agonist potency

Isoprenaline (1–300 nM), formoterol (0.3–100 nM), salbutamol (10–3000 nM) and salmeterol (0.3–30 nM), caused concentration-related relaxations of inherent tone in human bronchial smooth muscle. The results are summarized in Table 1, and composite cumulative concentration-effect curves are shown in Figure 1. There were no marked differences between the maximum responses obtained to isoprenaline, formoterol and salbutamol. Maximum responses were not achieved by salmeterol over the concentration-range tested, and relaxations at the highest concentration (30 nM) used were consistently lower than the isoprenaline maximum at 300 nM. The rank order of potency was: formoterol ≥ salmeterol > isoprenaline > salbutamol.

Potency, and times for onset and offset of action

In these experiments, two concentrations of each agonist (one causing < 50% relaxation, the other causing > 50% relaxation) were infused on one of each pair of tissues and potency, time for onset of action and time for offset (duration) of action were determined. The results are summarized in Table 2. Although this method is less precise for determination of agonist potency than that described in the previous section, the rank order of potency for the agonists was similar. Thus, under these conditions, formoterol and salmeterol were 20 and 11 fold more potent than isoprenaline, whilst salbutamol was nine fold less potent than isoprenaline. Quinprenaline was a partial agonist of low potency, being at least 140 fold less active than isoprenaline and achieving only 40–50% of the isoprenaline maximum response. In one experiment, the relaxant response to quinprenaline was biphasic in nature, in that a second phase of relaxation was observed following termination of the infusion of the agonist.

Onset of action

Relaxant responses to isoprenaline, salbutamol, terbutaline, clenbuterol, fenoterol and formoterol were all rapid, Ot_{50} values ranging from 1.5–5.3 min (Table 2). However, relaxant responses to salmeterol were significantly slower, with a mean Ot_{50} value of ~36 min. A precise Ot_{50} value for quinprenaline could not be determined, mainly because of the shallow nature of the responses.

Table 1 Human isolated bronchus; relaxant potencies of a range of β-adrenoceptor agonists

Agonist	*Equieffective concentration* (Isoprenaline = 1)	n
Isoprenaline	1.0 (EC_{50} = 24.4 (17.7–35.6) nM)	8
Formoterol	0.08 (0.06–0.09)	7
Salbutamol	9.3 (4.1–21.2)	6
Salmeterol	0.15 (0.04–0.48)	4

Values are expressed as geometric means of (*n*) individual experiments (with 95% confidence limits).

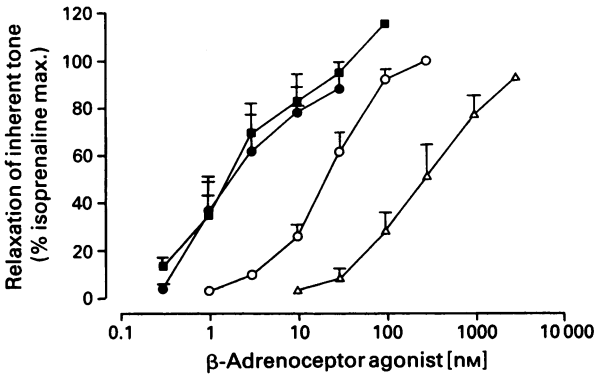

Figure 1 Human isolated, superfused bronchus; mean cumulative relaxant concentration-effect curves to isoprenaline (○), salmeterol (●), salbutamol (△) and formoterol (■). Each point is the mean ± s.e.mean of the least 4 determinations.

Table 2 Human isolated bronchus: relaxant potencies, onset of action and duration of action of a range of β-adrenoceptor agonists

Agonist	*Equieffective concentration* (Isoprenaline = 1)	*Onset* (Ot_{50} min)	*Recovery* (Rt_{50} min)	n
Isoprenaline	1.0 (EC_{50} = 26.6 (18.9–36.4) nM)	1.5 (± 0.1)	2.2 (± 0.3)	14
Clenbuterol	0.2 (0.02–1.36)	2.3 (± 0.9)	12.7 (± 5.4)	5
Fenoterol	0.9 (0.1–11.3)	2.1 (± 0.4)	4.6 (± 0.9)	4
Formoterol	0.05 (0.02–0.10)	5.3 (± 1.4)	6.6 (± 1.3)	5
Quinprenaline	141 [55–235]	–	⩾ 20 [20–>180]	3
Salbutamol	9 (2 – 44)	3.3 (± 0.3)	6.8 (± 1.3)	5
Salmeterol	0.09 (0.06 – 0.13)	35.6 (± 4.6)	>275	8
Terbutaline	5.4 (3.9 – 7.6)	3.0 (± 0.4)	6.9 (± 1.9)	4

Potency values are expressed as geometric means (with 95% confidence limits) or [range]. Onset and duration values are expressed as arithmetic means (± s.e.mean) or [range].

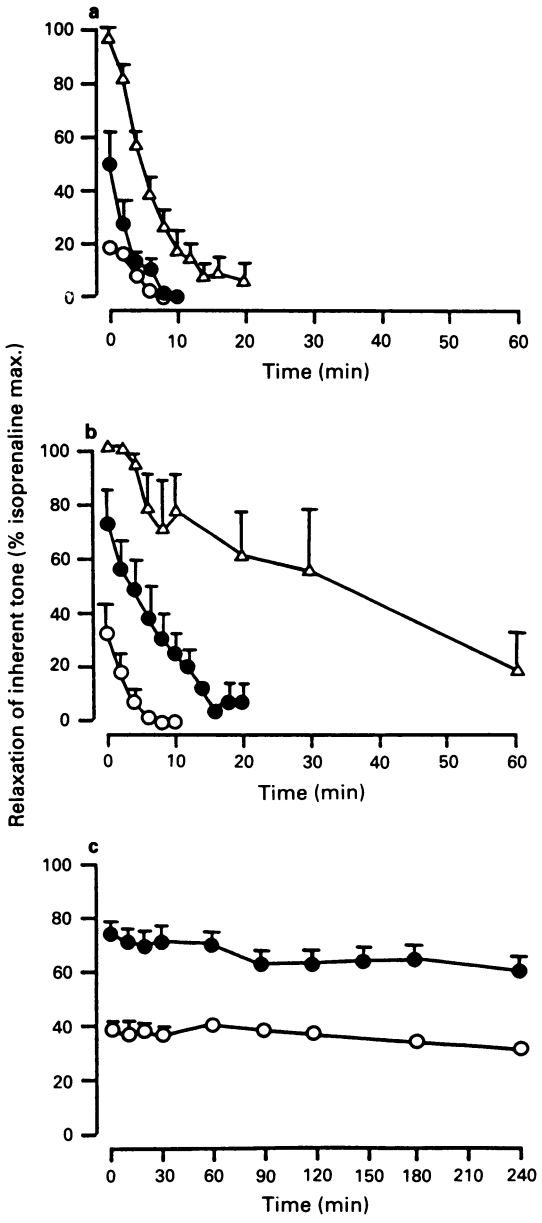

Figure 2 Human, isolated, superfused bronchus: relationship between duration of action of relaxant responses and concentration of agonist (a) salbutamol (10 nM, ○; 100 nM, ●; 1 000 nM, △); (b) formoterol (1 nM, ○; 10 nM, ●; 100 nM, △); and (c) salmeterol (1 nM, ○; 10 nM, ●) Data are expressed as arithmetic means ± s.e.mean.

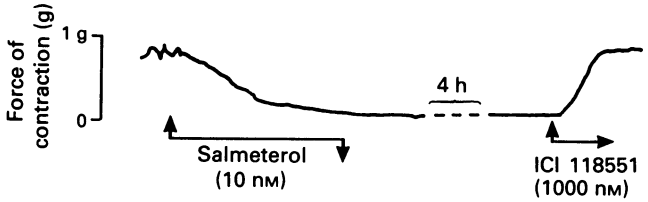

Figure 3 Human, isolated, superfused bronchus: a typical relaxant response to an infusion of salmeterol and its subsequent reversal by infusion of the β_2-adrenoceptor blocking drug, ICI 118551.

Duration of action

The relaxant responses to isoprenaline, salbutamol, terbutaline, clenbuterol, fenoterol and formoterol were short-lived, with Rt_{50} values ranging from 2.2–12.7 min. The duration of action of quinprenaline was somewhat longer, with an Rt_{50} value of ⩾ 20 min. In contrast, the effects of salmeterol were long-lasting, with an Rt_{50} value of >275 min, no appreciable recovery being observed during the time course of the experiment (Figures 2c and 3).

The durations of action of both salbutamol and formoterol were concentration-related (Figure 2a and b). At a concentration of 10 nM, relaxant responses to formoterol ranged from 70–85% of the isoprenaline maximum, and were of 4–13 min duration following wash out. As the concentration was increased ten fold, responses were all ⩾ 100%, and the duration increased to 54–196 min. In contrast, the duration of action of salmeterol was not concentration-related, as little or no recovery was obtained for at least 240 min from concentrations (1, 10 nM), producing approximately 40% and 75% relaxation respectively (Figure 2c).

The relaxant responses to salmeterol were, however, fully reversed by infusions of the β-adrenoceptor blocking drugs, (±)-propranolol (0.1–1 μM) or ICI 118551 (0.1–1 μM; Figure 3).

Discussion

The aim of these experiments was to evaluate the potency, onset and duration of action of a range of β-adrenoceptor agonists in human bronchial smooth muscle *in vitro*, and to compare the results with those previously obtained in guinea-pig trachea. The β-adrenoceptors mediating relaxation of human bronchial smooth muscle have previously been shown to be of the β_2-subtype (Goldie *et al.*, 1984).

The guinea-pig trachea has been extensively used to study the potency and duration of action of a wide range of β-adrenoceptor agonists (Castillo & DeBeer, 1947; Foster, 1966; Coleman & Nials, 1986), and was particularly impor-

tant in the identification of novel, long-acting β_2-adrenoceptor agonists such as salmeterol (Ball *et al.*, 1991). Guinea-pig tracheal smooth muscle exhibits a pronounced level of inherent tone, which is mediated almost exclusively by prostanoids. The evaluation of β-adrenoceptor agonists against this tone in guinea-pig trachea is complicated by the fact that they can enhance prostanoid release in this preparation (Coleman & Farmer, 1971; Farmer *et al.*, 1972) which would tend to offset their relaxant activity. For the evaluation of long-acting β_2-adrenoceptor agonists, such an effect could make the results difficult to interpret. Spasmogen-induced contractions of guinea-pig trachea preparations, in the presence of a cyclo-oxygenase inhibitor such as indomethacin to prevent endogenous prostanoid release, are poorly maintained for periods in excess of 30 min, and are therefore similarly unsuitable for the evaluation of long-acting β_2-adrenoceptor agonists. For this reason, we developed the electrically-stimulated preparation on which the potencies and durations of action of such agonists can be determined for periods of at least 7 h (Ball *et al.*, 1991).

In order, therefore, to compare the profiles of β-adrenoceptor agonists in human bronchus with those in guinea-pig trachea, we attempted to use electrical stimulation. Initial experiments showed, however, that despite the inclusion of indomethacin in the bathing solution, human bronchial preparations still exhibit marked inherent tone and as a result, β-adrenoceptor agonists not only inhibited the electrically-induced contractile responses, but also relaxed the inherent tone. Furthermore, the inherent tone was more sensitive to the relaxant effects of β-adrenoceptor agonists, and at low concentrations, while an inhibitory effect on inherent tone was observed; this was associated with an apparent enhancement of electrically-induced contractions (Coleman *et al.*, 1993). This same phenomenon has previously been observed in guinea-pig trachea, but inherent tone in this tissue can be abolished by indomethacin thereby enabling this complication to be overcome (Coleman & Farmer, 1971; Farmer *et al.*, 1972).

The nature of inherent tone in human bronchial smooth muscle is unknown, although it has variously been reported to be due to prostanoids, histamine and 5-lipoxygenase products (Ito *et al.*, 1989). In our studies, although we included the cyclo-oxygenase inhibitor, indomethacin, and also phenoxybenzamine, which among its range of effects, is a histamine H_1-receptor blocking drug, we failed to inhibit the inherent tone. We concluded, therefore, that the electrically-stimulated preparation of human bronchus is not suitable for the evaluation of the airway relaxant effects of β-adrenoceptor agonists. However, we and others (Goldie *et al.*, 1984; Chideckel *et al.*, 1987; Ito *et al.*, 1990; Rabe *et al.*, 1992a), have found that inherent tone in the human airway is particularly stable and therefore permits the evaluation of both the potency and duration of action of spasmolytic drugs. Therefore, in the present study, we have used inherent tone of the human bronchus to evaluate the relaxant activity of β-adrenoceptor agonists.

Although we have evaluated β-adrenoceptor agonists against electrically-induced contractions in guinea-pig trachea, and against inherent tone in human bronchus, the results obtained are similar. Thus, as on guinea-pig trachea, all of the β-adrenoceptor agonists relaxed human bronchus in a concentration-related manner, and the rank order of agonist potency is the same in both preparations, with formoterol, salmeterol and clenbuterol being more potent than isoprenaline, fenoterol being approximately equipotent with isoprenaline, and the other agonists all being weaker. As in guinea-pig trachea, quinprenaline was more than 100 fold weaker than isoprenaline. This rank order of agonist potency and the observation that relaxant responses to salmeterol were reversed by the β_2-adrenoceptor blocking drug, ICI 118551, support the conclusion that relaxation of human bronchial smooth muscle is mediated by β_2-adrenoceptors (Goldie *et al.*, 1984). Interestingly, in one preparation, quinprenaline produced a biphasic effect, as previously reported for guinea-pig airways (Nials *et al.*, 1991), with only partial relaxation being observed on addition of the agonist, but a further complete relaxation developing following termination of infusion. This is the first report of the occurrence of this phenomenon in human airways. The explanation for such biphasic activity is not clear, but it has been suggested that quinprenaline induces a permanently-activated conformation of the β_2-adrenoceptor protein, which only manifests itself after removal of the agonist from the biophase (Jack, 1991). However, there is as yet no conclusive evidence that this actually occurs.

In addition to the similarity in both the absolute and relative agonist potencies of the various β-adrenoceptor agonists in guinea-pig and human tissue, there is also a similarity in their respective durations of action. As in guinea-pig tissues, salmeterol is by far the longest-acting agonist tested, although we did not attempt to follow the duration for such an extended period as previously carried out (Ball *et al.*, 1991). Nevertheless, it is clear that responses to salmeterol in human bronchial smooth muscle persist without decline for periods in excess of 4.5 h, despite continuous washing with agonist-free medium. Although quinprenaline is approximately 1000 fold weaker than salmeterol, it also exhibits an extended duration of action, responses persisting for periods of at least 20 min. This result is similar to that previously reported by Nials *et al.* (1990) on guinea-pig trachea. All of the other β-adrenoceptor agonists tested were of short duration, none having an Rt_{50} in excess of 13 min. The fact that these results closely resemble those previously obtained in guinea-pig trachea, both qualitatively and quantitatively, is encouraging in that it suggests that guinea-pig trachea may be predictive of the human bronchus in terms of *in vitro* responses to β-adrenoceptor agonists. However, the question remains of the utility of either preparation for the prediction of the *in vivo* bronchodilator activity of β_2-adrenoceptor agonists in man.

As far as salmeterol is concerned, both isolated preparations suggest that its effects at β_2-adrenoceptors are highly persistent, and it is now well established that salmeterol (50 μg) has a long duration of action after inhalation in man (Ullman & Svedmyr, 1988). Indeed, the duration of action after a single administration to asthmatic patients has been reported to be as long as 20 h (Rabe *et al.*, 1992b). Similarly, the *in vitro* data in both preparations with isoprenaline, salbutamol, clenbuterol, fenoterol and terbutaline are all consistent with their known relatively short durations of action in the clinic. One apparent inconsistency concerns formoterol. Although others have reported that formoterol has persistent relaxant effects in human bronchial smooth muscle (Advenier *et al.*, 1991), we have found this compound to be clearly short-acting *in vitro*, in both guinea-pig trachea (Nials *et al.*, 1990) and now in human bronchus (present study). There are two likely explanations for the persistent effects observed by Advenier *et al.* (1991): the first is that very high concentrations of formoterol were tested, and the other is that the authors used an immersion rather than a superfusion technique. The importance of the concentration used is apparent in the results of the present study, where we found that at low concentrations, there was little difference in the durations of action of salbutamol and formoterol, whereas at higher concentrations, the recovery time for formoterol was considerably extended when compared with that for salbutamol. The relevance of immersion rather than superfusion is that the washing of the tissues is likely to have been substantially less rigorous than that used in our studies; as formoterol is more lipophilic than salbutamol (but less than salmeterol), it is likely to wash out from superfused preparations rather more slowly than salbutamol, particularly with only intermittent washing as was used in the immersion experiments. Unlike salbutamol and formoterol, salmeterol is long-acting under both immersed and superfused conditions, irrespective of the concentration tested. No attempt was

made to determine the rates of recovery from the effects of the shorter-acting compounds, from which the kinetics of the process could possibly be derived, as rate is likely to be a complex function. A number of factors could contribute towards recovery rate, including receptor affinity and efficacy, and physico-chemical factors, such as membrane: water partition coefficients. Indeed the influence of concentration on the pattern of recovery from the responses to formoterol illustrate this, as the recovery from the highest concentration tested (100 nM), clearly does not follow first-order kinetics.

Formoterol is also relatively short-acting *in vivo* when administered by aerosol to conscious guinea-pigs, and in man, when administered by the oral route (Lofdahl & Svedmyr, 1989). However, it is also clear that after inhalation in man, formoterol exhibits a prolonged duration of action (Lofdahl & Svedmyr, 1989; Derom *et al.*, 1989; Larsson *et al.*, 1990). The explanation for the extended duration of effect, associated with a particular route of administration, almost certainly lies with the high local concentrations achieved in the lung after inhalation of the recommended therapeutic doses (12–24 μg). This would explain why we have demonstrated only a short duration of action in guinea-pigs after administration by the inhaled route, since these experiments were carried out using only threshold effective doses. The evidence suggests that the mechanism of the extended duration of action of formoterol is different from that of salmeterol, which exhibits a long duration of action, both *in vitro* and *in vivo* irrespective of dose and of route of administration.

In conclusion, therefore, we have shown that reduction in inherent tone of human superfused bronchial smooth muscle can be used to evaluate the potency and duration of action of β-adrenoceptor agonists. The results obtained with salmeterol are similar, both qualitatively and quantitatively, to those previously obtained in electrically-stimulated, superfused guinea-pig trachea and support its well-established clinical profile as a potent, long-acting bronchodilator (Ullman & Svedmyr, 1988). The similarity in the data with β-adrenoceptor agonists in the human and guinea-pig airways preparations suggests that the latter may be used instead of human bronchus in the *in vitro* evaluation of this class of compounds.

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Effects of a water-soluble forskolin derivative (NKH477) and a membrane-permeable cyclic AMP analogue on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of rabbit mesenteric artery

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1 Effects were studied of 6-(3-dimethylaminopropionyl) forskolin (NKH477), a water-soluble forskolin derivative and of dibutyryl-cyclic AMP, a membrane-permeable cyclic AMP analogue on noradrenaline (NA)-induced Ca^{2+} mobilization in smooth muscle strips of the rabbit mesenteric artery. The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), isometric force and cellular concentration of inositol 1,4,5-trisphosphate ($InsP_3$) were measured.

2 NA (10 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force in a solution containing 2.6 mM Ca^{2+}. NKH477 (0.01–0.3 μM) attenuated the phasic and the tonic increases in both $[Ca^{2+}]_i$ and force induced by 10 μM NA, in a concentration-dependent manner.

3 In Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+, NA (10 μM) produced only phasic increases in $[Ca^{2+}]_i$ and force. NKH477 (0.01 μM) and dibutyryl-cyclic AMP (0.1 mM) each greatly inhibited these increases.

4 NA (10 μM) led to the production of $InsP_3$ in intact smooth muscle strips and $InsP_3$ (10 μM) increased Ca^{2+} in Ca^{2+}-free solution after a brief application of Ca^{2+} in β-escin-skinned smooth muscle strips. NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) modified neither the NA-induced synthesis of $InsP_3$ in intact muscle strips nor the $InsP_3$-induced Ca^{2+} release in skinned strips.

5 In Ca^{2+}-free solution, high K^+ (40 and 128 mM) itself failed to increase $[Ca^{2+}]_i$ but concentration-dependently enhanced the amplitude of the increase in $[Ca^{2+}]_i$ induced by 10 μM NA with a parallel enhancement of the maximum rate of rise. The extent of the inhibition induced by NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) on the NA-induced $[Ca^{2+}]_i$ increase was inversely related to the maximum rate of rise of $[Ca^{2+}]_i$ induced by NA in Ca^{2+}-free solution containing various concentrations of K^+. These results suggest that the increase in the rate of Ca^{2+} release induced by NA can conceal the inhibitory action on NA-induced Ca^{2+} mobilization of agents that increase cyclic AMP.

6 Repetitive application of 10 μM NA in Ca^{2+}-free solution led to a disappearance of the NA-induced increase in $[Ca^{2+}]_i$, but NA could again increase $[Ca^{2+}]_i$ in Ca^{2+}-free solution after a brief application of Ca^{2+} with 40 mM K^+ ('Ca^{2+}-loading'). The magnitude of this NA-induced increase in $[Ca^{2+}]_i$ depended on the duration of the Ca^{2+}-loading. With application of dibutyryl-cyclic AMP (0.1 mM) during the Ca^{2+}-loading period, the loading duration required for the restoration of the maximum NA-response was shortened.

7 Cyclopiazonic acid (10 μM, an inhibitor of Ca^{2+}-ATPase at intracellular storage sites) attenuated the inhibitory action of dibutyryl-cyclic AMP on the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. When NA (10 μM) was applied twice for 30 s with a 10 min interval in Ca^{2+}-free solution, the amplitude of response to the second application was about one third of the first response. With application of 0.1 mM dibutyryl-cyclic AMP during the first application of NA, the increase in $[Ca^{2+}]_i$ induced by the first application of NA was inhibited, but the response induced by the second was enhanced. These results suggest that dibutyryl-cyclic AMP enhances Ca^{2+} uptake into the NA-sensitive storage sites.

8 We conclude that, in smooth muscle of the rabbit mesenteric artery, agents that increase cyclic AMP inhibit the NA-induced increase in $[Ca^{2+}]_i$ through an activation of Ca^{2+} uptake into the cellular storage sites.

Keywords: Cyclic AMP; forskolin derivative; vascular smooth muscle; agonist-induced synthesis of inositol 1,4,5-trisphosphate; agonist-induced Ca^{2+} release

Introduction

Activation of β-adrenoceptors in vascular smooth muscle inhibits agonist stimulated contraction by increasing the intracellular concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Bülbring & Tomita, 1987). Forskolin directly activates adenylate cyclase and has been used to study the roles of cyclic AMP in cellular functions in various types of cell (Seamon & Daly, 1983). A useful recent introduction (Hosono *et al.*, 1990) is the water-soluble forskolin derivative, N,N-dimetyl-β-alanine[3**R**-(3α, 4aβ, 5β, 6β, 6aα, 10α, 10aβ, 10bα)]-5(acetyloxy)-3-ethenyldodecahydro-10, 10b-dihydroxy-3, 4a, 7, 7, 10a-pentamethyl-1-oxo-1H-naphtho [2,1-b] pyran-6-yl ester hydrochloride (NKH477). This agent, like forskolin, directly activates adenylate cyclase and its potency has been found to be similar to, or greater than that of forskolin (Fujihara *et al.*, 1990). We recently found that, in smooth muscle of the porcine coronary artery, NKH477 is a more potent drug than forskolin for increasing

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the cellular concentration of cyclic AMP (Shafiq et al., 1992).

It has been found that agents that increase cyclic AMP produce vasodilatation with a decrease in $[Ca^{2+}]_i$ in the presence or absence of agonists (Bülbring & Tomita, 1987). This action is caused by an inhibition of Ca^{2+} influx (Muller & van Breemen, 1979; Meisheri & van Breemen, 1982), by an activation of Ca^{2+} sequestration (Itoh et al., 1982; Saida & van Breemen, 1984; Eggermont et al., 1988; Furukawa et al., 1988) or by an activation of Ca^{2+} extrusion to the extracellular space (Scheid et al., 1979). It has also been reported that cyclic AMP inhibits agonist-induced increases in $[Ca^{2+}]_i$ by reducing the synthesis of inositol 1,4,5-trisphosphate ($InsP_3$, Watson et al., 1984; Takenawa et al., 1986; Madison & Brown, 1988) or through the phosphorylation of the $InsP_3$ receptor (Supattapone et al., 1988; Yamamoto et al., 1989). However, the mechanism underlying the inhibition of agonist-induced Ca^{2+} mobilization by agents which increase cyclic AMP has not been well characterized in vascular smooth muscle.

To investigate this, we studied the effects of NKH477 and dibutyryl-cyclic AMP on the increases in $[Ca^{2+}]_i$ and force and $InsP_3$ synthesis induced by NA in smooth muscle strips of the rabbit mesenteric artery. The effects of NKH477 and dibutyryl-cyclic AMP on $InsP_3$-induced Ca^{2+} release were also studied in β-escin-skinned smooth muscle strips.

Methods

Male albino rabbits, weighing 1.9–2.3 kg, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.), and then exsanguinated. The third branch of the mesenteric artery was immediately excised and cleaned by removal of connective tissue in Krebs solution at room temperature.

Simultaneous recording of Ca^{2+} and force

To permit simultaneous recording of $[Ca^{2+}]_i$ and isometric force, 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 described previously (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 10 μM NA, as described previously (Itoh et al., 1992). The strip was transferred into a chamber of 0.3 ml volume and mounted horizontally on an inverted-microscope (Diaphot TMD with special optics for epifluorescence, Nikon). The resting force was adjusted so as to obtain a maximal contraction in 128 mM K^+.

To load Fura-2 into the smooth muscle cells of the strip, 1 μM acetoxy methyl ester of Fura-2 (Fura-2 AM) dissolved in dry dimethyl sulphoxide (1 mM stock solution) was applied for 1 h in Krebs solution at room temperature (23–25°C). The strip was moved to the centre of the field and a mask (0.04 mm square) placed in an intermediate image plane of the microscope to reduce background fluorescence. The Fura-2 fluorescence emission at 510 nm (passed through an interference filter centred at 510 nm with a full width at half transmission of 20 nm) was passed through the objective lens (20 x fluor, Nikon) and collected with a photomultiplier tube (R928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphot-TMD microscope. 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 were analyzed with customized software provided by Spex (DM-3000 CM).

The ratio of the Fura-2 fluorescence intensities excited by 340 and 380 nm was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) excited by 340 and 380 nm u.v.-light was measured after the experiment 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). 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; Becker et al., 1989). The ratio of maximum (F_{max}) to minimum fluorescence (F_{min}) was determined in the calibration solution after subtraction of background excited by either 340 or 380 nm, and the 380 signals of Fura-2 were assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects of the dye (Poenie et al., 1986; Becker et al., 1989). The K_d value for Fura-2 was assumed to be 200 nM (Becker et al., 1989). To enable comparison of the effects of drugs on intact and skinned smooth muscle strips and to prevent the extrusion of Fura-2 (Roe et al., 1990), the present experiments were performed at room temperature.

Experiment on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were prepared using β-escin (Itoh et al., 1992). The methods used to prepare the skinned muscle strips and the composition of the solutions used have been described elsewhere (Itoh et al., 1986; 1992). To enable measurement of Ca^{2+} release from intracellular storage sites, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 5 min (to load Ca^{2+} into the storage sites). Ca^{2+} was then removed from the bathing solution by the application of Ca^{2+}-free solution containing 4 mM EGTA for 0.5 min. A solution containing 50 μM EGTA and 2 μM Fura-2 was then applied for 5 min. Finally, 10 μM $InsP_3$ was applied for 2 min in a solution containing 50 μM EGTA and 2 μM Fura-2.

Measurement of $InsP_3$

Endothelium-denuded strips (15 mm length, 0.3–0.5 mm width, 0.1 mm thickness) were equilibrated for over 2 h in Krebs solution at 32°C. After this, the strips were transferred to Ca^{2+}-free solution containing 2 mM EGTA for 2 min and then 10 μM NA was applied for 10 s. NKH477 or dibutyryl-cyclic AMP was given as a pretreatment for 5 min in Krebs solution, for 2 min in Ca^{2+}-free solution and throughout the application of NA. The reaction was stopped by the addition of a large amount of ice-cold 8% trichloroacetic acid and the strips were homogenized. The homogenate was centrifuged and the supernatant fraction treated with water-saturated ether three times and assayed with a radioimmunoassay kit from Amersham International, as described previously (Itoh et al., 1992).

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^- 134.2 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, 3 μM guanethidine and 0.3 μM propranolol were present in the Krebs solution throughout the experiment. Ca^{2+}-free 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 was 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: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N*-*N'*-*bis*-(2-ethanesulphonic acid) (PIPES), 5.1 mM $Mg(MS)_2$, 5.2 mM ATP, 5 mM phosphocreatine and 4 mM ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N'N'-tetraacetic acid (EGTA). To enable measurement of Ca^{2+} release from skinned strips, the concentration of EGTA was reduced to 50 μM and 2 μM Fura-2 was 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.*, 1986). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were Fura-2, Fura-2 AM, $InsP_3$, EGTA, PIPES, HEPES and MOPS (Dojin, Japan), NA and cyclopiazonic acid (CPA), dibutyryl-cyclic AMP and β-escin (Sigma), guanethidine (Tokyo Kasei, Japan), ATP (Na salt; Kojin, Japan) and propranolol (Nacalai, Japan). N,N-dimethyl-β-alanine [3**R**- (3α, 4αβ, 5β, 6β, 6aα, 10α, 10aβ, 10bα)]-5 (acetyloxy)-3-ethenyldodecahydro-10, 10b-dihydroxy-3, 4a, 7, 7, 10a-pentamethyl-1-oxo-1H-naphthol[2,1-b]pyran-6-yl ester hydrochloride (NKH477) was kindly provided by Nippon Kayaku Co., Ltd., (Tokyo, Japan).

Statistics

The values recorded were expressed as mean ± s.d. and statistical significance was determined by a paired or unpaired Student's *t* test. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effects of NKH477 on increases in $[Ca^{2+}]_i$ and force induced by NA in the presence of Ca^{2+}

Figure 1a shows traces of the effect of NKH477 on the increases in $[Ca^{2+}]_i$ (i) and force (ii) induced by 10 μM NA in Krebs solution (containing 2.6 mM Ca^{2+}). NA (10 μM) was applied for 2 min at 30 min intervals. Under these conditions, the resting $[Ca^{2+}]_i$ was 84.0 ± 3.7 nM ($n = 4$). Application of 10 μM NA produced a phasic, then a tonic increase in $[Ca^{2+}]_i$ and force. The phasic increases in $[Ca^{2+}]_i$ and force took their levels to 243.5 ± 17.2 nM and 10.4 ± 4.6 mg, respectively and the tonic levels reached (measured 2 min into the application) were 133.2 ± 15.7 nM and 9.6 ± 4.7 mg, respectively ($n = 4$). NKH477 (0.01–0.3 μM) produced concentration-dependent attenuations of the NA-induced phasic (Figure 1b(i)) and tonic (Figure 1b(ii)) increases in $[Ca^{2+}]_i$ and force, the two variables being affected in parallel.

In smooth muscle of the rabbit mesenteric artery, the NA-induced phasic increase in $[Ca^{2+}]_i$ is thought to be caused by Ca^{2+} release from its intracellular storage sites (Ito *et al.*, 1991; Itoh *et al.*, 1992). Since NKH477 is a potent activator of adenylate cyclase (Shafiq *et al.*, 1992), these results suggest that NKH477 inhibits NA-induced Ca^{2+} release through an activation of cyclic AMP-dependent mechanisms.

Effects of NKH477 and dibutyryl-cyclic AMP on increases in $[Ca^{2+}]_i$ and force induced by NA in Ca^{2+}-free solution containing 5.9 mM K^+

To investigate more precisely the effect of agents that increase cyclic AMP on NA-induced Ca^{2+} release, the effects were studied of NKH477 and dibutyryl-cyclic AMP on the increases 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^+. NA (10 μM) was applied for 2 min in Ca^{2+}-free solution 2 min after removal of Ca^{2+}, the strips being kept in Krebs solution (containing 2.6 mM Ca^{2+}) for 25 min between the tests. Following 2 min removal of Ca^{2+}, $[Ca^{2+}]_i$ rapidly decreased to 72.0 ± 6.2 nM ($n = 4$) and an application of NA (10 μM) produced a transient increase in $[Ca^{2+}]_i$ (to 165.8 ± 23.5 nM) and in force (to 6.2 ± 1.9 mg) ($n = 4$). NKH477 (0.01 and 0.1 μM) did not change the resting $[Ca^{2+}]_i$ significantly but concentration-dependently inhibited the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA. Dibutyryl-cyclic AMP (0.1 mM) also inhibited the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free solution containing 5.9 mM K^+ without a significant change in the resting concentration of $[Ca^{2+}]_i$.

In Ca^{2+}-free solution containing 5.9 mM K^+, caffeine (5 mM) transiently increased both $[Ca^{2+}]_i$ and force, the maximum increases being to 298.3 ± 71.2 nM and 5.2 ± 1.1 mg, respectively ($n = 4$). Neither NKH477 (0.01 μM) nor dibutyryl-cyclic AMP (0.1 mM) had any effect on the caffeine-induced increases in $[Ca^{2+}]_i$ and force.

Effects of NKH477 on NA-induced $InsP_3$ production

In smooth muscle of the rabbit mesenteric artery, $InsP_3$ is thought to be a possible second messenger in the NA-induced release of Ca^{2+} from intracellular storage sites (Hashimoto *et al.*, 1986; Ito *et al.*, 1991; Itoh *et al.*, 1992). To investigate the sites involved in the inhibitory actions of NKH477 and dibutyryl-cyclic AMP on the NA-induced increase in $[Ca^{2+}]_i$, the effects of these agents on NA-induced $InsP_3$ synthesis were studied in Ca^{2+}-free solution containing 5.9 mM K^+. NA (10 μM) was applied for 2 min after 2 min removal of Ca^{2+}. Under these conditions, NA (10 μM) transiently increased the amount of $InsP_3$ at 10 s, the effect decaying within 30 s (Itoh *et al.*, 1992). The effects of NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) on NA-induced synthesis of $InsP_3$ were therefore measured 10 s after the application of NA.

The concentration of $InsP_3$ was 5.1 ± 3.1 pmol mg^{-1} protein ($n = 4$) before, and 13.6 ± 3.9 pmol mg^{-1} protein ($n = 4$) 10 s after application of 10 μM NA. Neither NKH477 (0.01 μM) nor dibutyryl-cyclic AMP (0.1 mM) had any effect on this NA-induced $InsP_3$ synthesis (14.7 ± 0.5 pmol mg^{-1} protein in the presence of 0.01 μM NKH477, $n = 4$; 18.2 ± 2.8 pmol mg^{-1} protein in the presence of 0.1 mM dibutyryl-cyclic AMP, $n = 4$).

Effects of NKH477 and dibutyryl-cyclic AMP on $InsP_3$-induced Ca^{2+} release in β-escin-treated skinned smooth muscle

To investigate further the sites involved in the inhibitory actions of NKH477 and dibutyryl-cyclic AMP on the NA-induced increase in $[Ca^{2+}]_i$, the effects of NKH477 and dibutyryl-cyclic AMP were observed on the $InsP_3$-induced Ca^{2+} release in β-escin-treated skinned smooth muscle strips. After the strips had been skinned by an application of 25 μM β-escin for 30 min, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 5 min to load Ca^{2+} into the storage sites ('Ca^{2+}-loading') and Ca^{2+}-free solution containing 4 mM EGTA was applied for 0.5 min to remove Ca^{2+} from the solution. Finally, 10 μM $InsP_3$ was applied for 2 min in Ca^{2+}-free solution containing 50 μM EGTA and 2 μM Fura-2 following a 5 min application of the same Ca^{2+}-free solution (containing 50 μM EGTA and 2 μM Fura-2). Under these conditions, the resting $[Ca^{2+}]_i$ was 83.7 ± 5.6 nM ($n = 4$) and $InsP_3$ (10 μM) increased $[Ca^{2+}]_i$ to 146.6 ± 24.6 nM ($n = 4$), possibly due to release of Ca^{2+} from its storage sites. Neither NKH477 (0.1 μM) nor dibutyryl-cyclic AMP (0.1 mM) modified the $InsP_3$-induced Ca^{2+} release when each drug was applied separately in Ca^{2+}-free solution after Ca^{2+}-loading (0.92 ± 0.11 times control in the presence of 0.01 μM NKH 477 and 1.06 ± 0.27 times control in the presence of 0.1 mM dibutyryl-cyclic AMP, $n = 6$). However, under these conditions, NKH477 (0.01 μM) and dibutyryl-cyclic AMP (0.1 mM)

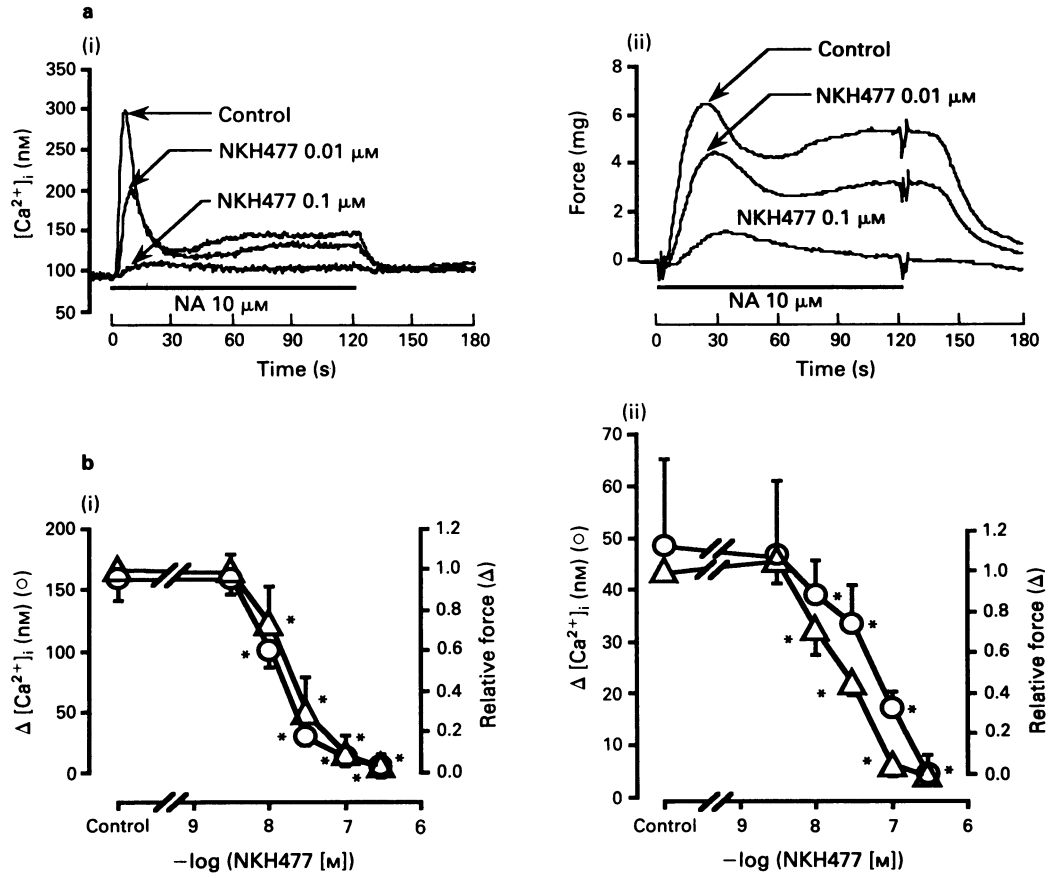

Figure 1 Concentration-dependent effects of NKH477 on increases in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA) in the presence of 2.6 mM Ca^{2+}. (a) (i) $[Ca^{2+}]_i$ and (a) (ii) force. NA (10 μM) was applied for 2 min (indicated by bar) at 30 min intervals. NKH477 (0.01 and 0.1 μM) was applied for 5 min before and during application of NA. The results illustrated were obtained from a single smooth muscle strip and are overlayed in the figure. (b) (i) Concentration-dependent effects of NKH477 on phasic increase in $[Ca^{2+}]_i$ (○) and force (Δ) induced by 10 μM NA. The amplitude of the increase in $[Ca^{2+}]_i$ induced by NA is expressed as '$\Delta [Ca^{2+}]_i$' (the peak minus the resting $[Ca^{2+}]_i$). The maximum force induced by 10 μM NA in the absence of NKH477 was normalized as a relative force of 1.0. (b) (ii) The effects on NA-induced tonic increase in $[Ca^{2+}]_i$ (○) and force (Δ). The tonic responses were measured 2 min after the application of 10 μM NA and the force induced by 10 μM NA in the absence of NKH477 was normalized as a relative force of 1.0. Results shown are each the mean ± s.d. of 4 observations. *Statistically significant difference from the value obtained in the absence of NKH477 ($P < 0.05$).

each inhibited the contraction induced by 0.3 μM Ca^{2+}, as reported previously for smooth muscle strips of the porcine coronary artery (Shafiq *et al.*, 1992). These results suggest that cyclic AMP does indeed have some inhibitory action on the contractile proteins in β-escin skinned smooth muscle strips.

Effects of NKH477 and dibutyryl-cyclic AMP on increase in $[Ca^{2+}]_i$ induced by NA in Ca^{2+}-free solution containing high K^+

The magnitude of the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution may depend on the rates of Ca^{2+} release and Ca^{2+} removal in the presence of NA in smooth muscle cells. If this were so, it would be expected that, when Ca^{2+} removal mechanisms are activated by NKH477 or dibutyryl-cyclic AMP, the size of the NA-induced increase in $[Ca^{2+}]_i$ would be reduced. Moreover, the inhibitory action of NKH 477 or dibutyryl-cyclic AMP on the NA-induced $[Ca^{2+}]_i$ increase might be concealed as the rate of the NA-induced Ca^{2+} release increases. We have previously found that K^+ concentration-dependently enhances the NA-induced increase in $[Ca^{2+}]_i$ without any increase in the NA-induced $InsP_3$ synthesis (Itoh *et al.*, 1992). To test the hypothesis, we studied the effects of NKH477 and dibutyryl-cyclic AMP on the increase in $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+}-free solution containing various concentrations of high K^+.

Ca^{2+}-free solution containing high K^+ (40 or 128 mM) was applied 1 min after application of Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. High K^+ itself failed to increase either $[Ca^{2+}]_i$ or force, but concentration-dependently enhanced the NA-induced increase in $[Ca^{2+}]_i$ with a corresponding increase in the maximum value of the rate of rise. NKH477 (0.01 μM) greatly inhibited the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution containing 5.9 mM K^+, but the inhibition was attenuated in Ca^{2+}-free solution containing 40 mM K^+ and abolished in Ca^{2+}-free solution containing 128 mM K^+ (Figure 2). Dibutyryl-cyclic AMP (0.1 mM) also inhibited the increases in $[Ca^{2+}]_i$ (0.14 ± 0.02 times control, $n = 4$) and force (0.18 ± 0.14 times control, $n = 4$) in Ca^{2+}-free solution containing 5.9 mM K^+, but the inhibition was attenuated in Ca^{2+}-free solution containing 40 mM K^+ (0.58 ± 0.08 times control for $[Ca^{2+}]_i$ and 0.61 ± 0.19 times control for force, $n = 4$) and in Ca^{2+}-free solution containing 128 mM K^+ (0.75 ± 0.02 times control for $[Ca^{2+}]_i$ and 0.86 ± 0.14 times control for force, $n = 4$).

Figure 3 shows the relationship between the maximum rate of rise of $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ in the presence of 10 μM NA in Ca^{2+}-free solution containing various concentrations of K^+ (5.9–128 mM). The relationship between the two was, to a first approximation, described by a linear function (correlation coefficient, $\gamma = 0.98$). NKH477 (0.01 μM, Figure 3a) and dibutyryl-cyclic AMP (0.1 mM, Figure 3b) each attenuated both the maximum rate of rise of $[Ca^{2+}]_i$

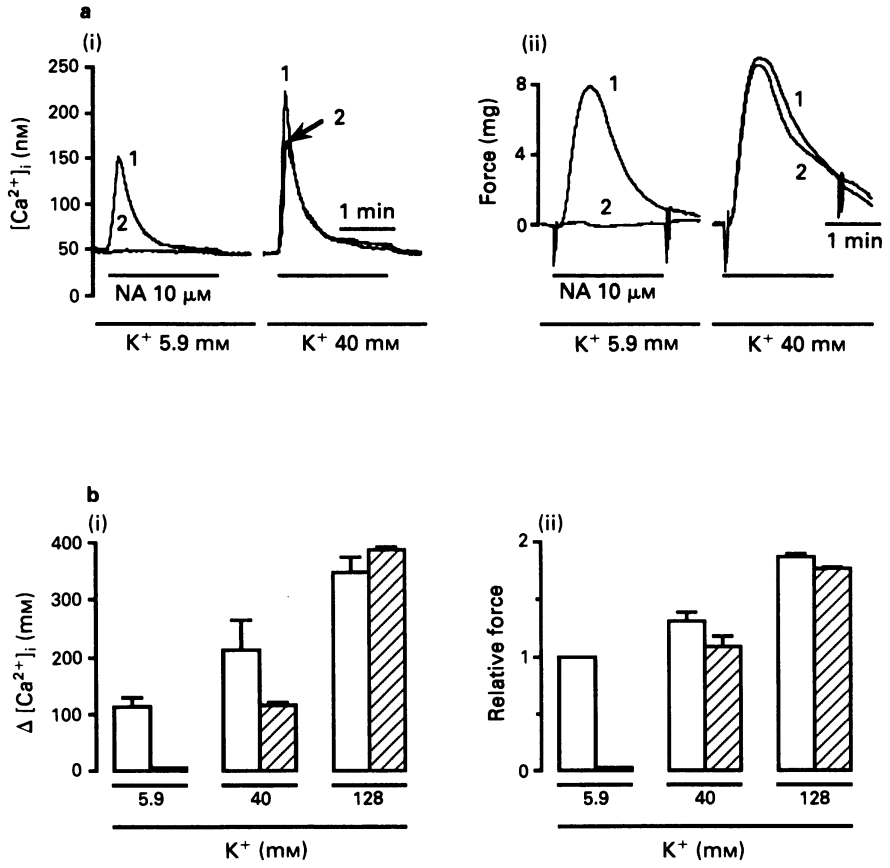

Figure 2 Effects of NKH477 on increases in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA) in Ca^{2+}-free solution containing 2 mM EGTA with high K^+. NA (10 μM) was applied for 2 min in Ca^{2+}-free solution after a 2 min removal of Ca^{2+}. Ca^{2+}-free solution containing high K^+ (40 or 128 mM) was applied 1 min after an application of Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. NA (10 μM) was applied for 2 min in Ca^{2+}-free solution containing various concentrations of K^+. NKH477 (0.01 μM) was applied for 3 min in Krebs solution, for 2 min in Ca^{2+}-free solution and throughout the application of 10 μM NA. (a) Shows actual traces of $[Ca^{2+}]_i$ (i) and force (ii). (1) Control; (2) in the presence of 0.01 μM NKH477. (b) The effects of 0.01 μM NKH477 on increase in $[Ca^{2+}]_i$ (i) and relative force (ii) induced by 10 μM NA in Ca^{2+}-free solution containing various concentrations of K^+ (5.9, 40 and 128 mM). Open columns: control; hatched columns: in the presence of 0.01 μM NKH477. Results shown are each the mean with s.d. of 3 observations.

and the increase in $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+}-free solution containing 5.9 mM or 40 mM K^+, but not 128 mM K^+.

High K^+ depolarizes the membrane in smooth muscle cells of the rabbit mesenteric artery, in a concentration-dependent manner (Itoh *et al.*, 1983). Thus, it would be expected that K^+ would attenuate the inhibitory actions of NKH477 and dibutyryl-cyclic AMP on the NA-induced increase in $[Ca^{2+}]_i$ through its membrane depolarizing action. To examine this possibility, the effects were studied of NKH477 and dibutyryl-cyclic AMP on the NA-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free solution containing 128 mM K^+ under conditions in which the amount of stored Ca^{2+} in the cells was varied. Following repetitive application of 10 μM NA in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+, the muscle strips were incubated with Ca^{2+}-free solution containing 5.9 mM K^+ for 5 min. Then, 2.6 mM Ca^{2+} with 40 mM K^+ was applied for various times ('Ca^{2+}-loading') and Ca^{2+}-free solution containing 5.9 mM K^+ subsequently applied for 9 min. Finally, NA (10 μM) was applied after 1 min of an application of Ca^{2+}-free solution containing 128 mM K^+ (Figure 4a). The amplitude of the NA-induced response increased in proportion with the duration of the Ca^{2+}-loading for times up to 60 s. Either NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) was applied in Ca^{2+}-free solution after the Ca^{2+}-loading period. Both NKH477 and dibutyryl-cyclic AMP inhibited the increase in $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+}-free solution containing 128 mM K^+ when the time set for the Ca^{2+}-loading was shorter than 60 s (Figure 4b). These results suggest that the membrane depolarization induced by high K^+ may not be responsible for the attenuation of the inhibitory action of NKH477 or dibutyryl-cyclic AMP on the NA-induced $[Ca^{2+}]_i$ increase which occurs in the presence of high K^+.

Since K^+ replaced an equimolar concentration of Na^+ in the high K^+ solution, a lack of Na^+ in the Ca^{2+}-free solution might have influenced the effect of NKH477 and dibutyryl-cyclic AMP. The effects of NKH477 were therefore observed on the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free, NA^+-deficient solution. NaCl (121.9 mM) was replaced by Tris-Cl (containing 15.5 mM Na^+). When the Ca^{2+}-free, Na^+-deficient solution was applied 1 min after removal of Ca^{2+} by Ca^{2+}-free solution containing 5.9 mM K^+ with 137.4 mM Na^+, the Na^+-deficient solution itself failed to increase either $[Ca^{2+}]_i$ or force but did somewhat enhance the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA. The peak values for the effects of NA (10 μM) on $[Ca^{2+}]_i$ and force were respectively 158.2 ± 24.9 nM and 4.2 ± 1.0 mg in Ca^{2+}-free solution containing 137.4 mM Na^+, and 251.2 ± 31.1 nM and 7.9 ± 1.8 mg in Ca^{2+}-free solution containing 15.5 mM Na^+ ($n = 4$). NKH477 (0.01 μM) inhibited the increase in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free, Na^+-deficient solution (116.4 ± 10.0 nM and 1.1 ± 0.4 mg in the presence of 0.01 μM NKH477, $n = 4$).

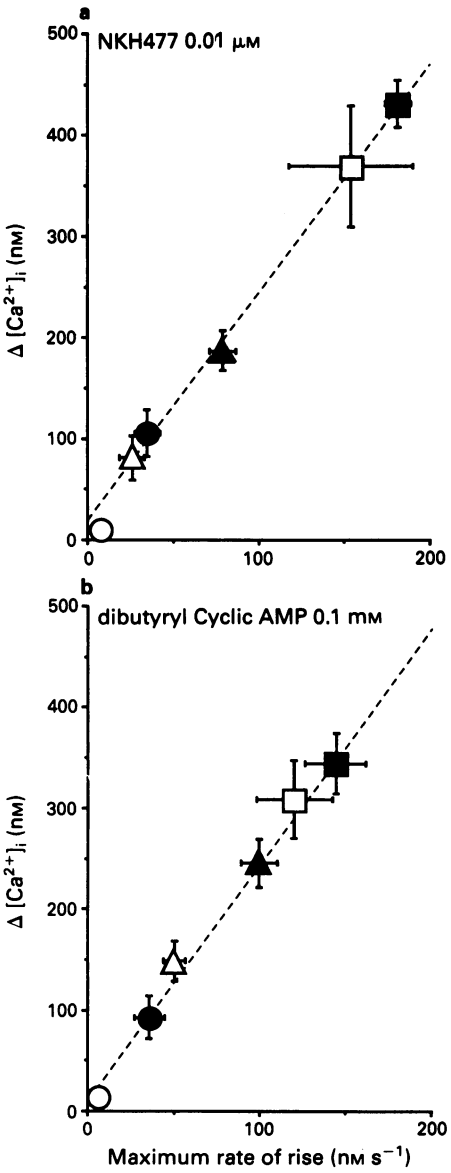

Figure 3 The relationship between the maximum rate of rise of $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+}-free solution containing various concentrations of K^+ in the absence or presence of NKH477 (a) or dibutyryl cyclic AMP (b). NA (10 μM) was applied for 2 min in Ca^{2+}-free solution containing 5.9 mM (●, ○), 40 mM (▲, △) or 128 mM (■, □) K^+ in the absence (solid symbols) or presence (open symbols) of 0.01 μM NKH477 or 0.1 mM dibutyryl-cyclic AMP. The amplitude of the maximum increase in $[Ca^{2+}]_i$ induced by NA is expressed as Δ $[Ca^{2+}]_i$. Results shown are each the mean with s.d. of 3 observations.

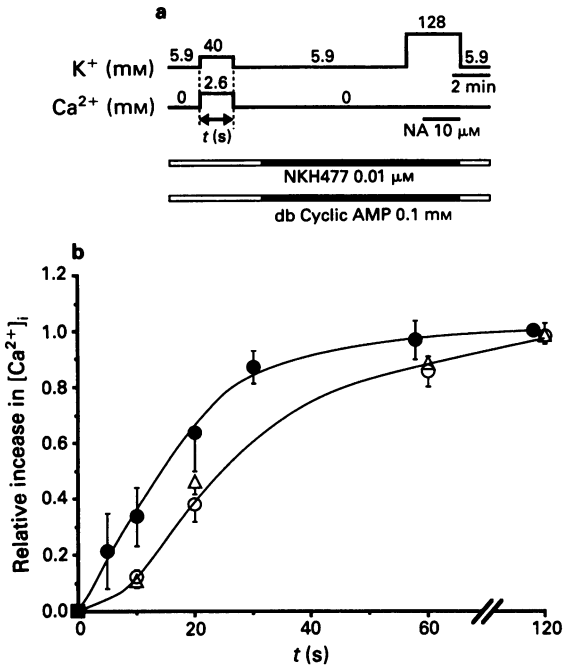

Figure 4 Effects of NKH477 and dibutyryl-cyclic AMP (db-Cyclic AMP) on increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+}-free solution containing 128 mM K^+ under conditions in which the amount of stored Ca^{2+} in the cells was varied. (a) Shows the experimental protocol. The muscle strips were initially kept in Krebs solution, followed by a 5 min exposure to Ca^{2+}-free solution containing 5.9 mM K^+. NA 10 μM was then repetitively applied before the experiment started. Ca^{2+} (2.6 mM) with 40 mM K^+ was applied for various durations (t(s)) and then Ca^{2+}-free solution containing 5.9 mM K^+ applied for 9 min. Finally, 10 μM NA was applied for 2 min after 1 min application of Ca^{2+}-free solution containing 128 mM K^+. (b) NA-induced increase in $[Ca^{2+}]_i$ as a function of the duration of the Ca^{2+}-application. The maximum increase in $[Ca^{2+}]_i$ induced by 10 μM NA following a 120 s application of 2.6 mM Ca^{2+} was normalized as a relative increase in $[Ca^{2+}]_i$ of 1.0. (●): Control. Either db-cyclic AMP (0.1 mM, ○) or NKH477 (0.01 μM, △) was applied before and during application of 10 μM NA in Ca^{2+}-free solution, as indicated in (a). Results shown are each the mean of 4 observations ± s.d. shown by a vertical bar.

Effects of dibutyryl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites

The effects of dibutyryl-cyclic AMP were studied on Ca^{2+} uptake into the storage sites using a protocol similar to that described in Figure 4a, except that the dibutyryl-cyclic AMP was applied during the Ca^{2+}-loading period (Figure 5a). When Ca^{2+} was applied simultaneously with 0.1 mM dibutyryl-cyclic AMP, the amplitude of the NA-induced increase in $[Ca^{2+}]_i$ was enhanced when the duration for Ca^{2+}-loading was set within 20 s ($P<0.05$, paired t test, $n=4$), but the maximum NA-induced response obtained with a 1 min application of 2.6 mM Ca^{2+} was not affected (Figure 5b). These results suggest that dibutyryl-cyclic AMP may increase not Ca^{2+} influx, but rather the rate of Ca^{2+} uptake into the NA-sensitive storage sites under the present experimental conditions.

The effect of NKH477 on the rate of Ca^{2+} uptake was not examined since the inhibitory action of NKH477 (0.01 μM) on the NA-induced increase in $[Ca^{2+}]_i$ continued for more than 30 min after washout.

To study further the effects of dibutyryl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites, the effects of dibutyryl-cyclic AMP were observed on the increase in $[Ca^{2+}]_i$ induced by repetitive application of NA in Ca^{2+}-free solution containing 5.9 mM K^+. Following 2 min removal of Ca^{2+}, NA (10 μM) was applied twice for 30 s with a 10 min interval in Ca^{2+}-free solution (Figure 6a). Under these conditions, the amplitude of the increase in $[Ca^{2+}]_i$ induced by the second application of 10 μM NA was one third that induced by the first (0.32 ± 0.09 times, $n=4$). When 0.1 mM dibutyryl-cyclic AMP was given during the first application of 10 μM NA, the amplitude of the first NA-response was reduced (0.30 ± 0.05 times the first NA-response without application of dibutyryl-cyclic AMP, $n=4$) but the second NA-response was increased (2.09 ± 0.32 times the second NA-response without application of dibutyryl-cyclic AMP, $n=4$). In accordance with the previous findings based on mechanical responses (Itoh *et al.*, 1983; 1985), these results suggest that some of the Ca^{2+} released induced by the first application of NA undergoes re-uptake into the storage sites and dibutyryl-cyclic AMP may enhance this Ca^{2+} uptake.

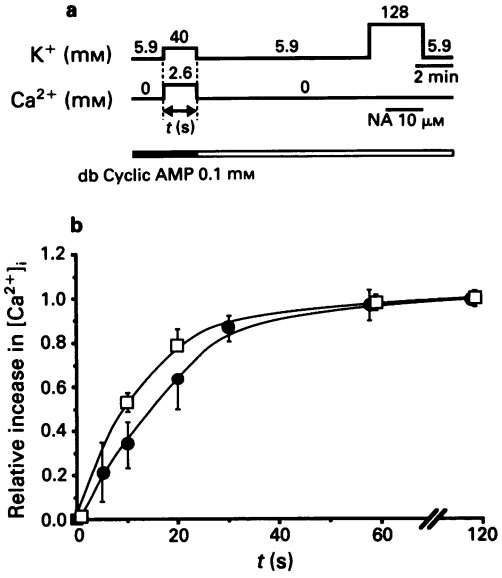

Figure 5 Effects of dibutyryl-cyclic AMP (db-cyclic AMP) applied during pre-incubation with Ca^{2+} on increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+}-free solution containing 128 mM K^+. (a) Shows the experimental protocol. Db-cyclic AMP (0.1 mM, □) was applied for 3 min in Ca^{2+}-free solution and throughout the application of Ca^{2+}, as indicated in (a). The rest of the experimental protocol was as described in Figure 4a. (b) NA-induced increase in $[Ca^{2+}]_i$ (normalized as in Figure 4b) as a function of the duration of the Ca^{2+}-application. Control responses shown in Figure 5b (●) are reproduced from Figure 4b. Results shown are each the mean of 4 observations with s.d. shown by a vertical bar.

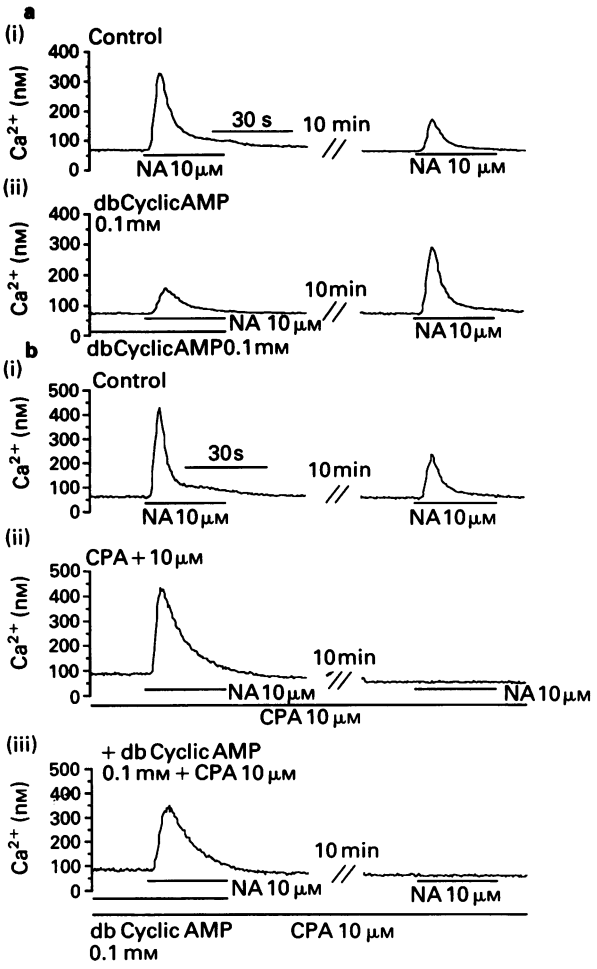

Figure 6 Effects of dibutyryl-cyclic AMP (db-cyclic AMP) on increase in $[Ca^{2+}]_i$ induced by repetitive application of noradrenaline (NA) in Ca^{2+}-free solution containing 5.9 mM K^+ in the absence (a) or presence (b) of cyclopiazonic acid (CPA). Following 2 min application of the Ca^{2+}-free solution, NA (10 μM) in Ca^{2+}-free solution was applied twice for 30 s with a 10 min interval. In (a) (i) control. (a (ii) DB-cyclic AMP (0.1 mM) was applied for 2 min in Ca^{2+}-free solution and throughout the first application of NA. Between traces a(i) and a(ii), Krebs solution (containing 2.6 mM Ca^{2+}) was applied for 25 min to ensure reproducible NA responses in Ca^{2+}-free solution. In (b): b(i) control; b(ii) in the presence of 10 μM CPA. CPA (10 μM) was applied 1 min after application of Ca^{2+}-free solution and was present for the rest of the experiment. Between traces b(i) and b(ii), Krebs solution containing 2.6 mM Ca^{2+} was applied for 25 min to ensure reproducible NA responses in Ca^{2+}-free solution. After the procedure shown in trace b(ii), CPA was washed out by Krebs solution and the muscle strip kept for 60 min in Krebs solution to ensure complete recovery of the NA-responses in Ca^{2+}-free solution. b(iii) Db-cyclic AMP (0.1 mM) was applied 1 min after application of Ca^{2+}-free solution and throughout the first application of NA. Results shown are representative of three other experiments.

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been reported to be a highly selective inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR) of skeletal muscle (Seidler *et al.*, 1989; Kurebayashi & Ogawa, 1991). To study further the effects of dibutyryl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites, the effects of dibutyryl-cyclic AMP were observed on the increase in $[Ca^{2+}]_i$ induced by repetitive application of NA in the presence of 10 μM CPA. Following 2 min removal of Ca^{2+}, NA (10 μM) was applied twice for 30 s with a 10 min interval in Ca^{2+}-free solution in the presence or absence of 10 μM CPA. When used, CPA (10 μM) was applied 90 s before the first application of 10 μM NA and was present throughout the rest of the experiment. CPA (10 μM) abolished the increase in $[Ca^{2+}]_i$ induced by the second application of NA without any change in the amplitude of the first NA-response (Figure 6b (ii)). These results suggest that CPA inhibits the re-uptake of Ca^{2+} into the storage sites during the Ca^{2+} mobilization induced by the first application of NA. Following a 60 min washout of CPA with Krebs solution, the increases in $[Ca^{2+}]_i$ induced by repetitive application of 10 μM NA recovered completely. When 0.1 mM dibutyryl-cyclic AMP was applied during the first application of 10 μM NA in the presence of 10 μM CPA, the inhibitory action of dibutyryl-cyclic AMP on the increase in $[Ca^{2+}]_i$ induced by the first application of NA was attenuated (0.60 ± 0.05 times the control in the presence of 10 μM CPA, $n = 4$ and see also Figure 6a(ii) for the action of dibutyryl-cyclic AMP in the absence of CPA) and there was no response to the second application of NA (Figure 6b(iii)).

Discussion

In the present experiments, NA produced a phasic followed by a tonic increase in both $[Ca^{2+}]_i$ and force in the presence of 2.6 mM Ca^{2+} in smooth muscle of the rabbit mesenteric artery. The NA-induced phasic increases in $[Ca^{2+}]_i$ and force persisted in Ca^{2+}-free solution, suggesting that these phasic responses are probably due to a release of Ca^{2+} from its intracellular storage sites. We have previously reported that, in smooth muscle of the rabbit mesenteric artery, NA activates the synthesis of $[^3H]$-$InsP_3$ through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), and $InsP_3$ releases ^{45}Ca from intracellular storage sites (Hashimoto *et al.*, 1986). In the present experiments, we partly confirmed these observations in that NA increased the synthesis of $InsP_3$ in intact smooth muscle strips (measured by radioimmunoassay)

and $InsP_3$ released Ca^{2+} from the cellular storage sites in β-escin-treated skinned smooth muscle (measuring by Fura-2 fluorescence). These results suggest that $InsP_3$ is a second messenger in the NA-induced release of Ca^{2+} from intracellular storage sites in smooth muscle of the rabbit mesenteric artery.

It has been found that elevation of cyclic AMP reduce agonist-mediated inositol phosphate formation in platelets (Watson *et al.*, 1984), neutrophils (Takenawa *et al.*, 1986) and A10 cultured vascular smooth muscle cells (Xuan *et al.*, 1991). It has also been reported that cyclic AMP-dependent protein kinase reduces the ability of $InsP_3$ to release stored Ca^{2+} through phosphorylation of the $InsP_3$ receptor in isolated vesicles, from brain synaptosome (Supattapone *et al.*, 1988). In the present experiments, NKH477 and dibutyryl-cyclic AMP each greatly inhibited the NA-induced increase in $[Ca^{2+}]_i$ without a corresponding change in the NA-induced synthesis of $InsP_3$. Further, in β-escin-treated skinned smooth muscle, neither NKH477 nor dibutyryl-cyclic AMP modified the $InsP_3$-induced Ca^{2+} release from the intracellular storage sites. These results suggest that, in smooth muscle of the rabbit mesenteric artery, NKH477 and dibutyryl-cylic AMP inhibit the NA-induced increase in $[Ca^{2+}]_i$ without either an inhibition of the NA-induced synthesis of $InsP_3$ or a reduction of the sensitivity of the receptor to $InsP_3$.

In smooth muscle cells, the magnitude of the increase in $[Ca^{2+}]_i$ induced by an agonist in Ca^{2+}-free solution may depend on the balance between the rates of Ca^{2+} release and Ca^{2+} removal (such as Ca^{2+}-uptake into the storage site or Ca^{2+}-extrusion to the extracellular space). When Ca^{2+} removal mechanisms are activated, the size of the increase in $[Ca^{2+}]_i$ induced by the agonist could be minimized. Conversely, the size of the agonist-induced increase in $[Ca^{2+}]_i$ would be enhanced as the rate of Ca^{2+} release induced by the agonist increases while the rate of Ca^{2+} removal remains constant. We have previously found that in Ca^{2+}-free solution, high K^+ concentration-dependently enhances the NA-induced increase in $[Ca^{2+}]_i$ without any increase in the amount of NA-induced $InsP_3$ synthesis (Itoh *et al.*, 1992). In the present experiments, high K^+ itself failed to increase $[Ca^{2+}]_i$ in Ca^{2+}-free solution but concentration-dependently enhanced both the maximum rate of rise of $[Ca^{2+}]_i$ and the amplitude of the increase in $[Ca^{2+}]_i$. The relationship between the maximum values for the increase in $[Ca^{2+}]_i$ and the rate of rise of $[Ca^{2+}]_i$ induced by NA was linear in Ca^{2+}-free solution in the presence of various concentrations of K^+ (5.9–128 mM). Although at present the mechanism of the high K^+-induced enhancement of the NA-induced Ca^{2+} mobilization in Ca^{2+}-free solution remains unclear, these results suggest that the magnitude of the $[Ca^{2+}]_i$ mobilization induced by NA increases in proportion to the maximum rate of rise of $[Ca^{2+}]_i$ in Ca^{2+}-free solution. NKH477 and dibutyryl-cyclic AMP each greatly attenuated both the maximum rate of rise of $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by NA in Ca^{2+}-free solution containing 5.9 mM K^+. These inhibitory actions of NKH477 and dibutyryl-cyclic AP were attenuated by high K^+, in a concentration-dependent manner and this attenuation is not due simply to the membrane depolarization induced by high K^+ because NKH477 inhibited the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution containing 128 mM K^+ when the amount of stored Ca^{2+} was set at a low value (Figure 4). These results indicate that the extent of the inhibition induced by NKH477 or dibutyryl-cyclic AMP on the NA-induced increase in $[Ca^{2+}]_i$ is inversely related to the rate of Ca^{2+} release induced by NA.

Caffeine (5 mM) transiently increased $[Ca^{2+}]_i$ in Ca^{2+}-free solution containing 5.9 mM K^+. The maximum rate of rise of $[Ca^{2+}]_i$ in the presence of 5 mM caffeine was almost five times larger than that for 10 μM NA in Ca^{2+}-free solution containing 5.9 mM K^+, but similar to that for 10 μM NA in Ca^{2+}-free solution containing 128 mM K^+. NKH477 had no effect on the caffeine-induced increase in $[Ca^{2+}]_i$. This lack of effect might be due to the greater rate of rise of $[Ca^{2+}]_i$ in the presence of caffeine than with NA in Ca^{2+}-free solution containing 5.9 mM K^+.

In saponin-treated skinned smooth muscle of the guinea-pig and rabbit mesenteric arteries, cyclic AMP increases the rate of Ca^{2+} re-uptake into its intracellular storage sites, as estimated from mechanical responses (Itoh *et al.*, 1982; 1985; Saida & van Breemen, 1984). In the present experiments, we found that dibutyryl-cyclic AMP increased the rate of Ca^{2+} uptake into the NA-sensitive storage sites (Figure 5). We also found that when 10 μM NA was applied twice for 30 s with a 10 min interval in Ca^{2+}-free solution, the second application of NA could still increase $[Ca^{2+}]_i$. CPA, an inhibitor of the Ca^{2+}-pump in the SR of skeletal, cardiac and smooth muscle cells (Seidler *et al.*, 1989; Kurebayashi & Ogawa, 1991; Uyama *et al.*, 1992), did not modify the increase in $[Ca^{2+}]_i$ induced by the first application of NA in Ca^{2+}-free solution, but abolished the increase induced by the second. With an application of dibutyryl-cyclic AMP during the first application of NA, the increase in $[Ca^{2+}]_i$ induced by this first application of NA was inhibited but the increase induced by the second was enhanced. CPA attenuated the inhibitory action of dibutyryl-cyclic AMP on the increase in $[Ca^{2+}]_i$ induced by the first application of NA. These results suggest that agents which increase cyclic AMP may inhibit the NA-induced increase in $[Ca^{2+}]_i$ by an activation of Ca^{2+} uptake into the storage sites.

Ca^{2+}-extrusion mechanisms (such as Na^+-Ca^{2+} exchange or the Ca^{2+}-pump on the plasma membrane) may be activated by cyclic AMP, as reported for toad stomach smooth muscle cells (Scheid *et al.*, 1979). However, since NKH477 (0.01 μM) inhibited the increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free, Na^+-deficient solution, the Na^+-Ca^{2+} exchange mechanism may not play an essential role in the inhibitory action of NKH477 on the NA-induced increase in $[Ca^{2+}]_i$.

In conclusion, NKH477 and dibutyryl-cyclic AMP each attenuate NA-induced Ca^{2+} mobilization with no change in either the NA-induced $InsP_3$ synthesis or the sensitivity of the receptor to $InsP_3$. Dibutyryl-cyclic AMP increases the rate of Ca^{2+}-uptake into storage sites. Thus, it can be concluded that, in smooth muscle of the rabbit mesenteric artery, agents that increase cyclic AMP attenuate the NA-induced increase in $[Ca^{2+}]_i$ possibly through an activation of Ca^{2+} uptake into its storage sites.

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Block of potassium currents in rat isolated sympathetic neurones by tricyclic antidepressants and structurally related compounds

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1 The block of K^+ currents by the tricyclic antidepressants (TCAs), imipramine and amitriptyline and three structurally related compounds, chlorpromazine, tacrine and carbamazepine was investigated in rat isolated sympathetic neurones by whole-cell voltage-clamp recording.

2 At a concentration of 10 μM, imipramine, amitriptyline and chlorpromazine all blocked the delayed rectifier K^+ current (I_{Kv}) by about the same extent, 54%, 47% and 53%. Tacrine was less effective (10%) while carbamazepine was ineffective at all concentrations tested.

3 The degree of block by the four effective compounds was relatively independent of the size of the voltage-step. Neither the activation nor the inactivation rates of I_{Kv} were altered by the blocking drugs.

4 Concentration-response relationships for imipramine and tacrine showed that imipramine was about 7 fold more potent than tacrine but that the maximum inhibition and the Hill slope were the same for both compounds.

5 Amitriptyline, chlorpromazine and imipramine (at 10 μM) were 2–3 fold more potent at inhibiting the sustained K^+ current (mostly I_{Kv}) than the transient K^+ current (mostly I_A). Tacrine, however, was equally effective in blocking both components.

Keywords: Tricyclic antidepressants; chlorpromazine; tacrine; carbamazepine; K^+ currents; rat sympathetic neurones; whole-cell voltage-clamp

Introduction

Potassium (K^+) channels are important regulators of neuronal membrane potential and neuronal excitability (Cook & Quast, 1990; Hille, 1992). Neurotransmitters and drugs which activate or block neuronal K^+ channels will, therefore, have profound effects on cellular signalling in the peripheral and central nervous systems. Unfortunately, apart from certain highly specific toxins (Strong, 1990), there are relatively few blocking drugs that are selective for one particular K^+ channel type (Cook & Quast, 1990). This hampers study of the precise role of the various K^+ channel subtypes in the CNS *in vivo* (Halliwell, 1990) as well as making comparisons of cloned and native K^+ channels virtually impossible using present pharmacological tools (Pongs, 1992a,b).

Recently, it has been suggested that the tricyclic antidepressant (TCA), imipramine, may selectively decrease the transient outward K^+ current (I_T) of rabbit isolated atrial myocytes and thereby lengthen the action potential of these cells (Delpón *et al.*, 1992). While there are a number of reports of blocking effects of TCAs on cardiac ion channels (e.g. Isenberg & Tamargo, 1985; Ogata & Narahashi, 1989a), little is known about the effects and selectivity, if any, of TCAs on mammalian neuronal K^+ channels (however see Ogata *et al.*, 1989). Bennett & Middleton (1975) reported that the TCA, desipramine, can prolong the preterminal action potential of rat sympathetic neurones by a mechanism that does not seem to involve the 'classical' action of TCAs, namely, inhibition of catecholamine uptake. This action could, however, be explained by a block of K^+ currents, so the aim of this study was to test directly for such a block on two particular K^+ channel-currents in isolated rat sympathetic neurones using the TCAs imipramine and amitriptyline. These currents are the delayed-rectifier K^+ current (I_{Kv}) and the transient K^+ current (I_A) (Freschi, 1983; Galvan & Sedlmeir, 1984; Belluzzi *et al.*, 1985a,b).

Additionally, we considered three related, tricyclic, compounds, chlorpromazine (a neuroleptic), carbamazepine (an anticonvulsant) and tacrine (9-amino-1,2,3,4-tetrahydroacridine, an anticholinesterase). Chlorpromazine has previously been shown to block a number of voltage- and ligand-gated ion channels (e.g. Ogata & Narahashi, 1989b; Ogata & Tatebayashi, 1989; Ogata *et al.*, 1990; Revah *et al.*, 1990; Bolotina *et al.*, 1992) including K^+ channels (Ogata *et al.*, 1989), while tacrine has also been shown to block certain K^+ channels (e.g. Robbins & Sim, 1990; DiFrancesco *et al.*, 1991). Carbamazepine, however, has been suggested to enhance rather than block K^+ currents in rat cortical neurones in culture (Zona *et al.*, 1990). It is of interest to compare the relative effects of these structurally related but pharmacologically distinct compounds on the two K^+ currents, I_{Kv} and I_A in a uniform population of neurones.

Some of these results have been published in abstract form (Wooltorton & Mathie, 1993).

Methods

Cell dissociation

Neurones were dissociated from rat superior cervical ganglia (SCG) by a method modified from one described previously (Beech *et al.*, 1991; Bernheim *et al.*, 1991). Sprague-Dawley rats of either sex (age 7–8 days) were killed by decapitation. The SCGs were removed and placed at 37°C in a modified Hank's solution containing 20 iu ml^{-1} papain for 20 min. The ganglia were then incubated in a mixture of 400 iu ml^{-1} collagenase (type 1) and 16 mg ml^{-1} dispase (grade 2) for 45 min and triturated every 15 min. Cells were then centrifuged and resuspended in Leibovitz L-15 medium. Isolated neurones were kept at 4°C and used for recording within 10 h.

Solutions

The external solution (in mM) was 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 8 glucose and the pH was

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adjusted to 7.4 with NaOH. Tetrodotoxin (TTX, 500 nM) was added when required (see below). The internal solution (in mM) was 145 KCl, 5 $MgCl_2$, 5 HEPES, 10 BAPTA and pH was adjusted to 7.4 with KOH. Preliminary experiments suggested that this high concentration of BAPTA was necessary to minimise the contribution of Ca^{2+}-activated-K^+ currents to the whole-cell current (Wooltorton & Mathie, 1993).

Stock solutions of the tricyclic compounds were made up in distilled water (except for carbamazepine which was made up in ethanol) and kept at − 20°C until use. They were then added to the external solutions at suitable concentrations shortly before the experiment. In the carbamazepine experiments an identical concentration of ethanol was added to the control solution. Solutions were applied to the cells by continuous perfusion of the chamber during recording. The perfusion rate was 4–5 ml min^{-1} and complete exchange of the bath solution occurred within 20–40 s.

All compounds were from Sigma except dispase (Boehringer Mannheim) and Leibovitz's L-15 (Gibco).

Current recording and analysis

Currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) at 20–23°C using an Axopatch 1D amplifier. Patch pipettes had resistances between 2 and 5 MΩ. During whole-cell recording, series resistance was 11.8 ± 0.6 MΩ and whole-cell capacitance was 17.4 ± 0.8 pF ($n = 79$).

Voltage protocols Protocol (1): To measure the delayed rectifier current (I_{Kv}) in isolation, cells were held at − 70 mV and stepped once every 6 s to − 50 mV for 30 ms to inactivate any residual transient current (I_A) then depolarized to a test potential of + 10 mV for 150 ms before stepping back first to − 50 mV for 30 ms then to the holding-potential. I_{Kv} was measured as the average current over 25 ms, 116.5 ms following the step to + 10 mV.

Protocol (2): To measure I_A and I_{Kv}, cells were held at − 30 mV and a conditioning pulse to − 120 mV was applied for 500 ms, once every 5 s, before a test pulse of 400 ms to 0 mV. The peak current, measured within 10 ms of the step to 0 mV was predominantly I_A, while the sustained current measured as the average current over 100 ms, 297 ms following the step was predominantly I_{Kv} (see results).

Na^+ currents inactivate rapidly in SCG neurones (Belluzzi & Sacchi, 1986) and could be ignored when using protocol 1. Experiments using protocol 2 were done in the presence of 500 nM tetrodotoxin (TTX). Under optimal recording conditions (5 mM $CaCl_2$ outside, 2 mM Na_2ATP inside, see Stansfeld & Mathie, 1993) the peak Ca^{2+} current in these cells from 7–8 day old rats was − 130 ± 24 pA at the peak of the current-voltage curve, 0 mV ($n = 5$) or about 5% of the transient K^+ current (see results) and inactivated rapidly. Under the conditions used in this study (above) the Ca^{2+} current will make a negligible contribution to the whole-cell current evoked by either of the two voltage protocols.

Currents were low-pass filtered at 2 or 5 kHz, digitized at 0.5–5 kHz and recorded and analysed using an IBM compatible p.c., pClamp version 5.5 with a Tl-1 labmaster interface (Axon Instruments) and Excel 4.0 (Microsoft). Statistical tests were carried out using a Student's t test. P values of 0.01 or less were regarded as significant. All data are expressed as mean ± s.e.mean and n is the number of cells.

Results

Structure of tricyclic compounds

The structures of the five compounds tested are shown in Figure 1. They are all tricyclic. The tricyclic antidepressants (TCAs), imipramine and amitriptyline, have a similar central chain substituent to chlorpromazine, however their respective ring structures differ. On the other hand, carbamazepine, orginally derived from the TCAs, has a similar ring structure but a much shorter central chain. Tacrine differs from the other compounds both in ring structure and by lacking a central chain substituent.

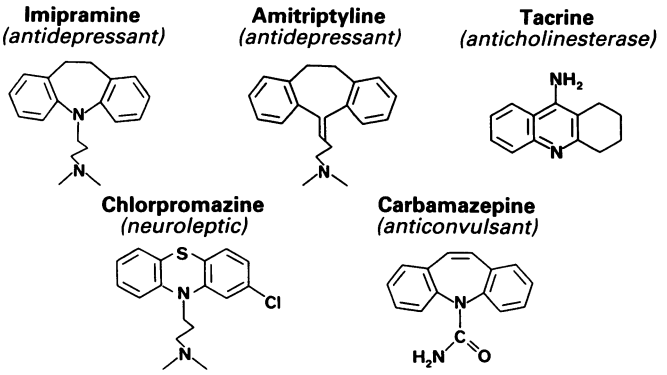

Figure 1 Chemical structures of the tricyclic compounds investigated.

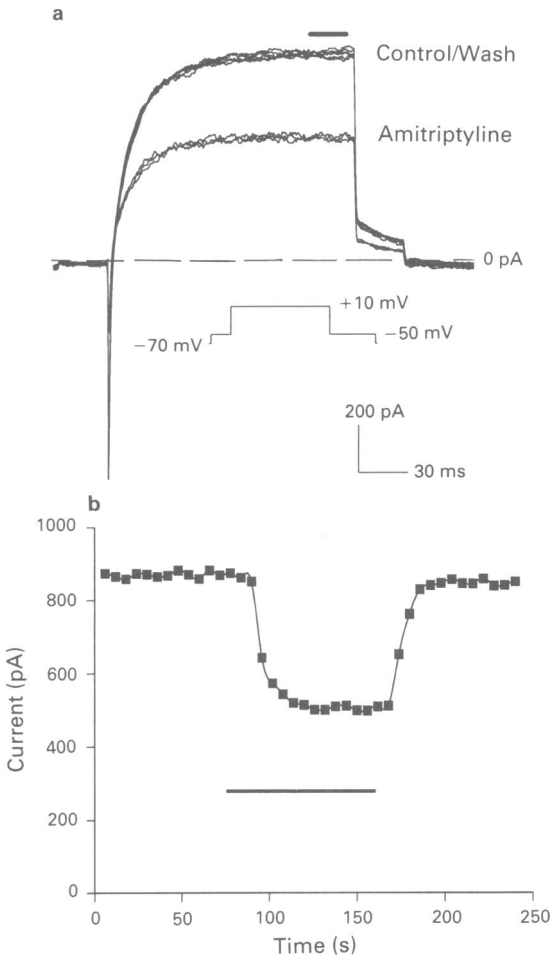

Figure 2 The effect of 10 μM amitriptyline on I_{Kv} is shown in (a) and (b). The currents were activated using stimulus protocol 1. In (a), only 9 episodes are shown for clarity, 3 traces are shown in control solution, 3 in 10 μM amitriptyline and 3 in wash. The dashed line denotes the zero current level. Each point in (b) is the mean current recorded over 25 ms, 116.5 ms following the depolarizing step (a). The bar in (b) indicates the time of application of amitriptyline. In this cell, the inhibition was 42%. Note the fast onset and washout of the drug's effect.

Block of I_{Kv} by amitriptyline

The sustained outward potassium current (I_{Kv}) was evoked by the protocol shown in Figure 2a and described in the

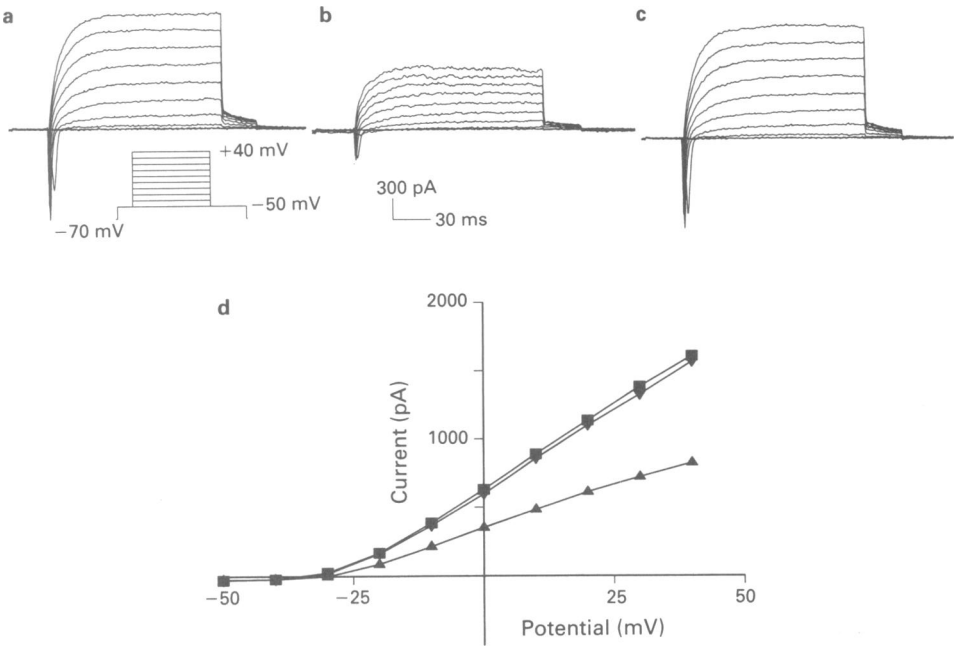

Figure 3 Shows the current-voltage relationship for I_{Kv} in (a) control, (b) 10 μM amitriptyline and (c) wash. Stimulus protocol 1 was adapted so that the test potential began at − 50 mV and incremented by 10 mV each trial; (d) illustrates the magnitude of I_{Kv} in control (▼), in the presence of amitriptyline (▲) and after wash (■) at the different test potentials.

methods (protocol 1). The outward current evoked was 1236 ± 67 pA ($n = 64$). Application of amitriptyline (10 μM), Figure 2b, inhibited outward current by 42% in this cell. In 13 cells, amitriptyline inhibited the current by 47.2 ± 2.5%. The onset of drug action was relatively fast and the wash-off was also rapid and complete. The tail current seen on stepping back to − 50 mV represents the slow deactivation of I_{Kv} and allows a measure of this current in isolation. Amitriptyline inhibited this tail current by 53.6 ± 4.0%. This suggests that under these recording conditions virtually all of the sustained outward current is carried by I_{Kv}. Furthermore, addition of 100 μM Cd^{2+} had no effect on the magnitude of I_{Kv} (1207 ± 144 pA, $n = 8$) or on the magnitude or kinetics of the tail current. The activation rate of I_{Kv} was unaffected by amitriptyline. This time constant of activation, fitted by a single exponential component (see Belluzzi *et al.*, 1985b), was 11.6 ± 0.7 ms ($n = 10$) at + 10 mV in control conditions and 10.5 ± 1.0 ms ($n = 10$) in the presence of amitriptyline. I_{Kv} showed virtually no inactivation over the time scale of the depolarising pulse applied here and this was clearly unaltered in the presence of amitriptyline. None of the blocking drugs were found to affect the rate of activation or induce inactivation of I_{Kv}.

Figure 3 shows the effect of amitriptyline (10 μM) on the current voltage relation for I_{Kv}. The degree of block was relatively independent of voltage, for example 43% at 0 mV and 46% at + 40 mV. A similar result was obtained for amitriptyline in 2 other cells and for imipramine ($n = 6$) and tacrine ($n = 2$). There is a noticeable increase in the noise level of the current trace in the presence of amitriptyline, compare Figure 3a and 3b. This shows that the kinetic behaviour of the underlying I_{Kv} channels has become more complicated in the presence of amitriptyline with the addition of a component which must have a relatively slow time constant in order to be visible in the limited bandwidth of the recording system (see discussion).

Comparative effects of the tricyclic compounds on I_{Kv}

Similar experiments to that shown in Figure 2 were carried out for all five of the tricyclic compounds at a concentration of 10 μM and the results are shown in Figure 4. The compounds fall into two groups. Amitriptyline, imipramine and chlorpromazine all inhibited I_{Kv} to a similar degree (47.2 ± 2.5%, $n = 13$, 54.0 ± 4.5%, $n = 9$ and 53.3 ± 2.3%, $n = 6$, respectively). Tacrine and carbamazepine at the same concentration were significantly less effective blockers of I_{Kv} (10.2 ± 2.0%, $n = 5$ and − 0.67 ± 7.7%, $n = 3$, respectively).

The concentration-response relations for one compound from each group, imipramine and tacrine, were investigated in detail and are shown in Figure 5. Data were fitted with the equation:

$$y = 100 - (max/(1 + (K_D/x)^s)) \quad (1)$$

where *max* is the maximum inhibition attainable and *s* is the slope factor (Hill coefficient). For imipramine, the K_D was 6.8 μM, *max* was 86.0% and *s* was 1.3. For tacrine, the K_D was 46.0 μM, *max* was 84.8% and *s* also 1.3. Therefore,

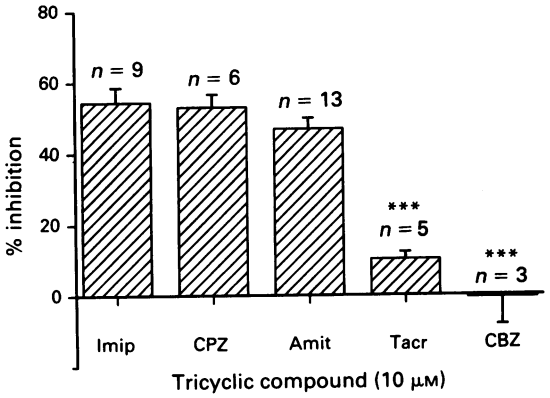

Figure 4 The percentage inhibitions of I_{Kv} by 10 μM concentrations of imipramine (Imip), chlorpromazine (CPZ), amitriptyline (Amit), tacrine (Tacr), and carbamazepine (CBZ) are shown. Tacrine (10.2 ± 2.0%, $n = 5$) and carbamazepine (− 0.67 ± 7.7%, $n = 3$) are significantly different from chlorpromazine (53.3 ± 2.3%, $n = 6$; *** = $P < 0.001$). However, imipramine (54.0 ± 4.5%, $n = 9$) and amitriptyline (47.2 ± 2.5%, $n = 13$) are not significantly different from chlorpromazine.

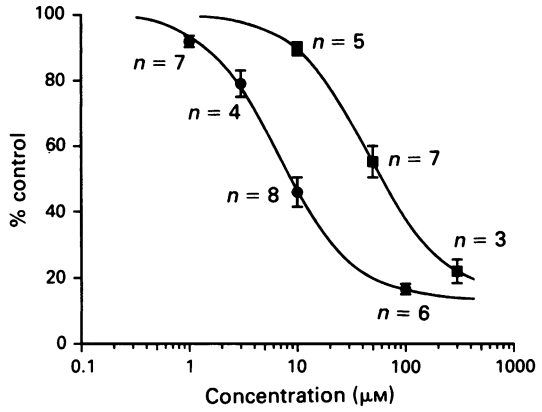

Figure 5 The dose-dependence of I_{Kv} inhibition by imipramine (●) and tacrine (■) is shown. Data are fitted using equation 1 in the text. For imipramine, $K_D = 6.8\ \mu M$, s (the Hill slope) = 1.3 and *max* (the maximum inhibition) = 86.0%, and for tacrine, $K_D = 46.0\ \mu M$, $s = 1.3$, and *max* = 84.8%.

tacrine is about 7 fold less potent than imipramine at inhibiting I_{Kv}; however, the maximum response attainable and the slope factor were the same for both compounds which may suggest a common site of action.

Of the five compounds tested, carbamazepine was the most unusual. Even at 50 μM it produced little inhibition of I_{Kv} (0.44 ± 4.2%, $n = 3$). Higher concentrations could not be tested due to problems with carbamazepine solubility. Previous reports have suggested that carbamazepine may enhance rather than block potassium currents (Zona *et al.*, 1990; Rogawski & Porter, 1990). We saw no consistent evidence for this, however, in 3 out of 6 cells there was an apparent enhancement of the outward current. This occurred after some delay following drug application compared to the speed of block produced by the other four compounds and was maintained after wash out. This effect was not studied further here.

Effect of tricyclic compounds on transient and sustained components of K^+ current

As stated in the introduction, it was of interest to compare the effects of these compounds on the transient K^+ current in SCG neurones, I_A (Belluzzi *et al.*, 1985a) with those on I_{Kv}. Figure 6a illustrates a protocol which elicits both I_A and I_{Kv} (protocol 2, see methods). The peak current measured within 10 ms of the step to 0 mV was 2537 ± 226 pA ($n = 30$). It is important to consider exactly how the 'transient' and 'sustained' components evoked by this protocol related to I_A and I_{Kv}. I_A decays with a time constant of 27.9 ± 3.2 ms ($n = 9$) at 0 mV in our experiments at room temperature (see also Belluzzi *et al.*, 1985a), so the sustained component measured after 297 ms will be almost entirely I_{Kv}. I_{Kv} activates with a time constant of 14.6 ± 1.6 ms ($n = 10$) on depolarization to 0 mV (see also Belluzzi *et al.*, 1985b), so it only reaches approximately 29% of its maximum after 5 ms. As the peak transient current is reached well within 5 ms and the maximal size of I_{Kv} is 720 ± 69 pA at 0 mV or 28.4% of the peak transient current, it follows that even after 5 ms only about 8% of the outward current will be carried by I_{Kv} and the major part of this current (92%) is I_A. Additionally, in four cells we used a 'prepulse subtraction protocol' (see Belluzzi *et al.*, 1985a). Responses were evoked at 20 mV increments from a starting test potential of − 60 mV following prepulses to − 50 mV. These were subtracted from responses obtained at the same test potentials following prepulses to − 120 mV. This protocol showed that the peak current evoked on stepping to − 40 mV and − 20 mV was almost completely I_A. With a test pulse to − 40 mV, peak outward current was

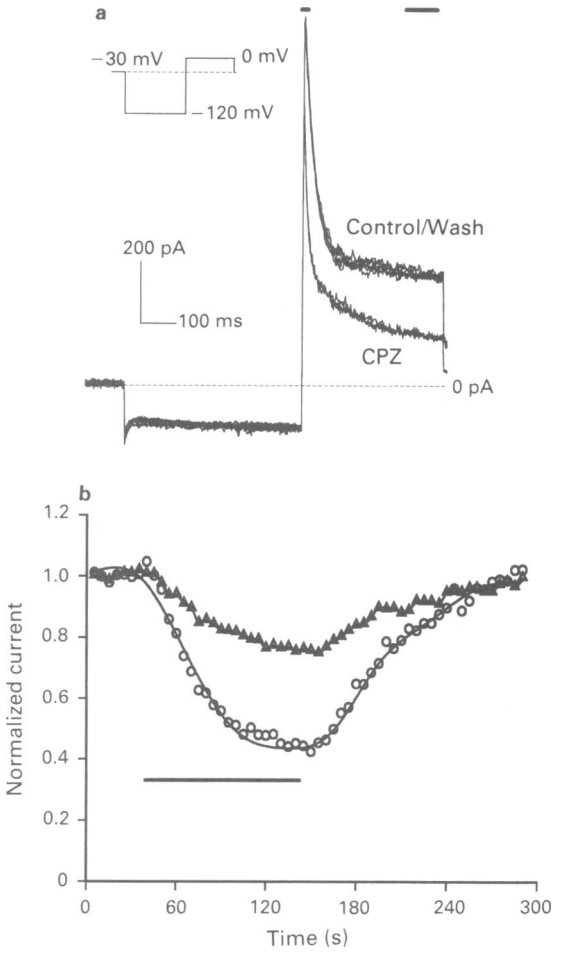

Figure 6 Panel (a) shows the effect of 10 μM chlorpromazine on the transient and sustained components of the outward current evoked by stimulus protocol 2. The dashed line shows the zero current level. Panel 6(b) shows the peak current attained within 10 ms of the depolarizing step (▲) and the average sustained current measured over 100 ms, 297 ms after the depolarizing step (○) indicated by the bars in (a). Current amplitudes were normalised with respect to the first measurement for clarity. The bar in (b) indicates the time of chlorpromazine application. In this cell, inhibition by 10 μM chlorpromazine of the transient and sustained components was 22% and 54%, respectively.

415 ± 45 pA ($n = 4$) of which 98 ± 2% was I_A while with a test pulse to − 20 mV the peak outward current was 1314 ± 91 pA of which 97 ± 1% was I_A. Even with test steps to 0 mV, I_A made up 93 ± 1% of the outward current at its peak (2404 ± 192 pA).

Concentrations of tricyclic compound which inhibited I_{Kv} by approximately 50% were studied in most detail. Figure 6 shows the results obtained in a single cell with 10 μM chlorpromazine. In this cell, chlorpromazine produced a 54% inhibition of the sustained current and a 22% inhibition of the transient current. The mean results are shown in Figure 7. Chlorpromazine (10 μM) and amitriptyline (10 μM) produced inhibitions of the sustained component of 54.8 ± 2.0% ($n = 3$) and 48.3 ± 4.1% ($n = 5$) respectively, but significantly less inhibition of the transient component; only 20.3 ± 1.8% and 18.2 ± 1.2%. Note that the inhibition of the sustained component compares very well with the results obtained for these compounds in Figure 4 for inhibition of I_{Kv} using protocol 1. In one cell, imipramine (10 μM) produced a similar effect, 47.7% inhibition of the sustained component but only 17.6% inhibition of the transient compound. The ratios of the effectiveness of amitriptyline, chlorpromazine and imipramine on the transient compared to the sustained

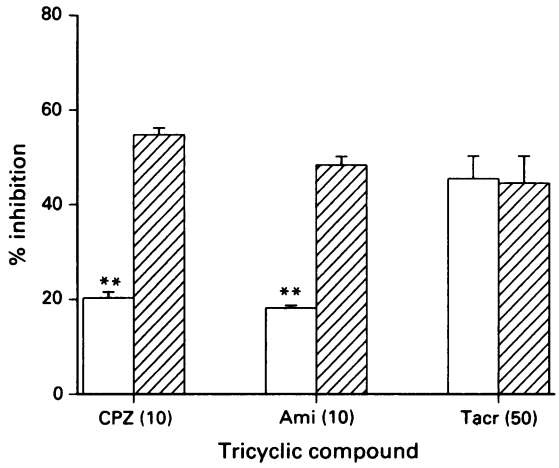

Figure 7 Shows the relative effects of 10 μM chlorpromazine, 10 μM amitriptyline, and 50 μM tacrine on the transient (open bars) and sustained (hatched bars) components of the outward current. Chlorpromazine (CPZ) and amitriptyline (Ami) (10 μM) produced inhibitions of 20.3 ± 1.8%; 54.8 ± 2.0% ($n = 3$) and 18.2 ± 1.2%; 48.3 ± 4.1% ($n = 5$), of the transient and sustained components respectively. Tacrine (50 μM) produced a similar inhibition of both components (transient: 45.5 ± 4.8%; sustained: 44.5 ± 5.8%, $n = 5$). For chlorpromazine and amitriptyline, inhibition of the transient component is significantly different from that of the sustained component (** denotes $P < 0.01$). However, the inhibition of the two components by tacrine was not significantly different.

components of the current were strikingly similar (1:2.66, 1:2.70 and 1:2.72, respectively). Amitriptyline (10 μM) inhibited I_A at −40 and −20 mV by 21.1% and 26.5%, respectively ($n = 2$) using the prepulse subtraction protocol above, compared with 53.2% inhibition of I_{Kv} at 0 mV when amitriptyline was applied earlier to the same two cells. At higher concentrations of the blocking drugs the selectivity, not surprisingly, becomes less marked as the block of I_{Kv} reaches it maximum. Thus 100 μM imipramine inhibited the sustained component by 80.1 ± 2.1% ($n = 3$) and the transient component by 57.5 ± 4.2% a ratio of only 1:1.39.

Tacrine, on the other hand, was equally effective at inhibiting both components of the current. Tacrine (50 μM) inhibited the sustained component by 44.5 ± 5.8% ($n = 5$) and the transient current by 45.5 ± 4.8%, a ratio of 1:0.98. At a concentration of 300 μM, tacrine inhibited the sustained component by 78.7% ($n = 2$) and the transient component by 75.7%.

Discussion

The five compounds tested can be divided into two groups in terms of their effectiveness at blocking I_{Kv}. Imipramine, amitriptyline and chlorpromazine were equally effective blockers of I_{Kv} but tacrine was about 7 fold less potent and carbamazepine was ineffective.

Imipramine and amitriptyline have a different tricyclic ring structure to chlorpromazine but have a similar three carbon central chain; indeed imipramine and chlorpromazine have an identical central chain. Carbamazepine, on the other hand, has a similar ring structure to the TCAs but a much shorter central chain. Imipramine, amitriptyline and chlorpromazine are weak bases (with pK_As of 9.5, 9.4 and 9.3; Jack, 1992) and are therefore mostly charged at pH 7.4. Carbamazepine and tacrine have no published pK_A values (Jack, 1992); however, carbamazepine is described as a neutral lipophilic substance (Kutt & Paris-Kutt, 1982). It is not clear whether amitriptyline, imipramine and chlorpromazine are effective blockers of I_{Kv} because of the length of the central chain *per se* or because the aromatic rings have less influence on the terminal tertiary amine. Theoretically, this could be answered by changing the pH of the recording solutions to alter the ratio of charged to uncharged molecules, however, this would be extremely difficult practically, as experiments would need to be done at pH 9–10.

Whichever is the case, it appears that the structure of the rings is relatively unimportant in determining blocking potency but the central chain structure is critical. This observation can be compared with the blocking potency of quaternary ammonium ion derivatives on the squid giant axon delayed rectifier K^+ channels where the length of the chain separating a hydrophobic benzene from a quaternary nitrogen determines the potency of these compounds and is optimal at a length of three carbon atoms (Armstrong, 1971). Furthermore, quinidine and its analogue quinacrine, block the fast transient outward current $I_K(f)$ of rat melanotrophs and have a broadly similar structure to the TCAs and chlorpromazine. For these compounds it has been suggested that the aromatic rings play an important anchoring role at, or close to, the site where they bind to the channel; however, the exact structure of the ring may not be critical since quinidine and quinacrine are both effective blockers yet have quite different ring structure (Kehl, 1991).

The mechanism of block of I_{Kv} by the tricyclic compounds has not been studied in detail here; however, there are some important points that can be made from our data. None of the tricyclic compounds tested altered the rate of activation of I_{Kv} or induced inactivation. This can be contrasted with tetrapentylammonium block of squid giant axon delayed rectifier K^+ current (French & Shoukimas, 1981) or clofilium block of I_{Kv} in NG108-15 cells (Reeve & Peers, 1992) where the drugs promote inactivation of I_{Kv}. Compounds which block in this way are thought to be open-channel blockers acting at a site inside the pore of the channel thereby mimicking the normal mechanism of inactivating K^+ channels (Armstrong, 1966; 1971). It may be that the tricyclic compounds tested here act in a similar way, but like TEA, block so quickly that 'inactivation' is not seen (Hille, 1992). The block that the tricyclic compounds produce is relatively voltage-independent, however, and this would argue against such a mechanism. The TCAs and chlorpromazine are much more potent blockers of I_{Kv} than TEA with K_Ds in the micromolar rather than millimolar range. Furthermore, the increased noise level of the current trace in the presence of the blocking drugs suggests that the drugs dissociate relatively slowly from the channels (k_{-1} of a few ms or longer) compared to TEA. Experiments using single-channel recording would be necessary in order to obtain further insight in to the mechanism of block of I_{Kv} by these tricyclic compounds.

The TCAs and chlorpromazine show some degree of selectivity for I_{Kv} over I_A compared to tacrine; however, at higher drug concentrations both currents are blocked. Imipramine and chlorpromazine have been shown to block I_{Kv} in neuroblastoma cells or I_T in cardiac cells with about the same potency as their action here on I_{Kv} in sympathetic neurones (Ogata *et al.*, 1989; 1990; Delpón *et al.*, 1992). Similarly, tacrine blocks I_{Kv} in heart cells by the same degree as here and it is a slightly more potent blocker of I_f in heart cells (K_D ~18 μM; DiFrancesco *et al.*, 1991). On the other hand, tacrine seems much less effective at blocking I_A in NG108-15 cells, $K_D = 0.17$ mM (Robbins & Sim, 1990) than was found here for block in sympathetic neurones. To add to the complexity, imipramine and chlorpromazine are both able to block Na^+ and Ca^{2+} currents in neuroblastoma cells being most effective on Na^+ currents (K_D ~3 μM) and least effective on Ca^{2+} currents (K_D ~27 μM) (Ogata *et al.*, 1989; 1990). The block of Ca^{2+} channels has been suggested to be mediated through a *pertussis* toxin-sensitive G-protein (Choi *et al.*, 1992).

It seems then, that these compounds show some potency differences for block between ion channels but that they are not selective blockers and are able to inhibit a number of

different ion channels. This may have some advantages in terms of the design of selective drugs for targeting particular ion channels.

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 chlorpormazine binding site and the surrounding structure of nicotinic receptors with known sequences of *Shaker*-like K^+ channels (Pongs, 1992a) shows 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 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.

Thus compounds which block a number of different ion channel-types may provide important structural information about the different channels that they block and illustrate regions of homology between them. Furthermore, it is clear from this study that relatively small changes to the structure of tricyclic blocking compounds can have profound effects on their blocking potency, so it may be possible to discover or design more selective compounds knowing the sequence of amino-acids in the region of protein to which they bind.

The results presented here for the TCAs can explain the experimental observation of Bennett & Middleton (1975) who showed that TCAs could prolong the preterminal action potential of sympathetic neurones. At the concentration they used (about 10 μM) I_{Kv} would be blocked by the TCA by about 50%. Such a prolongation of the action potential is a direct consequence of potassium channel block by a number of different drugs (e.g. Robbins & Sim, 1990; Delpón *et al.*, 1992; Zhang *et al.*, 1992).

Carbamazepine did not block I_{Kv} in sympathetic neurones and in some cases it has been suggested to enhance K^+ currents (Zona *et al.*, 1990). At first sight this would seem to be a requirement for such a drug since any increase in excitability would be counter-productive to its anti-convulsant action. Surprisingly then, the anticonvulsants valproate (Van Erp *et al.*, 1990) and U-54494A (Zhu *et al.*, 1992) have been shown to block neuronal delayed-rectifier K^+ currents.

It is difficult to be certain of the clinical importance of these effects of the TCAs and chlorpromazine on K^+ channels, due to variations in published values for therapeutic plasma concentrations of these drugs. A best estimate for imipramine is about 0.5–1 μM under therapeutic conditions, rising to >3 μM in overdose (see Benet & Williams, 1990). Similar values would seem to be appropriate for chlorpromazine; however, these drugs may accumulate in ganglia or brain tissue by up to 20 fold. Even at therapeutic concentrations of around 1 μM, significant (10–15%) inhibition of I_{Kv} will occur which could profoundly alter the excitability and firing frequency of neurones and contribute to some of the therapeutic or adverse effects of these drugs.

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The effects of β-adrenoceptor activation on contraction in isolated fast- and slow-twitch skeletal muscle fibres of the rat

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1 The aim of the experiments was to examined the effects of β-adrenoceptor activation on twitch and tetanic contractions in fast- and slow-twitch mammalian skeletal muscle fibres. Isometric force was recorded from bundles of intact fibres isolated from the normal and denervated slow-twitch soleus and normal fast-twitch sternomastoid muscles of the rat.

2 Terbutaline (10 μM), a β_2-adrenoceptor agonist, induced an average 15% potentiation of peak twitch and peak tetanic force in normal soleus fibres and abbreviated twitch and tetanic relaxation. In white- and red-sternomastoid fibres, 10 μM terbutaline potentiated peak twitch force by about 7% and slowed twitch relaxation.

3 The potentiation of twitches and tetani by terbutaline was quantitatively similar in normal and denervated soleus fibres. However, in contrast to the normal soleus, terbutaline slowed twitch relaxation and had no effect on tetanic relaxation in denervated soleus fibres.

4 Adrenaline (10 μM) increased peak tetanic force by about 7% in both normal and denervated soleus fibres.

5 Exposure to (±)-propranolol (0.1 μM), a general β-adrenoceptor blocker, completely abolished the tetanus potentiation by terbutaline.

6 Dibutyryl-cyclic AMP (2 mM) mimicked the effects of 10 μM terbutaline on peak tetanic force and tetanic relaxation in normal and denervated soleus fibres. Dibutyryl-cyclic AMP also potentiated peak twitch force in denervated soleus fibres but only after a brief period of twitch depression: the twitch depression might be due to butyrate.

7 The results suggest that the increase in peak twitch and tetanic force and abbreviation of tetanic relaxation induced by terbutaline depend on the activation of β-adrenoceptors and a consequent increase in the myoplasmic cyclic AMP concentration.

Keywords: β-Adrenoceptor; terbutaline; cyclic AMP; skeletal muscle contraction

Introduction

Sympathomimetic-amines modulate contraction and relaxation in mammalian skeletal muscles (reviewed by Tomita, 1975; Bowman, 1980; Williams & Barnes, 1989). The nature of the response depends on the fibre-type composition of muscles. In fast-twitch muscles (a) peak twitch force is increased by 10–20% and twitch relaxation is slowed (Goffart & Ritchie, 1952; Bowman & Zaimis, 1958; Bowman *et al.*, 1962; Tashiro, 1973; Tomita, 1975; Holmberg & Waldeck, 1980) and (b) the amplitude of unfused tetani is increased by up to 60% and the degree of fusion increased (Bowman & Zaimis, 1958; Bowman *et al.*, 1962; Holmberg & Waldeck, 1977; 1980). In contrast, peak twitch force is depressed by 10–25% in slow-twitch muscles and twitch relaxation is accelerated (Bowman & Zaimis, 1958; Bowman *et al.*, 1962; Tashiro, 1973; Tomita, 1975). The amplitude of unfused tetani is decreased by as much as 60% and fusion is reduced (Bowman & Zaimis, 1958; Bowman & Nott, 1970; Holmberg & Waldeck, 1979; 1980). However, under some conditions force is increased in slow-twitch muscles (Tashiro, 1973; Tomita, 1975; Holmberg & Waldeck, 1980). Sympathomimetics in these previous studies had little or no effect on peak tetanic force *in vivo* (Goffart & Ritchie, 1952; Bowman & Zaimis, 1958; Bowman & Nott, 1970; Williams & Barnes, 1989).

Most previous studies have examined the effects of sympathomimetic-amines on twitches and unfused tetani evoked by nerve stimulation in anaesthetized animals or in directly stimulated whole muscles *in vitro*. The aim of the present study was to investigate the direct effects of β-adrenoceptor activation on isometric contractions by using small bundles of fibres isolated from various rat skeletal muscles. The use of these preparations allowed us to: (i) eliminate indirect sympathomimetic effects on contraction via neuromuscular transmission; (ii) reduce drug diffusion problems to inner fibres of whole muscles; (iii) distinguish between effects in different fibre-types.

Adrenoceptors in the sarcolemma of mammalian skeletal muscle are predominantly of the β_2-subtype (Hirata *et al.*, 1986; Elfellah & Reid, 1987). Consequently, terbutaline, a selective β_2-adrenoceptor agonist (Holmberg & Waldeck, 1977; McArdle & D'Alonzo, 1981), was chosen as the principal drug for the present study. Novel findings of this study were that terbutaline increased peak tetanic force in all types of preparation studied and abbreviated the relaxation of tetanic force in normal slow-twitch soleus fibres. Twitch force was increased and the relaxation of the twitch was faster in the presence of terbutaline. Furthermore, dibutyryl cyclic AMP mimics the effects of β-receptor activation on contractile force and relaxation, suggesting that cyclic AMP is the second messenger responsible for the effects of terbutaline on contraction.

Preliminary accounts of some of this work have been reported elsewhere (Cairns & Dulhunty, 1989a,b).

Methods

Biological preparations

Adult male Wistar rats (250–550 g) were killed with an overdose of either halothane or CO_2. Soleus or sternomastoid muscles were isolated, pinned by their tendons

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into a Petri dish lined with Sylgard (Dow Corning) and bathed in a modified Krebs solution (Solution A, Table 1) at room temperature (18–22°C). Small bundles of 5–50 fibres were dissected from tendon-to-tendon. Soleus muscles contain predominantly slow-twitch type I fibres (McArdle & D'Alonzo, 1981; Williams *et al.*, 1984) whereas red- or white-sternomastoid muscle segments contain predominantly fast-twitch type IIa or IIb fibres, respectively (Dulhunty & Dlutowski, 1979).

Denervation procedure

Rats were anaesthetized with ether and a 4–5 mm segment of the sciatic nerve was removed just distal to the sciatic notch. Soleus muscles were usually removed about 3–6 weeks later and denervation confirmed by: (i) whole muscle atrophy (Festoff *et al.*, 1977; Dulhunty, 1985); (ii) fibrillations (Smith & Thesleff, 1976; Dulhunty, 1985); (iii) a higher than normal twitch-to-tetanus ratio (Dulhunty, 1985).

Force recording and stimulation

Fibre bundles were mounted horizontally in a small volume (1.4 ml), rapid flow bath, with a solution change-over time of 700 ms. One tendon was held in spring-loaded clamping forceps and the other tendon was attached to a piezo-resistive force transducer (AME, 0–6 g, Horton, Norway). Isometric contractions were evoked by massive transverse electric field stimulation via platinum electrodes. Supramaximal stimulation was achieved by setting the voltage pulses to 0.5 ms duration and 1.1–1.3 times the voltage amplitude which just produced maximum twitch force. The pulse parameters were set using an isolated stimulator constructed in our laboratory. The length of the preparation was adjusted until maximum twitch force was obtained. Temperature was monitored by a thermistor placed in the bathing solution near the preparation and was set at 24.0°C, maintained to within 0.3°C, with a circulating water jacket.

Tetani were evoked at 100 Hz (unless stated otherwise) for a duration that ensured a force plateau was established (usually 1 s in soleus fibres and 250–300 ms in sternomastoid fibres). Stimulation was initiated by computer (Digital PDP 11/03) and pulse parameters set with an isolated stimulator constructed in our laboratory. Force was recorded continuously on a chart recorder (Hewlett Packard 7402A) and selected twitches and tetani sampled by the computer for later analysis.

Solutions and drugs

Most experiments were done in Solution A (Table 1). Solutions were usually bubbled with 100% O_2, although the absence of 100% O_2 did not influence contractile force or the viability of the fibres, suggesting that sufficient atmospheric O_2 equilibrated with the rapidly flowing solutions to maintain contractile function. All fine dissection took place in Solution A since it took about 1 h for the fibres to equilibrate in this solution.

Table 1 Composition of bathing solutions (in mM)

Solution	*NaCl*	*Na_2SO_4*	*KCl*	*K_2SO_4*	*$CaCl_2$*	*$CaSO_4$*	*Sucrose*
A	16	32.25	–	1.75	–	7.6	170
B	16	67.00	–	1.75	–	7.6	
Normal Krebs	150	–	3.5	–	2.5	–	–

In addition all solutions contained: 1 mM $MgSO_4$; 11 mM glucose; 2 mM TES(N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) pH buffer. The pH was adjusted to 7.4 ± 0.1 pH units with NaOH. The measured free Ca^{2+} concentration in solutions A and B was 2 mM.

Drugs were added to the bathing solution. Force was monitored until the maximum effect was seen and the drug was then washed out of the bath. Drugs used and sources were: terbutaline sulphate (ASTRA Pharmaceuticals); adrenaline hydrogen tartate (BDH Chemicals Ltd); N^6,-2'-O-dibutyryl adenosine 3':5'-cyclic adenosine monophosphate sodium salt (Sigma); (±)-propranolol hydrochloride (Sigma); n-butyric acid sodium salt (Sigma); ethylenediaminetetraacetic acid sodium salt (EDTA, AJAX); (+)-tubocurarine chloride (Boehringer). Terbutaline, adrenaline, dibutyryl-cyclic AMP and propranolol were all stored in anhydrous form in light-proof containers at 4°C. EDTA (50 μM) was added to all solutions to prevent the oxidation of sympathomimetic-amines. EDTA has no direct effect on force.

Analysis

The contractile properties measured were: peak twitch and peak tetanic force; 80–20% relaxation time; 20–80% rise time (twitch) and 20–60% rise time (tetanus). Maximum drug-induced changes in peak force or rise times were usually expressed relative to control contractions recorded immediately prior to drug application. However, the effect of terbutaline on peak tetanic force in sternomastoid fibres was difficult to quantify because of a progressive run-down of force with repetitive tetanic stimulation. Consequently, the control value for peak tetanic force was estimated from the values for peak tetanic force recorded immediately prior to and following wash-out terbutaline. A constant rate of force run-down was assumed and the control value was determined by interpolation to the time of the maximum terbutaline-induced effect. Changes in relaxation times were compared to the mean of controls obtained immediately prior to drug administration and following wash-out, since relaxation slowed progressively during the experiment. The effect of dibutyryl cyclic AMP (dbcyclic AMP) on relaxation was compared with the initial control since the effect of this drug on force did not readily reverse on wash-out. Values presented in the text are the mean ± s.e.mean of n preparations. Student's t test (paired data) was used to examine if drug-induced effects were significant and ANOVA (paired + unpaired data) was used to compare quantitatively the effects of different drugs or the effect of a drug in different types of preparation.

Results

Effects of terbutaline on slow-twitch soleus fibres

Terbutaline (10 μM) did not induce contractures, but induced positive inotropic effects (i.e. an increase in peak twitch and tetanic force) in slow-twitch soleus fibres (Figure 1). Maximum potentiation of the twitch and tetanus took 5–15 min and this potentiation was usually maintained until terbutaline was washed out of the bath. Full recovery from the effects of terbutaline were seen within 10–15 min after wash-out.

The average increase in peak twitch force was 14.6 ± 2.9% ($n = 15$, $P < 0.001$). Holmberg & Waldeck (1980) reported that terbutaline induced an initial depression of twitch amplitude in soleus muscles followed by potentiation. An early depression was seen in 3 out of 15 preparations in the present study but it was small (never more than 4% of peak twitch force): the decrease in force reversed and a potentiation was observed by 5 min.

An average increase in peak tetanic force of 14.6 ± 1.9% ($n = 30$, $P < 0.001$, Table 2) was recorded. This was a surprising result because sympathomimetic-induced tetanus potentiation of this magnitude had not been seen previously (Bowman, 1980; Williams & Barnes, 1989). The considerable variation in tetanus potentiation (s.d. = 10.5%, maximum increase 38.1%) could not be explained by submaximal receptor activation, preparation thickness, age of the animal,

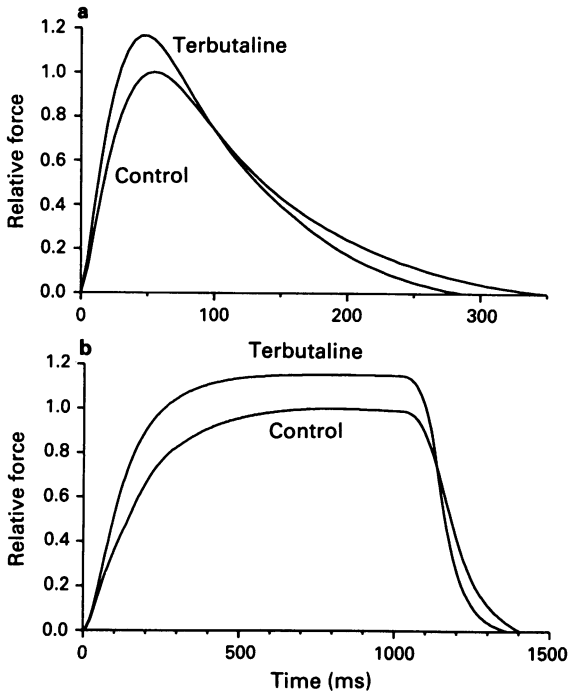

Figure 1 Effects of 10 μM terbutaline on (a) twitch force and (b) tetanic force in slow-twitch soleus fibres. Solution A, 24°C. Control, is the force record obtained immediately prior to the addition of terbutaline. Terbutaline, is the force record showing the maximum effect with terbutaline.

Table 2 Effects of terbutaline, adrenaline and dibutyryl-cyclic AMP on peak tetanic force in normal and denervated soleus fibres

	Peak tetanic force (% increase)	
Drug	*Normal soleus*	*Denervated soleus*
Terbutaline (10 μM)	14.6 ± 1.9% ($n = 30$)	13.5 ± 1.1% ($n = 44$)
Adrenaline (10 μM)	7.8 ± 2.6%* ($n = 4$)	6.9 ± 2.3% ($n = 6$)
dbCyclic AMP (2 mM)	15.2 ± 2.0% ($n = 5$)	16.0 ± 2.8% ($n = 7$)
Terbutaline** (10 μM)	12.7 ± 4.6%	17.0 ± 3.8%

Values shown are the mean ± s.e.mean of n preparations. The maximum increase in peak tetanic force (100 Hz) was expressed as a percentage increase relative to the immediately preceding control tetanus. Solution A, 24°C. Drug induced effects were tested by paired t test.
All values were significant for $P < 0.001$ except * where $P < 0.05$; **indicates paired with dbcyclic AMP.

season, or the extent of run-down (as in isolated cardiac muscle, Kurihara & Konishi, 1987). Indeed, the same variation was observed when different preparations from the same muscle were used, indicating a biological origin.

Although sympathomimetics have been reported to depress the amplitude of unfused tetani in slow-twitch muscles (Bowman & Nott, 1970; Holmberg & Waldeck, 1979), 10 μM terbutaline in our hands induced a 7–37% potentiation of unfused tetani (evoked at 20 Hz for 1 s at 31°C ($n = 2$) or 15 Hz for 1 s at 24°C ($n = 1$)).

Since sympathomimetic-amines facilitate neuromuscular transmission by stimulating α-adrenoceptors (Bowman *et al.*, 1962; Bowman & Nott, 1970; Tomita, 1975), it was unlikely that terbutaline (a β_2-agonist) potentiated force by acting at the neuromuscular junction. This was confirmed when terbutaline, applied in the presence of 1.5 μM tubocurarine, potentiated tetanic force in the usual way ($n = 2$), suggesting that the drug acted directly on the muscle fibres.

Terbutaline also abbreviated the time-course of twitch and tetanic relaxation (Figure 1): the 80–20% relaxation time was abbreviated from 169 ± 15 ms to 153 ± 7 ms ($n = 9$, $P < 0.05$) for the twitch and from 156 ± 8 ms to 119 ± 6 ms ($n = 20$, $P < 0.001$) for the tetanus. The effect of terbutaline on tetanic relaxation has not been described previously and was significantly greater than the effect on twitch relaxation ($P < 0.05$). No significant changes were observed in rise times for either the twitch or tetanus.

Effects of terbutaline on fast-twitch sternomastoid fibres

No previous studies have compared the effect of sympathomimetic-amines on contraction in different types of mammalian fast-twitch fibres. Terbutaline (10 μM) increased peak twitch force in both white- and red-sternomastoid fibres (Figure 2). Twitch potentiation was the same, i.e. 7.3 ± 2.1% ($n = 8$, $P < 0.025$) and 7.2 ± 2.8% ($n = 5$, $0.1 < P < 0.5$) in white- and red-sternomastoid fibres, respectively and the average pooled potentiation (7.3 ± 1.6) was significantly smaller than in slow-twitch soleus fibres ($P < 0.05$). Peak tetanic force was increased by terbutaline in two preparations (5.1% and 7.5%) while in seven other preparations the run-down was slowed. The average terbutaline-induced increase in peak tetanic force was 6.5 ± 1.3% ($n = 9$, $P < 0.001$, maximum increase 13.5%) following correction for run-down.

Twitch relaxation was prolonged with terbutaline in sternomastoid fibres (Figure 2), in contrast to the faster twitch relaxation in soleus. The average 80–20% relaxation time increased from 44.5 ± 3.5 ms to 50.0 ± 3.0 ms ($n = 8$, $P < 0.01$) in white-sternomastoid fibres and from 60.0 ± 11.0 ms to 64.0 ± 11.0 ms ($n = 5$, $P < 0.05$) in red-sternomastoid fibres. There was no significant change in the rise time for twitches. We noted that, although the average relaxation time increased, relaxation times in some individual preparations remained unchanged in the presence of terbutaline, despite significant twitch potentiation. This suggested that the pro-

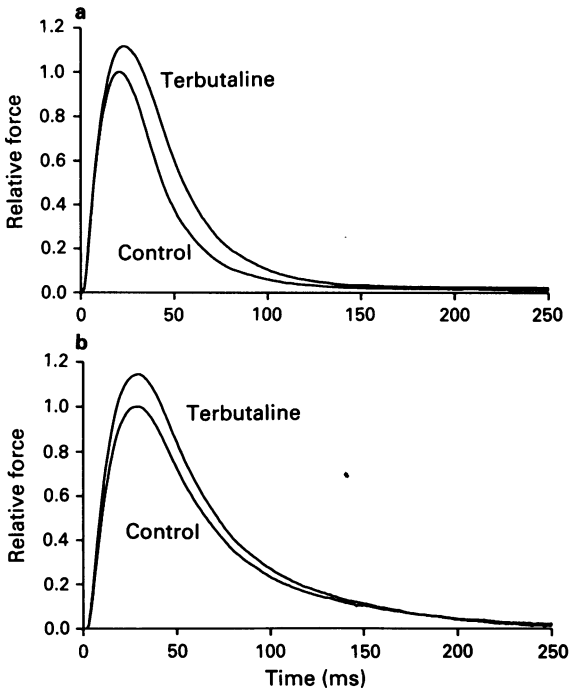

Figure 2 Effects of 10 μM terbutaline on isometric twitches in (a) fast white-sternomastoid and (b) fast red-sternomastoid fibres. Solution A, 24°C. Note the prolonged relaxation with terbutaline in (a) but no change in the relaxation time-course in (b). Terbutaline and control are defined in the legend of Figure 1.

cess responsible for twitch potentiation was independent of the process responsible for prolonged relaxation.

Effects of terbutaline on denervated soleus fibres

Skeletal muscle is thought to become supersensitive to catecholamines following chronic denervation as catecholamine-induced contractures have been reported (Bowman & Raper, 1965; Evans & Smith, 1976). It was possible that the denervated muscles may also become supersensitive to terbutaline. Thus we investigated the possibilities that contractures might be induced by terbutaline and that potentiation of force would be enhanced in denervated fibres. Terbutaline (10 μM) did not induce contractures. Instead it caused a small depression of resting force in 50% of the preparations and reduced the amplitude of fibrillations (asynchronous twitches produced by the spontaneous firing of action potentials in some fibres, Smith & Thesleff, 1976; Dulhunty, 1985).

Terbutaline did potentiate twitches and tetani in denervated soleus fibres (Figure 3). The average increase of peak twitch force of 13.7 ± 2.1% ($n = 23$, $P < 0.001$) was not significantly different from that in normal soleus fibres ($P > 0.1$). As in normal soleus fibres, maximum twitch potentiation occurred 10–15 min after the drug was applied and the effect was reversed when terbutaline was washed out of the bath. However, in contrast to normal soleus fibres, relaxation of the twitch in the denervated soleus was prolonged by terbutaline (Figure 3a). On average the 80–20% relaxation time increased from 114 ± 6 ms to 151 ± 9 ms ($n = 19$, $P < 0.001$). The potentiation of peak tetanic force of 13.5 ± 1.1% ($n = 44$, $P < 0.001$, Table 2) was not different from that in the normal soleus ($P > 0.1$). Thus, denervation did not alter the extent of twitch or tetanus potentiation, nor the variability in tetanus potentiation (s.d. = 7.4%, maximum increase 29.7%). Terbutaline did not influence tetanic relaxation in denervated soleus fibres. The 80–20% relaxation times were 92 ± 3 ms (control) and 95 ± 3 ms (terbutaline) ($n = 37$, $P > 0.1$).

There was another notable change in the response to terbutaline following denervation. Repeated exposure to terbutaline (at least 20 min between applications) induced similar increases in peak tetanic force in denervated soleus fibres. In contrast, normal soleus fibres displayed progressively smaller response with successive applications, i.e. tachyphylaxis. The effect of terbutaline on peak tetanic force on the third application was 80 ± 5% ($n = 12$) of the increase on the first application in denervated fibres, but was only 44 ± 7% ($n = 5$) in normal fibres. Thus denervation resulted in some protection against tachyphylaxis.

The effect of increasing concentrations of terbutaline on peak twitch force (not shown) was similar to the effect on peak tetanic force (Figure 4) and these effects were quantitatively similar in four preparations examined. Terbutaline (0.1 μM) first slightly depressed peak force (the largest decrease was 8%) and then augmented force. Progressively increasing the concentration of terbutaline caused further potentiation; the maximum increase was with 10 μM terbutaline.

Tetanus potentiation by terbutaline under different experimental conditions

The tetanus potentiation with terbutaline was very much greater than reported previously for other sympathomimetic amines (Bowman, 1980). We considered the possibility that the large tetanus potentiation was a consequence of our experimental conditions. The effects of the composition of the bathing solution and stimulation frequency during the tetanus were examined.

The action of terbutaline in different bathing solutions The amplitude of action potentials during tetanic stimulation may have been reduced in the modified Krebs solution which contained 80.5 mM Na^+ (Solution A), compared with the normal 150 mM Na^+ (Table 1). A smaller action potential amplitude would have reduced peak tetanic force to allow room for greater potentiation with terbutaline. However, this was not the case because 10 μM terbutaline produced a similar increase in peak tetanic force in the normal Krebs solution ($n = 5$). In three denervated soleus preparations, where paired data were obtained, terbutaline potentiated peak tetanic force by 19.4 ± 10.1% in the normal Krebs solution and then by 15.2 ± 4.7% in the 80.5 mM Na^+ solution. In addition, peak tetanic force was increased when the bathing solution was changed from Solution A to one containing 196 mM Na^+ (Solution B, Table 1), but this did not prevent tetanus potentiation by terbutaline (Figure 5). Peak tetanic force was increased by 18.4 ± 5.0% ($n = 3$) when exposed to the 196 mM Na^+ solution and then by a further 8.9 ± 0.9% with terbutaline.

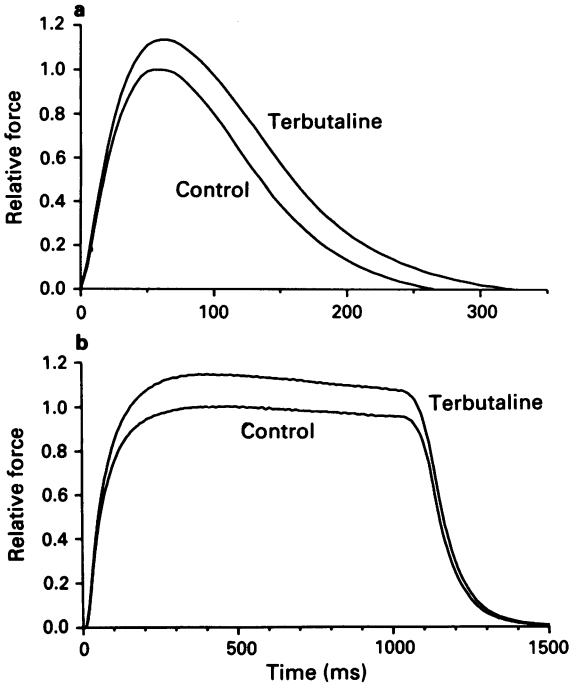

Figure 3 Typical effects of 10 μM terbutaline on (a) twitch force and (b) tetanic force in denervated soleus fibres. Solution A, 24°C. Terbutaline and control are defined in the legend of Figure 1.

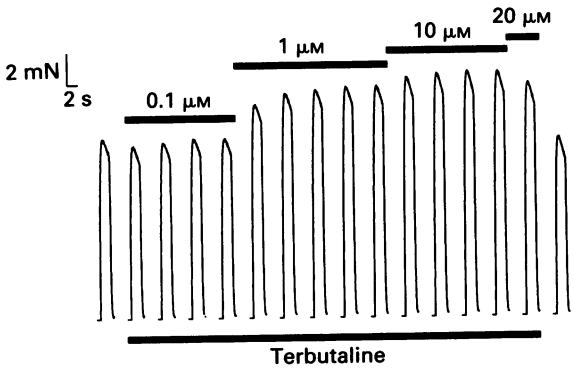

Figure 4 Concentration- and time-dependent effects of terbutaline on peak tetanic force in denervated soleus fibres. Tetani were evoked at 50 Hz for 2 s every 5 min. Solution A, 24°C. The concentration of terbutaline was progressively increased during the experiment with the period of exposure to terbutaline, at each concentration, indicated by the bars above the force records.

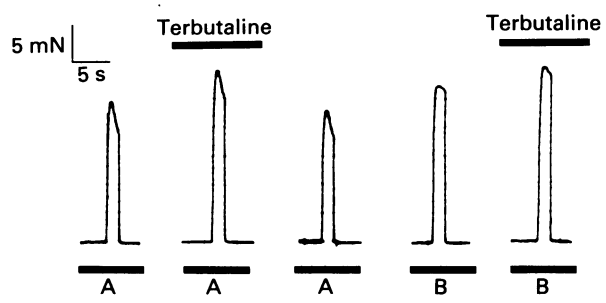

Figure 5 Chart recordings showing the effect of 10 μM terbutaline on peak tetanic force in Solution A ($[Na^+] = 80.5$ mM, $[Cl^-] = 16$ mM) and then in Solution B ($[Na^+] = 196$ mM, $[Cl^-] = 16$ mM) in a denervated soleus preparation. Tetani were evoked at 30 Hz for 2 s, 24°C. Terbutaline increased peak tetanic force by 22% in Solution A. The third tetanus was recorded in Solution A following washout of terbutaline. Exposure to Solution B increased peak tetanic force by 17% and then terbutaline potentiated peak tetanic force by 10% in Solution B.

The action of terbutaline at different frequencies of tetanic stimulation Although fully fused tetani were evoked at 100 Hz, a greater peak tetanic force was achieved at lower frequencies (around 50 Hz) in normal and denervated soleus fibres (in Solution A, 24°C). Since terbutaline may have acted by preventing this depression of force at 100 Hz, the effect of terbutaline was examined on tetani evoked at 100 and 50 Hz. The peak tetanic force at 100 Hz was 87.4 ± 2.0% ($n = 9$) of the 50 Hz tetanus in normal soleus fibres. Terbutaline increased the amplitude of tetani at 100 Hz by 14.6 ± 1.9% ($n = 30$) and by 8.6 ± 1.6% ($n = 9$) at 50 Hz. In paired experiments on seven denervated soleus preparations, the peak tetanic force at 100 Hz was 79.8 ± 1.8% of that at 50 Hz. Terbutaline potentiated the 100 and 50 Hz tetani by 15.0 ± 1.8% and 12.9 ± 1.6%, respectively. Therefore tetanus potentiation by terbutaline did not depend on the frequency of tetanic stimulation.

Effects of adrenaline on contraction

It was of interest to see if adrenaline, a physiological catecholamine, induced similar effects on contraction to those of terbutaline. Adrenaline (10 μM) potentiated tetani by about 7% in normal and denervated soleus fibres (Table 2), and potentiated twitches by 17.3 ± 3.4% ($n = 6$, $P < 0.01$) (pooled normal and denervated soleus fibres). There was no consistent effect on relaxation times. Adrenaline did not alter resting force in normal soleus fibres, but induced small contractions (associated with an increased amplitude of fibrillations) in denervated soleus preparations. This was in contrast to the decrease in resting force and the depressed fibrillations with terbutaline.

Effect of propranolol on tetanus potentiation by terbutaline

Since high concentrations of terbutaline were used, the drug may have produced effects that were independent of β-adrenoceptor stimulation, perhaps by activating α-adrenoceptors which may also be found in rat skeletal muscle (Hirata *et al.*, 1986). Propranolol, a general β-adrenoceptor blocker, was used to test this possibility. Addition of 0.1 μM propranolol alone had no direct effect on force. The tetanus potentiation with terbutaline was completely abolished following a 15 min exposure to 0.1 μM propranolol (Figure 6) in three denervated and one normal soleus. Thus force potentiation by terbutaline requires β-receptor activation.

Effects of dibutyryl cyclic AMP on contraction

Effects of β-agonists may occur via cyclic AMP (Oota & Nagai, 1977; Fellenius *et al.*, 1980b) or directly via G-proteins (Yatani *et al.*, 1988). We tested the hypothesis that an increased concentration of myoplasmic cyclic AMP ($[cyclic\ AMP]_i$) was responsible for the effects of terbutaline on contraction using, dibutyryl cyclic AMP (dbcyclic AMP), a derivative of cyclic AMP which is more membrane permeable and less susceptible to intracellular hydrolysis than is cyclic AMP (Skelton *et al.*, 1970; Drummond *et al.*, 1974).

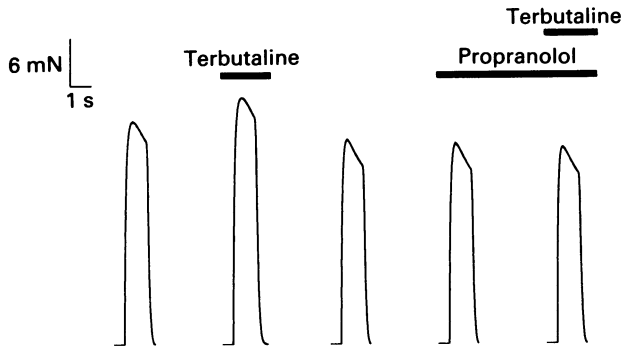

Figure 6 Effect of 10 μM terbutaline on peak tetanic force (100 Hz) in the absence and presence of 0.1 μM propranolol in a denervated soleus preparation. Solution A, 24°C. Terbutaline maximally increased peak tetanic force by 11% in 10 min under control conditions. Propranolol added for 15 min had no direct effect on force production and then terbutaline failed to potentiate force during exposure to propranolol when added for 20 min.

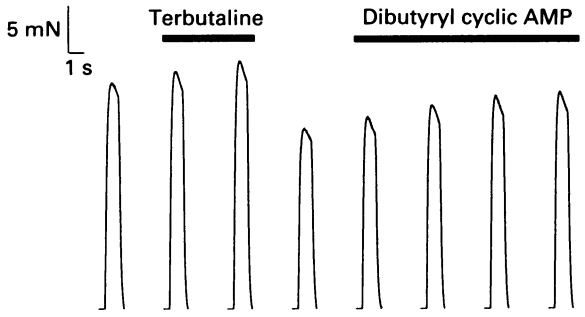

Figure 7 Representative effects of 10 μM terbutaline and 2 mM dibutyryl cyclic AMP on peak tetanic force (100 Hz) in a normal soleus preparation. Solution A, 24°C. Tetani were recorded every 5 min. Terbutaline maximally increased peak tetanic force by 10% in 10 min. The second control was obtained about 30 min after washout of terbutaline. Dibutyryl cyclic AMP maximally increased peak tetanic force by 19% in 20 min.

dbCyclic AMP (2 mM) mimicked the tetanus potentiation seen with 10 μM terbutaline in normal soleus fibres (Figure 7). Notably a longer time was required to develop maximum effects with dbcyclic AMP and these effects reversed only slowly on wash-out of the drug. Consequently, in paired experiments, terbutaline was added first and post-wash controls for dbcyclic AMP were not obtained. The tetanus potentiation by terbutaline and dbcyclic AMP was quantitatively similar in normal and denervated soleus fibres (Table 2). As with terbutaline, dbcyclic AMP had no effect on tetanic relaxation in denervated soleus fibres, but abbreviated relaxation in normal soleus fibres (Figure 8). The 80–20% relaxation time was reduced from the control 165 ± 15 ms to 126 ± 7 ms with dbcylic AMP ($n = 5$, $P < 0.001$).

Unexpectedly, dbcyclic AMP reduced peak twitch force by 7.4 ± 2.0% ($n = 6$, $P < 0.05$) at 5 min; an effect seen with 0.1 μM terbutaline, but seldom seen with 10 μM terbutaline (see above). Twitch potentiation slowly developed and the initial twitch depression was reversed (Figure 9). In nine paired experiments, maximum twitch potentiation with dbcyclic AMP (10.3 ± 4.1%) was similar to that with ter-

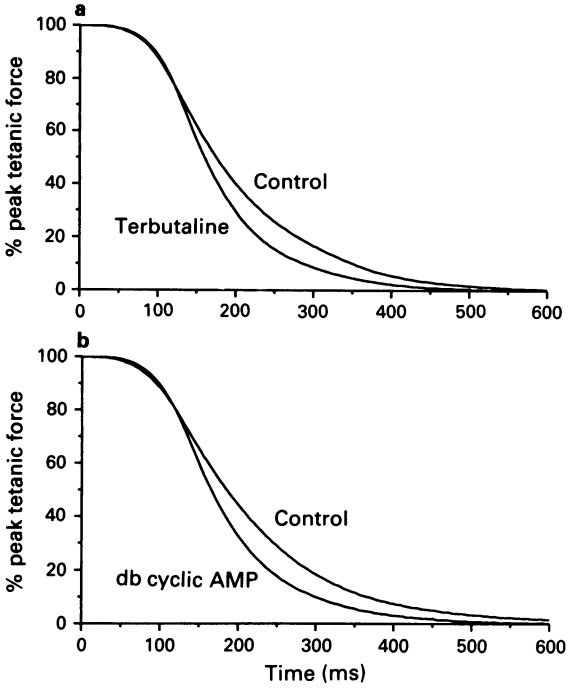

Figure 8 Typical effects of (a) 10 μM terbutaline and (b) 2 mM dibutyryl cyclic AMP on tetanic relaxation in normal soleus fibres. The relaxation portion of the force records (from the end of the stimulation period) were normalized and superimposed. Tetani were evoked at 100 Hz for 1 s, Solution A, 24°C. The control in (b) was obtained following wash-out of terbutaline in (a).

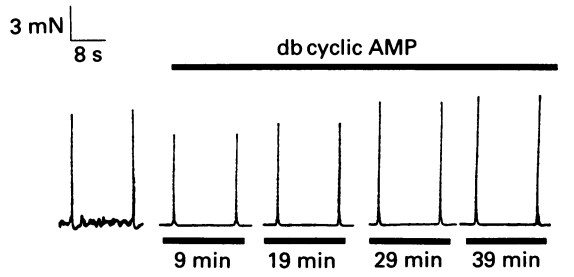

Figure 9 Chart recordings showing the effect of 2 mM dibutyryl cyclic AMP (dbcyclic AMP) on twitches and fibrillations in a denervated soleus preparation. Solution A, 24°C. The twitches were depressed by 9% in 9 min and showed a maximal potentiation of 22% at 39 min.

butaline (10.9 ± 2.2%) dbcyclic AMP slowed twitch relaxation in the same way as terbutaline.

Some effects of dbcyclic AMP might have been due to butyrate which is formed following deacylation of dbcyclic AMP (Skelton *et al.*, 1970; Kurihara & Konishi, 1987). Exposure to butyrate (2 mM) depressed peak twitch force by 7.5 ± 0.8% ($n = 3$) and either depressed or had no effect on peak tetanic force. Therefore force potentiation by dbcyclic AMP cannot be attributed to butyrate. However the initial reduction of force with dbcyclic AMP might be caused by butyrate.

Discussion

The results of this study demonstrate that (a) β-adrenoceptor agonists potentiate force in all types of mammalian skeletal muscle fibres – a clear potentiation of tetanic force is shown for the first time; (b) β-adrenoceptor agonists alter the rate of relaxation of twitch and tetanic contractions in a manner that varies with the type of muscle fibre; (c) dbcyclic AMP mimics the effects of β-receptor activation, providing strong evidence for a role of cyclic AMP in the end effects β-adrenoceptor activation on contraction.

Effects of β-adrenoceptor activation on contraction in different fibre-types and after denervation

The greater twitch potentiation by terbutaline in soleus than in sternomastoid is consistent with a greater density of β-adrenoceptors in slow-twitch fibres (Williams *et al.*, 1984) and greater enhancement of adenylate cyclase activity (Festoff *et al.*, 1977; Fellenius *et al.*, 1980a; Williams *et al.*, 1984). The similar twitch potentiation in different types of fast-twitch sternomastoid fibre indicates similar β-adrenoceptor densities and activation of adenylate cyclase. Terbutaline induced a dramatic acceleration of tetanic relaxation which had not been reported previously. The smaller acceleration of twitch relaxation with terbutaline in soleus fibres and slowing of relaxation in sternomastoid fibres was in agreement with earlier studies (Bowman & Zaimis, 1958; Bowman *et al.*, 1962; Tashiro, 1973; Tomita, 1975).

The increase of peak twitch and tetanic force by terbutaline and adrenaline was unaltered by denervation in soleus fibres, although relaxation of the twitch force was slowed with terbutaline, rather than accelerated. Tachyphylaxis with repeated drug application was reduced, possibly because β-adrenoceptors in denervated fibres are less susceptible to desensitization, or because adenylate cyclase activity is enhanced (Hashimoto *et al.*, 1989). Changes in resting force were also observed in denervated fibres. Catecholamines induce contractures in denervated muscle (Bowman & Raper, 1965; Evans & Smith, 1976) and adrenaline induced contractures with increased fibrillations in this study. In contrast, terbutaline and dbcyclic AMP reduced resting force and depressed fibrillations (Figure 9). These changes in resting force could be due to changes in membrane potential and spontaneous depolarizations, which cause fibrillations and initiate action potentials (Bowman & Raper, 1965). The increased frequency of spontaneous depolarizations with adrenaline (Bowman & Raper, 1965; Smith & Thesleff, 1976) could cause a contracture by synchronizing fibrillations in different fibres. Terbutaline and dbcyclic AMP hyperpolarize fibres (McArdle & D'Alonzo, 1981; Cairns & Dulhunty, unpublished observations) and spontaneous depolarizations no longer trigger action potentials (Cairns & Dulhunty, unpublished observations).

The role of β-adrenoceptors and myoplasmic cyclic AMP

Sympathomimetics activate β-adrenoceptors in mammalian skeletal muscle (Bowman & Nott, 1970; Holmberg & Waldeck, 1977; 1980; Bowman, 1980; McArdle & D'Alonzo, 1981). Propranolol abolished the tetanus potentiation by terbutaline. This is consistent with previous studies with various β-blockers (including selective β_2-blockers) and demonstrated the involvement of β-adrenoceptors in the effects that we describe.

The gradual development of inotropic effects with terbutaline suggested the involvement of a second messenger. Terbutaline and other sympathomimetics increase [cyclic AMP]$_i$ in skeletal muscle (Festoff *et al.*, 1977; Fellenius *et al.*, 1980a; Williams *et al.*, 1984). dbCyclic AMP has been shown to potentiate twitches in frog twitch fibres and in rat diaphragm (Oota & Nagai, 1977; Varagić & Kentara, 1978). dbCylic AMP in the present study mimicked most of the effects of terbutaline, again suggesting that cyclic AMP is the second messenger. The slow action of dbcyclic AMP was attributed to time required for dbcyclic AMP to enter the

cells (Drummond *et al.*, 1974). Studies using phosphodiesterase inhibitors (Bowman & Nott, 1974) or forskolin (Prostran & Varagić, 1986) support the involvement of cyclic AMP in sympathomimetic-induced potentiation of force in fast-twitch fibres.

Mechanism for the terbutaline-induced effects on relaxation

The cyclic AMP-dependent phosphorylation of phospholamban, a 22 kD sarcoplasmic reticulum (SR) protein which regulates the SR Ca^{2+}-pump, is thought to abbreviate relaxation of cardiac twitch force (Kirchberger & Tada, 1976; Lindemann *et al.*, 1983; Kurihara & Konishi, 1987; Tada *et al.*, 1988). Phospholamban is present in slow-twitch, but not fast-twitch, skeletal musle (Jorgensen & Jones, 1986; Tada *et al.*, 1988) and is probably the 22 kD protein phosphorylated by cyclic AMP-protein kinase to accelerate Ca^{2+} uptake into SR from slow-twitch, but not from fast-twitch, fibres (Kirchberger & Tada, 1976; Salviati *et al.*, 1982).

The cyclic AMP/phospholamban system might also be responsible for the terbutaline-induced acceleration of relaxation in normal soleus fibres. The absence of phospholamban would explain the inability of terbutaline to abbreviate relaxation in sternomastoid fibres and also possibly in denervated soleus. Faster twitch relaxation in cardiac muscle with sympathomimetics may also be due to altered myofilament function following phosphorylation of troponin-I or C-protein (England *et al.*, 1984; Hartzell, 1984). However, this is not the case in skeletal muscle since neither protein is phosphorylated during β-adrenoceptor activation (England *et al.*, 1984). Furthermore, sympathomimetics decrease the resting myoplasmic calcium concentration in slow-twitch fibres (Ballanyi & Grafe, 1988) in a manner that is consistent with increased calcium uptake by the SR, but not consistent with a change in myofilament function (i.e. with faster calcium dissociation from troponin). The effect of terbutaline on relaxation is unlikely to reflect a modulation of cross-bridge function by changes in metabolite concentrations. Intracellular pH in soleus fibres is not altered by adrenaline (Ballanyi & Grafe, 1988) and changes in the concentration of other metabolites due to enhanced glycolysis (Fellenius *et al.*, 1980a,b) may account for the slower relaxation of fast-twitch fibres with terbutaline, but cannot account for the faster relaxation in soleus fibres (Fellenius *et al.*, 1980b; Ballanyi & Grafe, 1988).

Mechanism for the positive inotropic effect of terbutaline

Force potentiation by terbutaline, adrenaline and dbcyclic AMP was not due to an effect at the neuromuscular junction. The drugs apparently modulated a cellular process involved in contraction initiated by action potentials since they did not trigger contractures by themselves. Phosphorylation of phospholamban may have contributed to force potentiation in slow-twitch fibres, possibly by increasing the Ca^{2+} loading of the SR, but phospholamban is not found in fast-twitch fibres (Jorgensen & Jones, 1986; Tada *et al.*, 1988) and hence cannot be involved in those fibres. Force potentiation occurred irrespective of the changes in the rate of relaxation; therefore at least two separate processes were influenced by terbutaline. Sympathomimetics could, in principal, potentiate force by enhancing myofilament function, although cyclic AMP-protein kinase does not influence the myofilaments in skinned fast-twitch fibres (Fabiato & Fabiato, 1978). There may be greater action potential depolarization (Smith & Thesleff, 1976; McArdle & D'Alonzo, 1981) or cyclic AMP might modulate a process in excitation-contraction coupling (Oota & Nagai, 1977; Fabiato & Fabiato, 1978; Yatani *et al.*, 1988; Williams & Barnes, 1989). Indeed, Brum *et al.* (1990) demonstrated that adrenaline increases Ca^{2+} release in voltage-clamped frog fibres.

Why were twitches and unfused tetani in slow-twitch fibres potentiated by terbutaline in this study but depressed in previous studies?

The increased peak force during twitches and unfused tetani in soleus fibres was in striking contrast to most previous reports where sympathomimetic-amines reduce the amplitude of submaximal contractions in slow-twitch muscles (Bowman & Zaimis, 1958; Bowman *et al.*, 1962; Tashiro, 1973; Tomita, 1975; Holmberg & Waldeck, 1979). We think that the differences between our results and those of others arise from the concentration of terbutaline used and the smaller size of the preparations. It is unlikely that the discrepancies were due to the external solution used. Holmberg & Waldeck (1980), using a standard bicarbonate buffered solution, demonstrated that 2.3 μM terbutaline in guinea-pig soleus initially depressed force by 15% before producing a final potentiation of 25%. This was similar to the effect of 0.1 μM terbutaline and to dbcyclic AMP in the present study. One possibility is that the force depression seen with 0.1 μM terbutaline and with dbcyclic AMP was due to the same process that induced twitch depression in other studies.

We suggest that there may be a biphasic response to sympathomimetics if they activate separate processes that firstly depress and later potentiate force, and the depression is activated at lower [cyclic AMP]$_i$ than the potentiation (see Figure 4). This hypothesis is based on the observation of the small (maximum 8%) and transient depression of force, followed by force potentiation, at lower concentrations of terbutaline (Figure 4 above) and with dbcyclic AMP. The hypothesis is also consistent with the twitch depression prior to potentiation noted by Holmberg & Waldeck (1980) with 2.3 μM terbutaline in whole muscles. Diffusion limitations with the whole muscle preparation would mean that concentrations of terbutaline (and [cyclic AMP]$_i$) away from the surface of the muscle would be significantly less than 2.3 μM for some time after this concentration of the drug had been applied to the external solution.

The depression of force has been attributed to enhanced sodium-pump activity (Tashiro, 1973; Tomita, 1975; Holmberg & Waldeck, 1980) or to increased Ca^{2+} removal by the SR during contraction (Bowman, 1980), but could also be due to reduced myofilament calcium sensitivity. We seldom saw twitch depression with terbutaline, and when we did it rapidly reversed and potentiation was seen. Therefore the force potentiating mechanism dominated in our system.

Sympathomimetic-induced potentiation of peak tetanic force

Terbutaline and adrenaline potentiated peak tetanic force in soleus fibres. This was in contrast to *in vivo* studies where sympathomimetics either reduce, or have no effect on, peak tetanic force (Goffart & Ritchie, 1952; Bowman & Zaimis, 1958; Bowman & Nott, 1970): tetanus depression has been associated with vasoconstriction (Bowman & Zaimis, 1958; Bowman & Nott, 1970). A small tetanus potentiation in soleus, induced by isoprenaline in anaesthetized cats, was attributed to vasodilatation or to a contribution from fast-twitch fibres (Bowman & Nott, 1970).

The lack of tetanus potentiation in previous studies may have been due to the use of *in vivo* preparations with anaesthetics which directly potentiate force (Holmberg & Waldeck, 1979) and may thus prevent the force potentiating effects of the sympathomimetics from being seen. In addition, circulating hormones may increase cyclic AMP levels to mask the effects of added sympathomimetics. Indeed, the [cyclic AMP]$_i$ is considerably higher in resting muscle *in vivo* than *in vitro* (Fellenius *et al.*, 1980b).

Moreover, it has been thought that hormones cannot increase peak tetanic force by increasing calcium release from the SR since it is assumed that troponin-C is saturated with

Ca^{2+} during the tetanus (Blinks *et al.*, 1978). However this is not true in mammalian fibres since caffeine (Cairns & Dulhunty, 1989b; Fryer & Neering, 1989) and external solutions containing high potassium concentrations (Dulhunty & Gage, 1985) allow force production that is greater than the normal peak tetanic force. Therefore sympathomimetics could, in principal, increase force in a normal tetanus by increasing the myoplasmic Ca^{2+} concentration and allowing further Ca^{2+} activation of the myofilaments.

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Depression of primary afferent-evoked responses by GR71251 in the isolated spinal cord of the neonatal rat

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1 The pharmacological profile of GR71251, a new tachykinin receptor antagonist, and its effect on the responses evoked by stimulation of primary afferent fibres were studied in isolated spinal cord preparations of neonatal rats. Potential changes were recorded extracellularly from a lumbar ventral root (L3-L5).

2 Bath-application of substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) at 0.01–3 μM to the spinal cord induced depolarization of the ventral root in normal artificial cerebrospinal fluid (CSF). The NK_1 agonist, acetyl-Arg^6-septide, and the NK_3 agonist, senktide, at 0.01–3 μM, also had potent depolarizing actions whereas two NK_2 agonists, β-Ala^{8}NKA$_{4-10}$ and Nle^{10}NKA$_{4-10}$, showed little depolarizing effects at 1 μM.

3 GR71251 (0.3–3 μM) caused a rightward shift of the concentration-response curves for SP, acetyl-Arg^6-septide and NKA with pA_2 values of 6.14, 6.75 or 6.70, respectively. The effects of GR71251 were readily reversible within 15–30 min after its removal. By contrast, GR71251 (1–5 μM) had little effect on the depolarizing responses to NKB and senktide.

4 GR71251 (1–3 μM) did not depress the depolarizing responses to bombesin, neuromedin B and gastrin-releasing peptide in normal artificial CSF.

5 Application of capsaicin to the spinal cord induced a depolarizing response, which was partially depressed by GR71251 (3–10 μM).

6 In the isolated spinal cord preparation, intense electrical stimulation of a dorsal root evoked a slow depolarizing response of the contralateral ventral root of the same segment. A similar slow ventral root depolarization was evoked by electrical stimulation of the ipsilateral saphenous nerve in an isolated spinal cord-saphenous nerve preparation. GR71251 (0.3–10 μM) dose-dependently depressed these slow ventral root potentials.

7 In the spinal cord-peripheral nerve preparation, conditioning stimulation of the saphenous nerve evoked an inhibition of the muscle nerve-evoked monosynaptic reflex lasting about 20 s. The late part of the inhibition was markedly depressed by GR71251 (1–3 μM).

8 The present results indicate that GR71251 is a potent and specific antagonist for tachykinin receptors in the spinal cord. The present study further provides evidence for the involvement of SP and NKA in the slow ventral root depolarization and the prolonged inhibition of monosynaptic reflex that are evoked by primary afferent stimulation.

Keywords: Tachykinin receptor; tachykinin antagonist; GR71251; substance P; bombesin

Introduction

We have previously reported that activation of primary afferent fibres induces two types of prolonged response in the spinal cord of the neonatal rat: a depolarization of the ventral root elicited by electrical or chemical stimulation of primary afferents (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989), and an inhibition of the muscle nerve-evoked monosynaptic reflex elicited by electrical conditioning stimulation of the saphenous nerve (Yoshioka *et al.*, 1990). Both the slow ventral root depolarization and the prolonged inhibition of the monosynaptic reflex were markedly depressed by tachykinin antagonists, [D-Arg^1, D-Pro^2,D-Trp^{7,9},Leu^{11}]SP and/or spantide (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989; Yoshioka *et al.*, 1990), which suggests the involvement of tachykinins in these responses. To clarify the functional roles of tachykinins and their receptors, however, further improvement of tachykinin antagonists in terms of potency and selectivity is needed. For example, some tachykinin antagonists were shown to have local anaesthetic actions at high concentrations (Post *et al.*, 1985; Yoshizawa *et al.*, 1987), and to act as antagonists on bombesin receptors (Folkers *et al.*, 1984; Yachnis *et al.*, 1984; Jensen *et al.*, 1984; 1988; Mizrahi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Jensen & Coy, 1991).

Ward *et al.* (1990) recently developed a new tachykinin receptor antagonist, GR71251, which was shown to be selective for NK_1 receptor (Hagan *et al.*, 1990; Ward *et al.*, 1990; Hall & Morton, 1991; Ireland *et al.*, 1991). Furthermore, GR71251 did not depress the action of bombesin in the guinea-pig gallbladder (Ward *et al.*, 1990). In the present study we therefore examined the pharmacological profile of GR71251 in the neonatal rat spinal cord and its effects on the responses evoked by activation of primary afferent fibres. Preliminary accounts of this study have been presented elsewhere (Guo *et al.*, 1992; 1993).

Methods

Preparations

Isolated spinal cord preparations and spinal cord-peripheral nerve preparations from neonatal Wistar rats (Nihon Rat Co., Japan) aged 1 to 3 days of either sex were used (Akagi *et al.*, 1985; Nussbaumer *et al.*, 1989; Yoshioka *et al.*, 1990). Spinal cords below thoracic segments were used in the

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experiments in which the effects of GR71251 on the dorsal root- and saphenous nerve-evoked slow depolarizing responses were examined. Hemisected spinal cords were used in other experiments. The preparation was placed in a recording chamber of 0.5 ml volume and perfused with artificial cerebrospinal fluid (CSF) saturated with 95% O_2:5% CO_2 at a flow rate of 2–4 ml min^{-1}. The compositon of artificial CSF was as follows (in mM): NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0. In the experiments in which the effects of GR71251 on the action of exogenous tachykinins and other peptides were examined (Figures 1, 2, 3 and 4), the concentration of $MgCl_2$ was increased to 2 mM in order to depress spontaneous activity. The temperature of the perfusion medium was kept at 27°C. Drugs were dissolved in artificial CSF and applied to the spinal cord by perfusion.

Electrophysiology and data analysis

Suction electrodes were used for electrical stimulation of and extracellular recording from L3-L5 nerve roots. Changes in potential of the ventral root were led to a d.c. amplifier and then to a pen recorder and a computer recording device (Axotape). Spinal reflexes of fast time-course were stored in a memory device and then displayed on a pen-recorder with a slower time scale.

Effects of drugs on the contralateral dorsal root-evoked slow ventral root potentials (VRPs) were investigated in the whole spinal cord preparation (Akagi *et al.*, 1985). Stimulation with five shocks (40 V intensity, 300 μs duration at 20 Hz) were given to a dorsal root every 90 s and the resultant reflex responses were recorded extracellularly from the contralateral ventral root of the same segment. The average areas (mV·s) of 3 consecutive responses were measured before and after adding each antagonist. GR71251 was applied in a cumulative manner (5 and 10 μM), for 20 min for each concentration. After washing out GR71251 for at least 30 min, spantide was applied for 30 min.

Effects of drugs on the cutaneous nerve-evoked slow depolarizing responses were investigated in the spinal cord-saphenous preparation (Nussbaumer *et al.*, 1989). Stimulation with two shocks (40 V intensity, 100 μs duration at 20 Hz) were given to the saphenous nerve every 3 min and the resultant reflex responses were recorded extracellularly from the ipsilateral L3 ventral root. The average areas (mV·s) of 3 consecutive responses were measured before and after adding GR71251.

To investiage the cutaneous nerve-evoked inhibition of monosynaptic reflex in the spinal cord-peripheral nerve preparation (Yoshioka *et al.*, 1990), single-shock test stimuli (a square pulse of 40–50 V in amplitude and 200–500 μs in duration, i.e. of supramaximum intensity for monosynaptic reflex) were applied to a nerve branch of the quadriceps femoris muscle every 120 s. The resulting reflex responses were recorded from the L3 ventral root. Conditioning stimuli (2–5 shocks of 200–500 μs duration, at 20–50 Hz and 40–50 V in intensity) were delivered to the saphenous nerve. Conditioning-test intervals were altered in a decreasing order usually starting at 20 s, and the amplitude of the conditioned monosynaptic reflex was expressed as a percentage of the control reflex amplitude (the amplitudes of monosynaptic reflexes immediately before the conditioning stimulation).

To examine the effects of GR71251 and spantide on the capsaicin-induced depolarization, capsaicin was bath-applied for 30 s every 30 min to the spinal cord preparation. GR71251 was applied at two concentrations (5 and 10 μM) for 10 min. After washing out GR71251 for at least 60 min, spantide (15 μM) was applied for 15 min.

Estimation of pA_2 values

Tachykinin agonists were bath-applied for 30 s at 10 to 30 min intervals to the spinal cord preparation and the area of the depolarization (mV·s) was calculated. The concentration-response curves were constructed in normal artificial CSF and then after equilibration of the preparation for 8 min with GR71251 or for 15 min with spantide at two different concentrations. The antagonist-induced displacement of agonist concentration-response curves was quantified as the ratio of equi-active molar concentrations at the half-maximum response level of the control concentration-response curve and the pA_2 value was determined from Arunlakshana-Schild plots (Arunlakshana & Schild, 1959).

Drugs

GR71251 ([D-Pro^9[spiro-γ-lactam]Leu^{10},Trp^{11}]SP) was synthesized as previously described (Ward *et al.*, 1990; Hagan *et al.*, 1990). Spantide ([D-Arg^1,D-Trp^{7,9},Leu^{11}]SP) was kindly supplied by Dr M. Fujino, Takeda Chemical Industries, Ltd. Japan. Acetyl-Arg^6-septide (acetyl-[Arg^6,Pro^9]SP_{6-11}, a water soluble form of septide with similar properties; Papir-Kricheli *et al.*, 1987) and senktide (succinyl-[Asp^6-MePhe^8]SP_{6-11}; Papir-Kricheli *et al.*, 1987) were gifts from Professor Z. Selinger, Department of Biological Chemistry, the Hebrew University of Jerusalem, Israel. SP, NKA, bombesin, gastrin-releasing peptide (GRP), neuromedin B and thyrotropin-releasing hormone (TRH) were purchased from Peptide Institute, Inc. Osaka, Japan. Neurokinin B (NKB), β-Ala^8NKA_{4-10} and Nle^{10}NKA_{4-10} were from Peninsula's Laboratory. Other drugs were obtained from various commercial sources.

Results

Effects of GR71251 and spantide on depolarization induced by tachykinin agonists in normal artificial CSF

Bath-application of SP, NKA and NKB at a concentration range of 0.01–3 μM to the spinal cord of the neonatal rat produced depolarization of ventral roots in normal artificial CSF. Acetyl-Arg^6-septide, a selective NK_1 agonist, and senktide, a selective NK_3 agonist, had also potent depolarizing effects, whereas β-Ala^8NKA_{4-10} and Nle^{10}NKA_{4-10}, which act as selective NK_2 agonists in peripheral tissues (Rovero *et al.*, 1989; Regoli *et al.*, 1990), had only minor depolarizing effects at 1 μM (Table 1).

Figure 1 illustrates the effect of GR71251 on the SP-induced depolarization, as compared with that of spantide, in the same preparation. Both GR71251 and spantide depressed the SP-induced response. The depressant effects of GR71251 at 3 μM and 5 μM were approximately the same as those of spantide at 10 μM and 15 μM, respectively (see Table 2). After removal of GR71251 the SP-induced response rapidly

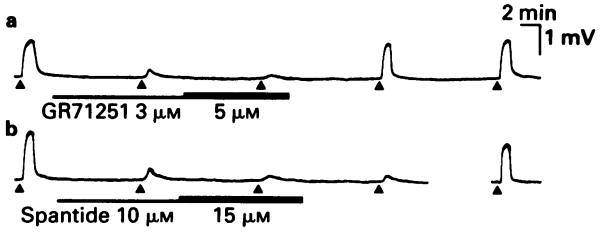

Figure 1 Comparison of the effects of GR71251 and spantide on the depolarization of the ventral root induced by substance P (SP). Extracellular recording was made from the L4 ventral root of a hemisected spinal cord preparation of 1 day-old rat. SP (0.1 μM) was bath-applied for 30 s at (▲) every 12 min. The effect of spantide was examined after complete recovery from the effect of GR71251 in the same preparation. (a) GR71251 at 3 and 5 μM was applied during the period shown by the thin and thick horizontal bars, respectively. (b) Spantide at 10 and 15 μM was applied during the period shown by the thin and thick horizontal bars, respectively. The trace at the right was recorded 120 min after removal of spantide.

Table 1 The potencies of tachykinin agonists in evoking ventral root depolarization

Agonist	*Area* (mV·s)	n
Substance P	203.5 ± 16.4	18
Neurokinin A	187.6 ± 19.4	9
Neurokinin B	253.3 ± 10.6	4
Acetyl-Arg^6-septide	529.5 ± 78.9	7
Senktide	355.8 ± 41.0	5
β-Ala^{8}NKA$_{4-10}$	3.8 ± 1.4	5
Nle^{10}NKA$_{4-10}$	3.1 ± 1.2	5

All agonists were bath-applied at 1 μM for 30 s. The areas of depolarization of the ventral root were measured and expressed as mean ± s.e.mean.

Table 2 pA_2 values and slopes of the Schild plot for GR71251 against tachykinin agonist-evoked depolarization in normal artificial CSF

Antagonist and Agonist	*pA_2*	*Slope*	n
GR71251			
Substance P	6.14 ± 0.03	0.97 ± 0.02	6
Neurokinin A	6.75 ± 0.03*	1.00 ± 0.01	4
Acetyl-Arg^6-septide	6.70 ± 0.08*	0.99 ± 0.03	5
Spantide			
Substance P	5.88 ± 0.03*	0.77 ± 0.04	6
Bombesin	5.29 ± 0.02	1.12 ± 0.09	4

The pA_2 values were determined from Aunlakshana-Schild plots. Data are expressed as mean ± s.e.mean.
*Significantly different from the value of GR71251 against substance P by unpaired t test at $P<0.001$.

returned to its original size in 10–20 min, whereas the recovery from the action of spantide occurred much more slowly and often incompletely within 1–2 h (Figure 1b). GR71251 or spantide alone did not alter the potential of the ventral root at a concentration range of 0.3–30 μM.

In the experiments shown in Figure 2 and Table 2, the effects of GR71251 on the depolarizing responses of the ventral root to SP, acetyl-Arg^6-septide, NKA, senktide and NKB were examined. GR71251 (0.3–3 μM) caused a rightward shift of the concentration-response curves for SP, acetyl-Arg^6-septide and NKA (Figures 2a,b and c). About 20 min after removal of GR71251, the curves for these agonists were similar to controls. The slope of the Schild plot for GR71251 against SP, acetyl-Arg^6-septide and NKA was close to unity (Table 2). The estimated pA_2 values for GR71251 against NKA, acetyl-Arg^6-septide were about the same (6.7–6.8) whereas the pA_2 against SP was slightly lower (6.14). In contrast, the depolarizing responses to NKB and senktide were little affected by GR71251 at 1–5 μM (Figure 2d,e). Thus, in the presence of GR71251 (3–5 μM), the depolarizing responses to NKB (0.3 μM) and senktide (0.3 μM) were 86.6 ± 10.4% ($n = 3$) and 78.3 ± 14.5% ($n = 3$) of the control responses (statistically not significant at $P<0.05$, by t test), respectively. The effects of GR71251 on β-Ala^{8}NKA$_{4-10}$ and Nle^{10}NKA$_{4-10}$ actions were not examined in the present study because of their minor effects in this preparation.

Specificity of GR71251

Figure 3 shows the effects of GR71251 on the responses to bombesin, and two mammalian bombesin-like peptides, GRP and neuromedin B. When these agonists were bath-applied for 30 s in normal artificial CSF, depolarizing responses were induced in ventral roots. GR71251 (1–3 μM) caused no shift of the concentration-response curves for these agonists. The response to bombesin at 0.01–0.03 μM in the presence of

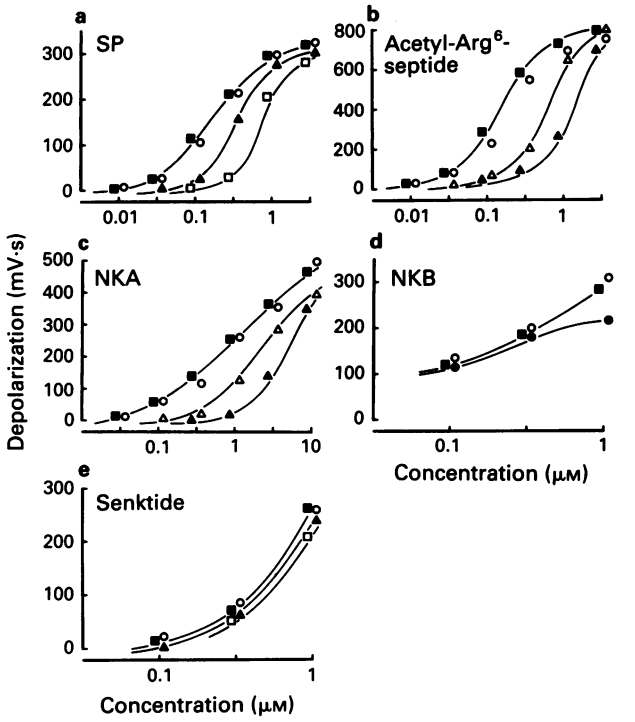

Figure 2 Effects of GR71251 on the concentration-response curves for substance P (SP), acetyl-Arg^6-septide, neurokinin A (NKA), senktide and neurokinin B (NKB) in normal artificial CSF. The tachykinin receptor agonists were applied by perfusion for 30 s. The area of depolarization of the ventral root is plotted against logarithmic concentrations of the agonists. The positions of symbols are horizontally adjusted to avoid their overlaps. (■) In normal artificial CSF; (△), (▲), (□) and (●) after addition of GR71251 at 0.3 μM, 1 μM, 3 μM and 5 μM, respectively; (○) 20–80 min (a,b,c) and 120–200 min (d,e) after removal of GR71251.

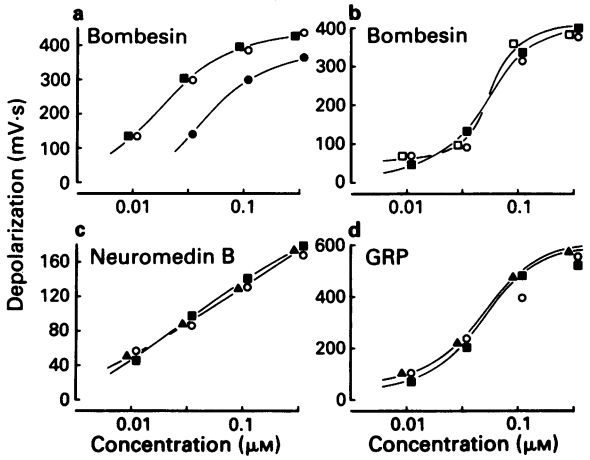

Figure 3 Effects of spantide (a) and GR71251 (b,c,d) on the concentration-response curves for bombesin, and bombesin-like peptides, neuromedin B and gastrin releasing peptide (GRP). These agonists were applied by perfusion for 30 s. The area of depolarization of the ventral root is plotted against logarithmic concentrations of the agonists. The positions of symbols are horizontally adjusted to avoid their overlaps: (a) (■) in normal artificial CSF; (●) after addition of spantide 15 μM; (○) 60–240 min after removal of spantide. (b,c,d) (■) In normal artificial CSF; (▲) and (□) after addition of GR71251 at 1 μM and 3 μM, respectively; (○) 60–240 min after removal of GR71251.

GR71251 at 3–5 μM was 103.2 ± 7.6% of the control responses ($n = 5$), whereas spantide at 15 μM caused a rightward shift of the concentration-response curve for bombesin (Figure 3a). The estimated pA_2 value for spantide against bombesin was 5.29 ± 0.02 ($n = 4$) (Table 1). After removal of

spantide the responses to bombesin returned to original sizes in 60–240 min.

The effects of GR71251 on the responses to other agonists, such as L-glutamate, acetylcholine, TRH, GABA and noradrenaline were examined in the presence of tetrodotoxin (TTX) at 0.3 μM. When these agonists were bath-applied for 30 s, they produced depolarizing responses. GR71251 at 1 μM did not affect the depolarization induced by L-glutamate (0.03–3 mM), acetylcholine (0.03–3 mM), GABA (0.03–3 mM) and noradrenaline (1–30 μM) (not shown). The depolarizing action of TRH was potentiated by GR71251 (1 μM). Thus, in the presence of GR71251 (1 μM), the depolarizing response to TRH at 0.3 μM was potentiated by 28.7 ± 2% ($n = 3$) and at 1 μM, by 13 ± 1% ($n = 3$), respectively, statistically significant at $P < 0.001$, by t test.

Effects of GR71251 on the capsaicin-induced depolarization

Bath-application of 0.3 μM-capsaicin for 20 s produced a depolarizing response similar to the response to SP at 20 nM applied for 30 s (Figure 4). While the SP-induced depolarization was almost completely depressed by GR71251 at 3–10 μM, the capsaicin-induced response was only partially depressed by GR71251: i.e. the initial part of the response was resistant to GR71251 at 3–10 μM, whereas the later part was completely depressed by GR71251 at 3 μM ($n = 4$). Spantide also exerted similar effects. However, the maximum depressant effect of GR71251, which was observed at 5–10 μM, was slightly but significantly smaller than that of spantide at 15 μM (Table 3).

Effects of GR71251 on the dorsal root-evoked spinal reflexes

Intense electrical stimulation of a dorsal root evoked in the contralateral ventral root of the corresponding segment a slow depolarizing response lasting about 20 s (Figure 5). This reflex response which is referred to as contralateral slow ventral root potential (VRP), has been shown to involve activation of primary afferent C-fibres and to be depressed by tachykinin antagonists, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985) and spantide (Otsuka & Yanagisawa, 1988). GR71251 (3–10 μM) markedly depressed the contralateral slow VRP and recovery occurred in 10–20 min after removal of the antagonist ($n = 4$) (Figure 5b). The depressant effect of GR71251 became maximum at 5 μM (Table 3). Spantide at 15 μM, which was approximately equipotent to GR71251 at 5 μM in antagonizing the depolarizing action of SP, showed a slightly but significantly more pronounced depressant effect than GR71251 at 5 μM on the contralateral slow VRP (Table 2). Recovery of the response after removal of spantide was much slower taking 60–90 min.

Single-shock stimulation of a dorsal root evoked in the ipsilateral ventral root of the same segment monosynaptic and polysynaptic reflexes of fast time course which were followed by a slow depolarization (ipsilateral slow VRP; Otsuka & Yanagisawa, 1988). GR71251 (1–3 μM) exerted a depressant effect on the ipsilateral slow VRP but did not affect the monosynaptic and polysynaptic reflexes of fast time course (not shown). The latter finding suggests that GR71251 at 3 μM has no local anaesthetic action.

Effects of GR71251 on the saphenous nerve-evoked depolarization

Our previous studies using isolated spinal cord-saphenous nerve preparation showed that electrical stimulation of the saphenous nerve at strength sufficient to activate C fibres evoked a slow depolarizing potential of approximately 20 s duration in the L3 ventral root and this potential was depressed by spantide (Nussbaumer *et al.*, 1989). GR71251

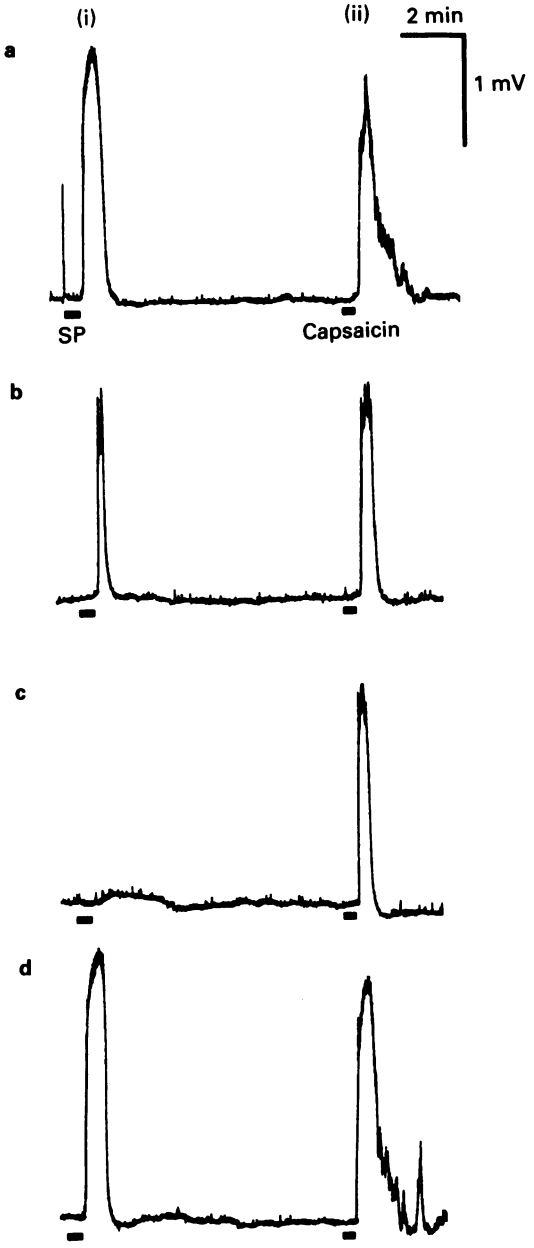

Figure 4 Effects of GR71251 on the responses to substance P (SP) and capsaicin. Responses were recorded from the L4 ventral root of a hemisected spinal cord preparation of 1 day-old rat. SP (10 nM) (i) and capsaicin (0.3 μM) (ii) were bath-applied for 30 s and 20 s, respectively, at 10 min intervals; (a) control responses; (b) and (c) 8–18 min after addition of GR71251 at 3 μM and 10 μM, respectively; (d) 40–50 min after removal of GR71251.

(0.3–3 μM) likewise depressed the saphenous nerve-evoked slow depolarization. This recovered rapidly after the removal of GR71251 ($n = 5$) (Figure 6).

Effects of GR71251 on the saphenous nerve-evoked inhibition of monosynaptic reflex

In the spinal cord-peripheral nerve preparation, conditioning electrical stimulation of the saphenous nerve inhibited (for about 20 s) the monosynaptic reflex elicited by stimulation of quadriceps femoris nerve. In the experiment illustrated in Figure 7, test monosynaptic reflexes were elicited by stimulations of the quadriceps femoris nerve and recorded from the L3 ventral root of an isolated spinal cord-peripheral nerve preparation. Conditioning stimulation with 2–5 shocks

Table 3 Effects of GR71251 and spantide on the capsaicin-induced depolarization and contralateral slow VRP

	GR71251 (5 μM)	*GR71251* (10 μM)	*Spantide* (15 μM)	n
Capsaicin-induced response	56.7 ± 5.1	57.7 ± 1.6	34.1 ± 5.9*†	4
Contralateral slow VRP	61.4 ± 3.0	60.8 ± 3.8	50.7 ± 4.0*	3

For the capsaicin-induced response the areas of depolarization induced by capsaicin (0.1–0.5 μM, 30 s) were measured before and after adding each antagonist. For the contralateral slow VRP evoked by the dorsal root the average areas of 3 consecutive responses were measured before and after adding each antagonist. Each value represents % of control response and is expressed as mean ± s.e.mean.

*Significantly different from the value for GR71251 (5 μM) by paired t test at $P<0.05$; †Significantly different from the value for GR71251 (10 μM) by paired t test at $P<0.05$. The values for GR71251 at 5 μM and 10 μM in inhibiting both the capsaicin-induced response and the contralateral slow VRP were not significantly different from each other at $P<0.05$ by paired t test.

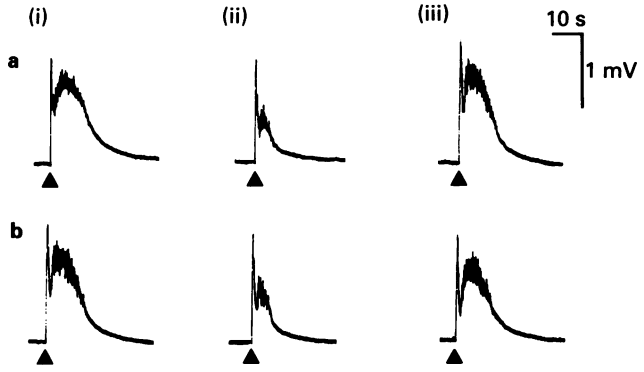

Figure 5 Effects of GR71251 and spantide on the contralateral slow ventral root potential (VRP). Records from an isolated spinal cord preparation of 2 day-old rat. Stimulation with five shocks (40 V intensity, 300 μs duration at 20 Hz) were given at (▲) to an L4 dorsal root every 90 s and the resultant reflex responses were recorded extracellularly from the L4 ventral root of the contralateral side. (i) Control response; (ii) 12–14 min after addition of spantide at 15 μM (a) and GR71251 at 10 μM (b); (iii) 120 and 30 min after removal of spantide (a) and GR71251 (b), respectively.

(40–50 V intensity and 200–500 μs duration at 50 Hz) was applied to the saphenous nerve every 120 s (Yoshioka *et al.*, 1990). GR71251 (1–3 μM) markedly reduced the saphenous nerve-evoked inhibition of the monosynaptic reflex, particularly at conditioning-test intervals of 5–20 s. This recovered rapidly after the removal of GR71251 ($n=6$).

Discussion

GR71251 had little depressant action on the depolarizing response to bombesin, GRP or neuromedin B in the neonatal rat spinal cord whereas spantide markedly depressed the response to bombesin (Figure 3; Otsuka & Yanagisawa, 1988; Jensen & Coy, 1991; Rouissi *et al.*, 1991). GR71251 had also virtually no antagonist action on the depolarization induced by NKB and senktide. Furthermore, GR71251 did not alter the concentration-response curves for L-glutamate, acetylcholine, TRH, GABA and noradrenaline. Therefore, the present study shows that GR71251 is more selective than

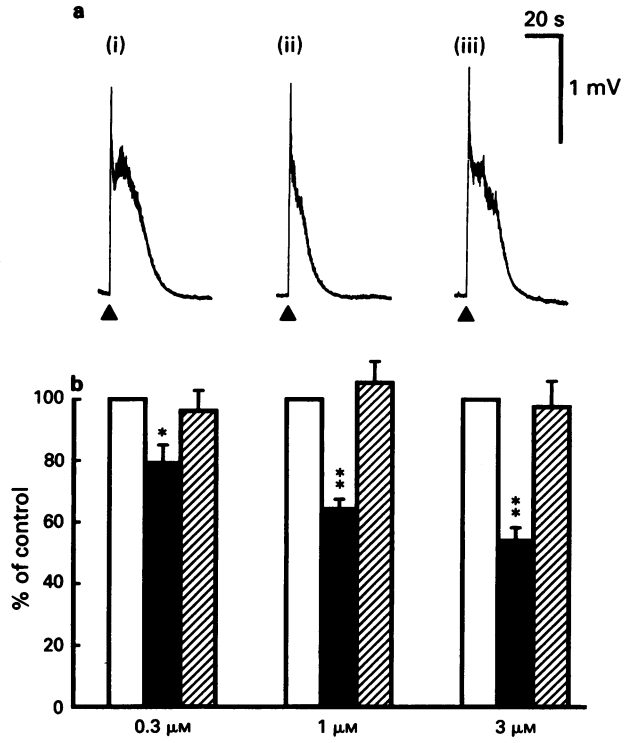

Figure 6 Effect of GR71251 on the saphenous nerve-evoked slow VRP. (a) Experiment in an isolated spinal cord-peripheral nerve preparation of a 2 day-old rat. The saphenous nerve was stimulated every 3 min at (▲) with 2 shocks (40 V intensity and 100 μs duration at 20 Hz) and the potential was recorded from the ipsilateral L3 ventral root. (i) Control response; (ii) after addition of GR71251 at 3 μM; (iii) after removal of GR71251. (b) Dose-dependently of the effect of GR71251 on the saphenous nerve-evoked slow VRP. The areas of the responses in mV·s were measured and shown as percentages of the average of control responses. Open columns represent control responses; solid columns, after addition of GR71251 at 0.3, 1 and 3 μM respectively; hatched columns, after removal of GR71251. Each column and vertical bar express mean ± s.e.mean ($n=5$). Significantly different from the control values at $*P<0.05$ and $**P<0.001$ by paired t test.

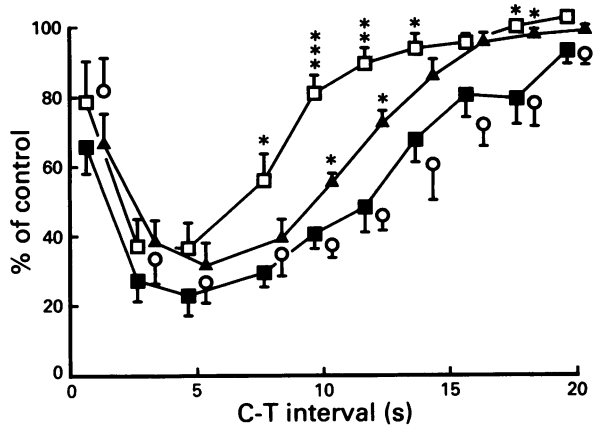

Figure 7 Effects of GR71251 on the saphenous nerve-evoked inhibition of monosynaptic reflex. Test monosynaptic reflexes were elicited by stimulations of the quadriceps femoris nerve and recorded from the L3 ventral root of an isolated spinal cord-peripheral nerve preparation. Conditioning stimulation with 2–5 shocks (40–50 V intensity and 200–500 μs duration at 50 Hz) was applied to the saphenous nerve every 120 s. The positions of symbols are horizontally adjusted to avoid overlapping. (■) Inhibition in normal artificial CSF; (▲) and (□) after addition of GR71251 at 1 μM and 3 μM, respectively; (○) 60 min after removal of GR71251. Each point expresses mean ± s.e.mean ($n=6$). Significantly different from the corresponding control values at $*P<0.05$; $**P<0.01$, and $***P<0.001$ by unpaired t test.

spantide as a tachykinin antagonist in the neonatal rat spinal cord. In addition, GR71251 is superior to spantide as an experimental tool in that it is more potent and its effect is readily reversed after removal.

Previous studies showed that both the dorsal root- or saphenous nerve-evoked slow VRP and the saphenous nerve-evoked inhibition of the monosynaptic reflex were depressed by the tachykinin antagonists, [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]-SP and/or spantide (Yanagisawa *et al.*, 1982; Akagi *et al.*, 1985; Otsuka & Yanagisawa, 1988; Nussbaumer *et al.*, 1989; Yoshioka *et al.*, 1990). The depolarization of ventral roots induced by application of capsaicin to the spinal cord was also depressed by spantide (Yoshioka *et al.*, 1990). These results suggested that tachykinins are involved in these responses. However, because of the blocking action of these antagonists on bombesin receptors (Folkers *et al.*, 1984; Otsuka & Yanagisawa, 1988; Jensen *et al.*, 1988), the possibility remained that bombesin-like peptides, but not tachykinins, contribute to these responses. Indeed, neuromedin B mRNA and GRP mRNA have been found in rat dorsal root ganglia and the spinal cord, respectively (Wada *et al.*, 1990). Bombesin-like immunoreactivity has also been demonstrated in a subpopulation of mammalian primary sensory neurones (Fuxe *et al.*, 1983; Panula *et al.*, 1983; Cameron *et al.*, 1988) and certain spinal neurones (Leah *et al.*, 1988), although the amounts of the immunoreactivity were much smaller than those of SP in the spinal cord and sensory ganglia (McGregor *et al.*, 1984; Yaksh *et al.*, 1988). In the present study, GR71251 depressed both the dorsal root- and saphenous nerve-evoked slow VRPs and the saphenous nerve-evoked inhibition of monosynaptic reflex. GR71251 also depressed the later slow component of the capsaicin-induced depolarization. The present study, therefore, adds further support for the involvement of SP and NKA in these responses. Spantide exerted a slightly greater depressant effect than GR71251 on the dorsal root-evoked contralateral slow VRP as well as the capsaicin-induced depolarization (Table 3). Whether this is due to the contribution to these responses of other peptides, such as NKB and bombesin-like peptides, remains to be clarified. The partial blockade of the capsaicin-evoked depolarization by GR71251 and spantide (Table 3) suggests that it is due to a release of both tachykinins and other sensory transmitters (cf. Urbán & Dray, 1992).

The pA_2 of GR71251 against the depolarizing action of NKA was close to the pA_2 against acetyl-Arg[6]-septide. This suggests that GR71251, NKA and acetyl-Arg[6]-septide bind to a single class of tachykinin receptors. The pA_2 value of GR71251 against NKA obtained in this study (6.75) was much higher than the pA_2 of GR71251 against the NK_2 receptor-mediated contractile action of NKA in the rat colon muscularis mucosae (4.8) (Ward *et al.*, 1990). Furthermore, β-Ala[8]NKA_{4-10} and Nle[10]NKA_{4-10}, two selective NK_2 agonists that selectively activate at NK_2 receptors in peripheral tissues (Rovero *et al.*, 1989; Regoli *et al.*, 1990), showed little depolarizing effect at 1 μM. Since it was known in peripheral tissues that acetyl-Arg[6]-septide and NKA preferentially act on NK_1 and NK_2 receptors, respectively, the receptor to which NKA and acetyl-Arg[6]-septide bind as observed in the present study appears to be distinct from NK_1, NK_2 or NK_3 receptor found in peripheral tissues. The existence of this novel type of receptor was suggested previously based on the similar antagonist profile of spantide against the depolarizing responses of motoneurones to NKA and acetyl-Arg[6]-septide (Yanagisawa & Otsuka, 1990). On the other hand, the significantly lower pA_2 of GR71251 against the SP-induced depolarizing responses than against the NKA- and acetyl-Arg[6]-septide-induced responses may reflect involvement of another type of tachykinin receptor in the SP-induced response. Furthermore, there was a slight tendency for the concentration-response curves to become steeper in the presence of increasing concentrations of GR71251 (Figure 2). This may indicate that the responses to these agonists involve more than one type of receptor. These possibilities need to be examined further by the use of other selective tachykinin agonists and antagonists.

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R56865 inhibits catecholamine release from bovine chromaffin cells by blocking calcium channels

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1 The effects of R56865 (a new class of cardioprotective agent which prevents Na^+ and Ca^{2+} overload in cardiac myocytes) on catecholamine release, whole-cell current through Ca^{2+} channels (I_{Ba}) and cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, have been studied in bovine chromaffin cells.

2 R56865 caused a time- and concentration-dependent blockade of catecholamine release from superfused cells stimulated intermittently with 5 s pulses of 59 mM K^+. After 5 min superfusion, a 3 μM concentration inhibited secretion by 20%; the blockade increased gradually with perfusion time, to reach 85% after 40 min. The IC_{50} to block secretion after 5 min periods of exposure to increasing concentrations of R56865 was around 3.1 μM. The blocking effects of R56865 were reversible after 5–15 min wash out. In high Ca^{2+} solution (10 mM Ca^{2+}), the degree of blockade of secretion diminished by 20% in comparison with 1 mM Ca^{2+}.

3 In electroporated cells, R56865 (10 μM) did not modify the secretory response induced by the application of 10 μM free Ca^{2+}.

4 R56865 blocked the peak I_{Ba} current in a concentration- and time-dependent manner; its IC_{50} was very similar to that obtained for secretion (3 μM). The compound not only reduced the size of the peak current but also promoted its inactivation; when the effects of R56865 were measured at the most inactivated part of the current, its IC_{50} was 1 μM. Both the inactivation and the reduction of the peak currents were reversible upon washing out the drug.

5 In fura-2-loaded single chromaffin cells the basal $[Ca^{2+}]_i$ of around 100 nM was elevated to a peak of 1.5 μM by the application of a 5 s pulse of 59 mM K^+. R56865 (10 μM) did not affect the basal $[Ca^{2+}]_i$ but blocked by 90% the K^+-evoked increase. This effect was fully reversible after 5–10 min of wash out.

6 The results are compatible with the idea that R56865 blocks Ca^{2+} entry into K^+-depolarized chromaffin cells by promoting the inactivation of voltage-dependent Ca^{2+} channels; this would lead to the limitation of the rise in $[Ca^{2+}]_i$ and of the release of catecholamines. The restriction of catecholamine release may favour indirectly the known direct beneficial cardioprotective actions of R56865.

Keywords: R56865; chromaffin cells; catecholamine release; calcium currents; cytosolic calcium

Introduction

R56865 (Figure 1), is a compound that belongs to a class of cardioprotective agents with a novel mode of action: the drug interferes with processes that underlie ischaemia-reperfusion injury, cardiac glycoside intoxication and triggered activity (Ver Donck *et al.*, 1993). At least three interactions at the cellular level may be responsible for protection in these conditions: (i) inhibition of excessive Na^+ entry into myocardial cells due to non-inactivating Na^+ channels in depolarized cells, thus preventing Na^+ overload and subsequent Ca^{2+} overload and cell death; (ii) inhibition of the transient inward current in Ca^{2+} overload cells, avoiding in this manner after depolarizations and triggered propagated contractions; and (iii) attenuation of K^+-efflux in Na^+ and Ca^{2+} loaded cells, probably preventing action potential shortening and inhomogeneous repolarization.

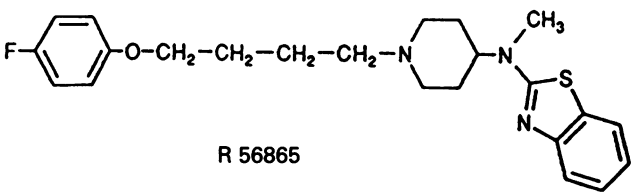
With this pharmacological profile, we thought that R56865 could interfere with Ca^{2+} homeostasis and secretion in chromaffin cells. Therefore, we decided to study its effects on K^+-evoked catecholamine release and cytosolic Ca^{2+} transients, as well as on whole-cell Ca^{2+} currents in bovine chromaffin cells. The results of this study are presented here.

Methods

Catecholamine release from intact cells

Bovine adrenal chromaffin cells were isolated and prepared as described by Moro *et al.* (1990). Cells (2.5×10^6) were placed in a microchamber and superfused at room temperature (25° ± 2°C) with Krebs-HEPES solution of the following composition (in mM): NaCl 144, KCl 5.9, $CaCl_2$ 1, $MgCl_2$ 1.2, HEPES 10 and glucose 10, pH 7.4.

The rate of perfusion was 1 ml min^{-1}; the liquid flowing from the perfusion chamber reached an amperometric detector through a thin polyethylene tube. Electrochemical detection of standard and released catecholamines was performed with a Methrom amperometric detector equipped with a



R 56865

Figure 1 Chemical structure of compound R56865, N-[1-(4-(4-flurophenoxy)butyl)]-4-piperidinyl-*N*-methyl-2-benzo-thiazolamine.

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glassy carbon working electrode, an Ag/AgCl reference electrode and a gold auxiliary electrode. Catecholamines were oxidized at a potential of + 0.65 V and the oxidation current signal was recorded in an Omni Scribe recorder (Borges *et al.*, 1986).

Known concentrations of mixtures of noradrenaline and adrenaline (10^{-8} to 10^{-6} μg ml^{-1}) were administered directly to the detector in order to obtain a standard curve. Catecholamine release was quantitated by measuring peak heights and comparing them to those obtained with noradrenaline and adrenaline standards.

Before starting the experiments, the cells were perfused for 20 to 30 min with Krebs-HEPES in order to reach equilibrium. After this, catecholamine secretion was induced by 5 s pulses of a Krebs-HEPES solution containing 50 mM K^+. The pulse duration and frequency were controlled by an electronic valve. K^+-rich solutions were made up by replacing appropriate amounts of NaCl by iso-osmotic concentrations of KCl.

Catecholamine release from electroporated cells

Cells were washed in Ca^{2+}-free Krebs-HEPES solution and resuspended (4×10^6 ml^{-1}) in an electroporation buffer of the following composition (in mM): potassium glutamate 140, $MgCl_2$ 3, EGTA 1, HEPES 20, pH 7, K-ATP 2 and 0.5% bovine serum albumin. Cells were rendered permeable by 10 discharges of 1,500 V at a capacitance of 3 μF using a Gene Pulser Apparatus (Bio-Rad) with 10 s in between the discharges, in a cuvette (0.8 ml) with two electrodes 0.4 cm apart.

Measurement and analysis of calcium currents

Membrane currents were measured by the patch-clamp technique (Hamill *et al.*, 1981) in the whole-cell configuration, using a List EPC-7 patch-clamp amplifier and pipettes of borosilicate glass with a resistance of 2 to 5 MΩ when filled with the standard CS/TEA intracellular solution.

The external bath solution contained (in mM): $BaCl_2$ 10, $MgCl_2$ 1, NaCl 155, HEPES 10 (pH adjusted to 7.3 with NaOH) and 2 μM tetrodotoxin. The patch pipette solution contained (in mM): CsCl 110, tetraethylammonium chloride 30, EGTA 20, HEPES 20 (adjusted to pH 7.3 with NaOH) and MgATP 5. External solutions were exchanged by a fast superfusion device consisting of a modified multi-barrelled ejection pipette (Carbone *et al.*, 1990). The pipette had an opening of 50–100 μm and was positioned 10–20 μm from the cell. Control and test solutions were changed with miniature electro valves (The Lee Company, Westbrook, CT, U.S.A.) operated manually. The flow rate (0.5–1 ml min^{-1}) was regulated by gravity to achieve complete replacement of the cell surrounding within 20 to 30 ms.

Current recordings were filtered at 3 to 10 KHz (– 3 dB, 8-pole Bessel filter) and digitized at sampling intervals of 100 μs using a 12 bit A/D Tecmar Lab Master board (125 kHz) interfaced with an IBM-compatible computer. Stimulation and acquisition were made with pClamp software (Axon Instruments, Forster City, CA, USA). Off line data analysis and curve fittings were made with pClamp and FIG PLOT software.

Cells were clamped at – 70 mV holding potential. Step depolarizations to 0 mV from this holding potential lasted 50 ms and were applied at intervals of 10 s to minimize the 'run-down' of Ca^{2+} currents (Fenwick *et al.*, 1982). Cells with pronounced run downs were discarded. Capacitative transients and leakage currents were compensated electronically and by subtracting Cd^{2+}-insensitive currents. Membrane current were always fully blocked by 200 μM Cd^{2+}, suggesting the absence of K^+ and Cl^- currents in our recordings. Data are expressed as mean ± s.e.mean for a given number of cells (*n*).

Intracellular Ca^{2+} measurements

Cells attached to glass coverslips were losed with fura-2/AM (2.5 μM for 40 min at 25°C, in the dark). Then, the cells were washed with Krebs-HEPES and kept 10 min at 37°C in an incubator before being placed on the stage of an inverted microscope, in a chamber allowing their continuous superfusion with Krebs-HEPES. High K^+ (59 mM)- and R56865-containing solutions were applied to the cell under investigation using the fast superfusion device employed in the electrophysiological studies. Only one experimental protocol was run in each single coverslip. Single cell fluorescence measurements were performed by exciting the fura-2-loaded cells with alternating 360 and 390 nm filtered light. The apparent $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescent signals (short over long wavelengths) according to Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_{eff}\ (R-R_o)/R_1-R) \qquad (1)$$

where K_{eff} is an 'effective binding constant', R_o is the fluorescence ratio at zero Ca^{2+} and R_1 is the limiting ratio at high Ca^{2+}. These calibration constants were experimentally determined as described by Almers & Neher (1985). Briefly, three intracellular calibration measurements were made in different cells dialyzed in the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981), with various EGTA-Ca^{2+} buffers added to a pipette filling solution containing (in mM): K^+-glutamate 135, NaCl 8, $MgCl_2$ 1, GTP 0.3, MgATP 0.5, fura-2-pentapotassium salt 0.1, KOH-HEPES 10, pH 7.2. The additions were either 10 mM EGTA or 10 mM $CaCl_2$ and the R_o and R_1 values, defined as above, were measured directly in the cells. Finally, the K_{eff} was calculated from equation (1) making a third measurement of the fluorescence ratio from a cell injected with a solution containing 6.6 mM Ca-EGTA plus 3.3 mM free EGTA, assuming that the $[Ca^{2+}]_i$ is under this condition, clamped to an apparent value of 0.3 μM (implying a K_D for EGTA at pH 7.2 of 0.15 μM). Experimental fluorescence data were sampled every 0.5 s by a computer which provided continuously the $[Ca^{2+}]_i$ values in nM.

Materials

R56865 was obtained from Janssen, Beerse, Belgium. Fura-2/AM was obtained from Molecular Probes, Eugene, Oregon, USA. Dulbecco's Modified Eagles Medium (DMEM), foetal calf serum and antibiotics were purchased from GIBCO, Madrid, Spain. Other chemicals were obtained either from Sigma or Merck, Madrid, Spain.

Statistical analysis

Results are expressed as means ± s.e.mean. The statistical differences between means of two experimental results were assessed by Student's *t* test. A value of *P* equal or smaller than 0.05 was taken as the limit of significance.

Results

Characteristics of the secretory responses to K^+

The spontaneous basal catecholamine release from superfused chromaffin cells was stabilized after 15 min of superfusion. At the maximum concentrations used (0.5%), the solvent for R56865 (ethanol) did not affect the basal rate of secretion. The compound itself did not increase the basal rate of secretion at concentrations 1–100 μM.

By increasing 10 fold the K^+ concentration of the superfusing Krebs solution (from 5.9 to 59 mM), the basal rate of secretion rose rapidly to a peak that was proportional to the time of exposure of the cells to this K^+-enriched solution (the K^+ pulse). With the aid of an electronic valve, pulses of 0.5

to 5 s duration were tried. The 5 s pulses, when applied at 5 min intervals, gave the most reproducible secretory responses. In Figure 2a, the peaks obtained in an experiment with 2.5×10^6 cells in the chamber are shown. After 15 such stimuli, peaks were kept fairly constant; 1 nA was equivalent to 71 ng ml^{-1} of noradrenaline at a perfusion rate of 1 ml min^{-1}.

Figure 2b shows the averaged results of 7 experiments carried out with cells from different batches stimulated with sequential 5 s pulses of 59 mM K^+ applied at 5 min intervals. At the 5th pulse, the secretory response was 96.7 ± 2.1% of the initial response; by the 10th pulse, secretion diminished to 83.6 ± 5.6% and by the end of the experiment (15th pulse) the release of catecholamines was 76.5 ± 6.3%. Thus, the secretory responses were fairly stable after 1–1.5 h stimulation of the cells at 5 min intervals. Therefore, different protocols, including concentration-response curves, could be designed and performed to test the effects of R56865 on K^+-evoked catecholamine secretion.

The effects of R56865 on catecholamine release

After obtaining 2–3 similar initial responses to 5 s pulses of 59 mM K^+, R56865 (10 μM) was superfused for 5 min in Krebs-HEPES solution and then the K^+ pulse was repeated in its presence (Figure 3a). Secretion was blocked by 81.4 ± 2.9% ($n = 9$). Ten min after washing out the compound, the secretory response recovered to 60.9 ± 5.4% of the initial response; after 15 min wash out, the response recovered further to 64.8 ± 6.3%.

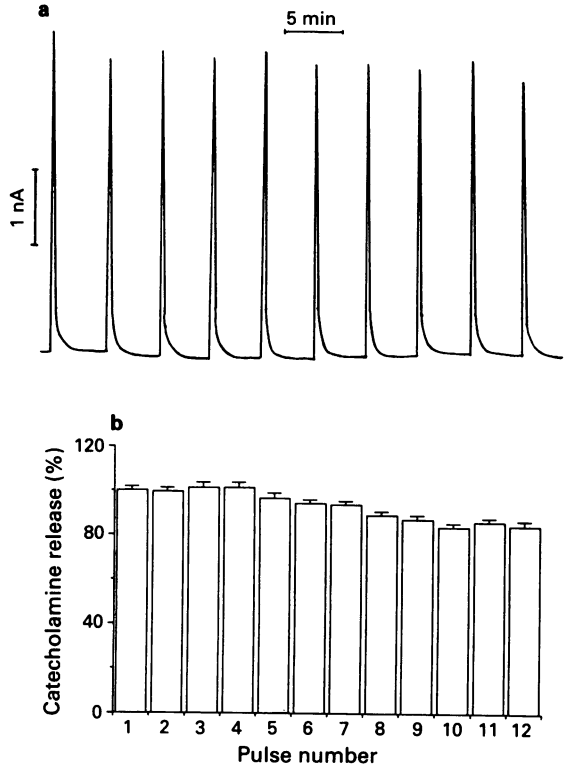

Figure 2 In (a) 2.5×10^6 cells were trapped in a small chamber and superfused at 1 ml min^{-1} for 15 min (equilibrium period) with Krebs-HEPES solution. Then, they were stimulated with 5 s pulses of a high K^+ solution (59 mM) at 5 min intervals. Catecholamines released were continuously monitored, on line, with an electrochemical detector. The records correspond to the originals of a typical experiment: 1 nA is equivalent to 71 ng ml^{-1} of noradrenaline at a perfusion rate of 1 ml min^{-1}. In (b) the averaged results of experiments similar to those shown in (a), are plotted. In every individual experiment, the secretion obtained during the first pulse was normalized to 100%; the response obtained in subsequent pulses was expressed as a % of the first pulse. Data are means ± s.e.mean of 7 experiments performed with cells drom different batches.

A concentration-response curve for the inhibitory effects of R56865 was obtained following the protocol described in the legend to Figure 3b. The threshold inhibitory concentration of R56865 was 0.3 μM (about 15% blockade of secretion). In this set of experiments, the inhibition induced by 10 μM was 84.9 ± 2.5%, a figure greater than that obtained in Figure 3a; this difference could be explained on the basis of different protocols (single concentration versus various concentrations) and the lipophilicity of the drug. Full blockade of secretion was achieved only at very high concentrations (100 μM). The IC_{50} of R56865 to block secretion, estimated by linear regression analysis, was 3.1 μM.

In the experiments described above, the concentration-dependence of the effects of R56865 on secretion were studied after short superfusion times (5 min). It was, therefore, desirable to see whether its blocking effects were

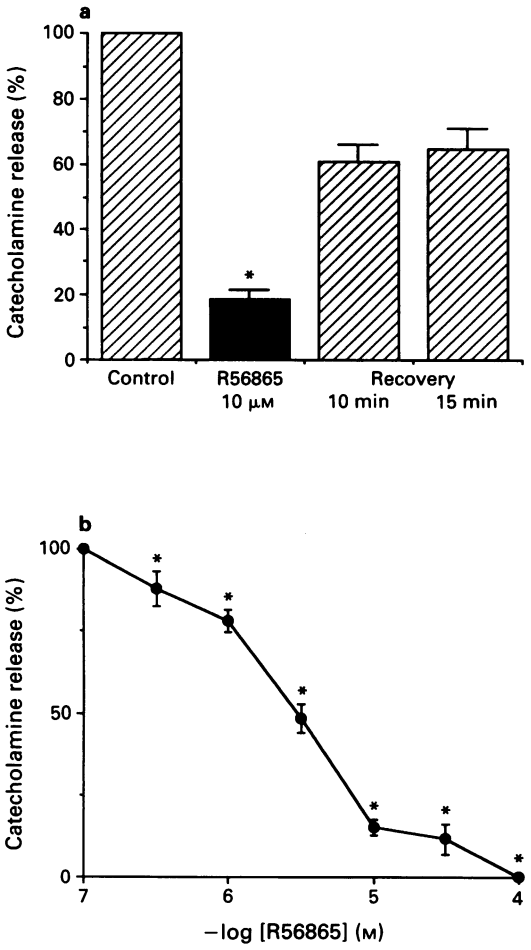

Figure 3 (a) Effects of R56865 on the release of catecholamines evoked by K^+ stimulation. Chromaffin cells were superfused with Krebs-HEPES solution and stimulated with 5 s pulses of 59 mM K^+ at 5 min intervals. After the initial secretory tests were reproducible, R56865 (10 μM) was introduced into the superfusion system and 5 min later the secretory response was re-tested. Recovery was tested 10 and 15 min after washing out the drug. Data are means ± s.e.mean of 9 experiments made with cells from different batches. *$P < 0.01$ with respect to control and to recoveries. (b) A concentration-response curve was determined according to the following protocol. Two K^+ pulses served to monitor the initial control response; a third pulse was applied 5 min after superfusion with the lowest concentration of R56865; then, two pulses were given at the fifth and tenth min of washing out the cells with drug-free medium. After recovery, the second concentration of drug was used and the recovery procedure repeated again. This was continued for the subsequent concentrations of R56865. The % of blockade for each concentration of drug was calculated, taking as 100% the secretory response obtained immediately before each drug concentration. Data are means ± s.e.mean of 5 experiments. *$P < 0.01$ with respect to its respective control secretory response.

also time-dependent. In the experiment shown in Figure 4, attempts were made to answer this question using a single concentration of R56865 (3 μM) close to its IC_{50} when added cumulatively (Figure 3b), to block secretion after 5 min exposure periods. The secretory response in the presence of the drug fell to 79.7 ± 5% after 5 min, to 47.2 ± 4.1% after 10 min and to 29.8 ± 3.8% after 15 min. From this time onwards the blockade developed very slowly; the response had decreased to 18.5 ± 3.6% after 40 min perfusion with R56865. After washing out the drug, the secretion recovered to 43.6 ± 5.5% of the intial in 5 min, and to 80.8 ± 12.1% after 20 min wash out. Thus, the inhibitory effects of R56865 were time-dependent and reversible. This time-dependence explains the lower potency of a single concentration of R56865 to block secretion using different protocols (compare Figures 3a and b).

The blocking effects of R56865 on secretion obtained in excess Ca^{2+} are shown in Figure 5. In 10 mM Ca^{2+} the secretory response to a 5 s pulse of 59 mM K^+ was 90.2 ± 7.9% of the response obtained in 1 mM Ca^{2+}. R56865 (10 μM) decreased secretion in high Ca^{2+} to 42.5 ± 4.5% of the high-Ca^{2+} response. Fifteen min after washing out the drug, the secretory response recovered to 82.3 ± 5.0% of the initial. Therefore, the degree of blockade of secretion was decreased by 20% in the presence of high extracellular Ca^{2+} concentrations ($P < 0.01$) while the recovery increased by around 20%.

Actions of R56865 on catecholamine release from electroporated cells

To discover a possible direct effect of R56865 beyond the plasma membrane, experiments were performed in electroporated cells. Cells (4×10^6 ml^{-1}) were electroporated as described in Methods, and then transferred to a superfusion chamber to estimate the on-line release of catecholamines. Once the rate of spontaneous secretion stabilized, in the nominal presence of around 50 nM Ca^{2+}, secretion was activated by superfusing an electroporation buffer containing 10 μM free Ca^{2+} for 2 min. Figure 6 shows two secretory profiles obtained in the absence (a) or in the presence (b) of 10 μM R56865. The initial rate of secretion and the maximum secretion were similar; in 5 experiments made in parallel the peak secretions were 66 ± 28 and 51 ± 28 nA respectively for control and R56865-treated cells.

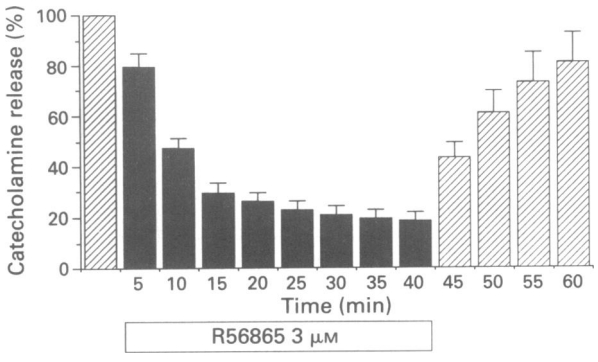

Figure 4 Time-dependence of the blocking effects of K^+-evoked catecholamine release by R56865. Pulses of 59 mM K^+ of 5 s duration were sequentially applied at 5 min intervals to chromaffin cells (2.5×10^6) superfused with Krebs-HEPES solution. After stabilization of the secretory response, R56865 (3 μM) was introduced (horizontal bar) and the K^+ pulses continued in its presence for 40 min. Then, 4 additional pulses were applied in the absence of the drug. Data are expressed as % of the secretory peak initially obtained; they are means ± s.e.mean of 5 experiments with different batches of cells.

Effects of R56865 on whole-cell Ca^{2+} currents

Bovine chromaffin cells (3–7 days old) were superfused with an extracellular solution containing 10 mM Ba^{2+}. A 50 ms test potential to 0 mV from a holding potential of −70 mV evoked I_{Ba} which averaged 472 ± 46 pA ($n = 5$). Only those cells which exhibited a stable current, with little run down (Fenwick *et al.*, 1982) were selected for use in experiments with R56865.

R56865 modified the I_{Ba} in two ways. On the one hand it decreased the peak current in a time- and concentration-

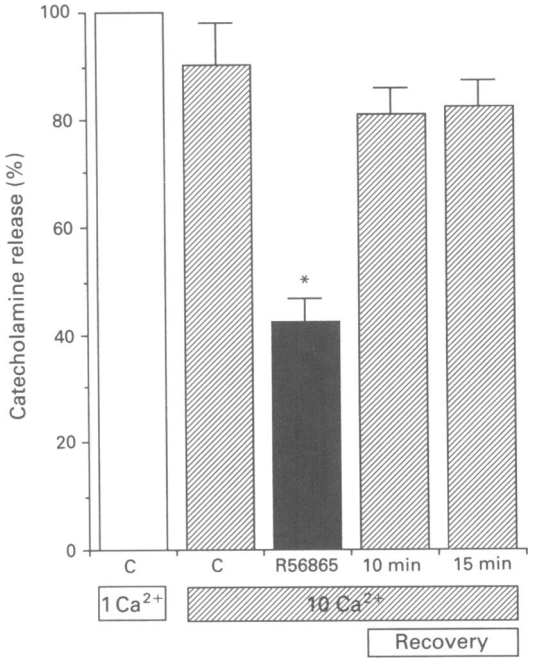

Figure 5 Blockade by R56865 of catecholamine release in the presence of high extracellular Ca^{2+} concentrations. Cells were initially stimulated as usual, in the presence of 1 mM Ca^{2+}. Then, Ca^{2+} in the superfusion medium was increased to 10 mM and the K^+ stimulation (59 mM for 5 s) repeated again. Then, still in the presence of 10 mM Ca^{2+}, R56865 (10 μM) was given and K^+ stimulation repeated 5 min later. Recovery was tested by superfusion with 10 mM Ca^{2+} at 10 and 15 min of washing out the drug. Data are expressed as % of the secretory response obtained in 1 mM Ca^{2+}; they represent means ± s.e.mean of 5 experiments. $^*P < 0.01$ with respect to the control in 10 mM Ca^{2+}.

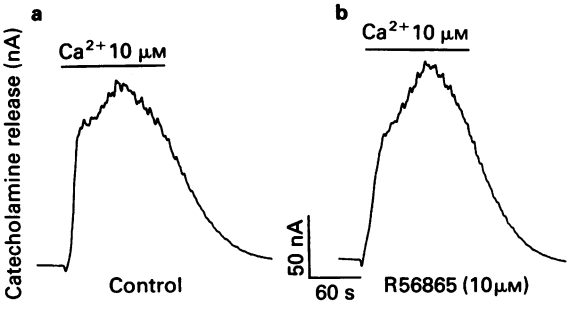

Figure 6 Actions of R56865 on catecholamine release from electroporated cells. Cells were electroporated as described in Methods. When added, R56865 (10 μM) was present during the electroporation procedure and during the entire superfusion period. One group of cells was stimulated with 10 μM free Ca^{2+} (top horizontal bars) in the absence of R56865 (a, control) and the other in its presence (b, R56865). Calibration bars express catecholamine release in nA and time.

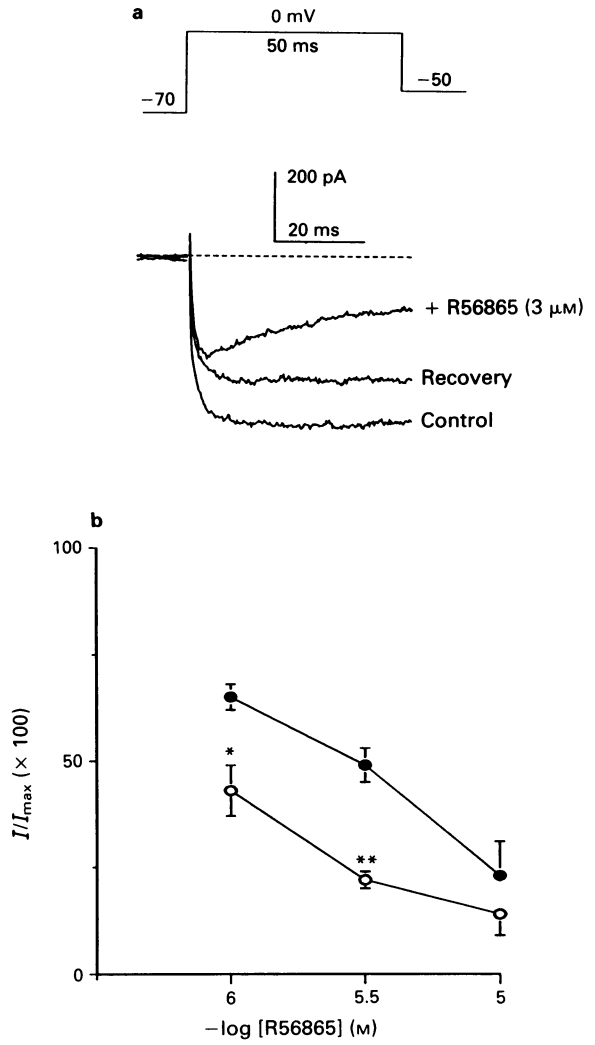

Figure 7 (a) Time course of effects of R56865 on whole-cell currents through Ca^{2+} channels. The holding potential was fixed at − 70 mV; currents were elicited by 50 ms test pulses to 0 mV applied at 15 s intervals to delay the run-down of the currents (see protocol on top of the figure). The charge carrier ion was Ba^{2+} (10 mM). Peak and late currents have been normalized with respect to maximum current in order to illustrate better the effects of the drug in increasing the inactivation of the current. Right panel shows current traces obtained at the points indicated in the figure. (b) Concentration-dependence of the blockade of I_{Ba} peak and at its most inactivated part, induced by R56865. The data to draw these curves were obtained by pooling the results obtained in various cells using the protocol shown at top of (a). Peak current (●) was measured at the maximum magnitude of I_{Ba} and inactivation of the current (○) in the presence of a given concentration of R56865 was calculated from the lowest point at the end of I_{Ba}. Data were normalized with respect to control values; they are means ± s.e.mean of 6 cells. $*P<0.05$ and $**P<0.005$ with respect to control values.

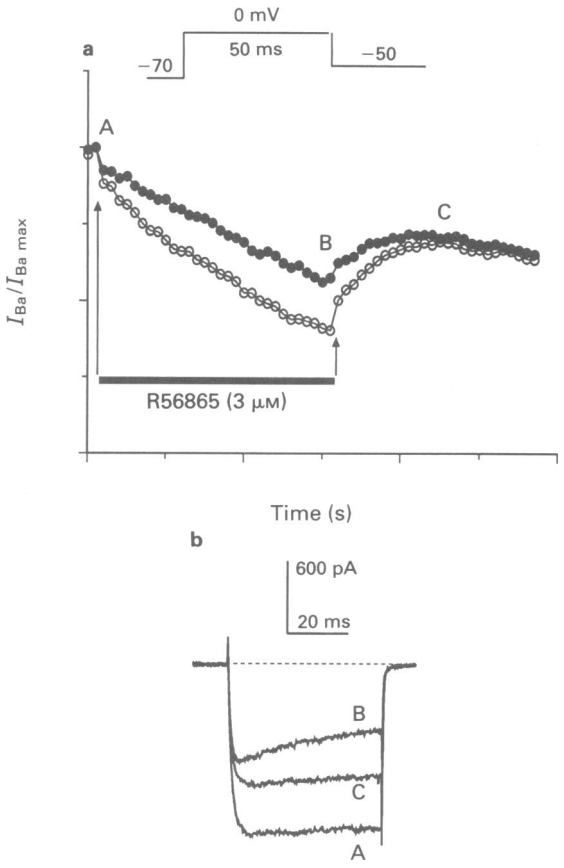

Figure 8 Time-dependence of R56865-induced blockade of I_{Ba}: (●) Peak current; (○) late current. The holding potential of the cell was maintained at − 79 mV. Test pulses to 0 mV were applied at 30 s intervals. R56865 (3 μM) was added to the superfusion solution, as shown by the horizontal bar. Inset, typical records of I_{Ba} at the indicated points A, B and C.

dependent manner; on the other, it promoted the inactivation of the current (Figure 7a). At 3 μM, the peak current was decreased by 51 ± 4% at the peak, and by 78 ± 2% at the most inactivated part of the current. At 10 μM, the peak current was further reduced by 77 ± 8%. After washing out the drug, the current was readily reversible. The reversal of inactivation was completed after 5 min of washing out the drug (Figure 7a), whatever the concentration used. However, after 5 min wash out, the blockade of the peak current was only partially reversed. More extensive wash out periods, similar to those used in the secretion experiments could not be performed because of the run-down of the current.

To estimate the IC_{50}, a given patch-clamped cell was exposed to two or three concentrations of the drug using a protocol similar to the secretion experiments. Control I_{Ba} current were determined 5 min before applying the drug, 5 min after exposure of the cell to a given concentration and 5 min after washing out R56865. The size of I_{Ba} before each drug concentration was taken as the 100% value for such a concentration; the effect of the drug at this concentration was calculated as % of its respective control. The concentration-response curve for the inhibition of the peak current exhibited an IC_{50} (3 μM) very close to that obtained in the secretion experiments. The inactivation of the current was achieved even at lower concentrations (Figure 7b); the IC_{50} to block I_{Ba} at its most inactivated part was around 1 μM.

The time-dependence of R56865 blockade of I_{Ba} was studied with the concentration used to study the time-dependence of its effects on secretion, which was similar to its IC_{50} (3 μM). To a voltage-clamped chromaffin cell, 50 ms depolarizing pulses to 0 mV (from a holding potential of − 70 mV) were applied at 30 s intervals. Once the evoked I_{Ba} was stable, R56865 was superfused; after 15 min, the cell was superfused back with a R56865-free solution. Figure 8 shows that R56865 blocks I_{Ba} in a time-dependent manner. Upon washing out the compound, I_{Ba} recovered partially.

Effects of R56865 on cytosolic Ca^{2+} transients

The effects of R56865 on cytosolic Ca^{2+} transients were studied with a protocol similar to that used in Figure 2 to study its effects on secretion. A single fura-2-loaded chromaffin cell was first perfused with Krebs-HEPES solution until a stable basal $[Ca^{2+}]_i$ was achieved (usually, around

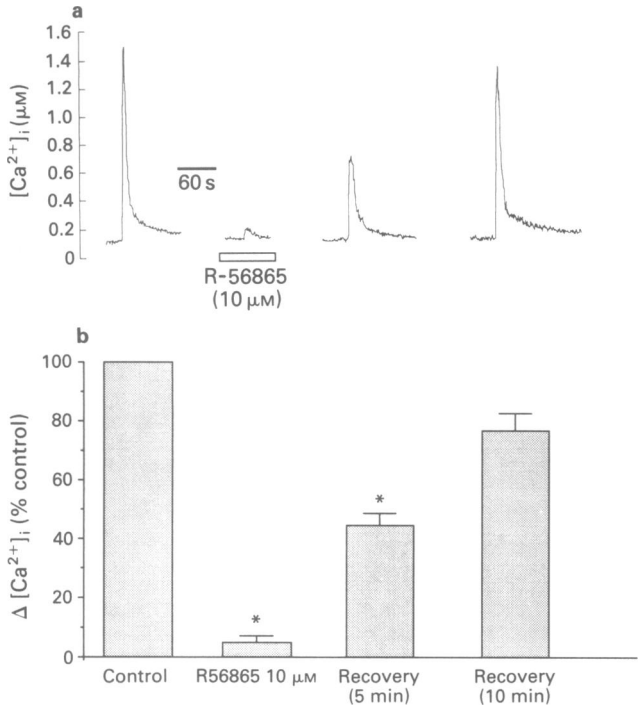

Figure 9 Effects of R56865 on the changes in cytosolic Ca^{2+} concentrations induced by K^+. Five second pulses of high K^+ (59 mM) were given to a fura-2-loaded single chromaffin cell at 5 min intervals (a). Between pulses, R56865 (10 μM) was introduced and a K^+ pulse given in its presence 5 min later. Recovery of the Ca_i^{2+} signal was tested 5 and 10 min after washing out the drug. The blockade and recovery of the K^+-evoked increase of $[Ca^{2+}]_i$ could be repeated several times in the same cell. Part (b) of the figure shows the averaged results of several K^+ pulses given to 3 different cells; data are means ± s.e.mean of 6 K^+ pulses. $^*P<0.01$ with respect to control.

100 nM). At 10 μM, R56865 did not affect the basal $[Ca^{2+}]_i$. Application of 5 s pulses of 59 mM K^+ solution containing 1 mM Ca^{2+} produced a rapid increase of $[Ca^{2+}]_i$ to a peak of 1.5 μM (Figure 9a); the $[Ca^{2+}]_i$ quickly returned to basal levels. The peaks were very reproducible on sequential applications of K^+ at 5 min intervals.

R56865 (10 μM) blocked the K^+-evoked increase of $[Ca^{2+}]_i$ by 90%. On washing out the drug, the increase of $[Ca^{2+}]_i$ returned to control pre-drug levels after 10 min. This protocol (alternate periods of drug introduction and washing out) could be repeated in the same cell 2–3 times with identical results. The averaged results obtained with eight K^+ stimuli performed in three separate cells are presented in Figure 9b. R56865 blocked by 95 ± 2.4% the increase in $[Ca^{2+}]_i$ evoked by K^+ pulses. The recovery (after 10 min wash out) was 76 ± 5.6% of the initial response. R56865 itself did not affect the basal $[Ca^{2+}]_i$.

Discussion

The experiments presented here demonstrate that R56865 blocks the release of catecholamines from chromaffin cells stimulated with depolarizing concentrations of K^+. The effect is concentration- and time-dependent, and readily reversible upon washing out the drug. The effects of R56865 extend over a wide range of concentrations (almost 3 logarithmic units). Some blockade was already evident at submicromolar concentrations but full blockade could not be established until a concentration of 100 μM was reached. The graded effects of increasing concentrations speak in favour of a selective action at a specific target involved in the control of secretion. The fact that even 100 μM R56865 did not itself increase the spontaneous release of catecholamines also support its specificity. Finally, the reversibility of its action strengthens this view.

An interesting feature was the time-dependence of the blocking effects of R56865, which can be explained on the basis of its lipophilicity. Inhibition of secretion by 80% took 40 min when using 3 μM and only 5 min at 10 μM. The protection against veratradine-induced Ca^{2+} overload in ventricular myocytes was also time-dependent: a maximal degree of protection was observed with 10^{-6} M within 1 min, whereas a similar effect was obtained with 10^{-7} M after about 30 min (Ver Donck & Borgers, 1991). Slowly progressing inhibition of the cardiac transient inward current (Leyssens & Carmeliet, 1991) and of the upstroke velocity of the Na^+ current (Carmeliet & Tytgat, 1991) were also observed. All these data are compatible with a time-dependent progressive accumulation of the drug in target cells. This time-dependent inhibition of secretion correlates well with a similar time-dependent blockade of I_{Ba}, suggesting that the progressive blockade by R56865 of catecholamine release is due to a progressive blockade of Ca_o^{2+} entry through Ca^{2+} channels.

Depolarization of bovine chromaffin cells with high K^+ concentrations causes parallel increases of Ca^{2+} entry, $[Ca^{2+}]_i$ and secretion (Kilpatrick *et al.*, 1982; Knight & Kesteven, 1983; Artalejo *et al.*, 1986; Michelena *et al.*, 1993). R56865 inhibited the increase of $[Ca^{2+}]_i$ (Figure 8). Because the compound caused a marked inhibition of I_{Ba} ($IC_{50} = 3$ μM; Figures 7 and 8), it seems that blockade of catecholamine release ($IC_{50} = 3.1$ μM; Figure 3b) can be explained best by suppression of Ca^{2+} entry through Ca^{2+} channels during cell depolarization. The fact that R56865 did not affect the Ca^{2+}-evoked secretion from electro-permeabilized chromaffin cells strengthens the view that the target for this compound is the chromaffin cell plasma membrane.

As in neurones (Bean, 1989; Hess, 1990; Tsien *et al.*, 1991; Swandulla *et al.*, 1991; Miller, 1992), multiple types of voltage-dependent Ca^{2+} channels have been discovered in bovine chromaffin cells. For instance, they contain around 18 000 CgTx-sensitive Ca^{2+} channels per cell (Ballesta *et al.*, 1989; Artalejo *et al.*, 1992) and 2000 L-type Ca^{2+} channels per cell (Castillo *et al.*, 1989). A third pathway for Ca^{2+} entry, which is resistant to DHPs and to CgTx has also been described (Rosario *et al.*, 1989). DHP antagonists and CgTx block partially the whole-cell Ca^{2+} currents (Artalejo *et al.*, 1991; Bossu *et al.*, 1991) and catecholamine release from bovine adrenal medullae (Gandía *et al.*, 1990; Jiménez *et al.*, 1993) and chromaffin cells (Boarder *et al.*, 1987; but see Ceña *et al.*, 1983). R56865 inhibited almost completely the whole-cell I_{Ba} in bovine chromaffin cells. The drug also blocks L- as well as T-type Ca^{2+} channels in cardiac cells (Himmel *et al.*, 1990; Leyssens & Carmeliet, 1991). Thus, it seems that R56865 exhibits a wider spectrum than most Ca^{2+} channel blockers known to date. These findings have two implications: on the one hand, they add to the growing evidence in favour of the presence of multiple Ca^{2+} entry pathways in bovine chromaffin cells; on the other, R56865 might be acting on a site different from DHP- or CgTx-binding sites, which might be common to various Ca^{2+} channel subtypes. In this sense, it could eventually become a probe to investigate molecular features common to such diverse channels. This is strengthened by the fact that in contrast to DHPs and to CgTx, R56865 blocks not only the peak Ca^{2+} current but also causes its inactivation even with short depolarizing test pulses (Figure 7).

For a cardioprotective drug such as R56865, the blockade of catecholamine release from catecholamine-storing cells might constitute an additional therapeutic advantage. One of the mechanisms of cardioprotection, the blockade of cardiac β-adrenoceptors is based on the prevention of the arrhythmogenic affects of the catecholamines, limiting the increased oxygen demand produced by their strong inotropic

effects. A limited supply of released catecholamines during ischaemia or arrhythmias may benefit the therapeutic profile of R56865.

In conclusion, R56865, a novel cardioprotective agent, blocks the release of catecholamines by blocking various subtypes of Ca^{2+} currents in bovine chromaffin cells, thus preventing the increase of $[Ca^{2+}]_i$ after their depolarization.

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Characterization of somatostatin receptors in guinea-pig isolated ileum, vas deferens and right atrium

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1 Somatostatin$_{14}$ (SS_{14}) inhibits neurogenically mediated contractile responses in guinea-pig ileum and vas deferens and exerts a direct negative inotropic action in guinea-pig spontaneously beating right atrium. In this study, the receptors mediating these inhibitory effects have been characterized by comparing the potencies of several cyclic somatostatin analogues.

2 In the guinea-pig ileum, SS_{14}, somatostatin$_{28}$ (SS_{28}), somatostatin$_{25}$ (SS_{25}) and several smaller cyclic somatostatin analogues including octreotide, angiopeptin and CGP 23996, inhibited neurogenically mediated contractile responses, each being of similar potency.

3 In contrast, in the guinea-pig vas deferens and right atrium, SS_{28} was about 30 times more potent than SS_{14}. However, although angiopeptin was nearly as potent as SS_{14} as an agonist in the vas deferens, in guinea-pig atrium angiopeptin had low intrinsic activity and antagonized the negative inotropic action of both SS_{14} and SS_{28} (pK_B values of 7.4 and 7.2, respectively). CGP 23996 was 2–7 times weaker than SS_{14} in guinea-pig vas deferens and atria.

4 Phosphoramidon (1 μM) and amastatin (10 μM) did not influence the potency of SS_{14} or SS_{28} in either the guinea-pig ileum or right atrium. In the guinea-pig vas deferens, phosphoramidon and amastatin did not affect the potency of SS_{28}, but enhanced the potency of SS_{14} about 5 fold. Despite the presence of phosphoramidon and amastatin, SS_{28} was still more potent than SS_{14} in the vas deferens.

5 The putative somatostatin receptor blocking drug, cyclo(7-aminoheptanoyl Phe-D-Trp-Lys-Thr[Bzl]) (CPP; 1 μM), did not antagonize the effects of either SS_{14} or SS_{28} in ileum, vas deferens or atrial preparations.

6 Somatostatin$_{14}$ did not modify the contractile action of carbachol or α,β-methylene ATP in the ileum and vas deferens respectively, suggesting that the site of the inhibitory effects on neurogenically mediated contractile responses in both preparations was pre-junctional. Consistent with this conclusion was the observation that the inhibitory effect of SS_{14} was markedly and inversely related to the external Ca^{2+} concentration. The inhibitory effect of SS_{14} in guinea-pig atrium was only partly dependent on the external Ca^{2+} concentration.

7 The somatostatin receptors mediating the inhibitory effect of SS_{14} in the ileum and vas deferens can be distinguished by the differential relative potencies of SS_{14} and SS_{28}. In the former, SS_{14} and SS_{28} have similar potency whilst in the latter SS_{28} is much more potent. In this respect, the somatostatin receptor mediating negative inotropy in the guinea-pig right atrium appears similar to that identified in the vas deferens.

8 We suggest that the somatostatin receptor mediating inhibition of neurogenic contraction in the ileum is similar to the recently cloned $SSTR_2$ receptor. In contrast, the somatostatin receptor mediating negative inotropy in the atrium and inhibition of neurotransmission in the vas deferens appears similar to the $SSTR_4$ receptor which recognises SS_{28} with higher affinity than SS_{14}.

Keywords: Somatostatin receptors; vas deferens; ileum; atrium; somatostatin$_{14}$; somatostatin$_{28}$

Introduction

The naturally occurring cyclic tetradecapeptide, somatostatin$_{14}$ (SS_{14}) originally isolated from hypothalamic extracts (Brazeau *et al.*, 1973) exerts a wide range of pharmacological actions in both the central nervous system and in peripheral tissues. The ability of SS_{14} to inhibit neurogenically mediated contractions in a range of isolated smooth muscle preparations is well documented (Cohen *et al.*, 1978; Furness & Costa, 1979; Magnam *et al.*, 1979; McIntosh *et al.*, 1986; Priestley & Woodruff, 1988). However, the somatostatin receptors mediating these inhibitory actions remain poorly characterized. Few if any specific somatostatin receptor blocking drugs exist and with some notable exceptions (e.g. Priestley & Woodruff, 1988), most agonist potency comparisons have been restricted to comparison of the potencies of SS_{14} and its N-terminally extended analogues SS_{25} and SS_{28}. Furthermore, the potential influence of endogenous aminopeptidases on reactivity to these peptides has largely been ignored.

Despite the fact that at least five different types of somatostatin receptor have recently been cloned (for review see Bell & Reisine, 1993 and also Bruno *et al.*, 1992) and the patterns of distribution of their mRNA studied by *in situ* hybridization, remarkably little is known about the different receptors which mediate the diverse biological actions of somatostatin.

In the present study, we have compared and characterized the somatostatin receptors which mediate inhibition of neurogenically mediated contractions in guinea-pig isolated ileum and vas deferens by comparing the relative potencies of several different cyclic somatostatin analogues of varying sizes. The guinea-pig ileum was chosen as a standard for comparison since inhibitory effects of somatostatin have been documented (McIntosh *et al.*, 1986) and the cholinergically mediated twitch response to electrical stimulation well characterized. The guinea-pig vas deferens was chosen for comparison since neurogenically mediated contractions in this preparation are predominantly mediated via the activation of purinergic nerves (Sneddon & Burnstock, 1984), and to our

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knowledge the effects of somatostatin on purinergic neurotransmission have not been investigated. In addition, the effects of these peptides have also been studied on guinea-pig isolated atria where somatostatin has been shown to exert a direct negative inotropic action through a pertussis toxin insensitive mechanism (Endou *et al.*, 1989).

Preliminary accounts of some of these findings have been presented to the British Pharmacological Society (Feniuk *et al.*, 1993a,b).

Methods

Guinea-pig isolated ileum and vas deferens

Male Dunkin Hartley guinea-pigs (220–450 g) were humanely killed by cervical dislocation and pairs of vas deferens and lengths of distal ileum (approximately 15 cm from the ileocaecal junction) were removed and placed in a modified Krebs solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, D-glucose 11.1, $CaCl_2.6H_2O$ 1.3, at room temperature and gassed with 95% O_2/5% CO_2. Preparations were cleared of adhering connective and vascular tissue. Each of the pair of vas deferens and four segments of ileum were mounted between a pair of parallel platinum electrodes and placed in 15–20 ml organ baths for recording isometric tension changes, using a Dynamometer UF1 force transducer, from a resting tension of 0.5 g. Bath temperature was maintained at 37–38°C. Following an initial equilibration period of approximately 30 min, transmural electrical stimulation was applied to guinea-pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Once responses to electrical stimulation had been established, preparations were washed with Krebs solution and responses to electrical stimulation re-established for a period of at least 5 min to allow responses to electrical stimulation to stabilize. Preliminary studies showed that reponses to SS_{14} were subject to tachyphylaxis when administered in a cumulative manner. Consequently, concentration-effect curves to SS_{14} were obtained by a non-cumulative addition of agonist and drug was washed from the organ bath once the inhibitory response to each concentration of agonist had reached a plateau. At least 10 min was allowed between each successive application of SS_{14}. In order to conserve the supply of peptides, the maximum concentration of agonist tested was normally 1 μM. At least 30 min was allowed to elapse before concentration-effect curves to another agonist were established by use of the same protocol as described for SS_{14}. One ileal preparation and one of the pair of vas deferens acted as a control in order to monitor spontaneous changes in sensitivity to the effects of SS_{14}. Equi-effective molar ratios (EMR) were measured from the concentration-effect curves at a point corresponding to 50% of the second agonist maximum (EC_{50}) or, in the case of SS_{14} in vas deferens, 50% of the response obtained at the highest concentration examined (1 μM).

Effect on contractile responses to carbachol or α,β-methylene ATP

In some experiments, the effects of SS_{14} on contractile responses to a submaximal concentration of carbachol were examined in the guinea-pig isolated ileum. SS_{14} was administered at a single concentration when the contraction produced by carbachol had reached a plateau. In other experiments, the effects of SS_{14} on the contractile response to a submaximal concentration of α,β-methylene ATP were examined in the guinea-pig vas deferens. SS_{14} was administered approximately 3 min prior to administration of α,β-methylene ATP.

Guinea-pig isolated right atrium

Male Dunkin Hartley guinea-pigs (220–450 g) were humanely killed by cervical dislocation and the hearts rapidly removed. Spontaneously beating right atria were cleared of ventricular and adhering connective tissue, mounted on glass tissue holders and placed in 10 ml organ baths containing modified Krebs, gassed with 95% O_2/5% CO_2 at 32°C.

Each preparation was connected to a Dynamometer UF1 isometric force transducer and spontaneous isometric contractions measured from a resting tension of 1 g. All preparations were allowed to equilibrate for 60 min during which time tension was re-adjusted to 1 g and washed every 15 min. Cumulative concentration-effect curves for SS_{14} (1 nM–1 μM) were obtained on each preparation, successive doses being added when the response to the previous concentration had reached a plateau. Following a 60 min interval during which preparations were washed every 15 min, a second cumulative concentration-effect curve to SS_{14} or another agonist was obtained as previously described. Responses were expressed as percentage change in initial developed tension. The agonists were examined in a random sequence following a Latin square design.

Influence of peptidase inhibitors and antagonists

In some experiments, the influence of a combination of the peptidase inhibitors, amastatin (10 μM) and phosphoramidon (1 μM) on the inhibitory effect of SS_{14} and some other cyclic somatostatin peptides was examined. In the guinea-pig ileum and vas deferens, agonist concentration-effect curves were obtained before and after a 15 min exposure to the peptidase inhibitors which were in contact with the preparation throughout the second concentration-effect curve.

In the atrial studies where cumulative agonist concentration-effect curves were obtained, the contact time was 30 min. One preparation always acted as a control to monitor spontaneous changes in agonist sensitivity. Unless otherwise stated, an identical protocol was used to assess the effects of antagonists in all preparations. However, 'antagonist/inhibitor' experiments in atria were carried out in Krebs solution containing 2.6 mM Ca^{2+} rather than 1.3 mM Ca^{2+}.

Influence of external calcium concentration

Control concentration-effect curves to SS_{14} were obtained as previously described and then repeated after a 30 min period of equilibration in Krebs solution containing different concentrations of Ca^{2+}. As in previous studies, one of the preparations acted as a control and the second concentration-effect curve repeated in the presence of 'normal' calcium (1.3 mM).

Calculations and statistics

All values stated are mean ± s.e.mean of n observations, except for EC_{50} values which are geometric means (95% confidence limits). pA_2 values were determined according to the method of Arunlakshana & Schild (1959) and provided an estimate of the $\log_{10}$ of the dissociation constant (pK_B) when the slope of the Schild plot was constrained to unity. Tests for statistically significant differences were carried out using an unpaired Students t test, or where the data was not normally distributed a Mann-Whitney test at $P = 0.05$.

Drugs

The following drugs were obtained from Sigma Chemical Co. Ltd; somatostatin$_{14}$, somatostatin$_{28}$, somatostatin$_{25}$, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) (CPP), cyclo (D-Trp-Lys-Thr-Phe-Pro-Phe), cyclo (D-Trp-Lys-Thr-Phe-Pro-Tyr), angiopeptin (β- (2-napthyl) -D-Ala-Cys-Tyr-D-Trp-Lys-

Val-Cys-Thr amide), phosphoramidon, amastatin, atropine methylnitrate, tetrodotoxin, carbamylcholine chloride (carbachol) and N^6 cyclohexyladenosine.

CGP 23996 (cyclo [Ahep-Lys-Asn-Phe-Trp-Lys-Thr-Tyr-Thr-Serl]) was synthesized by Dr J. Kitchin's team, Chemistry Division, Glaxo Group Research Ltd.

Octreotide (Sandostatin®) was purchased from a pharmaceutical supplier.

All peptides were initially dissolved in distilled water, divided into aliquots and stored at − 20°C. No samples were thawed and subsequently refrozen, fresh aliquots being used on each experimental day. Samples were kept on ice during the experiment.

Results

Agonist studies

Guinea-pig ileum Transmural electrical stimulation of guinea-pig isolated ileum (0.1 Hz, 0.1 ms continuously) caused contractions of the ileum which were abolished by atropine (0.1 μM) or tetrodotoxin (0.3 μM). SS_{14} (1 nM–0.3 μM) caused concentration-dependent inhibition of these neurogenically mediated contractile responses (Figure 1). Although the maximum inhibitory effect of SS_{14} varied from preparation to preparation (39–100% inhibition), the effect of SS_{14} was reproducible in a given preparation. In twelve control preparations used to determine relative agonist potencies, the EC_{50} value obtained from the second control concentration-effect curve was 5.2 (3.7–7.4) nM with a 1.5 ± 0.3 fold rightward displacement of the second concentration-effect curve from the first (Figure 1).

SS_{28} and a range of stable cyclic somatostatin hexapeptide and octapeptide analogues also inhibited neurogenically mediated contractile responses of the guinea-pig isolated ileum and were of similar potency to SS_{14} (Table 1). The maximum inhibitory effect produced by each of the agonists examined was similar, except for angiopeptin which produced a significantly smaller maximum.

SS_{14} (1 μM) had little effect on the contractile response produced by a submaximal concentration of carbachol (0.3 μM). When SS_{14} was added at the sustained phase of contraction produced by carbachol (1.67 ± 0.13 g) it was reduced by 8 ± 2% ($n = 4$).

Guinea-pig vas deferens Transmural electrical stimulation of guinea-pig vas deferens (5 Hz, 0.5 ms pulse width, 1.5 s every 30 s) with supramaximal currents produced contractile responses which were abolished by tetrodotoxin (0.3 μM). Responses were unaffected by phentolamine (0.3 μM) but were abolished by prior exposure to a desensitizing concentration of α,β-methylene ATP (10 μM).

The non-cumulative addition of SS_{14} caused a concentration-dependent inhibition of neurogenically mediated contractile response in the guinea-pig vas deferens but the potency of SS_{14} was approximately 20 fold lower than that observed in ileum (Figure 1, Table 1). In order to conserve drug supply, the highest concentration of SS_{14} examined in these studies was 1 μM. In 10 out of 26 control studies, neurogenically mediated contractile response in the vas deferens were abolished by this concentration of SS_{14} and the mean inhibition observed from all control experiments was 84 ± 3%. Assuming that maximum inhibition was obtained at 1 μM SS_{14}, the approximate EC_{50} value for SS_{14} was 83 (63–111) nM ($n = 26$). Concentration-effect curves to SS_{14} were reproducible, there being only a 1.2 ± 0.3 fold rightward displacement between the first and second SS_{14} concentration-effect curves ($n = 26$ experiments) (Figure 1).

The effect of a range of other cyclic somatostatin analogues was also examined in the guinea-pig vas deferens. Both SS_{28} and SS_{25} were much more potent than SS_{14} and abolished responses to electrical stimulation in all experi-

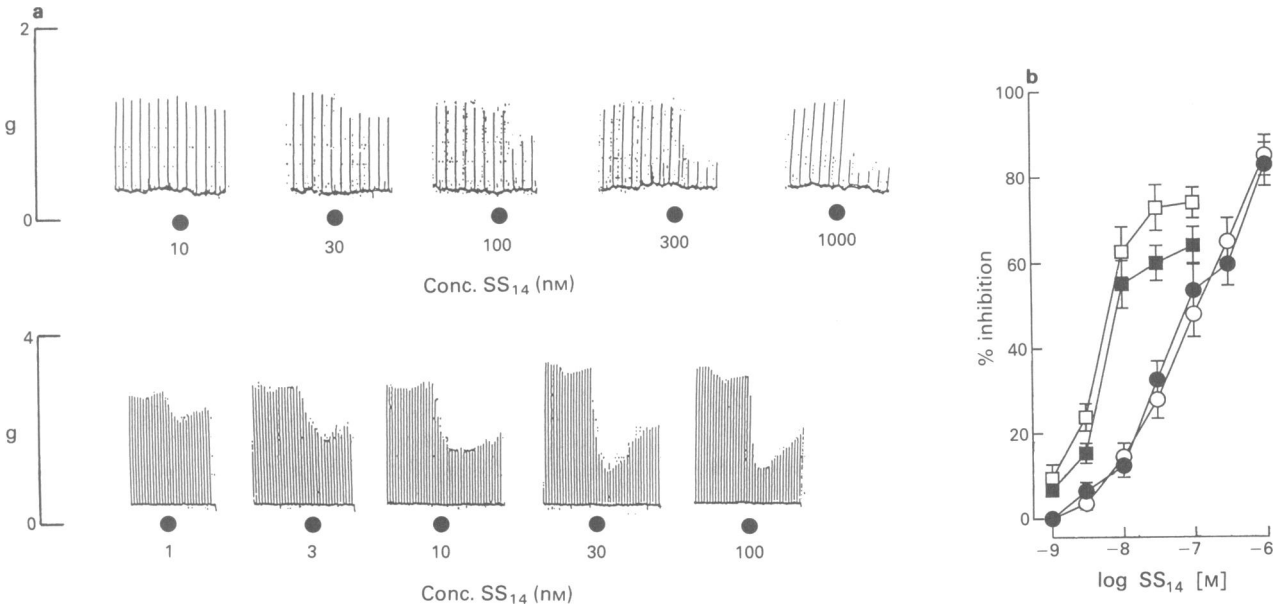

Figure 1 (a) Experimental recordings demonstrating inhibitory effect of somatostatin$_{14}$ (SS_{14}) in guinea-pig isolated vas deferens (top tracing) and guinea-pig isolated ileum (bottom tracing). (b) Reproducibility of concentration-effect curves to somatostatin$_{14}$ in guinea-pig isolated vas deferens (circles) and guinea-pig isolated ileum (squares). First concentration-effect curves (open symbols). Values are mean ± s.e.mean from 12 and 26 experiments in ileum and vas deferens respectively.

Table 1 A comparison of the potencies of some cyclic somatostatin analogues in guinea-pig isolated ileum, vas deferens and right atrium

	Guinea-pig ileum			*Guinea-pig vas deferens*			*Guinea-pig right atrium*		
	EC_{50} (nM)	*Max (%) inhibition*	*EMR*	*EC_{50} (nM)*	*Max (%) inhibition*	*EMR*	*EC_{50} (nM)*	*Max (%) inhibition*	*EMR*
SS_{14}	5.2 (3.7–7.4)	68 ± 5	1.0	83 (62–111)†	84 ± 3	1.0	188 (140–251)	55 ± 3	1.0
SS_{28}	8.0 (5.3–12.1)	69 ± 8	2.2 ± 0.6	4.3 (2.1n8.7)	100 ± 0*	0.03 ± 0.005	16 (10–25)	76 ± 4*	0.06 ± 0.01
SS_{25}	6.7 (3.8–11.8)	65 ± 8	1.2 ± 0.3	16 (11–24)	100 ± 0*	0.07 ± 0.02	14 (7–27)	66 ± 2*	0.06 ± 0.01
c[D-Trp-Lys-Thr-Phe-Pro-Phe]	9.7 (3.4–27.4)	54 ± 10	2.1 ± 0.5	22 (8–25)	65 ± 3*	0.28 ± 0.09			
c[D-Trp-Lys-Thr-Phe-Pro-Tyr]	4.4 (3.0–6.3)	62 ± 9	1.9 ± 0.9	12 (3–41)	65 ± 15	0.16 ± 0.01			
Angiopeptin	3.1 (1.1–8.8)	48 ± 6*	1.0 ± 0.3	18 (13–25)	67 ± 7	0.41 ± 0.16	19 (9–378)	23 ± 4*	1.3 ± 0.7
Octreotide	1.9 (0.8–4.6)	69 ± 4	1.0 ± 0.5	39 (14–105)	82 ± 9	0.27 ± 0.10	91 (30–260)	22 ± 2*	1.2 ± 0.4
CGP 23996	6.1 (3.3–11.0)	74 ± 6	1.7 ± 0.3	185 (105–329)	97 ± 3*	6.7 ± 4.7	268 (131–547)	47 ± 3	2.0 ± 0.6

Except for SS_{14}, values shown are geometric mean (95% confidence limits) or arithmetic mean ± s.e.mean from 4–5 experiments. Values for SS_{14} are for the second concentration-effect curves in the control preparations ($n = 12$ for ileum, $n = 26$ for vas deferens and $n = 16$ for atrium). †Approximate EC_{50} (see text).
*Significant difference in maximum response compared with that for SS_{14}.

Table 2 Effect of phosphoramidon (1 μM) and amastatin (10 μM) on the inhibitory effect of SS_{14} and some synthetic cyclic analogues in guinea-pig ileum, vas deferens and atrium

	Ileum		*Vas deferens*		*Right atrium*	
	Control EMR	*Test EMR*	*Control EMR*	*Test EMR*	*Control EMR*	*Test EMR*
SS_{14}	1.3 ± 0.5	0.9 ± 0.2	1.0 ± 0.3	0.2 ± 0.1*	0.9 ± 0.1	0.6 ± 0.1
SS_{28}	1.4 ± 0.6	0.8 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.7 ± 0.2
Octreotide	2.1 ± 0.5	2.5 ± 1.1	2.4 ± 0.7	2.0 ± 0.6	–	–
cyclo-D-Trp-Lys-Thr-Phe-Pro-Phe	1.5 ± 0.2	2.5 ± 0.8	1.2 ± 0.5	1.6 ± 0.8	–	–

Values are mean ± s.e.mean from 4 experiments. Control equi-effective molar ratio (EMR) represents spontaneous change in agonist sensitivity in time matched control preparations not treated with phosphoramidon and amastatin.
*Significant difference in test EMR compared with control EMR.

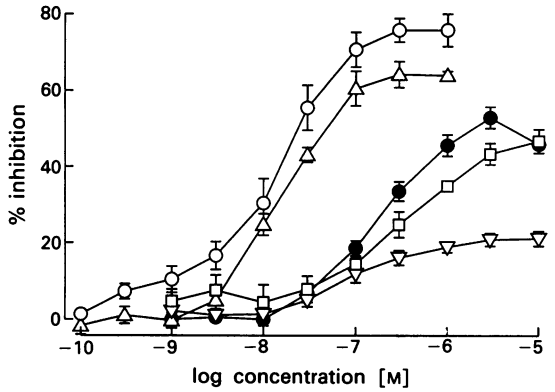

Figure 2 Negative inotropic action of somatostatin$_{14}$ (●), SS_{25} (△), SS_{28} (○), CGP 23996 (□) and octreotide (▽) in spontaneously beating guinea-pig isolated atria. Values are mean ± s.e.mean from 4 experiments except SS_{14} where $n = 16$.

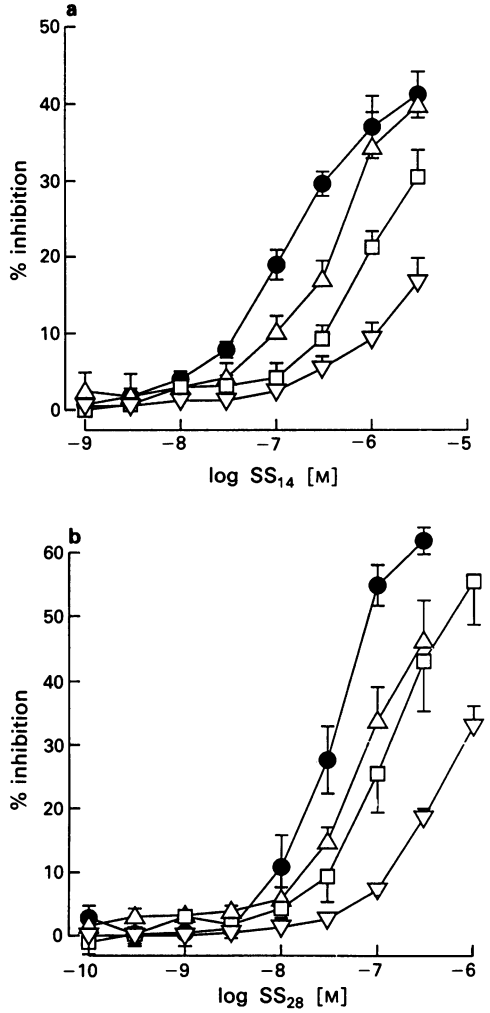

Figure 3 Negative inotropic effect of (a) somatostatin$_{14}$ and (b) somatostatin$_{28}$ in spontaneously beating guinea-pig atria in the absence (●) and presence of 0.1 μM (△), 0.3 μM (□) and 1 μM (▽) angiopeptin. Values are mean ± s.e.mean from 4 experiments.

ments. The effects of the other peptides are summarised in Table 1. Although cyclo-D-Trp-Lys-Thr-Phe-Pro-Phe, cyclo-D-Trp-Lys-Thr-Phe-Pro-Tyr and angiopeptin were also more potent than SS_{14}, their maximum effects appeared lower. The reduction in maxima was only statistically significant in the case of cyclo D-Trp-Lys-Thr-Phe-Pro-Phe.

SS_{14} (0.1 μM) had no effect on the contractile response to a submaximal concentration of α,β-methylene ATP (0.1 μM). In the presence of SS_{14}, contractile responses to α,β-methylene ATP were 100 ± 5% of the response obtained in the absence of SS_{14} ($n = 3$).

Guinea-pig isolated right atrium SS_{14} (1 nM–10 μM) caused a concentration-dependent negative inotropic action in guinea-pig isolated right atrial preparations. Concentration-effect curves were reproducible, there being a 1.2 ± 0.3 fold rightward displacement of the concentration-effect curves in twelve control experiments.

SS_{28} and SS_{25} were about 15 times more potent whilst CGP 23996 was approximately 2 times weaker than SS_{14} at decreasing tension in guinea-pig right atria (Figure 2 and Table 1). The maximum inhibitory effect produced by SS_{28} and SS_{25} was significantly greater than that produced by SS_{14}. Octreotide (0.1 nM–1 μM) and angiopeptin (0.1 nM–1 μM) were agonists with very low intrinsic activity producing only a 22 ± 2% and 23 ± 4% decrease in force respectively at the highest concentration tested ($n = 4$).

In view of the low intrinsic activity of angiopeptin, it was tested as a potential somatostatin receptor blocking drug. These studies were carried in Krebs solution containing 2.6 mM Ca^{2+}. Under these conditions, angiotpeptin produced only a 4.0 ± 2.4% decrease in developed tension of spontaneously beating atria which was smaller than that produced by octreotide in similar experiments (data not shown). Angiopeptin (0.1–1 μM) produced a concentration-dependent antagonism of the negative inotropic action of SS_{14} and SS_{28} in guinea-pig right atria producing rightward displacements of the agonist concentration-effect curves to SS_{14} and SS_{28} (Figure 3). Schild analysis, at the 20% inhibition level yielded a pA_2 value for angiopeptin against SS_{14} of 7.47 ± 0.13 with a slope of 0.99 ± 0.12 ($n = 4$) and a pA_2 value of 7.35 ± 0.16 with a slope of 0.98 ± 0.11 when SS_{28} was used as the agonist ($n = 4$). When the Schild slopes were constrained to unity the respective pK_B values were 7.44 ± 0.06 and 7.18 ± 0.08. Angiopeptin (1 μM) had no effect on the negative inotropic action of carbachol or cyclohexyladenosine, agonist concentration-ratios were 1.0 ± 0.1 and 1.5 ± 0.3 respectively ($n = 4$).

Influence of peptidase inhibitors

The influence of a combination of phosphoramidon (1 μM) and amastatin (10 μM) on the potency of SS_{14}, SS_{28}, octreotide and cyclo-D-Trp-Lys-Thr-Phe-Pro-Phe was examined in guinea-pig vas deferens and ileum as was their influence on the potency of SS_{14} and SS_{28} in guinea-pig right atrium (Table 2). Phosphoramidon and amastatin did not influence the potency of any of the peptides examined in either the ileum or atrial preparations. In contrast, phosphoramidon and amastatin caused a significant leftward displacement (approximately five fold) of the concentration-effect curve to SS_{14} in the vas deferens but had little effect on concentration-effect curves to SS_{28}, octreotide or cyclo-D-Trp-Lys-Thr-Phe-Pro-Phe (Table 2). However, in the presence of phosphoramidon and amastatin, SS_{28} was still much more potent than SS_{14} at inhibiting neurogenically mediated contractions of the vas deferens (Figure 4).

Influence of antagonists

The putative somatostatin receptor blocking drug CCP (1 μM) had no antagonistic effect on the inhibitory action of SS_{14} or SS_{28} in either ileum or vas deferens preparations (agonist concentration-ratios were 1.1 ± 0.5; 2.5 ± 1.4; 1.2 ± 0.1 and 0.9 ± 0.1 respectively; $n = 4$ for each). CPP even at a concentration as high as 10 μM had no antagonistic effect on the negative inotropic action of SS_{14} in guinea-pig right atrial preparations (agonist concentration-ratio was 1.1 ± 0.1, $n = 4$).

Naloxone (0.1 μM, 30 min contact time) had no antagonistic effect on the inhibitory action of SS_{14} in either the guinea-pig ileum or vas deferens preparations (agonist concentra-

tion-ratios were 0.9 ± 0.1 and 1.1 ± 0.3 respectively, $n = 4$ and 3, respectively).

Influence of changes in external calcium concentration

When the external Ca^{2+} concentration was decreased from 1.3 mM to 0.65 mM, there was a decrease in the magnitude of the response to electrical stimulation which was most marked in the vas deferens (Table 3). Spontaneously beating atria arrested when the calcium concentration was reduced to 0.65 mM and further studies could not therefore be performed at this low Ca^{2+} concentration. Increasing the Ca^{2+} concentration increased the magnitude of contraction in all preparations though this was maximal at 2.6 mM Ca^{2+} in both the ileum and the vas deferens.

The inhibitory effect of SS_{14} in both the guinea-pig ileum and vas deferens was very dependent on the concentration of Ca^{2+} in the bathing medium (Figure 5). In the vas deferens, decreasing the concentration of Ca^{2+} caused a leftward displacement of the SS_{14} concentration-effect curve, whilst in the guinea-pig ileum and to a lesser extent in the right atrium the maximum inhibitory effect of SS_{14} was increased with decreasing Ca^{2+} concentrations.

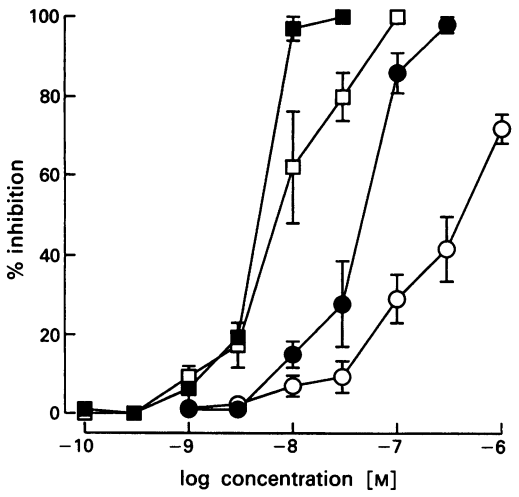

Figure 4 Concentration-effect curves to somatostatin$_{14}$ (SS_{14}) (○) and SS_{28} (□) in guinea-pig isolated vas deferens in the absence and presence (●, ■) of phosphoramidon (1 μM) and amastatin (10 μM). Values are mean ± s.e.mean from 4 experiments.

Table 3 Effect of changing the external Ca^{2+} concentration on neurogenic contractions in guinea-pig isolated ileum and vas deferens and the force of developed tension in spontaneously beating guinea-pig isolated right atrium.

	External calcium concentration			
	0.65 mM	1.3 mM	2.6 mM	5.2 mM
G-pig ileum	0.39 ± 0.08 g	0.73 ± 0.29 g	0.93 ± 0.16 g	0.88 ± 0.23 g
G-pig vas deferens	0.09 ± 0.03 g	0.75 ± 0.10 g	1.49 ± 0.17 g	1.13 ± 0.16 g
G-pig spontaneously beating right atrium	0	0.18 ± 0.04 g	0.36 ± 0.11 g	0.62 ± 0.05 g

Values are mean ± s.e.mean from 4 experiments.

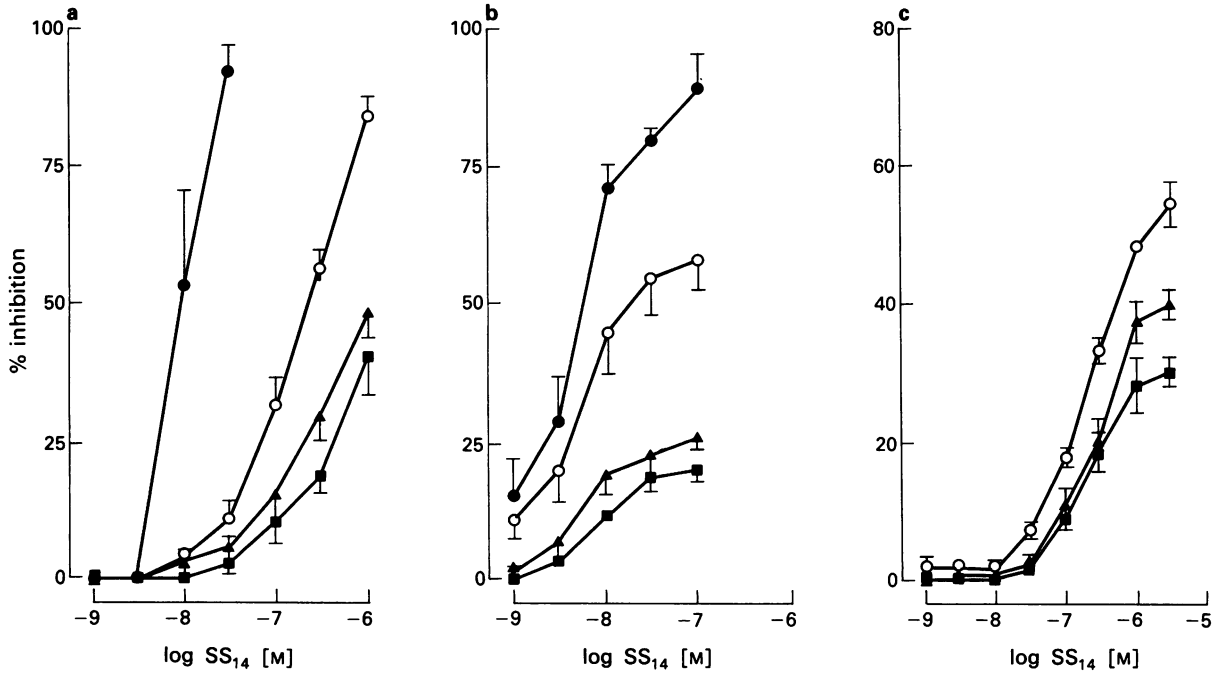

Figure 5 Inhibitory effects of somatostatin$_{14}$ in (a) guinea-pig vas deferens, (b) guinea-pig ileum and (c) guinea-pig spontaneously beating atria in Krebs solution containing (●) 0.65 mM Ca^{2+}; (○) 1.3 mM Ca^{2+}; (▲) 2.6 mM Ca^{2+} and (■) 5.2 mM Ca^{2+}. Values are mean ± s.e.mean from 4 experiments.

Discussion

The major objective of the present study was to characterize the somatostatin receptor types mediating inhibition of neurogenic contractions in guinea-pig isolated ileum and vas deferens as well as the negative inotropic action in guinea-pig isolated right atrium.

Early evidence for the potential existence of subtypes of somatostatin receptors was based largely on the relative potencies of SS_{14} and SS_{28} (Meyers *et al.*, 1980; Brazeau *et al.*, 1981; Hirst *et al.*, 1982) but the potential importance of metabolism was largely ignored (Gu *et al.*, 1992). The relative binding affinities of metabolically more stable analogues such as octreotide (SMS 201-995) and seglitide (MK 678) (Tran *et al.*, 1985; Raynor & Reisine, 1989; 1992) has been used as the basis for the subclassification of somatostatin receptors into $SRIF_1$ and $SRIF_2$. However, the recent cloning of five different somatostatin receptor isoforms and their subsequent expression in mammalian cells has revealed distinct structure activity patterns (Bell & Reisine, 1993; Bruno *et al.*, 1992) allowing functional responses in central and peripheral tissues to be characterized and compared with the operational characteristics of the cloned receptors.

In this study, a comparison of the agonist potencies in the isolated ileum, vas deferens and atrium revealed two distinct profiles of action. In the ileum, there was less than a three fold difference in potency between all of the agonists examined. In contrast, SS_{28} was much more potent than SS_{14} in both the vas deferens and atrium. Although the absolute potency of SS_{14} was lower in both the atrium and vas deferens compared with ileum, this difference cannot be attributed to preferential degradation of SS_{14} since the potency of SS_{14} or SS_{28} was not altered by phosphoramidon and amastatin in either the ileum or atrial preparations. Phosphoramidon and amastatin selectively enhanced the potency of SS_{14} approximately five fold in the vas deferens but even in the presence of these peptidase inhibitors, SS_{28} was still about ten times more potent than SS_{14} (see Figure 4). These data on relative agonist potencies suggest that the somatostatin receptor mediating negative inotropy in the guinea-pig isolated atrium and inhibiting neurogenic contractions in the vas deferens are similar but different from the somatostatin receptor inhibiting neurogenic contractions in the ileum.

An interesting finding with the synthetic peptide agonists was that octreotide and angiopeptin, which were quite potent agonists in the ileum and vas deferens, had low intrinsic activity in the guinea-pig atrium. Indeed, when the calcium concentration was increased and the intrinsic activity of angiopeptin reduced further, angiopeptin behaved as a specific and competitive somatostatin receptor blocking drug. Angiopeptin produced a concentration-dependent antagonism of the negative inotropic action of both SS_{14} and SS_{28}. The pK_B values obtained when either SS_{14} or SS_{28} was used as the agonist were similar (7.4 and 7.2, respectively) suggesting that both SS_{14} and SS_{28} mediate their effect via a common receptor. The antagonism was specific since the negative inotropic action of either carbachol or cyclohexyladenosine remained unchanged in the presence of angiopeptin. To our knowledge this is the first report of somatostatin receptor blockade by angiopeptin and also partial agonist activity of octreotide. It would seem that this profile of action in the isolated atrium is a reflection of a relatively low somatostatin receptor density and/or poor receptor-effector coupling efficiency. We have recently shown (Dimech *et al.*, 1993) that the potent cyclic hexapeptide somatostatin agonist, seglitide, also acts as a somatostatin receptor antagonist in guinea-pig atria but is approximately 20 times weaker than angiopeptin in this respect. It remains to be seen whether these smaller peptide analogues of somatostatin behave as antagonists, rather than agonists at other somatostatin receptor sites. The much higher intrinsic activity of angiopeptin in the guinea-pig vas deferens precluded attempts to use the drug as an antagonist, but interestingly the EC_{50} values for angiopeptin in both atrium and vas deferens were similar, data consistent with the view that the somatostatin receptors in both preparations are similar.

The putative somatostatin receptor blocking drug, CPP, increases growth hormone, insulin and glucagon release in the rat (Fries *et al.*, 1982), inhibits the negative inotropic actin of SS_{14} in guinea-pig isolated papillary muscles (Endou *et al.*, 1989) and inhibits the ability of SS_{14} to enhance acetylcholine release from rat hippocampal slices (Araujo *et al.*, 1990). However, in the present study CPP had no effect on the inhibitory action of SS_{14} or SS_{28} in either guinea-pig isolated ileum or vas deferens and did not antagonize the negative inotropic action of SS_{14} in the right atrium. This difference in the antagonist profile of CPP may be a reflection of somatostatin receptor heterogeneity (see below).

Somatostatin has been shown to interact with opiate receptors (Terenius, 1976). However, such an interaction seems unlikely in the present study since naloxone had no effect on the inhibitory action of SS_{14} in either the guinea-pig ileum or vas deferens.

As already mentioned, at least five different somatostatin receptor isoforms have recently been cloned and expressed in mammalian cells (Rens-Domiano *et al.*, 1992; Yasuda *et al.*, 1992; O'Carroll *et al.*, 1992; Bruno *et al.*, 1992). Although the binding potencies of only a few somatostatin analogues have been studied on four of these receptors transfected into different cells (Bell & Reisine, 1993; Table 4), they do allow a comparison of relative potencies to be made with the data obtained in the functional studies described here. The inhibitory effect of SS_{14} in the guinea-pig ileum, vas deferens and the negative inotropic action in the atrium would appear not to be mediated by the activation of either $SSTR_1$ or $SSTR_3$ receptors for two reasons. First, octreotide has extremely low affinity in displacing [^{125}I]-Tyr^{11}-SS_{14} binding from $SSTR_1$ and $SSTR_3$ receptors but was a potent agonist

Table 4 A comparison of the relative potencies of some somatostatin analogues in functional studies with their relative affinities in binding studies using cloned somatostatin receptor isoforms

	Functional studies[1]			*Ligand binding studies*[2]			
	G-pig ileum	*G-pig vas deferens*	*G-pig atrium*	*$SSTR_1$*	*$SSTR_2$*	*$SSTR_3$*	*$SSTR_4$*
SS_{14}	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SS_{28}	2.2	0.03	0.06	0.2	3.4	0.8	0.04
Octreotide	1.0	0.27	1.2	>500	6.3	192	0.08
CGP 23996	1.7	6.7	2.0	50	12.5	0.77	1.8
CPP	Inactive at 1 μM	Inactive at 1 μM	Inactive at 10 μM	100% inhibition at 1 μM	Inactive at 1 μM	100% inhibition at 1 μM	Not tested

[1]Data are relative EC_{50} values (EMRs) as agonists from this study, except for CPP which was tested as an antagonist.
[2]Data are relative IC_{50} values from Bell & Reisine (1993); absolute IC_{50} values for SS_{14} were 2.1, 0.08, 0.78 and 2.6 nM for $SSTR_1$–$SSTR_4$ respectively).
Values of less than one unit indicate a higher relative potency or affinity.

relative to SS_{14} in both the ileum and the vas deferens. It also showed similar potency to SS_{14} in the atrium although it had lower intrinsic activity than SS_{14} and SS_{28}. Secondly, the putative somatostatin antagonist CPP which abolishes [^{125}I]-Tyr^{11}-SS_{14} binding to $SSTR_1$ and $SSTR_3$ receptors at a concentration of 1 μM (Rens-Domiano *et al.*, 1992; Yasuda *et al.*, 1992) had no effect on the inhibitory action of SS_{14} in the preparations we have studied. An additional factor excluding an action at $SSTR_1$ receptors was the fact that CGP 23996 which was of similar potency to SS_{14} in the ileum and only slightly weaker than SS_{14} in the vas deferens and atria shows low affinity in displacing [^{125}I]-Tyr^{11}-SS_{14} binding at $SSTR_1$ receptors (Table 4).

As in the functional experiment described here, $SSTR_2$ and $SSTR_4$ receptors can be differentiated by the relative affinities of the two naturally occurring forms of somatostatin, SS_{14} and SS_{28}. Radioligand binding studies on $SSTR_2$ receptors have shown that SS_{14} has only approximately three times higher affinity than SS_{28} (Rens-Domiano *et al.*, 1992) whilst at $SSTR_4$ receptors, SS_{28} has about 25 times higher affinity than SS_{14} in displacing [^{125}I]-Tyr^{11} SS_{14} binding (O'Carroll *et al.*, 1992; Table 4). This is consistent with the possibility that $SSTR_2$ receptors mediate an inhibition of neurogenic contractions in the guinea-pig ileum whilst $SSTR_4$ receptors similar to those described by O'Carroll *et al.* (1992) mediate inhibition of neurogenic contractions in the guinea-pig vas deferens as well as mediating the negative inotropic action of SS_{14} in guinea-pig right atrium. This conclusion is largely based on the relative potencies of the agonists which have been studied in the functional experiments presented here and the relative binding affinities at the cloned somatostatin receptors following their expression in different cell lines (Table 4). Not only was SS_{28} the most potent of the agonists examined in the guinea-pig isolated atrium and vas deferens but it also showed the highest intrinsic activity in the guinea-pig atrium. Indeed SS_{14}, octreotide and angiopeptin all produced significantly lower maxima and should be considered to be partial agonists in the atrium. Since the maximum response produced by partial agonists demands full receptor occupancy, then in the atrium the EC_{50} value of the peptides with low intrinsic activity should provide an approximation of their dissociation constants for the somatostatin receptor mediating negative inotropy in the atrium. Consistent with this was the finding that the pEC_{50} value of 7.73 for angiopeptin in the atrium was close to its estimated pK_B value of 7.44 when used as an antagonist against SS_{14}.

Although the high potency of SS_{28} compared with SS_{14} in both atria and vas deferens provides some evidence that the somatostatin receptor in these preparations more closely resembles the $SSTR_4$ receptor cloned by O'Carroll *et al.* (1992) than the other cloned receptors, there is an important discrepancy. The IC_{50} values of the somatostatin agonists, determined in radioligand binding studies with radiolabelled peptide agonists on transfected cloned $SSTR_4$ receptors (Bell & Reisine, 1993) are at least 10 times lower than their potency (EC_{50} values) in the functional studies described here. The extent to which the estimates of agonist affinities on the cloned receptors provide a 'fingerprint' for $SSTR_4$ receptors occurring naturally in cells still remains to be determined. Certainly the different cloned muscarinic receptor isoforms can display marked differences in agonist and antagonist affinities, even when the same receptor is expressed in different cell lines (Richards, 1991). Further studies determining the affinities of different somatostatin analogues, on receptors expressed in a variety of cell lines are clearly needed.

Recently, a fifth somatostatin receptor has been cloned (Bruno *et al.*, 1992) which like the receptor cloned by O'Carroll and colleagues (1992) was termed $SSTR_4$ by the authors. The amino acid sequence of this receptor is distinct from that of other cloned somatostatin receptor isoforms although the affinity of SS_{14} and SS_{28} for this receptor was similar. Since octreotide in concentrations up to 1 μM did not displace SS_{14} binding to this fifth receptor (Bruno *et al.*, 1992), it is unlikely that this receptor is similar to those identified in the functional studies described here.

The precise site and mechanism of the inhibitory action of SS_{14} in both the ileum and vas deferens would appear to be pre-junctional, involving inhibition of neurotransmitter release (see Teitelbaum *et al.*, 1984), since SS_{14} did not modify the contractile response to carbachol in the ileum or α,β-methylene ATP in the vas deferens. To determine whether this action of somatostatin in the preparations used was directly on the cholinergic/purinergic nerve terminals or indirectly mediated via the release of an inhibitory neurotransmitter such as γ-aminobutyric acid (Takeda *et al.*, 1989) requires further study. Nevertheless, this inhibitory effect of SS_{14} in ileum and vas deferens and the negative inotropic effect in the atrium was inversely related to the external Ca^{2+} concentration, consistent with the view that the mechanism of inhibition of the release of neurotransmitter involves the limitation of the availability of Ca^{2+} at the nerve terminal (Priestley & Woodruff, 1988) and that decreases in atrial contractility also involved the limitation of Ca^{2+} entry (Quirion *et al.*, 1979; Ohmura *et al.*, 1990).

In conclusion, the somatostatin receptor mediating the inhibitory effect of SS_{14} in the guinea-pig ileum and vas deferens can be clearly differentiated by the relative potencies of SS_{14} and SS_{28}. In the former, SS_{14} and SS_{28} have similar potency whilst in the latter SS_{28} is much more potent. In this respect the somatostatin receptor mediating negative inotropy in the guinea-pig isolated right atrium is similar to the somatostatin receptor in the vas deferens. The somatostatin receptor in the atrium and vas deferens would appear to be similar to the recently cloned $SSTR_4$ receptor (O'Carroll *et al.*, 1992) whilst the somatostatin receptor inhibiting neurotransmission in the ileum seems similar to the recently cloned $SSTR_2$ receptor (Bell & Reisine, 1993). To explore these suggestions further, potent and selective antagonists are needed for the various somatostatin receptor types for use in both functional studies and to provide improved radioligands for binding studies.

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Effects of the novel potassium channel opener, UR-8225, on contractile responses in rat isolated smooth muscle

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1 The effects of UR-8225 [(1,2-dihydro-4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-1-oxonaphthalen-6-carbonitrile)] and levcromakalim were studied on the electrical and contractile responses induced by noradrenaline and KCl and on $^{86}Rb^+$ efflux in rat aortic rings and on spontaneous mechanical activity in rat portal vein segments.

2 UR-8225 and levcromakalim, 10^{-9} M–10^{-5} M, relaxed the contractile responses induced by noradrenaline ($IC_{50} = 2.7 \pm 0.4 \times 10^{-6}$ M and $6.6 \pm 1.3 \times 10^{-7}$ M, respectively) or 30 mM KCl ($IC_{50} = 1.4 \pm 0.2 \times 10^{-7}$ M and $9.4 \pm 1.3 \times 10^{-8}$ M, respectively) more effectively than those induced by 80 mM KCl. The relaxant effect on noradrenaline-induced contractions was independent of the presence or absence of functional endothelium.

3 The vasorelaxant effect of UR-8225 and levcromakalim can be competitively antagonized by glibenclamide, an ATP-sensitive K^+ channel blocker. There were no differences in the calculated pA_2 values for glibenclamide to inhibit UR-8225- and levcromakalim-induced relaxations (7.61 ± 0.08 and 7.69 ± 0.10, respectively). The slope of the Schild plot yielded values not significantly different from unity (0.95 ± 0.06 and 0.96 ± 0.05, respectively).

4 UR-8225 (10^{-5} M) hyperpolarized the resting aortic membrane potential from -50.7 ± 0.7 mV to -66.0 ± 2.0 mV and stimulated $^{86}Rb^+$ efflux.

5 UR-8225 and levcromakalim inhibited the contractions induced by Ca^{2+} in aortae incubated in Ca^{2+}-free PSS containing methoxyverapamil in the presence of noradrenaline.

6 Both drugs inhibited the amplitude of spontaneous activity in portal veins ($IC_{50} = 5.1 \pm 1.4 \times 10^{-8}$ M and $1.5 \pm 0.7 \times 10^{-8}$ M, respectively), this effect being competitively antagonized by glibenclamide.

7 These results indicated that UR-8225 exhibited qualitatively similar, but slightly less potent, vasorelaxant effects than those exerted by levcromakalim, which suggests that they can be related to its ability to activate ATP-sensitive K^+ channels in vascular smooth muscle cells.

Keywords: UR-8225; levcromakalim; rat aorta; portal vein; potassium channels; vascular smooth muscle

Introduction

Potassium channel openers constitute a class of vasodilator drugs with a novel mechanism of action. The vasorelaxant properties of this class of drugs have been initially attributed to the activation of ATP-sensitive potassium channels and the subsequent hyperpolarization of the smooth muscle membrane which prevents the opening of voltage-activated Ca^{2+} channels (Quast & Cook, 1989; Hamilton & Weston, 1989; Edwards & Weston, 1990). As potent peripheral vasodilators these drugs are expected to be useful in the treatment of several cardiovascular disorders, such as hypertension, angina pectoris, peripheral arterial diseases, cerebral ischaemia and congestive heart failure (Cook, 1988; Hamilton & Weston, 1989; Weston, 1989; Escande & Cavero, 1992; Sanguinetti, 1992).

UR-8225 is a new compound [(1,2-dihydro-4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-1-oxonaphthalen-6-carbonitrile)] that stems from a structure-activity study carried out at the Uriach Research Center (Almansa *et al.*, 1992). The key feature of the molecule is a naphthalenone ring replacing the conventional benzopyrane nucleus present in levcromakalim (formerly BRL 38227) and other related compounds (Figure 1). In preliminary experiments, it has been found that UR-8225 exhibits potent vasodilator properties possibly related to its potassium channel opener properties (García-Rafanell *et al.*, 1992). Therefore, the purpose of the present paper was: (1) to analyze the vasorelaxant effects of UR-8225 in rat isolated vascular smooth muscle, and (2) to compare its effects with those of levcromakalim. A preliminary report of some of the results of this study has already been published (Casis *et al.*, 1993).

Methods

Experimental procedure

Sprague-Dawley rats (either sex, 250–300 g) were killed by a blow on the head. The descending thoracic aorta and portal veins were rapidly dissected and placed in a physiological saline solution (PSS) of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.2 and glucose 11. After excess of fat and connective tissue were removed, the aortae were cut into rings (4–5 mm in length). Aortic rings were mounted under the tension of 1 g by two parallel L-shaped stainless-steel holders inserted into the lumen and longitudinal portal vein segments (15 mm in length) were mounted vertically under the basal tension of 1 g in 20 ml organ baths containing PSS and attached to a force-displacement transducer (Grass FT07) to measure isometric contractile force as previously described (Pérez-Vizcaíno *et al.*, 1991; 1993). The tissue bath was maintained at 37°C and bubbled with 95% O_2:5% CO_2 gas mixture. For the experiments in which Ca^{2+}-free medium was used, Ca^{2+} was omitted from normal PSS and 0.03 mM EDTA was added. For most of the experiments care was taken not to damage the endothelium. In some experiments, endothelial cells were gently removed by rubbing the internal surface of the vessels with a small metal rod. The absence of functional endothelium was confirmed by the inability of the

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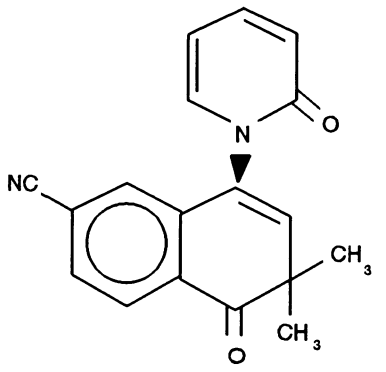
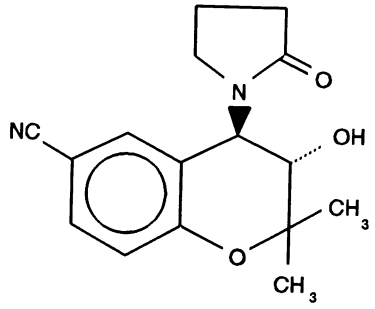


UR-8225



Levcromakalim

Figure 1 Chemical structure of UR-8225 and levcromakalim.

preparation precontracted with 10^{-5} M noradrenaline to relax in response to 10^{-6} M acetylcholine. Each preparation was allowed to equilibrate for at least 90 min, prior to initiation of experimental procedures, and during this period the incubation media were changed every 30 min before addition of drugs.

After equilibration the following experiments were performed: (a) in some experiments, aortic rings were exposed to single submaximal concentrations of KCl (20 mM). Control contractile responses were obtained at the beginning of the experiment every 30 min until two successive responses were almost identical in height. This was followed by exposure to UR-8225 or levcromakalim for 30 min before the addition of KCl. The results of these experiments are expressed as a percentage of the maximal control agonist-induced contractile responses. (b) Aortic rings were contracted by addition of noradrenaline (10^{-5} M) or KCl (30 or 80 mM). When the contractile tonic response to either agonist was stable, cumulative inhibitory concentration-response curves were obtained for UR-8225 or levcromakalim. The relaxant effect of each concentration was allowed to reach a stable level before the next addition was made. The ability of glibenclamide, a blocker of ATP-sensitive K^+ channels (Ashcroft, 1988), to antagonize the relaxant responses of UR-8225 was tested on the contractions induced by 30 mM KCl. Glibenclamide was added to the bath after the high KCl contraction had developed and 20 min before the addition of UR-8225 or levcromakalim. In another group of experiments, muscles were exposed to 80 mM KCl and when the contractile response reached the steady-state, 10^{-5} M noradrenaline was added to the bathing media. These results were expressed as a percentage of the maximal control agonist-induced responses. (c) In additional experiments, after equilibration, cumulative concentration-response curves were obtained for KCl (15–85 mM). Once the contractile response curve for a given agonist became stable, preparations were exposed to different concentrations of UR-8225 or levcromakalim for 30 min and a new concentration-response curve was obtained. (d) The effects of UR-8225 and levcromakalim on noradrenaline-stimulated Ca^{2+} entry were studied according to the following experimental protocol. Aortic rings were initially contracted with 10^{-5} M noradrenaline. After washing, rings were incubated in Ca^{2+}-free PSS containing 10^{-5} M methoxyverapamil and 0.03 mM EDTA for 10 min. At this time, the addition of 10^{-5} M noradrenaline induced a transient contraction. After 30 min, when the basal tension was reached, the concentration of Ca^{2+} in the bathing media was increased to 2 mM Ca^{2+} and a tonic contraction was recorded. In experimental muscles, UR-8225 or levcromakalim were added 30 min before the addition of Ca^{2+}. Results are expressed as a percentage of the initial noradrenaline-induced contraction. (e) To study the effects of UR-8225 and levcromakalim on the spontaneous portal vein contractions, cumulative concentration-response curves were obtained in the absence or in the presence of glibenclamide.

Appropriate parallel control experiments were always carried out in order to correct for the possible effects caused by vehicle alone.

Measurement of membrane potential

Cleaned, endothelium-free, aortic segments were pinned down in a Lucite chamber with the lumen side up. The muscle was continuously superfused with oxygenated PSS maintained at 34°C. Membrane potentials were recorded conventionally through glass microelectrodes filled with 3 M KCl (tip resistance 30–50 MΩ) as previously described (Deplón *et al.*, 1992). The microelectrode was connected via Ag–AgCl wire to high-input impedance capacity neutralizing amplifiers (WPI model 701, World Precision Instruments Inc., New Haven, CT, U.S.A.). Membrane potential was displayed on a storage oscilloscope (Tektronix 5104N, Tektronix Inc., Beaverton, OR, U.S.A.) and photographed with a Kymographic Grass camera (Model C-4, Grass Instrument Company, Quincy, MA, U.S.A.).

$^{86}Rb^+$ efflux

The effects of UR-8225 on $^{86}Rb^+$ efflux were determined as described by Tulenko & Cox (1991). Aortic rings were equilibrated for 10 min in a PSS of the following composition (mM): NaCl 140, KCl 4.75, $CaCl_2$ 1.5, $MgSO_4$ 1.0, glucose 11 and HEPES 10 at pH 7.4 bubbled with 100% O_2 at 37°C. Then rings were loaded for 3 h in PSS containing $^{86}Rb^+$ (5 μCi ml^{-1}). Afterwards the muscles were dipped quickly into PSS to remove excess radioactivity and then transferred through a series of vials (every 10 min for the first 30 min and every 3 min thereafter) each containing 0.8 ml of PSS for the first 51 min and PSS containing UR-8225 (10^{-6} M or 10^{-5} M) thereafter. At the end of the experiment, the radioactivity remaining in the aorta was determined by dissolving the vessel in 200 μl of a solution containing equal parts perchloric acid (37% w/v) and H_2O_2 (30 volumes) heated for 15 min at 75°C. After cooling, 5 ml of Aquasol-2 (Dupont, Boston, MA, U.S.A.) was added. The $^{86}Rb^+$ activity in the vials and that extracted from the tissues were measured by Cerenkov counting. The results were expressed in terms of efflux rate constants which reflect the permeability of the cell membrane to Rb^+. Rate constants (k) during each time interval were calculated using the following equation: $k = \ln(A1/A2)/(t2-t1)$, where A1 and A2 represent the total tissue counts at time points t1 and t2, respectively.

Drugs

The following drugs were used: UR-8225 (Laboratorios, Uriach, Barcelona), levcromakalim (SmithKline Beecham Pharmaceuticals Betchworth, U.K.), (−)-noradrenaline bitartrate and glibenclamide (Sigma Ltd. Co., London), methoxyverapamil (D600, Knoll AG, Ludwigshafen/Rhein, Germany). Glibenclamide was diluted in dimethyl sulphoxide to make a stock solution of 10^{-2} M. All other drugs were dissolved in distilled deionized water to prepare a 10^{-3} M stock solution and further dilutions were made in PSS. The final concentra-

tion of solvent had no measurable effect on contractile responses or $^{86}Rb^+$ efflux. Ascorbic acid (10^{-5} M) was added to each stock solution of noradrenaline, made up freshly each day.

Statistics

Throughout the paper values are expressed as mean ± s.e.mean and statistical analysis was performed with Student's *t* test. The differences between control and experimental values were considered significant when $P<0.05$. Dose-response slopes were analyzed to give the concentration of UR-8225 or levcromakalim producing a 50% inhibition of the maximal contractile response (IC_{50}) using a linear regression analysis over the response range of 20 to 80% of the maximal inhibition. pA_2-values were calculated by Schild-plot analysis (Arunlakshana & Schild, 1959).

Results

Effects on spontaneous and noradrenaline-induced contractions in the portal vein

In 16 portal vein segments the control amplitude of spontaneous contractions was 784.3 ± 133.4 mg. Figure 2 shows that UR-8225 and levcromakalim (10^{-9} M–10^{-5} M) inhibited the amplitude of these contractions in a concentration-dependent manner and at 2×10^{-7} M and 10^{-7} M, respectively, they suppressed the spontaneous activity. In 5 muscles, the IC_{50} values for UR-8225 and levcromakalim to inhibit the myogenic activity were $5.1 \pm 1.4 \times 10^{-8}$ M ($n = 5$) and $1.5 \pm 0.7 \times 10^{-8}$ M ($n = 5$), respectively. The ability of glibenclamide to reverse the inhibitory effects of UR-8225 and levcromakalim on the amplitude of spontaneous contractions was studied in 6 portal veins. In the presence of glibenclamide (3×10^{-7} M, 10^{-6} M and 3×10^{-6} M) there was a rightward shift of the curve for UR-8225 and levcromakalim (Figure 2). Thus, in the presence of 3×10^{-6} M glibenclamide the IC_{50} values for UR-8225 and levcromakalim were $1.5 \pm 0.5 \times 10^{-6}$ M and $3.2 \pm 0.6 \times 10^{-7}$ M, respectively. There were no differences in the calculated pA_2 values for glibenclamide to inhibit UR-8225- and levcromakalim-induced inhibitions (6.76 ± 0.05 and 6.80 ± 0.18, respectively). The slope of the Schild plot yielded values not significantly different from unity (1.14 ± 0.07 and 1.07 ± 0.22, respectively) which indicates that the inhibition was competitive. Addition of 10^{-5} M noradrenaline to portal vein segments induced a tonic contraction averaging 1312 ± 193 mg ($n = 6$). Cumulative addition of UR-8225 (10^{-7} M–10^{-5} M) induced a concentration-dependent inhibition of these contractions, the IC_{50} value being $6.9 \pm 3.3 \times 10^{-6}$ M.

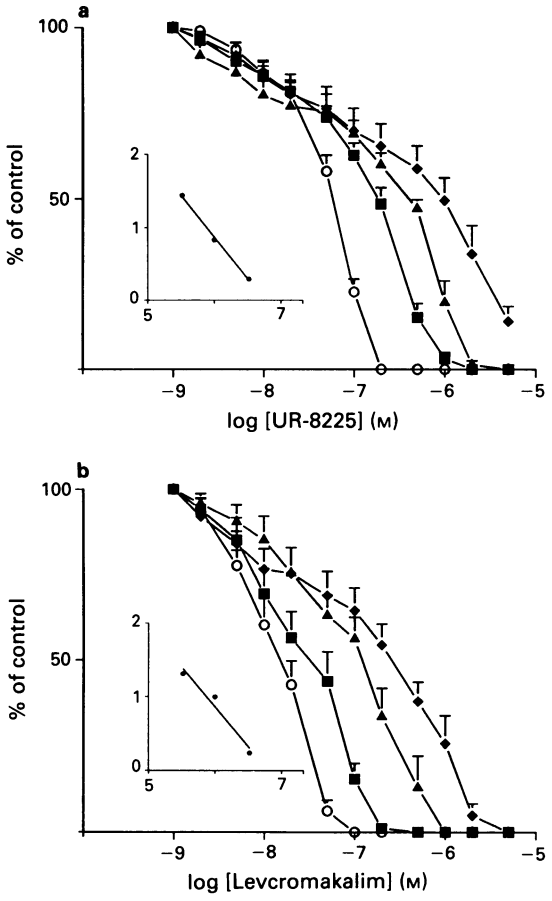


Figure 2 Effects of UR-8225 (a) and levcromakalim (b) added in a cumulative fashion on the amplitude of spontaneous contractions in rat portal vein segments. Results were obtained in the absence (○) and in the presence of glibenclamide 3×10^{-7} M (■), 10^{-6} M (▲) and 3×10^{-6} M (◆). Ordinate scale: percentage of control values. Abscissa scale: log UR-8225 or levcromakalim concentration (M). Each point represents the mean ± s.e.mean of 6 experiments. Insets: Schild-plot analysis. Ordinate scale: log(dose ratio − 1); abscissa scale: negative logarithm of glibenclamide concentration (M).

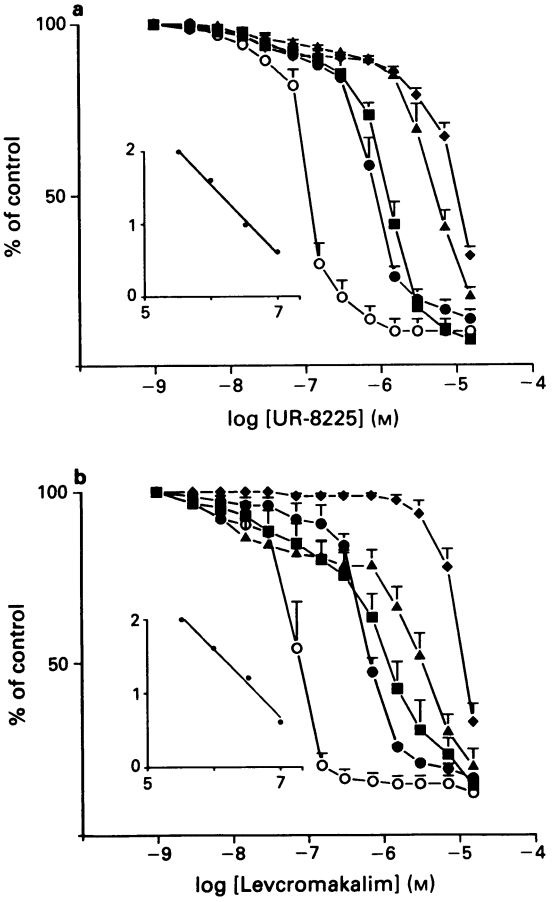


Figure 3 Effects of UR-8225 (a) and levcromakalim (b) added in a cumulative fashion on the 30 mM KCl-induced contractions in rat aortic rings. Results were obtained in the absence (○) and in the presence of glibenclamide 10^{-7} M (●), 3×10^{-7} M (■), 10^{-6} M (▲) and 3×10^{-6} M (◆). Ordinate scale: percentage of control values. Abscissa scale: log UR-8225 or levcromakalim concentration (M). Each point represents the mean ± s.e.mean of 6 experiments. Insets: Schild-plot analysis. Ordinate scale: log(dose ratio − 1); abscissa scale: negative logarithm of glibenclamide concentration.

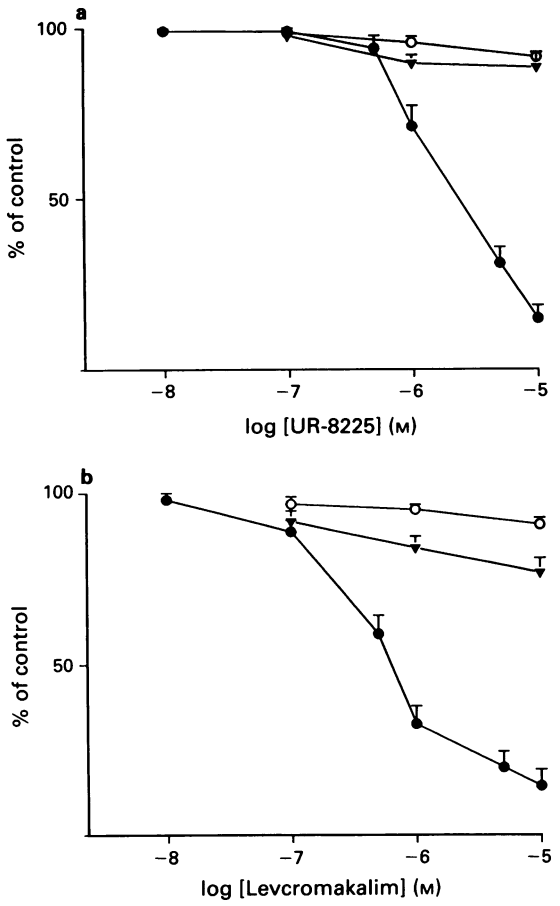


Figure 4 Effects of UR-82225 (a) and levcromakalim (b) added in a cumulative fashion on the contractions induced by 80 mM KCl (▼), 10^{-5} M noradrenaline (●) or 80 mM KCl plus 10^{-5} M noradrenaline (○) in rat aortic rings. Ordinate scale: percentage of control values. Abscissa scale: log UR-8225 or levcromakalim concentration (M). Each point represents the mean ± s.e.mean of 6–8 experiments.

Relaxant effects on KCl- and noradrenaline-induced contractions

At concentrations up to 10^{-5} M, UR-8225 or levcromakalim had no effect on baseline tension in aortic rings. In 14 aortae the contractile response produced by 20 mM KCl averaged 862.1 ± 107.9 mg. UR-8225 and levcromakalim, 10^{-9} M–10^{-5} M, produced a concentration-dependent inhibition of this contractile response, the IC_{50} values being $9.2 \pm 6.1 \times 10^{-8}$ M ($n = 8$) and $3.2 \pm 0.3 \times 10^{-8}$ M ($n = 6$).

As shown in Figure 3, UR-8225 and levcromakalim also relaxed the contractions previously induced by 30 mM KCl, the IC_{50} values being $1.4 \pm 0.2 \times 10^{-7}$ M ($n = 6$) and $9.4 \pm 1.3 \times 10^{-8}$ M ($n = 6$). The figure also shows that glibenclamide (10^{-7} M–3×10^{-6} M) shifted to the right these concentration-relaxation curves for UR-8225 and levcromakalim against these contractile responses. Thus, in the presence of 3×10^{-6} M glibenclamide, the IC_{50} values for UR-8225 and levcromakalim were $1.4 \pm 0.4 \times 10^{-5}$ M and $1.1 \pm 0.1 \times 10^{-5}$ M, respectively. There were no differences in the calculated pA_2 values of glibenclamide to inhibit UR-8225- and levcromakalim-induced relaxations (7.61 ± 0.08 and 7.69 ± 0.10, respectively). The slope of the Schild plot yielded values not significantly different from unity (0.95 ± 0.06 and 0.96 ± 0.05, respectively) which indicates that the inhibition was competitive.

Addition of KCl (80 mM), noradrenaline (10^{-5} M) or both, to aortic rings produced a contractile response which averaged 1756 ± 254 mg ($n = 15$), 2183 ± 530 mg ($n = 15$) and 2831 ± 342 ($n = 10$), respectively. Figure 4 shows the relaxant effects of UR-8225 and levcromakalim (10^{-8} M–10^{-5} M) when added cumulatively to aortic rings previously contracted with these agonists. UR-8225 and levcromakalim inhibited in a concentration-dependent manner the contractile responses induced by 10^{-5} M noradrenaline in endothelium intact rings, the IC_{50} being $2.7 \pm 0.4 \times 10^{-6}$ M ($n = 7$) and $6.6 \pm 1.3 \times 10^{-7}$ M ($n = 8$), respectively. In endothelium-denuded rings, UR-8225 also relaxed noradrenaline-induced contraction, the IC_{50} being $1.9 \pm 1.1 \times 10^{-6}$ M ($n = 4$, not significantly different compared to endothelium-intact rings). Pretreatment with glibenclamide (10^{-7} M–3×10^{-6} M) also shifted the concentration-responses to the right (not shown). In contrast, at 10^{-5} M, both drugs inhibited the 80 mM KCl-induced contractions by only 11.7 ± 4.5% ($P > 0.05$, $n = 6$) and 20.5 ± 4.3% ($P < 0.05$, $n = 6$), respectively. In another group of experiments, the muscles were firstly exposed to 80 mM KCl and when the contractile response reached a steady-state, 10^{-5} M noradrenaline was added to the bathing media. Figure 4 shows that under these conditions, UR-8225 or levcromakalim, 10^{-7} M–10^{-5} M, only slightly inhibited these contractions: thus, at 10^{-5} M these responses were inhibited by 8.7 ± 1.3% ($n = 6$) and 9.4 ± 2.0% ($n = 6$), respectively. These results indicated that both agents were not only almost ineffective against 80 mM KCl-induced contractions but also that a strong depolarization inhibited the effects of both drugs on noradrenaline-induced contractions.

Effects on concentration-response curves to KCl

Potassium channel openers relax contractions induced by 20 mM KCl but are ineffective against those induced by

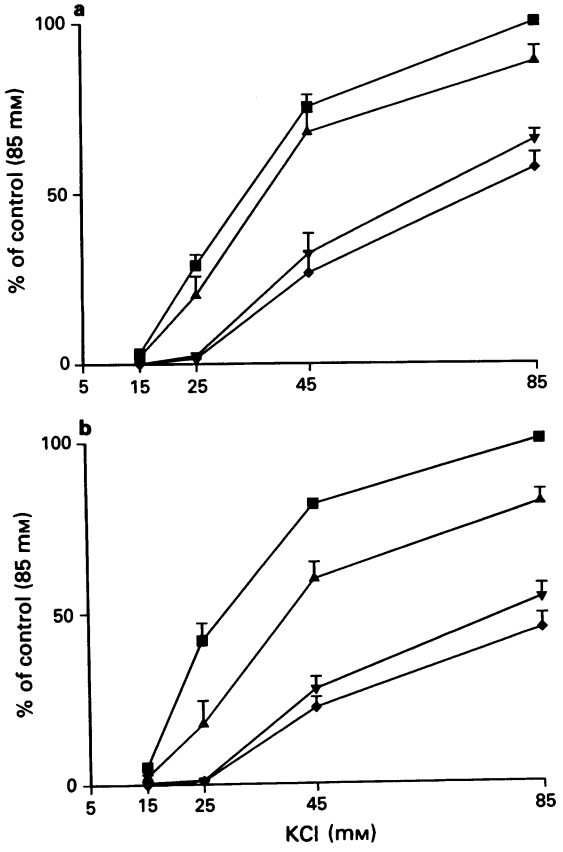


Figure 5 Effects of UR-8225 (a) and levcromakalim (b) on the contractions of aortic rings by addition of KCl (15–85 mM). Ordinate scale: percentage of the maximum control contraction obtained with 85 mM KCl in each experiment. Abscissa scale: KCl concentration (mM). Each point represents the mean ± s.e.mean of 6 experiments. (■) Controls; after UR-8225 or levcromakalim, 10^{-7} M, (▲), 10^{-6} M (▼) and 10^{-5} M (◆).

80 mM KCl (Hamilton & Weston, 1989). Cumulative increases in KCl concentration (15–85 mM) to aortic rings in a Ca^{2+}-containing PSS induced a concentration-dependent increase in developed tension. Figure 5 shows that both UR-8225 and levcromakalim (10^{-7} M–10^{-5} M), produced a concentration-dependent inhibition of these contractile responses, but this inhibitory effect was more marked against the responses induced by low concentrations of KCl ($\leq$ 30 mM) which were almost abolished, than against the contractions induced by 45 or 85 mM KCl. Thus, the greater the KCl concentration the less the effect induced by UR-8225 and levcromakalim.

Effects on noradrenaline-induced contractions in Ca^{2+}-free solution

In another group of experiments, the effects of UR-8225 or levcromakalim were studied on the contractile responses induced by $CaCl_2$ (2 mM) in aortic rings incubated in Ca^{2+}-free PSS containing 0.03 mM EDTA and 10^{-5} M methoxyverapamil. Under these conditions addition of 10^{-5} M noradrenaline induced a phasic contraction resulting from the release of intracellular Ca^{2+}. After 30 min, 2 mM $CaCl_2$ was added to the bathing media resulting in a tonic contractile response which averaged 54.0 ± 6.1% of the initial noradrenaline-induced contraction in the absence of EDTA and methoxyverapamil. In some aortic rings run in parallel, UR-8225 or levcromakalim (10^{-7} M–10^{-5} M) was added 30 min before the addition of $CaCl_2$. As is shown in Table 1, both drugs inhibited these tonic contractile responses induced by Ca^{2+}, but levcromakalim was significantly more potent than UR-8225 ($P<0.05$).

Table 1 Effects of UR-8225 and levcromakalim on the contractions induced by addition of 2 mM $CaCl_2$ to a Ca^{2+}-free (0.03 mM EDTA) medium in the presence of 10^{-5} M noradrenaline and 10^{-5} M methoxyverapamil expressed as a percentage of the contraction of control rings

	10^{-7} M	10^{-6} M	10^{-5} M
UR-8225	83.1 ± 11.0	83.0 ± 15.1	61.1 ± 13.3*
Levcromakalim	92.5 ± 22.6	54.0 ± 14.0*	27.1 ± 9.1**

Each value is the mean ± s.e.mean of 7–8 rings.
*$P<0.05$, **$P<0.01$.

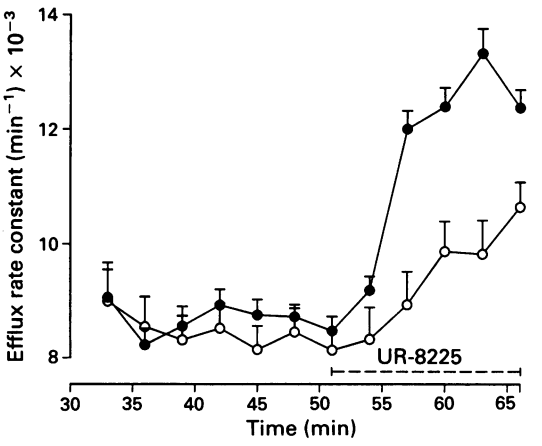


Figure 6 Effects of UR-8225 on the $^{86}Rb^+$ efflux in rat non-stimulated aortic rings. Ordinate scale: efflux rate constant ($min^{-1} \times 10^{-3}$ M). $^{86}Rb^+$ loaded rings were placed in PSS for the first 51 min and thereafter in PSS containing UR-8225, 10^{-6} M (○) or 10^{-5} M (●). Abscissa scale: time (min). Each point represents the mean ± s.e.mean of 6 experiments.

Effects of UR-8225 on membrane potential

The resting membrane potential of aortic smooth muscle cells averaged − 50.7 ± 0.7 mV ($n = 7$). Addition of 10^{-5} M UR-8225 hyperpolarized the cells by almost 16 mV (− 66.0 ± 2.0 mV, $n = 7$). Upon washing, the cells slowly repolarized to their normal resting potential.

Effects of UR-8225 on $^{86}Rb^+$ efflux

The magnitude of the hyperpolarization produced by UR-8225 strongly suggested that it could be due to an increase in K^+ conductance. To study this possibility $^{86}Rb^+$ was used as a substitute for $^{42}K^+$. UR-8225 produced a concentration-dependent increase in the rate constant of $^{86}Rb^+$ efflux from the rat aorta (Figure 6). The rate of onset of the effect was also concentration-dependent.

Discussion

In the present study we have compared in isolated vascular smooth muscle of the rat the effects of UR-8225, a novel vasodilator agent, to those of levcromakalim, a drug which relaxes vascular smooth muscle by opening K^+ channels (Weston, 1989; Weston *et al.*, 1990). The results indicated that the vasorelaxant effects of UR-8225 were qualitatively similar, but slightly less potent, than those exerted by levcromakalim. Thus, in rat isolated aortae UR-8225: (1) inhibits the contractile responses induced by noradrenaline or low KCl concentrations ($\leq$ 30 mM) more effectively than those induced by high (80 mM) KCl. These vasorelaxant effects do not appear to depend critically on the release of endothelial factors since the same inhibition was observed in the presence and absence of functional endothelium. (2) Decreases the contractile responses induced by Ca^{2+} in aortae incubated in Ca^{2+}-free PSS containing methoxyverapamil, a calcium channel blocker, in the presence of noradrenaline; (3) hyperpolarizes the aortic membrane potential; (4) stimulates $^{86}Rb^+$ efflux. Furthermore, UR-8225 suppresses the spontaneous activity as well as the contractile response induced by noradrenaline in portal veins. In addition, the vasorelaxant effect of UR-8225 and levcromakalim can be competitively antagonized by glibenclamide, an ATP-sensitive K^+ channel blocker (Ashcroft, 1988). All of these results indicated that, as previously suggested with levcromakalim (Weston, 1989; Weston *et al.*, 1990) the vasorelaxant effects of UR-8225 could be related to its ability to activate ATP-sensitive K^+ channels in vascular smooth muscle cells.

Potassium channel openers increase the permeability of the vascular smooth muscle cell to K^+, resulting in membrane hyperpolarization (Cook, 1988; Weston, 1989). In rat aortic smooth muscle cells, UR-8225 induced a hyperpolarization of up to 16 mV shifting the membrane potential towards the predicted K^+ equilibrium potential for the rat aorta (Hirst & Edwards, 1989) but far from the potential at which depolarization (voltage)-dependent L-type Ca^{2+} channels are activated (− 45 mV). Thus, the hyperpolarization induced by UR-8225 may reduce the intracellular concentration of free Ca^{2+} and cause vasorelaxation by preventing the opening of voltage-activated calcium channels by excitatory agonists (Chiu *et al.*, 1988; Nelson *et al.*, 1988). To confirm whether the hyperpolarization produced by UR-8225 was due to an increase in membrane permeability to K^+ the effects of the drug were studied on $^{86}Rb^+$ efflux. UR-8225 produced a concentration-dependent increase in the rate of $^{86}Rb^+$ efflux from rat aorta, which confirmed that UR-8225 hyperpolarizes the membrane potential and causes vascular relaxation in aortic smooth muscle through an increase in outward K^+ conductance.

In addition, the sulphonylurea glibenclamide, a potent and selective blocker of ATP-sensitive K^+ channels in vascular

smooth muscle (Ashcroft, 1988; Standen *et al.*, 1989), competitively antagonized UR-8225- and levcromakalim-induced vasorelaxation. In fact, the pA_2 values for glibenclamide to inhibit the relaxations induced by UR-8225 and levcromakalim were very similar, indicating that both drugs probably act at the same site. These results further support the contention that the population of potassium channels involved in the vasodilatation induced by UR-8225 could be the ATP-sensitive K^+ channels (Standen *et al.*, 1989: Quast & Cook, 1989).

If the hypothesis that the vasorelaxant effect of UR-8225 is due to the opening of K^+ channels leading to hyperpolarization and alteration in the magnitude of agonist-induced depolarization is correct, it should be markedly reduced under circumstances where the membrane potential is maintained constant. In fact, a major characteristic of K^+ channel openers is that they inhibit the contractions induced by 10–30 mM KCl, whereas they are almost ineffective against the contractions induced by 80 mM KCl or noradrenaline plus high KCl (Lawson & Cavero, 1989; Weston *et al.*, 1990). At low KCl concentrations UR-8225, like other K^+ channel openers, hyperpolarized the membrane potential decreasing the open state probability of L-type Ca^{2+} channels. In fact, cromakalim inhibited the increase in intracellular Ca^{2+} concentration induced by low concentrations (< 30 mM) of KCl in coronary arterial smooth muscle due to the closure of voltage-activated Ca^{2+} channels (Yanagisawa *et al.*, 1990). At high extracellular KCl concentrations the cell membrane is depolarized to a level far from the K^+ equilibrium potential (approximately − 20 mV in the presence of 80 mM KCl, Hamilton & Weston, 1989). Under these conditions K^+ channel openers do not hyperpolarize the smooth muscle cells and therefore, their vasorelaxant effect is negligible (Hamilton *et al.*, 1986; Bray *et al.*, 1987). In addition, the finding that UR-8225 has no effect on high KCl-induced contractions suggests that it does not act as a conventional Ca^{2+} channel blocker and excludes that its vasorelaxant effect can be related to a direct effect on contractile proteins.

The rat portal vein exhibits spontaneous myogenic activity which is due to depolarization induced by the influx of Na^+ and Ca^{2+} and is insensitive to tetrodotoxin (Johansson & Somlyo, 1980). Both levcromakalim and UR-8225 inhibited the frequency and the amplitude of spontaneous contractions. The ionic event terminating electrical excitation is a K^+ outward current through voltage and/or Ca^{2+}-dependent K^+ channels (Johansson & Somlyo, 1980). Therefore, the opening of potassium channels and the subsequent hyperpolarization can be responsible for the inhibitory effect of UR-8225 and levcromakalim on myogenic activity of rat portal veins (Hamilton *et al.*, 1986).

In rat aorta, the tonic component of noradrenaline-induced contractions is due to the activation of Ca^{2+} entry from the extracellular space via dihydropyridine-sensitive and insensitive pathways (Cauvin & Malik, 1984). UR-8225 and levcromakalim inhibited the tonic contraction induced by adding Ca^{2+} to a Ca^{2+}-free medium containing EDTA and methoxyverapamil in the presence of noradrenaline, which suggests that both drugs inhibit the agonist-induced Ca^{2+} entry (Cook, 1988; Bray *et al.*, 1991). Since this tonic contraction was generated in the presence of methoxyverapamil, it can be concluded that the inhibitory effect of UR-8225 and levcromakalim on K^+ conductance does not require the entry of Ca^{2+} through dihydropyridine-sensitive channels (Kreye & Weston, 1986). The inhibitory effect on noradrenaline-induced tonic contractions can be explained because noradrenaline increases open state-probability of single voltage-activated Ca^{2+} channels (Nelson *et al.*, 1988; Pacaud *et al.*, 1991), whereas K^+ channel openers oppose this action by hyperpolarizing the membrane potential (Hamilton *et al.*, 1986; Bray *et al.*, 1991). Other possible explanations are that the hyperpolarization induced by UR-8225 and levcromakalim may inhibit the ability of depleted intracellular Ca^{2+} stores to refill after Ca^{2+} release has occurred and/or the synthesis of inositol 1,4,5-trisphosphate (IP_3)-induced by noradrenaline. The former possibility has been previously reported with cromakalim in rabbit aorta (Chiu *et al.*, 1988; Bray *et al.*, 1991) and the latter with levcromakalim in rabbit mesenteric arteries (Ito *et al.*, 1991). In fact, in skinned skeletal muscles IP_3 induced Ca^{2+} release from the sarcoplasmic reticulum is voltage-dependent (Donaldson *et al.*, 1988).

In conclusion, the present results demonstrate that in rat vascular smooth muscle UR-8225 produced vasorelaxant responses qualitatively similar to those of levcromakalim. This vasorelaxant action seems to be mediated via hyperpolarization of the membrane by activation of ATP-activated K^+ channels.

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Blockade of human atrial 5-HT_4 receptors by GR 113808

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1 The mode of antagonism of 5-hydroxytryptamine (5-HT)-induced positive inotropic effects by the highly selective 5-HT_4 receptor antagonist GR 113808 ({1-[2-methlylsulphonylamino ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate) was investigated on isolated preparations of human right atrium.

2 GR 113808 caused concentration-dependent (2–100 nM) surmountable antagonism of the effects of 5-HT with a pK_B (M) of 8.8.

3 The affinity of GR 113808 for human atrial 5-HT_4 receptors, together with its high selectivity for 5-HT_4 receptors comprise useful properties for investigating the question of 5-HT_4 receptor subtypes.

Keywords: Human atrium; 5-HT_4 receptors; 5-hydroxytryptamine; GR 113808

Introduction

The existence of human atrial 5-HT_4 receptors was first shown by Kaumann *et al.* (1990). They demonstrated on right atrial preparations that tropisetron (ICS 205-930), used to block neuronal 5-HT_4 receptors (reviewed by Bockaert *et al.*, 1992), antagonized with moderate affinity ($pK_B = 6.7$) the positive inotropic effects of 5-hydroxytryptamine (5-HT). Subsequently a variety of drugs were found to antagonize competitively 5-HT-induced inotropic effects in human atrium. These compounds include the partial agonists renzapride and cisapride (Kaumann *et al.*, 1991) and the antagonists DAU 6285 (Turconi *et al.*, 1991) and SDZ 205-557 (Zerkowski *et al.*, 1993). The affinity of these drugs for human atrial 5-HT_4 receptors is, however, modest ($pK_B \leqslant 7.7$) and all but SDZ 205-557 are potent antagonists of 5-HT_3 receptors. The situation improved with the introduction of SB 203186 which blocks human atrial 5-HT_4 receptors with a pK_B of 8.7 and is 10 fold selective for 5-HT_4 receptors (Parker *et al.*, 1993). The effort to develop high affinity 5-HT_4 receptor antagonists appeared to culminate with GR 113808 which has subnanomolar affinity for rodent gut 5-HT_4 receptors ($pA_2 = 9.2–9.5$) and is 3000 fold selective for 5-HT_4 receptors with respect to 5-HT_3 receptors and other 5-HT receptors (Grossman *et al.*, 1993). GR 113808 has been designated as a prototype 5-HT_4 receptor antagonist (TIPS Receptor Nomenclature, 1993) but its affinity for human atrial 5-HT_4 receptors is unknown.

The antagonism by GR 113808 of the positive inotropic effects of 5-HT in human atrium is now presented.

Methods

Right atrial appendages were obtained from 8 patients undergoing surgery at Papworth Hospital for coronary artery bypass grafts. All of the patients had received β-adrenoceptor blockers chronically until the day of surgery. Sex, age and β-adrenoceptor blockers used were: 2 female (73y bisoprolol, 72y atenolol) and 6 males (42y, 46y and 49y atenolol, 71y and 72y bisoprolol; 71y oxprenolol). Some of these patients had also been prescribed some of the following drugs: aspirin, isosorbide mononitrate, nifedipine, diltiazem, benzafibrate, ranitidine, lisinopril, frusemide, glyceryl trinitrate, enalapril and amiodarone. After excision the appendages were immediately placed into a modified oxygenated Krebs solution at room temperature containing (mM): Na^+ 125, 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 and equilibrated with 95% O_2/5% CO_2. The appendages were sectioned into 2 to 3 strips and set up to contract as described (Kaumann *et al.*, 1990) with two modifications: the tissues were paced at 1 Hz instead of 0.5 Hz and received 300 nM CGP 20712A to block β_1-adrenoceptors, instead of 400 nM propranolol. The tissues were set up in 50 ml organ baths in the solution described above supplemented with (mM): Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5, glucose 10 at 37°C. The tissues were attached to Swema SG 4-45 strain gauge transducers and force recorded on a Watanabe polygraph. After the determination of a length-tension curve, the length of each strip was set to obtain 50% of the resting tension associated with maximum developed force.

The antagonism of 5-HT-induced positive inotropic effects was investigated in the presence of cocaine 6 μM (to reduce tissue capture of 5-HT), ascorbate 200 μM (to reduce oxidation of 5-HT) (Kaumann *et al.*, 1990) and CGP 20712A 300 nM to block β_1-adrenoceptors as used on human heart tissues by Kaumann & Lemoine (1987). A single cumulative concentration-effect curve to 5-HT was determined per strip in the absence or presence of GR 113808, which was present for at least 90 min before a curve was begun.

The experiments were concluded by the administration of a β-adrenoceptor saturating concentration of (−)-isoprenaline (200 μM) and after an equilibrium response to (−)-isoprenaline by raising the Ca^{2+} concentration to 6.75 mM. To assess whether GR 113808 modified the slope of 5-HT concentration-effect curves, − log values of the EC_{10}, EC_{30}, EC_{50}, EC_{70} and EC_{90} were obtained by interpolation. To assess whether blockade by GR 113808 was surmountable the maximum effects of 5-HT in the absence and presence of antagonist were computed in two ways, as percentage of responses to (−)-isoprenaline and to 6.75 mM Ca^{2+} respectively. 5-HT concentration-ratios caused by GR 113808 were calculated at EC_{50} levels relative to a control strip from the same tissue. pK_B values were calculated following Schild regression analysis (Arunlakshama & Schild, 1959) by forcing unit slope through each data point. Results are expressed as mean ± s.e.mean.

The following drugs were gifts: GR 113808 ({1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate, maleate salt) from Dr P.P.A. Humphrey (Glaxo UK) and CGP 20712A (1-[2-((3-carbamoyl 4-hydroxy) phenoxy)ethylamino]-3-{4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol methanesulphonate from Dr L. Maître (Hoffman-La Roche, Basle, Switzerland). 5-Hydro-

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xytryptamine hydrochloride, (−)-isoprenaline bitartrate and cocaine hydrochloride were purchased from Sigma Chemical Co. (Poole, Dorset). All solutions of 5-HT were in 200 μM ascorbate.

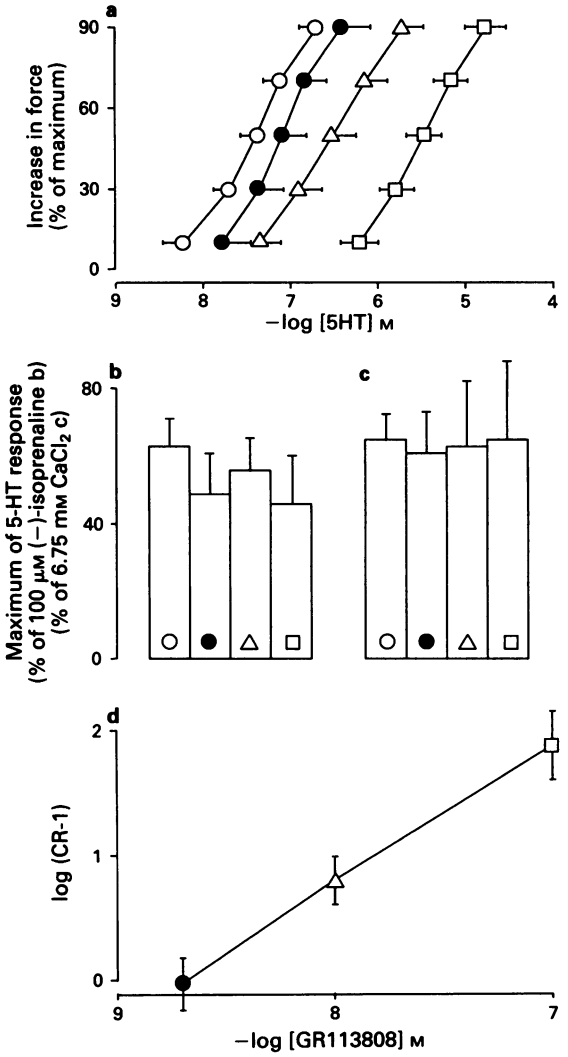

Figure 1 Antagonism of the positive inotropic effects of 5-hydroxytryptamine (5-HT) by GR 113808. Data obtained in the absence (○), or presence of GR 113808 2 nM (●), 10 nM (△) and 100 nM (□). (a) Concentration-effect curves to 5-HT in the absence and presence of GR 113808. (b) Maximum responses to 5-HT of the curves of (a), expressed as a percentage of the response to (−)-isoprenaline. (c) Maximum responses to 5-HT of the curves in (a) expressed a percentage of the response to 6.75 mM Ca^{2+}. (d) Schild-plot of the data in (a). The number of atrial strips was: (○) 8 from 8 patients; (●) 4 from 4 patients; (△) 6 from 6 patients and (□) 4 from 4 patients. For further details see Methods.

Results

GR 113808 did not modify the basal contractile force at the concentrations used (2–100 nM). GR 113808 caused a concentration-dependent shift to the right of the concentration-inotropic effect curves for 5-HT with little effect on their slope (Figure 1a). The maximum responses to 5-HT were not significantly changed by GR 113808, regardless of whether they were expressed as a percentage of the response to (−)-isoprenaline (Figure 1b) or of the response to 6.75 mM Ca^{2+} (Figure 1c). The nearly parallel shifts of the curves for 5-HT and the surmountable antagonism caused by GR 113808 suggest that it is a competitive antagonist. A slope of the Schild-plot of 1.11 ± 0.12 (not significantly different from 1) supports this suggestion. A pK_B of 8.79 ± 0.11 was estimated.

Discussion

GR 113808 and SB 203186 (Parker *et al.*, 1993) show the highest blocking potencies reported so far, for any compound acting at human atrial 5-HT$_4$ receptors. Other compounds studied on human isolated atrium include SDZ 205-557 (Zerkowski *et al.*, 1993), DAU 6285 (Turconi *et al.*, 1991), renzapride (partial agonist, Kaumann *et al.*, 1991), and cisapride (partial agonist, Kaumann *et al.*, 1991). The rank order of estimated affinities of these compounds for human atrial 5-HT$_4$ receptors is (pK_B values in parentheses): GR 113808 (8.8) ≥ SB 203186 (8.7) > SDZ 205-557 (7.7) > DAU 6285 (6.8) ≥ renzapride (6.7) ≥ tropisetron (6.7–6.3) ≥ cisapride (6.3). This rank order of affinities at the human atrial 5-HT$_4$ receptor can be used for comparison with data obtained from both binding (Grossman *et al.*, 1993) in cardiac and non-cardiac tissues and functional assays in non-cardiac tissues across species.

GR 113808 appears to have a somewhat lower affinity for human atrial 5-HT$_4$ receptors ($pK_B = 8.8$, this study) than for 5-HT$_4$ receptors of guinea-pig proximal colon ($pA_2 = 9.2$, Grossman *et al.*, 1993) and rat lower oesophagus ($pA_2 = 9.5$). Furthermore, binding of [^{3}H]-GR 113808 to guinea-pig cerebral 5-HT$_4$ receptors also revealed a higher affinity for these 5-HT$_4$ receptors ($pK_D = 9.7$) than that of unlabelled GR113808 for human atrial 5-HT$_4$ receptors. Possible differences between 5-HT$_4$ receptors of mouse embryonic colliculi neurones (Bockaert *et al.*, 1992) and of human atrium have previously been noted (Kaumann *et al.*, 1991). It remains to be investigated whether these differences are merely species-related or, more importantly, can be detected between the 5-HT$_4$ receptors located in different tissues of one species. The high affinity and selectivity of GR 113808 for 5-HT$_4$ receptors make it a powerful tool for discerning between species homologues of 5-HT$_4$ receptors and true 5-HT$_4$ receptor subtypes.

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Endothelin receptor subtypes in human and guinea-pig pulmonary tissues

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1 In this study the endothelin (ET) receptor subtypes mediating contractions produced by ET-1 in human and guinea-pig pulmonary tissues were investigated. In addition the receptor responsible for ET-1-induced prostanoid release in human bronchus was determined.

2 In human bronchus and human pulmonary artery ET-1 (0.1 nM–0.3 μM) was a potent and effective contractile agent ($pD_2 = 7.58 \pm 0.15$, $n = 6$, and 8.48 ± 0.11, $n = 7$, respectively). BQ-123 (1–10 μM), a potent and selective ET_A receptor antagonist, potently antagonized ET-1-induced contraction in human pulmonary artery ($pK_B = 6.8$ with 1 μM BQ-123, $n = 7$) but had no effect in human bronchus ($n = 6$).

3 Sarafotoxin S6c (0.1 nM–0.1 μM), the ET_B-selective agonist, did not contract human pulmonary artery ($n = 5$), but potently and effectively contracted human bronchus: $pD_2 = 8.41 \pm 0.17$, maximum response = 74.4 ± 3.1% of 10 μM carbachol; $n = 5$. BQ-123 (1–10 μM) did not antagonize sarafotoxin S6c-induced contraction in human bronchus ($n = 5$).

4 ET-1 potently contracted guinea-pig trachea, bronchus, pulmonary artery and aorta ($pD_2 = 8.15 \pm 0.14$, 7.72 ± 0.12, 8.52 ± 0.12, and 8.18 ± 0.12, respectively, $n = 6$–14). BQ-123 (0.1–10 μM) antagonized ET-1-induced contractions in guinea-pig pulmonary artery ($pK_B = 6.7$ with 1 μM BQ-123, $n = 6$), aorta ($pK_B = 7.1$ with 1 μM BQ-123, $n = 6$) and trachea ($pK_B = 6.2$ with 1 μM BQ-123, $n = 6$) but was without marked effect in bronchus ($n = 4$). In contrast, sarafotoxin S6c (0.1 nM–0.1 μM) did not contract guinea-pig aorta ($n = 4$) or guinea-pig pulmonary artery ($n = 6$) but potently and effectively contracted guinea-pig bronchus: $pD_2 = 8.55 \pm 0.1$; maximum contraction = 63.6 ± 3.1% of 10 μM carbachol, $n = 4$. Sarafotoxin S6c (0.1 nM–0.1 μM) was a much less effective agonist in guinea-pig trachea: maximum contraction = 13.9 ± 2.5% of 10 μM carbachol, $n = 4$; $P < 0.0001$, compared to bronchus. Contractions produced by sarafotoxin S6c in guinea-pig bronchus or trachea were unaffected by BQ-123 (10 μM, $n = 4$).

5 Significant differences were observed in the efficacy, relative to carbachol, but not the potency of sarafotoxin S6c in guinea-pig airways, with a much greater maximum contractile response in bronchus (69.6 ± 2.4% of 10 μM carbachol, $n = 6$) or lower region of the trachea (48.5 ± 5.9% of 10 μM carbachol, $n = 6$) than in the middle region of the trachea (14.4 ± 4.0% of 10 μM carbachol, $n = 6$) or the upper region of the trachea (19.3 ± 2.7% of 10 μM carbachol, $n = 6$). There were minimal regional differences in either ET-1-induced contraction or the potency of BQ-123 (3 μM) for inhibition of responses to ET-1 in guinea-pig airways.

6 Release of various prostanoids in human bronchus induced by ET-1 (0.3 μM) was essentially abolished with 10 μM BQ-123.

7 These data provide evidence that distinct ET receptors mediate ET-1-induced contraction in human pulmonary artery, guinea-pig pulmonary artery and guinea-pig aorta (ET_A subtype) compared with human bronchus and guinea-pig bronchus (non-ET_A, perhaps ET_B subtype). Contractions to ET-1 in guinea-pig trachea appear to involve both ET_A and non-ET_A (ET_B?) receptor subtypes. Furthermore, regional differences appear to exist in the relative distribution of ET receptor subtypes in guinea-pig airways. In human bronchus ET-1-induced prostanoid release, unlike the contractile response, appears to be mediated via ET_A receptor activation.

Keywords: Endothelin-1; sarafotoxin S6c; human bronchus; human pulmonary artery; guinea-pig airways; guinea-pig pulmonary artery; BQ-123; endothelin receptor subtypes; ET_A receptor; ET_B receptor

Introduction

In 1988 the isolation, purification and initial pharmacological characterization of a novel, potent vasoconstrictor peptide released from porcine cultured endothelial cells was described (Yanagisawa *et al.*, 1988). This 21-amino acid peptide, designated endothelin (ET), contains two intra-chain disulphide bridges (Yanagisawa *et al.*, 1988) and has a close structural and functional homology with a group of snake venom toxins, the sarafotoxins (Lee & Chiappinelli, 1988; Kloog *et al.*, 1988; Takasaki *et al.*, 1988b; Kloog & Sokolovsky, 1989). Subsequent research determined that there are different forms of ET, named ET-1 (which is the original porcine/human form that was isolated from endothelial cells), ET-2 (two amino acid substitution from ET-1) and ET-3, (six amino acid substitution from ET-1), which are encoded by three similar but distinct genes in the human genome (Inoue *et al.*, 1989; Yanagisawa & Masaki, 1989a,b; Masaki *et al.*, 1992).

The ET family exerts diverse biological effects which are thought to be mediated via an interaction with specific ET membrane receptors. However, some differences were apparent in the activities of ET-1, ET-2, ET-3 – for example, in their relative vasoconstrictor versus vasodilator effects – suggesting the existence of distinct ET receptor subtypes (Yanagisawa & Masaki, 1989a,b; Masaki *et al.*, 1992; Sakurai *et al.*, 1992). Subsequently, biochemical analysis, including ligand binding and cross-linking affinity labelling

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studies, provided further evidence for different ET receptors in various tissues including the lung (Kloog *et al.*, 1989; Masuda *et al.*, 1989; Watanabe *et al.*, 1989; Yanagisawa & Masaki, 1989a,b; Schvartz *et al.*, 1991; Takayanagi *et al.*, 1991; Masaki *et al.*, 1992; Sakurai *et al.*, 1992). For example, in rat lung membranes the existence of two ET receptors (44 kD and 32 kD molecular weights) which had different affinities for the ETs was proposed. The 44 kD receptor had a higher affinity for ET-1 or ET-2 compared with ET-3, whereas the 32 kD receptor exhibited selectivity for ET-3 (Masuda *et al.*, 1989). Similarly, ligand binding and affinity labelling studies in porcine lung suggested the presence of ET receptor subtypes (Takayanagi *et al.*, 1991).

Using a variety of systems, including rat and bovine lung, the cloning and expression of cDNA for a selective and a non-selective ET receptor, designated ET_A and ET_B, respectively, was described recently (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Sakamoto *et al.*, 1991; Masaki *et al.*, 1992). The ET_A receptor has a higher affinity for ET-1 or ET-2 compared with ET-3, whereas the ET_B receptor has equal affinity for the three members of the ET family. In rat cultured anterior pituitary cells and rat PC12 pheochromocytoma cells, evidence was provided for an ET receptor subtype which was selective for ET-3 (Martin *et al.*, 1990; Samson *et al.*, 1990), designated ET_C (Masaki *et al.*, 1992); the cDNA clone for this receptor has not yet been isolated. The solubilization of ET_A and ET_B receptors from rat lung (Kondoh *et al.*, 1991) and the purification of the ET_B receptor (52 kD) from bovine lung (Kozuka *et al.*, 1991; Hagiwara *et al.*, 1992) have been described.

There is some evidence from functional studies for distinct ET receptor subtypes. For example, the C-terminal hexapeptide, ET-(16-21) was reported to be a potent contractile agent in guinea-pig bronchus, but not in rat aorta (Maggi *et al.*, 1989); however, in guinea-pig trachea ET-(16-21) was a much less effective contractile agonist than ET-1 and did not affect [^{125}I]-ET-1 binding in airway smooth muscle (Tschirhart *et al.*, 1991). Analysis of the contractile activity of ET-1, ET-2 and ET-3, in addition to their ability to cause cross-desensitization, provided evidence that distinct ET receptors mediate ET-1-induced contraction in guinea-pig pulmonary artery and trachea (Cardell *et al.*, 1992). In pig coronary artery a comparison of the effects of different members of the ET and sarafotoxin families suggested the existence of a non-ET_A, non-ET_B receptor subtype which mediated, in part, the contractile response (Harrison *et al.*, 1992). Sarafotoxin S6c is a potent and selective ET_B receptor agonist (Williams *et al.*, 1991b) with greater than a 10 000 fold higher affinity for the ET_B versus ET_A receptor. Furthermore, BQ-123, a cyclic pentapeptide (D-Asp-L-Pro-D-Val-L-Leu,D-Trp) has been identified as a potent and selective ET_A receptor antagonist with affinity for the ET_A receptor in the high nM range and μM affinity for the ET_B receptor (Ihara *et al.*, 1992a). Utilizing these experimental tools, our laboratory has provided evidence that different ET receptors mediate ET-1-induced contraction in guinea-pig aorta (ET_A) and bronchus (non-ET_A, perhaps ET_B) (Hay, 1992). Recently it has been reported that FR 139317, a novel purported ET_A receptor antagonist, antagonized ET-induced contractions in guinea-pig pulmonary artery but not trachea (Cardell *et al.*, 1993).

The ETs exert several effects in the pulmonary systsem (Pons *et al.*, 1992; Hay *et al.*, 1993a), including contraction of human airway and vascular smooth muscle (Henry *et al.*, 1990; Brink *et al.*, 1991) and stimulation of prostanoid release from human bronchus (Hay *et al.*, 1993b) and have been postulated to be involved in the pathophysiology of pulmonary disorders including asthma and pulmonary hypertension (Cernacek & Stewart, 1989; Springall *et al.*, 1991; Mattoli *et al.*, 1991; Hay *et al.*, 1993a). If control of the actions of ET turns out to be a viable therapeutic strategy for pulmonary disorders, it will be important to characterize the receptors mediating the actions of the ETs in the pulmonary system including contraction of human pulmonary blood vessels and airway smooth muscles. Accordingly, one of the major objectives of the present series of experiments was, utilizing sarafotoxin S6c and BQ-123, to elucidate the ET receptor(s) responsible for ET-1-induced contraction in human pulmonary artery and human bronchus. The guinea-pig is used routinely as an *in vitro* and *in vivo* model of the human pulmonary system, and to ascertain the relevance of this species to man, with regard to the contractile effects of ET, a comparison was made between isolated guinea-pig and human pulmonary tissues. In addition, the ET receptors responsible for ET-1-induced contraction and prostanoid release in human bronchus were compared.

A preliminary account of this research has been presented previously to the British Pharmacological Society (Hay & Luttman, 1992).

Methods

Tissue preparation for contraction studies

Guinea-pigs Trachea, primary bronchus, aorta and pulmonary artery were removed carefully from male Hartley guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.; 450–650 g body weight) and placed in modified Krebs-Henseleit solution. Following careful removal of adherent fat and connective tissue, the trachea was cut open along its longitudinal axis, directly opposite the smooth muscle, and strips consisting of two adjacent cartilage rings were prepared. The epithelium was removed mechanically from trachea by gently rubbing the luminal surface with a cotton-tipped applicator. In some experiments, strip preparations were obtained from upper, middle and lower regions of the trachea and responses in the different areas were compared. Bronchus, aorta and pulmonary artery were placed around 21 g syringe needles, cleaned and cut into rings of approximately 3, 3 and 2 mm width, respectively. The epithelium and the endothelium was removed from bronchial and vascular tissues, respectively, by rotation of the tissues several times round the syringe needle.

The preparations were then placed in 10 ml water-jacketed, siliconized organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FT03C force-displacement transducers. Mechanical responses were recorded isometrically by multi-channel Grass polygraphs. Tissues were equilibrated under approximately 1.5 g resting load for trachea and bronchus and 2 g for aorta and pulmonary artery for at least 1 h, and washed every 15 min with fresh Krebs-Henseleit solution, before the start of each experiment. The composition of the Krebs-Henseleit solution, which was gassed with 95% O_2:5% CO_2 and maintained at 37°C, was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.5

Human lung Human lung tissue from organ donors who had no known history of respiratory disorders was obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA, U.S.A.) and the National Disease Research Interchange (NDRI, Philadelphia, PA, U.S.A.). Lungs were received within 24 h of removal. Bronchial tissue was removed from the lung by carefully placing a glass probe within individual segments and dissecting away lung parenchymal, fat and connective tissue, and also vascular tissue. First to fifth generation bronchial strips (approximately 4–15 mm diameter) were prepared; using this classification the main bronchus is regarded as the first generation airway. Similarly, a glass probe was placed in the pulmonary artery and following careful cleaning ring preparations of approximately 3–4 mm width were obtained. The tissues were placed under approximately 2 g of passive tension and they were treated as indicated above for guinea-pig tissues for the measurement of contractile responses.

Concentration-response curves

After the equilibration period, and before construction of agonist concentration-response curves, tissues were exposed to 10 μM carbachol for airway preparations and 10 μM phenylephrine for vascular tissues. Following plateau of this reference contraction, tissues were washed several times over 30–60 min until the tension returned to baseline level. The preparations were then left for at least 30 min before the start of the experiment. Agonist concentration-response curves were obtained by their cumulative addition to the organ bath in three fold increments according to the technique of Van Rossum (1963). Each drug concentration was left in contact with the preparation until the response reached a plateau before addition of the subsequent agonist concentration. At the end of the experiment, tissues were exposed again to the reference substance. In some studies, following completion of ET-1 and sarafotoxin S6c concentration-response curves, tissues were exposed to a maximally effective concentration of sarafotoxin S6c or ET-1, respectively. In experiments examining the effects of BQ-123, tissues were exposed to the compound for 30 min before addition of contractile agonists. Experiments were conducted in the presence of 1 μM sodium meclofenamate, the cyclo-oxygenase inhibitor, which was added 45 min before initiation of the curves. Only one agonist concentration-response curve was generated per tissue.

Tissue preparation for prostanoid release studies

Human lung tissues were obtained from organ donors as described above, and in addition some tissues were obtained from lung resections of anonymous lung cancer patients. The bronchi (2–12 mm diameter) were dissected free of parenchymal tissue with the aid of a dissecting microscope, cut into small pieces and divided into aliquots each containing approximately 175 mg (wet weight). The bronchial tissues were incubated in 2 ml of Krebs-Henseleit solution, which was gassed with 95% O_2/5% CO_2 and maintained at 37°C. The physiological buffer was replaced at 15 min intervals over 90 min. Following this equilibration period, 2 ml of Krebs-Henseleit, containing or lacking ET-1 (0.3 μM) in either the presence or absence of BQ-123 (10 μM), was added for 15 min and after this time the supernatant was collected for the measurement of prostanoid release.

Measurement of prostanoid release

Prostanoid release into the supernatant fluid was assayed using combined gas chromatography (negative ion chemical ionization) mass spectrophotometry (GC/MS) as described previously (Hubbard *et al.*, 1986). Briefly, a 100 μl aliquot of the supernatant was added to 250 μl of acetone in a silanized vial. A mixture containing a known quantity (about 1 ng) of 3,3,4,4-tetradeuterated PGE_2, PGD_2, $PGF_{2\alpha}$, TxB_2 and 6-keto-$PGF_{1\alpha}$ was added to provide internal standards for the identification and quantification of these prostanoids. In addition, the identification of 9α, 11β-PGF_2 was based on its retention times in relation to the tetradeuterated $PGF_{2\alpha}$. Samples were then dried under a stream of nitrogen and the residue was treated with 2% methoxymine hydrochloride dissolved in pyridine. Excess pyridine was evaporated under nitrogen and the residue was subjected to sequential procedures for the synthesis of pentafluorobenzyl ester and trimethylsilyl ether derivatives as described previously (Hubbard *et al.*, 1986). GC/MS analysis of the derivatized samples (1 μl volume) was performed with a Finnigan Model 9611 gas chromatograph interfaced with a Finnigan MAT 4610B EI/CI mass spectrophotometer (Finnigan MAT Corp., San José, CA, U.S.A.) supplied with a Superinocis data system. The sensitivity of this techique is <0.1 fmol/injection for each of the six prostanoids assayed.

Analysis of data

Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction obtained at the end of the experiment. Geometric mean EC_{50} values (pD_2s) were calculated from linear regression analyses of data. Where appropriate, antagonist potencies were calculated and expressed as pK_B; $pK_B = -\log$ [antagonist]/X − 1, where X is the ratio of agonist concentration required to elicit 50% of the maximal contraction in the presence of the antagonist compared with that in its absence. Results for control- and treated-tissues were analysed for differences in both the EC_{50}s and also the maximum contractile responses. Prostanoid release is expressed as ng g^{-1} tissue wet weight. All data are given as the mean ± s.e.mean. Statistical analysis was conducted using ANOVA or Student's two-tailed *t* test for paired samples where appropriate with a probability value less than 0.05 regarded as significant.

Drugs

The following drugs were used: endothelin-1 (human, porcine) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sarfotoxin S6c was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). BQ-123 was synthesized by Dr Michael Moore and colleagues, Department of Peptide Chemistry, SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Carbachol and phenylephrine were obtained from Sigma Chemical Co. and sodium meclofenamate was a generous gift from Warner Lambert (Ann Arbor, MI, U.S.A.).

Results

Contraction studies

Guinea-pig tissues ET-1 (0.1 nM–0.3 μM) potently contracted guinea-pig trachea, bronchus, pulmonary artery and aorta, with respective pD_2s of 8.15 ± 0.14, $n = 12$; 7.72 ± 0.12, $n = 14$; 8.52 ± 0.12, $n = 6$; 8.18 ± 0.12, $n = 12$ (Figure 1 and Table 1). ET-1 was an efficacious agonist in guinea-pig

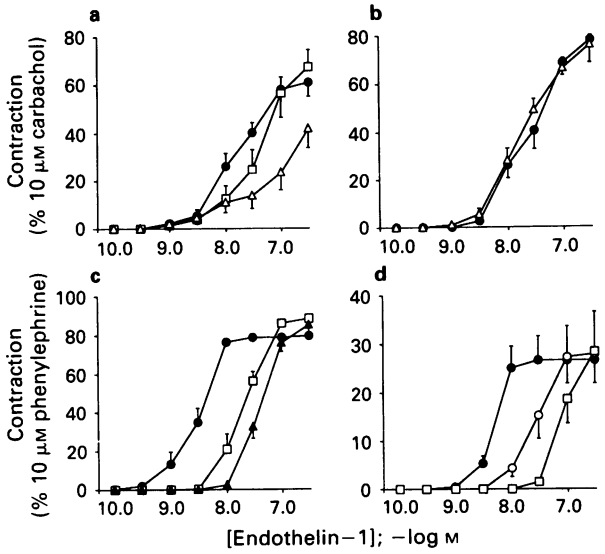

Figure 1 Effect of BQ-123 (0.1–10 μM) on endothelin-1 (ET-1) concentration-response curves in (a) trachea, (b) bronchus, (c) pulmonary artery or (d) aorta of the guinea-pig. Results are expressed as a percentage of the response to 10 μM carbachol and are given as the mean ± s.e.mean of (b) 4 or (a,c,d) 6 experiments; (●) control; (○) 0.1 μM BQ-123; (□) 1 μM BQ-123; (▲) 3 μM BQ-123; (△) 10 μM BQ-123. Studies were conducted in the presence of 1 μM sodium meclofenamate.

Table 1 Comparison of potencies and maximum contractile responses for endothelin-1 (ET-1) and sarafotoxin S6c in guinea-pig and human pulmonary tissues.

Tissue	*ET-1* Potency (pD_2)	*ET-1* Maximum contractile response (% 10 μM carbachol or phenylephrine)	n	*Sarafotoxin S6c* Potency (pD_2)	*Sarafotoxin S6c* Maximum contractile response (% 10 μM carbachol or phenylephrine)	n
Guinea-pig pulmonary artery	8.52 ± 0.12	79.4 ± 1.8	6	NE	NE	6
Guinea-pig aorta	8.18 ± 0.12	32.1 ± 3.8	12	NE	NE	4
Guinea-pig trachea (whole)	8.15 ± 0.14	55.7 ± 6.4	12	8.72 ± 0.11	13.9 ± 2.5	4
Guinea-pig bronchus	7.72 ± 0.12	76.5 ± 2.4	14	8.76 ± 0.05	69.6 ± 3.1	6
Guinea-pig trachea (upper third)	7.91 ± 0.17	59.8 ± 7.0	6	8.48 ± 0.08	19.3 ± 2.7	6
Guinea-pig trachea (middle third)	8.00 ± 0.08	53.7 ± 5.1	6	8.68 ± 0.05	14.4 ± 4.0	6
Guinea-pig trachea (lower third)	8.07 ± 0.13	61.7 ± 3.4	6	8.76 ± 0.05	48.5 ± 5.9	6
Human bronchus	7.58 ± 0.15	74.4 ± 3.4	6	8.41 ± 0.17	74.4 ± 3.1	5
Human pulmonary artery	8.48 ± 0.11	84.4 ± 5.2	7	NE	NE	5

Results are expressed as pD_2 (potency) and as a percentage of 10 μM carbachol or phenylephrine (maximum contractile response) and are given as the mean ± s.e.mean; n indicates the number of experiments are given in parentheses
NE = no effect.

trachea, bronchus or pulmonary artery, eliciting a maximum contraction which was about 60–80% of that produced by the reference agonist, whereas it was less effective in guinea-pig aorta producing a maximum response which was only about 30% of that elicited by 10 μM phenylephrine (Figure 1 and Table 1). The ET_A receptor antagonist, BQ-123 (0.1–10 μM) antagonized ET-1-induced contractions in guinea-pig trachea, pulmonary artery or aorta but was without effect in bronchus (Figure 1 and Table 2).

In contrast, sarafotoxin S6c (0.1 nM–0.1 μM) did not contract guinea-pig aorta ($n = 4$) or guinea-pig pulmonary artery ($n = 6$), although it potently and effectively contracted guinea-pig bronchus (Figure 2 and Table 1). Compared to guinea-pig bronchus, sarafotoxin S6c was a much less effective agonist in guinea-pig trachea, producing a maximum contraction, elicited by a concentration of 0.1 μM, which was only 13.9 ± 2.5% of that produced by 10 μM carbachol ($n = 4$; $P < 0.0001$ compared to bronchus) (Figure 2 and Table 1). In guinea-pig trachea, pulmonary artery and aorta, addition of a maximally effective concentration of ET-1 (0.3 μM) following completion of sarafotoxin S6c concentration-response curves produced a marked contractile response which represented 57.0 ± 1.4% of 10 μM carbachol ($n = 4$; $P < 0.001$ compared to sarafotoxin S6c), 87.8 ± 4.8% of 10 μM phenylephrine ($n = 6$; $P < 0.001$ compared to sarafotoxin S6c), and 37.8 ± 6.7% of 10 μM phenylephrine

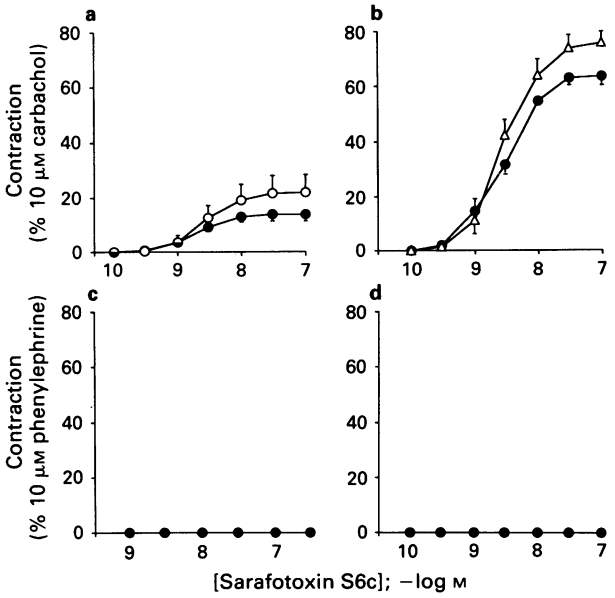

Figure 2 Comparison of sarafotoxin S6c concentration-response curves in (a) trachea, (b) bronchus, (c) pulmonary artery or (d) aorta of the guinea-pig and effects of BQ-123 (3 or 10 μM) on sarafotoxin S6c-induced contraction in (a) trachea and (b) bronchus. Results are expressed as a percentage of the response to 10 μM carbachol and are given as the mean ± s.e.mean of (a,b,d) 4 or (c) 6 experiments; (●) control; (○) 3 μM BQ-123; (△) 10 μM BQ-123. Studies were conducted in the presence of 1 μM sodium meclofenamate.

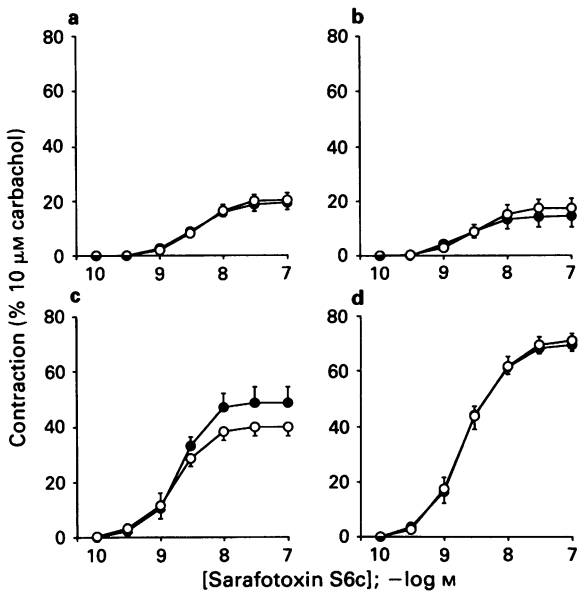

Figure 3 Effect of BQ-123 (3 μM) on sarafotoxin S6c concentration-response curves in (a) upper trachea, (b) middle trachea, (c) lower trachea and (d) bronchus of the guinea-pig. Results are expressed as a percentage of the response to 10 μM carbachol and are given as the mean ± s.e.mean of 6 experiments; (●) control; (○) 3 μM BQ-123. Studies were conducted in the presence of 1 μM sodium meclofenamate.

($n = 4$; $P < 0.005$ compared to sarafotoxin S6c), respectively. In contrast, in guinea-pig bronchus, addition of ET-1 (0.3 μM) at the end of sarafotoxin S6c concentration-response curves, elicited a smaller additional increase in tone, from 66.8 ± 2.5% to 88.8 ± 1.6% of 10 μM carbachol ($n = 8$; $P < 0.0001$ compared to sarafotoxin S6c). Contractions induced by sarafotoxin S6c in guinea-pig bronchus, and also trachea, were unaffected by BQ-123 (10 μM and 3 μM, respectively, $n = 4$) (Figure 2 and Table 2).

When comparing contractions in response to ET-1 and sarafotoxin S6c in different regions of guinea-pig airways, it was observed that there were appreciable regional differences in the efficacy, relative to carbachol, but not the potency, of sarafotoxin S6c. Thus, a significantly greater maximum contractile response ($P < 0.0001$), with 0.1 μM sarafotoxin S6c, was noted in bronchus or lower trachea than in middle trachea or upper trachea; there was no difference in respective potencies (Figure 3 and Table 1). Addition of ET-1 (0.3 μM) following completion of sarafotoxin S6c concentration-response curves produced a marked increase in tension in the three regions of the trachea, and a smaller increase in tone in bronchus (data not shown). BQ-123 (3 μM) did not antagonize sarafotoxin S6c-induced contraction in any region of the guinea-pig airways (Figure 3 and Table 2). In contrast, there were minimal regional differences in ET-1-induced contraction in terms of both potency and efficacy (Figure 4 and Table 1). Furthermore, the potency of BQ-123 (3 μM) for antagonism of responses to ET-1 was similar in the different regions of guinea-pig trachea (Figure 4 and Table 2).

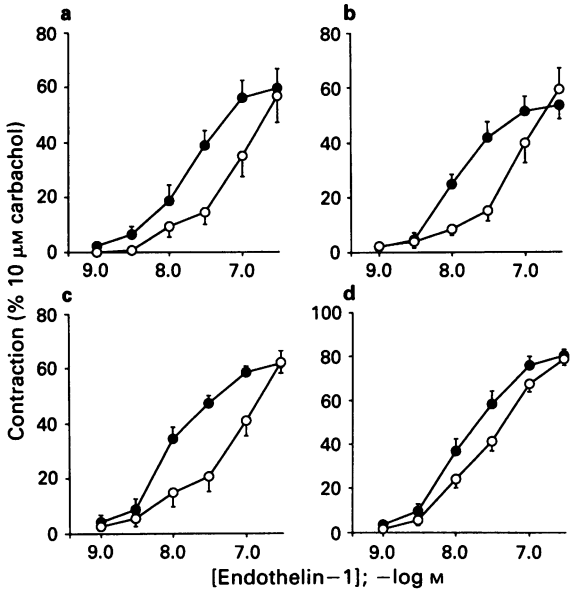

Figure 4 Effect of BQ-123 (3 μM) on endothelin-1 (ET-1) concentration-response curves in (a) upper trachea, (b) middle trachea, (c) lower trachea and (d) bronchus of the guinea-pig. Results are expressed as a percentage of the response to 10 μM carbachol and are given as the mean ± s.e.mean of 6 experiments; (●) control; (○) 3 μM BQ-123. Studies were conducted in the presence of 1 μM sodium meclofenamate. BQ-123 antagonized ET-1-induced contractions in all regions of the trachea but was without significant effect in the bronchus.

Table 2 Comparison of potencies of BQ-123 for inhibition of endothelin-1 (ET-1)- and sarafotoxin S6c-induced contractions in guinea-pig and human pulmonary tissues.

	ET-1		*Sarafotoxin S6c*	
Tissue	*Potency* (pK_B)	n	*Potency* (pK_B)	n
Guinea-pig pulmonary artery	6.7 (1 μM) 6.6 (3 μM)	6 6	ND	6
Guinea-pig aorta	7.6 (0.1 μM) 7.1 (1 μM)	6 6	ND	4
Guinea-pig trachea (whole)	6.2 (1 μM) 6.0 (10 μM)	6 6	NE (1-3 μM)	4
Guinea-pig bronchus	NE (1-10 μM)	4	NE (10 μM)	4
Guinea-pig trachea (upper third)	6.0 (3 μM)	6	NE (3 μM)	6
Guinea-pig trachea (middle third)	6.1 (3 μM)	6	NE (3 μM)	6
Guinea-pig trachea (lower third)	6.3 (3 μM)	6	NE (3 μM)	6
Human bronchus	NE (10 μM)	6	NE (10 μM)	5
Human pulmonary artery	6.8 (1 μM) 6.2 (10 μM)	7	ND	5

Results are expressed as pK_B and are given as the mean; the concentrations of BQ-123 are given in parentheses.
NE = no effect
ND = not determined as there was no contractile response to sarafotoxin S6c.

Human tissues In both human bronchus and pulmonary artery ET-1 (0.1 nM-0.3 μM) was a potent and effective contractile agent ($pD_2 = 7.58 \pm 0.15$, $n = 6$ and 8.48 ± 0.11, $n = 7$, respectively; maximum contraction with 0.3 μM ET-1 = 74.4 ± 3.4% 10 μM carbachol; and 84.4 ± 5.2% 10 μM phenylephrine, respectively) (Figure 5). BQ-123 (1 or 10 μM) antagonized ET-1-induced contraction in human pulmonary artery but had no effect, at 10 μM, on the response to ET-1 in human bronchus (Figure 5 and Table 2).

Sarafotoxin S6c (0.1 nM-0.1 μM) did not contract human pulmonary artery ($n = 5$) but potently and effectively contracted human bronchus (Figure 6 and Table 1). BQ-123

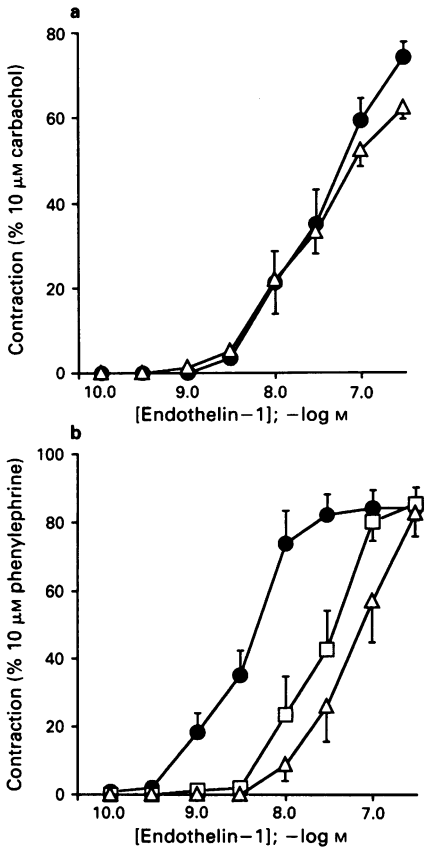

Figure 5 Effect of BQ-123 (1 or 10 μM) on endothelin-1 (ET-1) concentration-response curves in (a) human bronchus and (b) human pulmonary artery. Results are expressed as a percentage of the response to 10 μM carbachol (a) or 10 μM phenylephrine (b) and are given as the mean ± s.e.mean of (a) 6 and (b) 7 experiments; (●) control; (□) 1 μM BQ-123; (△) 10 μM BQ-123. Studies were conducted in the presence of 1 μM sodium meclofenamate.

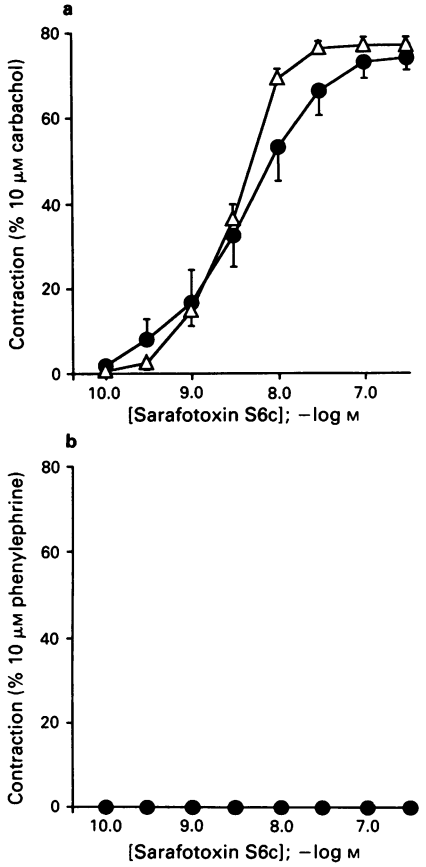

Figure 6 Sarafotoxin S6c concentration-response curves in (a) human bronchus and (b) human pulmonary artery and effect of BQ-123 (10 μM) on sarafotoxin S6c-induced contraction in the human bronchus (a). Results are expressed as a percentage of the response to 10 μM carbachol (a) or 10 μM phenylephrine (b) and are the mean ± s.e.mean of 5 experiments; (●) control; (△) 10 μM BQ-123. Studied were conducted in the presence of 1 μM sodium meclofenamate.

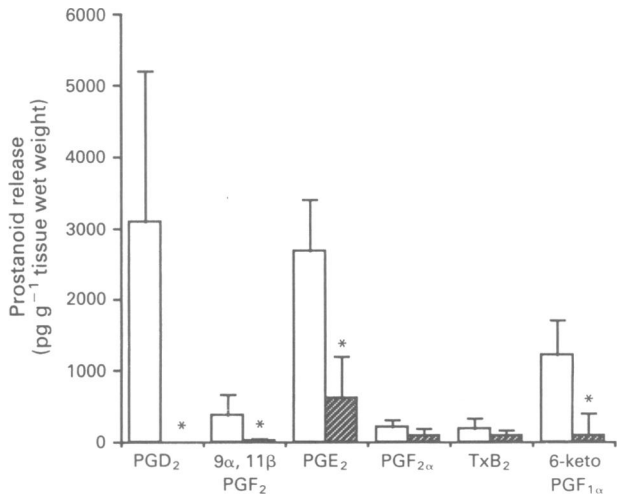

Figure 7 Effect of BQ-123 (10 μM) on prostanoid release in human bronchus induced by endothelin-1 (ET-1, 0.3 μM). Tissues (approximately 175 mg wet weight) were exposed to ET-1 for 15 min in the absence (open columns) or presence (hatched columns) of BQ-123 and levels of prostaglandin D_2 (PGD_2), 9α, 11β PGF_2 (PGD_2 metabolite), PGE_2, $PGF_{2\alpha}$, TxB_2 and 6-keto $PGF_{1\alpha}$ (PGI_2 metabolite) in the supernatant were measured using GC/MS as outlined in the Methods. Results, which represent the net release above basal levels, are expressed as ng g^{-1} tissue wet weight and are the mean ± s.e.mean of 4 tissues. *Indicates statistical significance, $P<0.05$.

(1–10 μM) did not antagonize sarafotoxin S6c-induced contraction in human bronchus ($n = 5$) (Figure 6 and Table 2). In human pulmonary artery addition of ET-1 (0.3 μM) following completion of sarafotoxin S6c concentration-response curves produced a substantial contractile response, which represented 94.1 ± 3.2% of that produced by 10 μM phenylephrine, $n = 5$ (data not shown).

Prostanoid release studies

In confirmation of the results of previous findings from our laboratory, (Hay *et al.*, 1993b), in human bronchus 15 min incubation with 0.3 μM ET-1 elicited a significant stimulation in the release of the various prostanoids measured, most notably PGD_2, PGE_2 and 6-keto $PGF_{1\alpha}$. BQ-123 (10 μM) inhibited the ET-1-induced release of these prostanoids by 100%, 77% and 91%, respectively (Figure 7). BQ-123 did not affect the spontaneous release of the prostanoids; PGD_2: control = 1363 ± 350 ng g^{-1} tissue wet weight; + BQ-123 = 1401 ± 376 ng g^{-1}; 9α, 11β PGF_2: control = 126 ± 42 ng g^{-1}; + BQ-123 = 89 ± 40 ng g^{-1}; PGE_2: control = 4985 ± 1110 ng g^{-1}; + BQ-123 = 3599 ± 667 ng g^{-1}; $PGF_{2\alpha}$: control = 773 ± 127 ng g^{-1}; + BQ-123 = 569 ± 34 ng g^{-1}; TxB_2: control = 212 ± 80 ng g^{-1}; + BQ-123 = 200 ± 58 ng g^{-1}; 6-keto $PGF_{1\alpha}$: control = 2566 ± 607 ng g^{-1}; + BQ-123 = 2141 ± 492 ng g^{-1}; $n = 4$; $P>0.05$.

Discussion

The major findings of the present study can be summarized as follows: (1) different ET receptor subtypes appear to mediate ET-1-induced contraction in pulmonary vascular smooth muscle (ET_A receptor) compared to airway smooth muscle (non-ET_A receptor, perhaps ET_B) for both human and guinea-pig preparations; (2) regional differences seem to exist in the relative distribution of ET_A and non-ET_A receptors in guinea-pig airways; (3) different receptors appear to mediate contraction (non-ET_A, perhaps ET_B) and mediator release (ET_A) in human bronchus; (4) overall guinea-pig pulmonary tissues seem to be good *in vitro* models of ET-1-induced contractions in human isolated pulmonary tissues.

Sarafotoxin S6c, a member of the sarafotoxin family of toxins derived from the venom of the middle Eastern burrowing asp, *Atractaspis engaddensis* (Takasaki *et al.*, 1988a; Kloog & Sokolovsky, 1989), has been shown to be a potent and very selective ET_B receptor agonist, inhibiting [^{125}I]-ET-1 binding in rat hippocampus and cerebellum with a K_i of approximately 20 pM compared to a K_i of about 5 μM in rat atria and aorta (Williams *et al.*, 1991b). Accordingly, it has been utilized as an experimental tool in the characterization and determination of the tissue distribution of ET receptor sub-types (Williams *et al.*, 1991a). In the present study sarafotoxin S6c, in concentrations up to 0.1 μM or 0.3 μM, was without effect on the level of tone in the three vascular tissues examined: human pulmonary artery, guinea-pig aorta and guinea-pig pulmonary artery. This was in contrast to the ability of ET-1 to induce contractions in these tissues. The lack of activity with sarafotoxin S6c in the pulmonary vascular tissues studied is in agreement with a previous report which observed that it produced a minimal contractile response in rabbit isolated aorta (Wollberg *et al.*, 1989). These data would suggest that ET-1-induced contraction in these vascular tissues is not mediated via stimulation of ET_B receptors. However, in contrast to these results in pulmonary vascular tissues sarafotoxin S6c was as potent and effective a contractile agonist as ET-1 in human bronchus and guinea-pig bronchus.

Further information on the ET receptor subtypes responsible for ET-1-induced contractions in the above preparations was provided by use of BQ-123, a cyclic pentapeptide analogue of a substance isolated from *Streptomyces misakiensis* (Ihara *et al.*, 1992a). Binding and functional studies

indicate that BQ-123 is a potent and selective ET_A receptor antagonist. Thus, BQ-123 possessed an IC_{50} of 7.3 nM for inhibition of [^{125}I]-ET-1 binding on porcine aortic smooth muscle cells (ET_A tissue), but was over 1 000 fold less potent for inhibition of [^{125}I]-ET-1 binding in an ET_B-rich tissue, porcine cerebellar membranes ($IC_{50} = 18\ \mu M$) (Ihara *et al.*, 1992a). BQ-123 potently and surmountably antagonized ET-1 concentration-response curves in human pulmonary artery, guinea-pig aorta and guinea-pig pulmonary artery. In contrast, BQ-123, even at a high concentration of 10 μM, was without effect on contraction elicited by ET-1 in human bronchus or guinea-pig bronchus. The potency of BQ-123 in some of the experiments and tissues used in the present study ($pK_B = 6.0-7.6$) was similar to that observed for antagonism of ET-1-induced contraction of porcine isolated coronary artery ($pA_2 = 7.4$) (Ihara *et al.*, 1992a), although the data of these two studies using this compound would perhaps suggest some differences in its potency in different tissues. However, further studies using different, selective ET_A antagonists are required to determine whether these preliminary findings actually indicate evidence for ET_A receptor subtypes which exhibit different sensitivity to compounds such as BQ-123.

Collectively, the results with sarafotoxin S6c and BQ-123 would suggest strongly that ET-1-induced contractions in human pulmonary artery, guinea-pig aorta and guinea-pig pulmonary artery are mediated via ET_A receptor activation, whereas responses in human bronchus and guinea-pig bronchus involve a non-ET_A receptor subtype. Regarding the latter tissues, one possibility is that contractions induced by ET-1 are mediated by ET_B receptor activation. However, the definitive test of this postulate requires the examination of the effects of a potent and selective ET_B receptor antagonist on ET-1-induced contractions in these tissues. It has been reported recently that IRL 1038, [Cys11–Cys15]-ET-1 (11-21), is a potent and selective ET_B receptor antagonist, with much greater affinity for the ET_B receptor ($K_i = 6-11$ nM) in various tissues than the ET_A receptor ($K_i = 0.4-0.7\ \mu M$) (Urade *et al.*, 1992). Functionally, IRL 1038 antagonized ET-3-induced contractions in guinea-pig ileum and guinea-pig trachea but was without effect on responses elicited by ET-3 in rat aorta (Urade *et al.*, 1992); the effects against ET-1-induced responses were not examined. The antagonism produced by IRL 1038 did not appear to be competitive in nature, as there was a significant decrease in the maximum contraction produced by ET-3. Another possibility is that ET-1-induced responses in human bronchus and guinea-pig bronchus involve a non-ET_A, non ET_B receptor subtype which is stimulated by sarafotoxin S6c. This may be similar to the ET receptor proposed by Harrison and co-workers in pig coronary artery, which appears to recognize sarafotoxin S6c and ET-3, but not ET-1 or sarafotoxin S6b and which was concluded to have properties of a non-ET_A or non-ET_B receptor (Harrison *et al.*, 1992). Alternatively, it is possible that it is the putative ET_C receptor (Martin *et al.*, 1990; Samson *et al.*, 1990; Masakai *et al.*, 1992). However, this appears unlikely as this receptor exhibits selectivity for ET-3, whereas ET-1, ET-2 and ET-3 all have been shown to contract human bronchus with a rank order for potency and efficacy of: ET-1 > ET-2 = ET-3 (Advenier *et al.*, 1990; Hemsén *et al.*, 1990).

From the results of the present study it would appear that, from a functional standpoint, the ET receptors in human bronchus and pulmonary artery are relatively homogeneous, being non-ET_A and ET_A, respectively. This contrasts with guinea-pig airways in which both receptor types are present, and, furthermore, there appear to be appreciable regional differences in the relative distribution of ET_A and non-ET_A receptors. Thus, descending from the upper trachea to the primary bronchus there is an increase in the effectiveness of sarafotoxin S6c for eliciting contraction. In addition, BQ-123 potently antagonized ET-1-induced contraction in all regions of guinea-pig trachea but was without significant effect in bronchus. It remains to be determined whether similar regional differences in the relative distribution of ET receptor subtypes also occurs in human airways.

A high concentration of ET-1 (0.01–0.3 μM) elicits the release of a variety of prostanoids in guinea-pig trachea and human bronchus (Hay *et al.*, 1993b,c; present study). Thus, in these tissues the ET-1 concentration-response curve for prostanoid release is shifted to the right compared with the curve for contraction. *In vivo* evidence suggests that a significant component of ET-1-induced bronchospasm in guinea-pigs is due to the release of cyclo-oxygenase products (Payne & Whittle, 1988; Nambu *et al.*, 1990), however, *in vitro* the role of prostanoids on the ET-1-induced contractile response in guinea-pig isolated airways appears to be minimal (Hay *et al.*, 1993b,c). In contrast to ET-1-induced contraction in human bronchus, the ability of ET-1 to stimulate prostanoid release was effectively antagonized by BQ-123, suggesting it is an ET_A-receptor-mediated event. Studies exploring the cellular site of release in guinea-pig airways suggest that it is not the epithelium or smooth muscle *per se* (Hay *et al.*, 1993c); one possibility is the blood vessels including the endothelium (Filep *et al.*, 1991). In agreement with the present set of experiments, in rat perfused lung ET-1-induced release of prostacyclin was substantially antagonized by BQ-123 (1 μM) (D'Orléans-Juste *et al.*, 1992), providing further evidence that ET-1-induced prostanoid release in airways is mediated via activation of ET_A receptors. In the same study, BQ-123 only partially inhibited, by about 60%, [^{125}I]-ET-1 binding in rat lung membranes, suggesting the existence of both ET_A and non-ET_A receptors.

There is considerable biochemical and molecular biological evidence for the existence of multiple ET receptor subtypes in lungs. Thus, in rat lung membranes two receptors were identified, one with a molecular weight of 44 kD, which possessed a higher affinity for ET-1 or ET-2 compared with ET-3, and another receptor (molecular weight = 32 kD) which had selectivity for ET-3 (Masuda *et al.*, 1989). Ligand binding and affinity labelling studies in porcine lung also provided evidence for different ET receptors which possessed different affinities for the ETs and sarafotoxins (Takayanagi *et al.*, 1991). In fact the lung has been employed frequently in molecular biological studies which have resulted in the identification, cloning and expression of cDNA for the designated ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Sakamoto *et al.*, 1991; Masaki *et al.*, 1992). In addition, rat and bovine lung have been used for the solubilization and purification of the ET_A and ET_B receptors (Kondoh *et al.*, 1991; Kozuka *et al.*, 1991; Hagiwara *et al.*, 1992). It is apparent that the lung is a rich source of various ET receptor subtypes, including ET_A and ET_B. As yet the precise cellular distribution and functional consequences of activation of these receptors remain largely undefined. The use of selective ligands for the various receptors, such as sarafotoxin S6c and BQ-123, as well as newer compounds, will be critical to answering these and other important questions related to the physiological and pathophysiological roles of the ETs in the pulmonary system. Recent work utilized BQ-123 and [Ala^{1,3,11,15}]-ET-1 (4Ala ET-1), the selective ET_B receptor ligand, to explore the distribution of ET receptor subtypes in porcine pulmonary tissues, and it was concluded that the blood vessels and bronchi possessed large amounts of ET_A receptors, whereas the lung parenchyma was rich in ET_B receptors (Nakamichi *et al.*, 1992). Thus, BQ-123 inhibited [^{125}I]-ET-1 binding in bronchus and lung parenchyma by about 65% and 30%, respectively, whereas with [Ala^{1,3,11,15}]-ET-1 the relative displacement was 25% and 60%; the combination of both compounds was required to inhibit completely [^{125}I]-ET-1 binding in both tissues.

Species differences appear to exist in the ET receptor subtypes responsible for ET-induced contraction of pulmonary tissues. Thus, in the present series of experiments, using guinea-pig and human pulmonary tissues, ET-1-induced contraction of vascular and airway smooth muscle is mediated predominantly via activation of ET_A and non-ET_A receptors,

respectively. In contrast, binding and contraction studies using various ligands provided evidence that contraction of rabbit pulmonary artery was produced predominantly via stimulation of non-ET_A receptors, which were designated as being of the ET_B receptor subtype (Ihara *et al.*, 1992b; Panek *et al.*, 1992). Furthermore, it was reported recently that bronchoconstriction elicited by aerosol administration of ET-1 in sheep *in vivo* was antagonized by a specific ET-1 receptor antagonist, and it was concluded that ET_A-like receptors predominate in sheep airways (Abraham *et al.*, 1993).

In conclusion, the potent and selective ligands sarafotoxin S6c and BQ-123 appear to be effective tools for the characterization of ET-receptor subtypes mediating ET-1-induced contraction in human and guinea-pig isolated pulmonary tissues. Thus, these data suggest differences in the ET receptor sub-types responsible for contraction in pulmonary vascular smooth muscle tissues (ET_A) compared to airway smooth muscle preparations (non-ET_A, perhaps, ET_B). Furthermore, contraction and prostanoid release induced by ET-1 in human bronchus appear to involve non-ET_A (ET_B?) and ET_A receptors, respectively. In addition, regional differences are apparent in the relative distribution of ET receptors in guinea-pig airways. Nevertheless, guinea-pig pulmonary tissues appear to be appropriate *in vitro* models of ET-1-induced contraction in human isolated pulmonary tissues. The availability of potent and selective agonists and, more importantly, antagonists for the various ET receptor subtypes will permit not only further characterization of their relative tissue distributions and functional effects, but should assist in the elucidation of the role of the ETs in the pathophysiology of disorders, including those of the pulmonary system, and may ultimately lead to the developement of novel therapeutics.

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Nitric oxide-mediated changes in vascular reactivity in pregnancy in spontaneously hypertensive rats

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1 To examine the mechanisms which may account for pregnancy-induced vasodilatation in spontaneously hypertensive rats (SHR), we have investigated the changes in vascular reactivity and the effects of endothelial nitric oxide (NO) inhibition in the *in situ* blood-perfused, mesenteric resistance vessels of 18–20 day pregnant SHR. The effects of N^G-nitro-L-arginine (L-NOARG) were compared in pregnant and nonpregnant SHR and gestation matched normotensive Wistar-Kyoto (WKY) rats.

2 Intra-arterial mean blood pressures (MBP) were similar in pregnant and nonpregnant SHR. Basal perfusion pressures (BPP) were decreased in pregnant compared with nonpregnant SHR. Pregnant WKY had lower MBP and BPP than either pregnant or nonpregnant SHR.

3 Vasoconstrictor responses to electrical stimulation (ES) and intra-arterial noradrenaline (NA) were decreased in pregnant compared with nonpregnant SHR. These responses were still greater in pregnant SHR when compared with pregnant WKY. Vascular reactivity to angiotensin II (AII) in pregnant SHR was reduced to a similar level to that in pregnant WKY.

4 L-NOARG (5 mg kg^{-1}, i.v.), an inhibitor of nitric oxide synthase, increased MBP and BPP in all groups. After L-NOARG, BPP were equalized between pregnant and nonpregnant SHR. Pregnant WKY still showed lower MBP and BPP than SHR groups.

5 L-NOARG potentiated vascular responses to ES, NA and AII in all groups. The blunted vascular responses to NA and ES were normalized and the reactivity to AII was only partially reversed in pregnant SHR compared with nonpregnant SHR. Pregnant WKY still had much lower vascular responses to ES and NA than either pregnant or nonpregnant SHR. L-NOARG enhanced vascular responses to AII to a greater extent in pregnant SHR than in pregnant WKY.

6 These results demonstrate that blunted responses to NA and ES were NO-dependent, while diminished reactivity to AII was only partially dependent on NO in the *in situ* blood perfused mesenteric resistance vessels of pregnant SHR.

7 The present results in pregnant SHR differ from our previous finding with pregnant normotensive WKY, in which blunted responses to NA, but not to ES, were equalized by L-NOARG. Pregnancy-induced vasodilatation in hypertensive rats appears to be more dependent on endothelial NO than in normotensive WKY. A defect of the endothelial NO generating pathway which promotes vasodilatation in pregnancy may contribute to the predisposition of women with essential hypertension to develop pre-eclampsia.

Keywords: Vasoconstrictor response; N^G-nitro-L-arginine, nitric oxide; blood perfused mesenteric resistance vessels; pregnant spontaneously hypertensive rat

Introduction

Late pregnancy in normotensive rats is characterized with lower systemic arterial mean blood pressure (MBP), widespread vasodilatation and decreased pressor responses to various vasoconstrictor agents (Paller, 1984; Molnar & Hertelend, 1992; Chu & Beilin, 1993). These changes appear to be, at least in part, related to the increase in or the sensitivity to endogenous endothelial nitric oxide (NO). In studies with conscious pregnant rats, decreased MBP and reduced systemic pressor responses to intravenous administration of noradrenaline (NA), arginine vasopressin (AVP) and angiotensin II (AII) have been reported to be restored by N^G-nitro-L-arginine (L-NOARG), a potent nitric oxide synthase inhibitor (Molnar & Hertelendy, 1992). However, in our previous study with an *in situ* blood-perfused mesenteric bed of pregnant WKY, we found that L-NOARG completely reversed the blunted vascular reactivity to NA, but did not normalize the lower MBP and the diminished mesenteric vascular responses to electrical stimulation of sympathetic nerves (ES) and AII (Chu & Beilin, 1993). These findings suggested that endothelial NO, generated from L-arginine, contributes to pregnancy-induced vasodilatation by modulating the effect of circulating NA, but that other, as yet unidentified mechanisms, are also involved in modulating the effects of sympathetic nerve activity and circulating AII in pregnant normotensive rats.

Pre-existing essential hypertension predisposes to pre-eclampsia in human pregnancy (Butler & Alberman, 1958). The mechanisms underlying this predisposition are unclear. The spontaneously hypertensive rat (SHR) mimics some aspects of human essential hypertension, with higher systemic blood pressure, increased vascular reactivity and altered endothelial function (Mulvany & Halpern, 1977; Luscher & Vanhoutte 1986). Furthermore, pregnancy-induced renal vasodilatation is defective in midterm gestational SHR as compared with normotensive pregnant WKY rats (Baylis, 1989). In view of the differences in systemic blood pressure and the differing changes in renal haemodynamics between pregnant SHR and WKY, we postulated that mesenteric resistance vessels of SHR might show different characteristics from WKY during pregnancy, with less blunting of vascular reactivity, and possibly different effects with regard to different agonists. In this study, we have examined the changes in vascular reactivity and the effects of endothelial

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NO inhibition by L-NOARG in the *in situ* blood perfused mesentery of 18–20 day pregnant SHR as compared with age-matched nonpregnant SHR, and age- and gestation-matched, pregnant WKY. A nonpregnant WKY group was not included in the present study as we used pregnant and nonpregnant WKY in our previous work (Chu & Beilin, 1993). Some of this work has been presented previously in abstract form (Chu & Beilin, 1992a).

Methods

Pregnant rat models

Eighteen to 20 day pregnant SHR (14 week old), age-matched virgin SHR and age- and gestation-matched pregnant WKY rats were used. Pregnancy was produced by mating an oestrous phase female with a normal male rat. The day when a plug was found was labelled as day 1 of pregnancy. Pregnancy was further confirmed by examining the pups during the experiment. Fifteen animals were initially designated to each group. Results from unsuccessful pregnant animals (no pups, dead pups and/or reabsorbed pups) were discarded.

Vascular reactivity

The *in situ* blood perfused mesenteric preparations and the methods for obtaining dose-effect curves have been described elsewhere (Chu & Beilin, 1993), and are briefly outlined here. The preparation is based on the method of Jackson & Campbell (1980). Animals were anaesthetized with α-chloralose (150 mg kg^{-1}, s.c.). The left femoral artery was cannulated for intra-arterial mean blood pressure measurement with a pressure transducer connecting to a Grass model 7B polygraph. The left femoral vein was also cannulated for continued 0.9% NaCl supplementation (0.1 ml min^{-1}) and intravenous drug administration. After complete haemostatis had been achieved by use of electrical cautery, heparin was administered (1500 units kg^{-1}, i.v.). Blood was perfused from left carotid to superior mesenteric artery by a roller pump at a constant flow rate of 2 ml min^{-1}. Vascular reactivity was indicated by the changes of perfusion pressure in this constant flow system. In order to abolish central nervous influences on the preparation, the mesenteric artery proximal to the cannulation point and the surrounding tissues were severed. Bipolar platinum electrodes were then placed around superior mesenteric artery for sympathetic nerve stimulation. Finally the abdomen was covered with moistened gauze and the body temperature was maintained at 37°C by a lamp above the animal.

Vascular responses to noradenaline and electrical stimulation After 30 min equilibration, dose-dependent responses to NA (100–560 ng 0.05 ml^{-1}) were constructed by bolus injection at 3 min intervals. The preparation was allowed to wash out for a 20 min period and frequency-dependent responses to ES (3–9 Hz, 15 V, 1 ms, until maximal response) were obtained at 3 min intervals. The animal was then treated with L-NOARG (5 mg kg^{-1}, i.v.) and after 30 min the response curves to NA and ES were repeated.

Contractile reactivity to angiotensin Responses to AII were studied in separate groups of animals. Dose-response curves to AII (10–300 ng 0.05 ml^{-1}) were constructed at 10 min intervals starting at 30 min equilibration. The dose-response curves were then repeated after pretreatement with L-NOARG (5 mg kg^{-1}, i.v.) for 30 min.

Drugs

Noradrenaline bitartrate, AII and L-NOARG were purchased from Sigma chemicals. Heparin sodium was obtained from Delta West, Western Australia. α-Chloralose was from BDH chemicals.

All drugs were dissolved in 0.9% NaCl and solutions were made up freshly each day.

Statistical analysis

Data are expressed as mean ± s.e.mean with n = number of rats. Differences in blood pressures or basal perfusion were first analysed by one way analysis of variance (ANOVA). If a significant group effect was found, the differences between the three groups were further tested by an unpaired two tail t test.

To compare dose-dependent responses to NA and AII or frequency-dependent responses to ES, areas under individual curves were calculated mathematically as described previously (Matthews *et al.*, 1990; Chu & Beilin, 1993). Then the means of the areas of the three groups were analysed by ANOVA. The significance of differences between groups was further examined by unpaired two tailed t test. A P value <0.05 was considered to be significant.

Results

Blood pressures

MBP were similar between pregnant and nonpregnant SHR (Table 1). Pregnant WKY had lower MBP than either pregnant or nonpregnant SHR. L-NOARG significantly increased MBP in all three groups.

Vascular reactivity

Basal perfusion pressure (BPP) was decreased in pregnant compared with nonpregnant SHR (Table 1). Pregnant WKY had lower BPP than either pregnant or nonpregnant SHR groups. L-NOARG increased BPP in all three groups and abolished the difference between pregnant and nonpregnant SHR.

Mesenteric vascular responses to NA were reduced in pregnant (area = 208 ± 9, n = 13) compared with nonpregnant (area = 275 ± 9, n = 15, $P<0.01$) SHR (Figure 1a). Pregnant WKY showed decreased responses to NA (area = 129 ± 7, n = 14) as compared with either pregnant ($P<0.01$) or nonpregnant ($P<0.01$) SHR. In the preparations pretreated with L-NOARG, responses were enhanced in all three groups and the difference between pregnant (area = 373 ± 31, n = 13) and nonpregnant (area = 405 ± 16, n = 15) SHR was abolished. After L-NOARG, pregnant WKY still showed lower reponses (area = 168 ± 7, n = 14, $P<0.01$) than SHR groups (Figure 1b).

Reactivity to ES was also blunted in pregnant (area = 170 ± 14, n = 13) compared with nonpregnant (area = 241 ± 27, n = 15, $P<0.01$) SHR (Figure 2a). Pregnant WKY had lower responses (area = 34 ± 5, n = 14) than either pregnant ($P<0.01$) or nonpregnant ($P<0.01$) SHR. After pretreating the animals with L-NOARG the reactivity was increased in all three groups and was equalized between pregnant (area = 413 ± 22, n = 13) and nonpregnant (area = 413 ± 28, n = 15) SHR groups. Pregnant WKY still had diminished reactivity (area = 94 ± 11, n = 14, $P<0.01$) compared with SHR groups (Figure 2b).

AII-induced contractile responses were attenuated to a similar level between pregnant SHR (area = 102 ± 9, n = 12) and pregnant WKY (area = 88 ± 6, n = 11) when compared with nonpregnant SHR (area = 184 ± 17, n = 10, $P<0.01$) (Figure 3a). L-NOARG potentiated the contractile responses in all three groups but the differences between pregnant (area = 199 ± 15, n = 12) and nonpregnant (area = 275 ± 26, n = 10, $P<0.05$) SHR were still evident and the responses in pregnant SHR became greater than in pregnant WKY (area = 132 ± 9, n = 11, $P<0.05$). In order to examine

Table I Effects of N^G-nitro-L-arginine (L-NOARG, 5 mg kg^{-1}) on intra-arterial mean blood pressures (MBP) and mesenteric basal perfusion pressure (BPP) in pregnant rats

	MBP (mmHg)		*BPP* (mmHg)	
	L-NOARG (−)	L-NOARG (+)	L-NOARG (−)	L-NOARG (+)
Nonpregnant SHR	147 ± 5 (n = 14)	204 ± 4 (n = 14)	54.1 ± 1.2 (n = 15)	63.6 ± 3.2 (n = 15)
Pregnant SHR	146 ± 5 (n = 13)	205 ± 3 (n = 13)	48.3 ± 1.1* (n = 13)	63.2 ± 3.8 (n = 13)
Pregnant WKY	95 ± 2† (n = 12)	150 ± 4† (n = 12)	30.6 ± 1† (n = 14)	39 ± 2† (n = 14)

*$P<0.01$ when compared with nonpregnant SHR group.
†$P<0.01$ when compared with either pregnant or nonpregnant SHR groups.
L-NOARG significantly increased MPB and BPP in all groups ($P<0.01$).

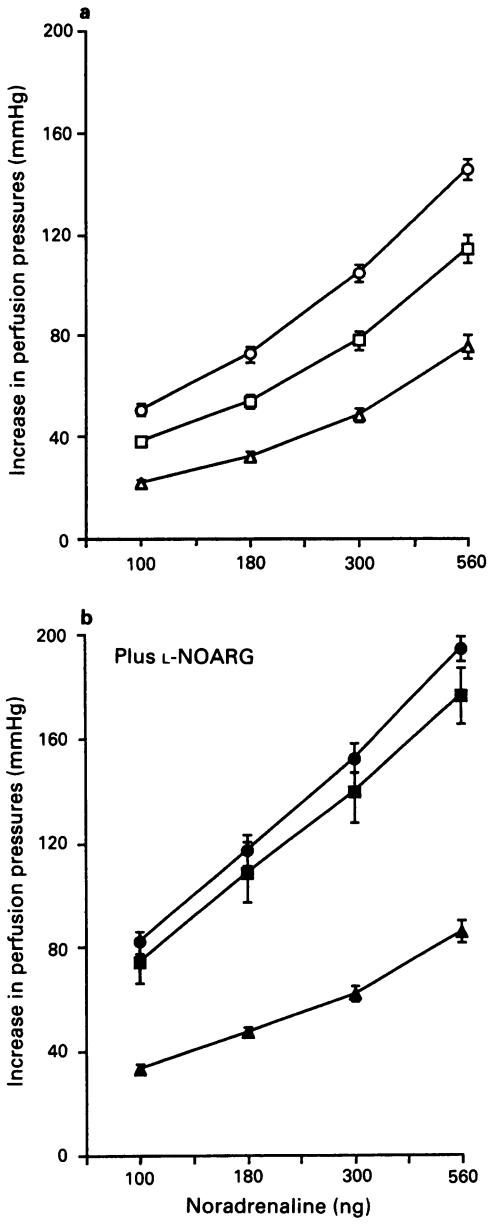

Figure 1 (a) Responses to noradrenaline (NA) were blunted in pregnant SHR (□, n = 13) compared with nonpregnant SHR (○, n = 15, $P<0.01$). Pregnant WKY (△, n = 14) had reduced responses when compared with either pregnant SHR ($P<0.01$) or nonpregnant SHR ($P<0.01$) groups. (b) N^G-nitro-L-arginine (L-NOARG) potentiated vascular responses in all three groups and abolished the difference between pregnant SHR (■, n = 13) and nonpregnant SHR (●, n = 15). After L-NOARG, pregnant WKY (▲, n = 14) still had much lower responses than either pregnant SHR ($P<0.01$) or nonpregnant SHR ($P<0.01$).

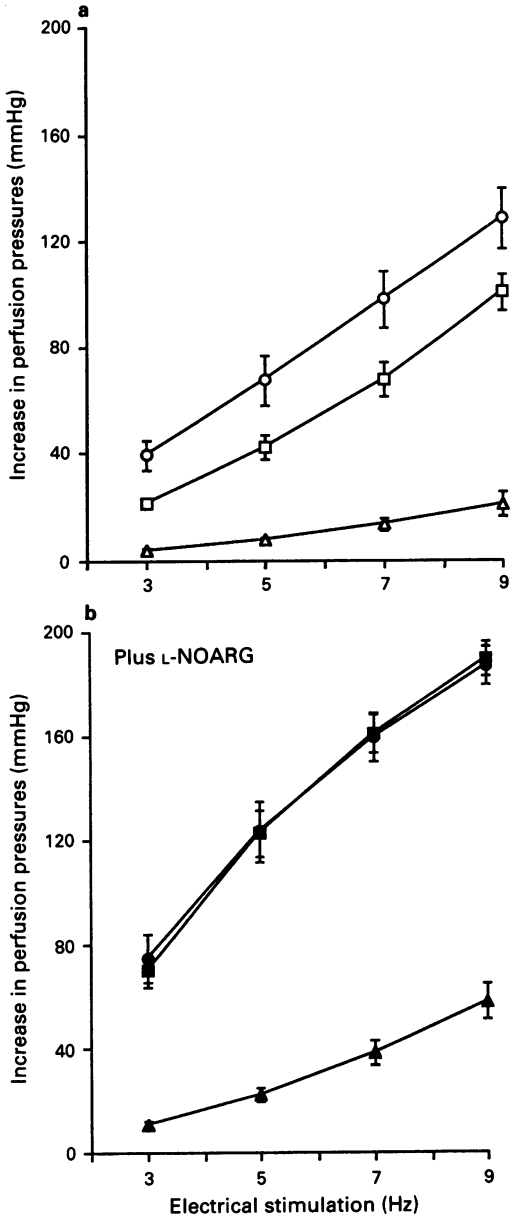

Figure 2 (a) Reactivity to electrical stimulation (ES) was diminished in pregnant SHR (□, n = 13) compared with nonpregnant SHR (○, n = 15, $P<0.01$). Pregnant WKY (△, n = 14) showed obviously reduced responses when compared with either pregnant SHR ($P<0.01$) or nonpregnant SHR ($P<0.01$). (b) N^G-nitro-L-arginine (L-NOARG) enhanced the reactivity in all groups and abolished the differences between pregnant SHR (■, n = 13) and nonpregnant SHR (●, n = 15) groups. After L-NOARG the responses were still decreased in pregnant WKY (▲, n = 14) compared with SHR groups ($P<0.01$).

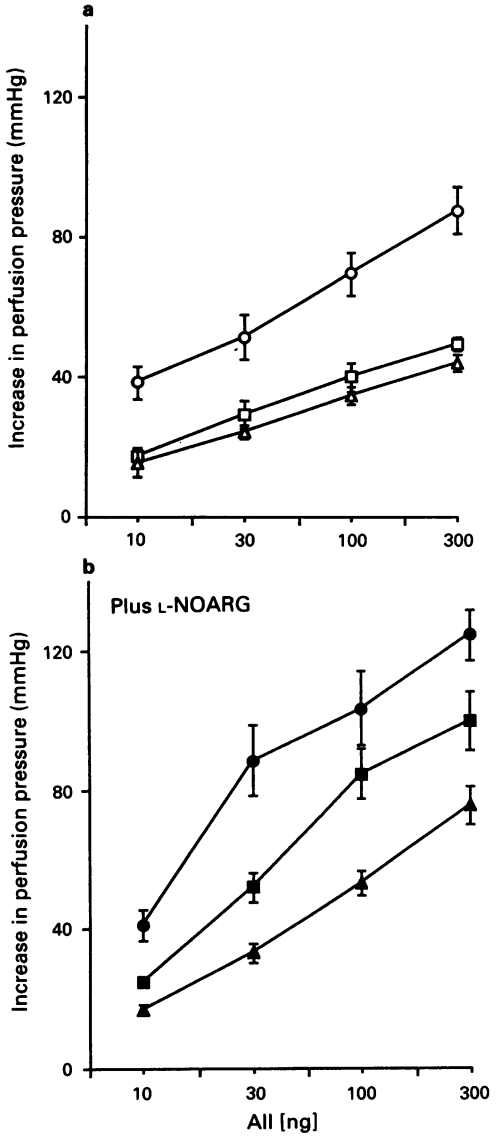

Figure 3 (a) Responses to angiotensin II (AII) in pregnant SHR (□, $n = 12$) were attenuated to a similar level as in pregnant WKY (Δ, $n = 11$) when compared with nonpregnant SHR (○, $n = 10$, $P < 0.01$). (b) In preparations pretreated with L-NOARG, the responses were elevated in all groups. The blunted responses were partly reversed in the pregnant SHR group, but the differences between pregnant SHR (■, $n = 12$) and nonpregnant SHR (●, $n = 10$, $P < 0.05$) were still evident. The responses in pregnant WKY (▲, $n = 11$) were lower than either pregnant SHR ($P < 0.05$) or nonpregnant SHR ($P < 0.01$).

whether the blunted responses to AII are partially reversed by L-NOARG in pregnant SHR, we have made a further comparison between the changes in AII responses after L-NOARG (calculated as percentage of intial response prior to L-NOARG), and found that the responsiveness to AII was increased to a greater extent in pregnant (94.3 ± 12.0%) than in nonpregnant (54.6 ± 13.7%, $P < 0.05$) SHR.

Discussion

In near-term pregnant SHR, reduced tail-cuff systolic blood pressures have been well-documented in previous studies (Lorenz *et al.*, 1984; Yong *et al.*, 1992), while intra-arterial blood pressures varied according to whether an anaesthetic is used. In awake, late pregnant SHR, MBP have been reported to be decreased (Lundgren *et al.*, 1979). In the present study we failed to detect a decrease in MBP in α-chloralose-anaesthetized, pregnant SHR, which is consistent with findings in previous studies with Nembutal plus Brietal sodium anaesthetized pregnant SHR (Yong *et al.*, 1992). L-NOARG substantially increased MBP and BPP in all groups in the present and in our previous studies (Chu & Beilin, 1983). These observations demonstrate that basal endogenous NO plays an important role in the regulation of blood pressure in both pregnant and nonpregnant rats of either normotensive or hypertensive breeds.

Our previous study with *in situ* blood-perfused mesenteric resistance vessels (Chu & Beilin, 1993) and the study by others with conscious animals (Molnar & Hertelend, 1992) have demonstrated that endogenous NO, at least in part, contributes to the blunted vascular reactivity in pregnant normotensive rats. Less information is available whether NO also plays a role in pregnancy-induced vasodilatation in SHR. In the present study, we found that L-NOARG equalized the blunted mesenteric vascular responses to NA and ES, but only partially reversed the reduced responses to AII in pregnant SHR. These results demonstrate that endogenous NO is responsible for the decreased mesenteric vascular responses to NA or ES, and partially involved in reduced reactivity to AII. Furthermore, the effects of L-NOARG on ES responses contrast to our previous findings with pregnant WKY (Chu & Beilin, 1993), in which the diminished responses to ES were not normalized by L-NOARG. These results indicate that an as yet unidentified NO-independent vasodilator mechanism(s) is responsible for the decreased mesenteric vascular reactivity to ES in pregnant WKY while in hypertensive rats, pregnancy-associated vasodilatation appears to be more dependent on NO.

The initial reactivity to AII prior to L-NOARG decreased to a similar level in pregnant SHR as compared with pregnant WKY, but L-NOARG enhanced the responses to a greater extent in pregnant SHR. The greater increases in the mesenteric vascular responses to AII after L-NOARG in pregnant SHR compared with pregnant WKY also suggests that the blunted reactivity to AII is likely to be more dependent on NO in pregnant SHR than pregnant WKY.

What NO-independent mechanism(s) accounts for the attenuated responses to ES seen in pregnant WKY is unclear. It has been well documented that endogenous NA released from sympathetic nerve endings mediates ES-induced vasoconstrictions in rat mesenteric resistance vessels (Jackson & Campbell, 1980; Chu & Beilin, 1993). The release of NA from mesenteric sympathetic nerves is unlikely to be impaired in pregnancy as ES-induced NA spillover was unchanged in the blood perfused mesenteric circulation of pregnant WKY (Chu & Beilin, 1992b). We suggested that an as yet unidentified vasodilator neurotransmitter, which is increased in pregnancy and released during ES, accounts for the blunted vascular responses to ES in pregnant normotensive rats, and may be defective in pregnant hypertensive rats. Calcitonin gene-related peptide (CGRP), may be a candidate for this as CGRP containing fibres in mesentery have been found to be decreased in adult SHR compared with WKY (Kawasaki *et al.*, 1990).

In the present study, basal perfusion pressure was slightly but significantly decreased and vascular reactivity to various vasoconstrictor stimuli was attenuated in the *in situ* blood-perfused mesenteric resistance vessels of 18–20 day pregnant SHR as compared with nonpregnant SHR. These results are consistent with our previous study with pregnant WKY in which a similar blood-perfused, mesenteric preparation was used (Chu & Beilin, 1993), but contrast with our previous findings in which a Krebs-perfused isolated mesenteric preparation was used (Yin *et al.*, 1992). Using the isolated preparation, we found that there were no differences in constrictor responses to noradrenaline, endothelium-dependent relaxations to acetylcholine (ACh) and endothelium-independent relaxations to sodium nitroprusside (SNP) between 12–13 day pregnant and nonpregnant rats.

Yong *et al.* (1992) using a similar isolated mesenteric preparation have also demonstrated that in pregnant SHR, responses to intraluminal noradrenaline were reduced 4 days before delivery but not 1 day before delivery and the reactivity to ES was unchanged on either occasion. In pregnant WKY the responses to intraluminal noradrenaline were unaltered 1 or 4 days before delivery, while the reactivity to ES was decreased only on day 4 before delivery.

The reasons why different results have been obtained from *in vivo* and *in vitro* experiments are not fully understood. Interestingly, vascular responses to prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) also contrast depending on whether *in vivo* or *in vitro* mesenteric vascular preparations are used (Jackson & Campbell, 1980). Several factors may contribute to the differences. Firstly, in the present experiments much smaller doses of agonists or frequency of ES have been applied due to much higher sensitivity of the *in vivo* preparation. Secondly, the *in vivo* blood-perfused mesenteric vascular bed is under more physiological conditions with regard to circulating vasoactive substances, antioxidants and oxygen pressure; while the *in vitro* Krebs-perfused preparation is exposed to a very basic physiological environment. Finally, the effects of any circulating factors originating from the uteroplacental unit or other part of the body in pregnancy may be undetectable in isolated preparations, particularly if they have a short biological half life. Considering the differences between *in vivo* and *in vitro* studies in pregnancy, it is reasonable to speculate that vasodilator substances with relatively short half life originating from foetal/placental unit are responsible for the decreased mesenteric vascular reactivity in pregnant rats.

In summary, the present study demonstrate that blunted mesenteric vascular responses to NA and ES are NO-dependent and may reflect increased vascular NO synthesis in pregnant SHR. The diminished reactivity to AII is only partially dependent on NO in the *in situ* blood perfused mesentery of pregnant SHR and may be partially due to down regulation of AII receptors in response to increased circulating AII levels in pregnancy (Broughton Pipkin *et al.*, 1978; Brown &Venuto, 1986).

These observations taken in conjunction with the lack of pregnancy-induced renal vasodilatation described in hypertensive rats (Baylis, 1989; Baylis & Rechelhoff, 1991) provide one possible mechanism by which women with essential hypertension may be predisposed to pre-eclampsia, as they may be more dependent on NO-mediated pregnancy-induced vasodilatation and may have a defective capacity for endothelial NO synthesis sufficiently to allow adequate systemic, renal and uterine vasodilatation in pregnancy.

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The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat

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1 The effect of endotoxin (*E. coli* lipopolysaccharide) on the induction of nitric oxide synthase (NOS) and the changes in vascular permeability in the colon and jejunum over a 5 h period have been investigated in the rat.

2 Under resting conditions, a calcium-dependent constitutive NOS, determined by the conversion of radiolabelled L-arginine to citrulline, was detected in homogenates of both colonic and jejunal tissue.

3 Administration of endotoxin (3 mg kg^{-1}, i.v.) led, after a 2 h lag period, to the appearance of calcium-independent NOS activity in the colon and jejunum *ex vivo*, characteristic of the inducible NOS enzyme.

4 Administration of endotoxin led to an increase in colonic and jejunal vascular permeability after a lag period of 3 h, determined by the leakage of radiolabelled albumin.

5 Pretreatment with dexamethasone (1 mg kg^{-1} s.c., 2 h prior to challenge) inhibited both the induction of NOS and the vascular leakage induced by endotoxin.

6 Administration of the NO synthase inhibitor N^{G}-monomethyl-L-arginine (12.5–50 mg kg^{-1}, s.c.) 3 h after endotoxin injection, dose-dependently reduced the subsequent increase in vascular permeability in jejunum and colon, an effect reversed by L-arginine (300 mg kg^{-1}, s.c.).

7 These findings suggest that induction of NOS is associated with the vascular injury induced by endotoxin in the rat colon and jejunum.

Keywords: Nitric oxide; inducible nitric oxide synthase; endotoxin; vascular permeability; intestinal inflammation; corticosteriods; NO synthase inhibitor; N^{G}-monomethyl-L-arginine

Introduction

The formation of the endothelium-derived vasodilator mediator, nitric oxide (NO) from L-arginine, by the calcium-dependent constitutive enzyme NO synthase (NOS) is involved in the regulation of the cardiovascular system (Palmer *et al.*, 1987; 1988; Palmer & Moncada, 1989; Moncada *et al.*, 1991). NO thus plays a physiological role in the regulation of gastro-intestinal blood flow (Pique *et al.*, 1989; 1992a,b; Walder *et al.*, 1990). NO is also involved in the regulation of gastric mucosal integrity, interacting with other local protective mediators (Whittle *et al.*, 1990). In addition, NO is involved in the modulation of intestinal vascular integrity under physiological conditions (Kubes & Granger, 1992) as well as pathological situations following acute endotoxin challenge (Hutcheson *et al.*, 1990). Thus, the inhibitor of NOS, N^{G}-monomethyl-L-arginine (L-NMMA), markedly enhances the vascular permeability and haemorrhage produced after 15 min in the jejunum by high doses of endotoxin (Hutcheson *et al.*, 1990), effects reversed by the nitrosothiol NO donor, S-nitroso-*N*-acetyl penicillamine (Boughton-Smith *et al.*, 1990; 1992b).

Endotoxin and some cytokines can induce a calcium-independent NOS in vascular tissue (Busse & Mulsch, 1990; Radomski *et al.*, 1990; Szabo *et al.*, 1993). The induction of NOS, leading to the excessive production of NO has also been implicated in the endothelial cell damage *in vitro* brought about by prolonged incubation with endotoxin and cytokines (Palmer *et al.*, 1992). Experimental and clinical studies suggest that excessive NO production has an important pathological role in the hypotension, hyporesponsiveness to vasoconstrictors and the cardiovascular collapse associated with septic shock (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Fleming *et al.*, 1991; Nava *et al.*, 1991; Petros *et al.*, 1991; Wright *et al.*, 1992; Szabo *et al.*, 1993). The anti-inflammatory corticosteroid, dexamethasone inhibits the induction of NOS by endotoxin and cytokines both *in vitro* and *in vivo* in vascular tissue (Radomski *et al.*, 1990; Knowles *et al.*, 1990), an effect that may contribute to beneficial actions of corticosteroid pretreatment in experimental septic shock (Wright *et al.*, 1992).

In the present study, the relationship between the induction of NOS and the increase in vascular permeability produced in the rat jejunum and the colon over a 5 h period following administration of endotoxin has been determined in the rat. The effects of dexamethasone on the induction of NOS and vascular leakage produced by endotoxin have also been investigated. In addition, the actions of L-NMMA, administered at a time of detectable expression of the inducible NOS, on the intestinal vascular injury has been evaluated.

A preliminary account of this work has been presented to the British Pharmacological Society (Boughton-Smith *et al.*, 1992a).

Methods

Nitric oxide synthase activity

Lipopolysaccharide (LPS) from *E. coli* (3 mg kg^{-1}) or the vehicle, isotonic saline (2 ml kg^{-1}) was administered via the tail vein, to male Wistar rats (225–275 g) under halothane anaesthesia. This dose of LPS was selected from previous dose-response studies as near-maximal for the induction of NOS (Knowles *et al.*, 1990).

Nitric oxide synthase activity (NOS) in the jejunal and colonic tissues was measured as the conversion of L-[^{14}C]-arginine monohydrochloride to [^{14}C]-citrulline, based on the

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method described by Knowles et al. (1990). Intestinal tissue was taken at the various time intervals over 5 h, and homogenized (20 s, Ultra-Turrax; 5 mm blade) in buffer (250 mg ml^{-1}) containing HEPES (40 mM), sucrose (32 mM), DL-dithiothreitol (1 mM), leupeptin (10 $\mu g\ ml^{-1}$), soybean trypsin inhibitor (10 $\mu g\ ml^{-1}$) and aprotonin (2 $\mu g\ ml^{-1}$).

Following centrifugation (10 000 g, 20 min 4°C), an aliquot of the supernatant (40 µl) was added to a tube containing 100 µl of pre-warmed (37°C) incubation buffer containing (final concentration) potassium phosphate (50 mM; pH 7.4); L-valine (6 mM); NADPH (100 µM); $MgCl_2$ (1 mM) and $CaCl_2$ (200 µM), L-arginine (20 µM) and L-[^{14}C]-arginine monohydrochloride (0.271 µCi, 11.8 GBq $nmol^{-1}$) and incubated for 10 min at 37°C. The reaction was terminated by the addition of 500 µl of a mixture of H_2O:Dowex-AG50W (1:1 v/v, 200–400, 8% cross-linked, Na^+ form), prepared by washing H^+ form of the resin with 1 M NaOH, four times and then washing with purified water until the pH was less than 7. The resin-incubate mix was dispersed and diluted by the addition of 860 µl of purified water. The resin was allowed to settle (30 min) and 975 µl of supernatant taken for scintillation counting (2 ml Pico-Fluor; Beckman LS400).

Characterization of nitric oxide synthase

Product formation that was inhibited by *in vitro* incubation with L-NMMA (300 µM) was taken as an index of NOS activity and calculated from the total of added substrate, as the formation of citrulline, nmol $min^{-1}\ g^{-1}$ of tissue.

The activity of NOS in the intestinal tissue was further characterized *in vitro* by incubation with EGTA (1 mM) to determine the dependence of the enzymic activity on calcium. The calcium-dependent activity under control conditions was taken as constitutive NOS, while that not inhibited by EGTA incubation following endotoxin challenge was taken as a calcium-independent inducible isoform of NOS (Salter et al., 1991).

Plasma leakage

Intestinal vascular permeability was determined as the leakage into the jejunal and colonic tissue of [^{125}I]-labelled human serum albumin ([^{125}I]-HSA) administered intravenously (0.2 ml; 0.5 µCi, 1.85 GBq) immediately after either LPS or isotonic saline. At various times (1 to 5 h) after LPS or saline administration, segments of jejunal and colonic tissue were ligated and removed. The intestinal tissues were rapidly washed, blotted dry and weighed. Blood (0.5 ml) from the abdominal aorta was collected into tubes containing trisodium citriate (0.318% final concentration) and plasma prepared by centrifugation (10 000 g × 10 min). The [^{125}I]-HSA content in segments of whole tissue and in aliquots of plasma (100 µl), was determined in a gamma spectrometer (Nuclear Enterprises NE1600). The total content of plasma in the intestinal tissues was expressed as µl g^{-1} of tissue.

Intravascular volume

Changes in intravascular volume in the intestinal tissue was determined in an additional group of rats by administering [^{125}I]-HSA (0.5 µCi) intravenously via the tail vein, at each time point, 2 min before tissue removal. The tissue and plasma content of radiolabel was determined and intravascular volume expressed as µl g^{-1} tissue. This value was subtracted from that obtained in the plasma leakage studies to obtain a measure of the intestinal plasma albumin leakage.

Effect of dexamethasone or L-NMMA

Groups of rats were pretreated with dexamethasone (1 mg kg^{-1}, s.c.) 2 h before intravenous administration of saline or LPS, in a dose derived from previous dose-response studies on the inhibition of the induction of NOS by endotoxin in the rat (Knowles et al., 1990). The NOS activity and vascular leakage of radiolabelled albumin were determined 4 h after LPS administration.

In a further study, L-NMMA (12.5–50 mg kg^{-1}, s.c.) was administered 3 h after LPS, at a time when induction of NO synthase was detectable. Vascular leakage of radiolabelled albumin in jejunum or colon was determined 1 h later (i.e. 4 h after LPS). In an additional group, L-arginine (300 mg kg^{-1}, s.c.) was administered 15 min prior to L-NMMA (50 mg kg^{-1}). In control experiments in the absence of LPS, the effects of L-NMMA (50 mg kg^{-1}, s.c.) on vascular leakage in jejunum and colon were determined 1 h after administration.

Drugs and materials

N^G-monomethyl-L-arginine (L-NMMA) was synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. *E. coli* lipopolysaccharide (0111:B4), and L-arginine hydrochloride were from Sigma Chemical Company (Poole, Dorset), L-[U-^{14}C] arginine monohydrochloride and [^{125}I]-labelled human serum albumin were from Amersham International (U.K.). Dexamethasone was supplied as the injectable form (Decadron, Merck Sharp & Dohme Herts). All other reagents were from the Sigma Chemical Company.

Statistical analysis

The data are expressed as the mean ± s.e.mean of (n) rats per experimental group. Statistical comparisons were made

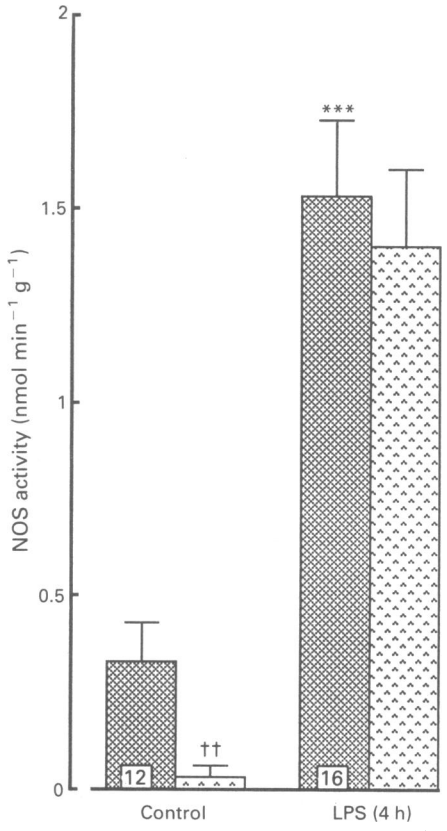

Figure 1 Increase in nitric oxide synthase (NOS) activity in rat jejunal tissue 4 h following challenge with LPS (*E. coli*, 3 mg kg^{-1}, i.v.). NOS activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol $min^{-1}\ g^{-1}$ tissue) that is abolished *in vitro* by N^G-monomethyl-L-arginine (L-NMMA, 300 µM), in supernatents of jejunal homogenates incubated in the absence (cross hatched columns) and presence (speckled columns) of EGTA (1 mM), is expressed as the mean values ± s.e.mean, of 12 and 16 experiments. A significant increase in NOS activity is given as ***$P < 0.001$, and significant inhibition of NOS activity by incubation with EGTA is shown by ††$P < 0.01$.

by Student's t test for paired and unpaired data as appropriate.

Results

Induction of nitric oxide synthase

Basal NOS activity, that was abolished by incubation *in vitro* with L-NMMA (300 μM), was detected in the supernatants of homogenates of segments of jejunum or colon, being 0.28 ± 0.08 ($n = 20$) and 1.18 ± 0.07 ($n = 12$) nmol min^{-1} g^{-1} of tissue, respectively. This activity in the supernatants from the jejunum (Figure 1) and colon (Figure 2) was abolished by incubation with EGTA (1 mM).

In jejunal tissue, elevated NOS activity was detected 4 h after LPS administration, and that was not significantly inhibited by incubation with EGTA, as shown in Figure 1. In the study on the time-course of NOS induction, administration of LPS (3 mg kg^{-1}, i.v.) had no effect on NOS activity in the colonic tissue when determined 1 and 2 h after challenge (Figure 2). However, 3 to 5 h after LPS administration, NOS activity was significantly ($P < 0.01$) increased in a time-dependent manner (Figure 2). This NOS activity, observed after 3 and 4 h, was only partially inhibited by incubation *in vitro* with EGTA (1 mM), the low level of EGTA-sensitive activity remaining corresponding to the level of calcium-dependent NOS activity seen under resting conditions (Figure 2). At 5 h after LPS administration, incubation with EGTA did not significantly inhibit the elevated colonic NOS activity (Figure 2).

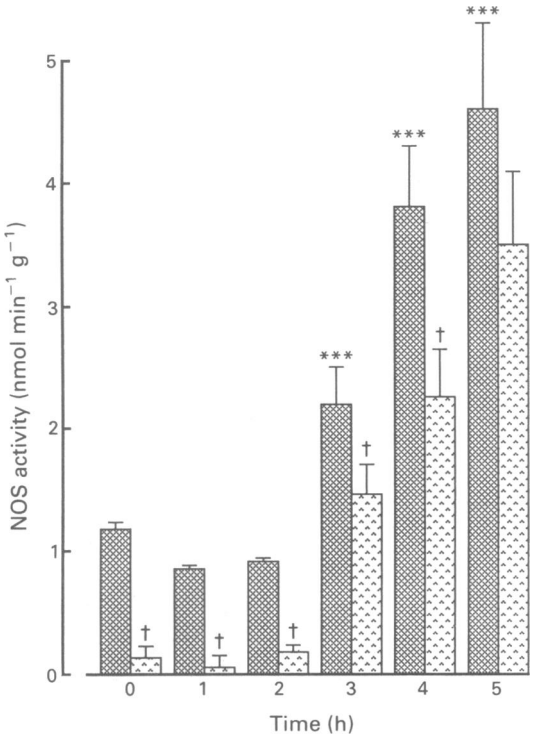

Figure 2 Induction of nitric oxide synthase (NOS) in rat colonic tissue over a 5 h period following challenge with LPS (*E. coli*, 3 mg kg^{-1}, i.v.). NOS activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol min^{-1} g^{-1} tissue), that is abolished *in vitro* by N^G-monomethyl-L-argine (L-NMMA, 300 μM), in supernatant of colonic homogenates incubated in the absence (hatched columns) and presence (speckled columns) of EGTA (1 mM), is expressed as the mean values ± s.e.mean, of 6–8 experiments for each time point. A significant increase in total NOS activity is given as $***P < 0.001$, and significant inhibition of this activity by incubation with EGTA is shown by $\dagger P < 0.01$.

Blood volume and plasma leakage

In control rats receiving bolus intravenous injection of isotonic saline (2 ml kg^{-1}), the intravascular blood volume in the jejunum and colon, determined by the tissue level of radiolabelled human serum albumin injected 2 min prior to tissue removal, did not significantly change over a 5 h period (Table 1). Likewise, intravascular blood volume in the jejunum and colon did not significantly change during the 5 h observation period following administration of LPS (3 mg

Table 1 Intravascular blood volume in rat jejunum and colon over a 5 h period following administration of *E. coli* lipopolysaccharide (LPS)

	Intestinal blood volume (μl g^{-1} tissue)			
	Control		*LPS*	
Time (h)	*Jejunum*	*Colon*	*Jejunum*	*Colon*
0	45 ± 2	66 ± 10	–	–
1	63 ± 9	87 ± 10	56 ± 1	59 ± 16
3	41 ± 8	55 ± 10	47 ± 10	60 ± 11
4	47 ± 6	52 ± 8	39 ± 3	64 ± 5
5	47 ± 2	64 ± 10	53 ± 5	39 ± 9

Intravascular blood volume in the intestinal segments was determined by injection of radiolabelled albumin 2 min prior to the removal of the tissue over a 5 h period in control rats and following administration of LPS (3 mg kg^{-1}, i.v.). Results, shown as blood volume (μl g^{-1} tissue), are mean ± s.e.mean of 4 experiments for each time point. There was no significant difference between control or LPS treatment in either the jejunum or colon at any time.

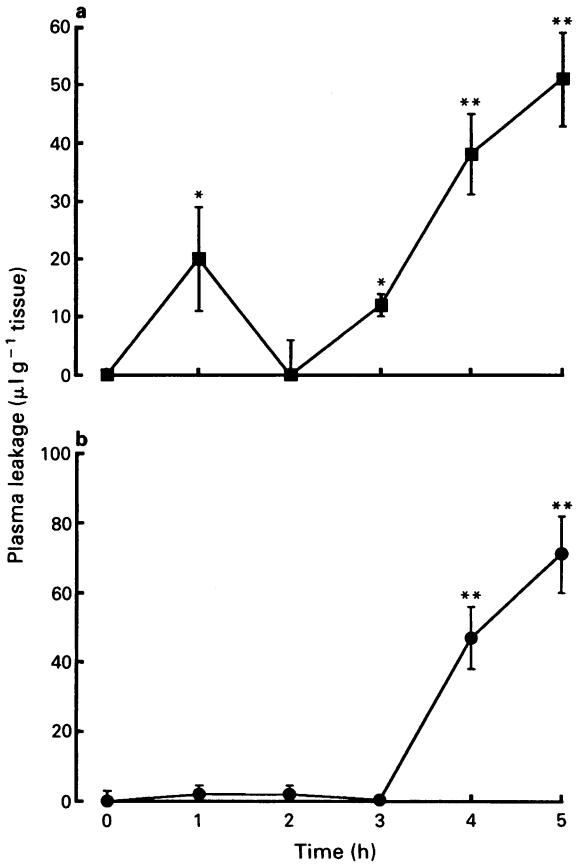

Figure 3 Extravasation of plasma into the rat jejunum (a) and the colon (b) over a 5 h period following challenge with *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.). Results, expressed as the leakage of radiolabelled albumin (μl g^{-1} tissue), are shown as the mean values ± s.e.mean of 6–8 experiments at each time point, where significant difference from control is given as $*P < 0.05$, $**P < 0.01$.

kg^{-1}, i.v.), and furthermore, were not significantly different from those under control conditions (Table 1).

Following administration of LPS (3 mg kg^{-1}, i.v.), there was no change in the plasma leakage into the colon, 1, 2 or 3 h after administration, which remained similar to the low resting levels. However, 4 and 5 h after LPS administration, there was a substantial ($P<0.01$) increase in the plasma leakage (Figure 3). There was also a significant increase in plasma leakage in the jejunum, 3, 4 and 5 h after LPS administration, with a transient initial increase also being observed 1 h after challenge which returned to the resting value after 2 h (Figure 3).

Effect of dexamethasone on nitric oxide synthase activity and plasma leakage

In control rats, pretreatment with dexamethasone (1 mg kg^{-1}, s.c.) 2 h prior to administration of isotonic saline (2 ml kg^{-1}, i.v.) had no significant effect on the calcium-dependent NOS activity in colonic or in jejunal tissue, determined 4 h later (Figures 4 and 5). However, the increase in NOS activity induced by LPS determined 4 h after challenge, was significantly ($P<0.001$) suppressed by pretreatment with dexamethasone in both colonic (Figure 4) and jejunal tissue (Figure 5).

Pretreatment with dexamethasone (1 mg kg^{-1}, s.c.), 2 h prior to LPS challenge abolished the increase in plasma leakage induced by LPS in both the colon and jejunum, determined 4 h after challenge (Figures 4 and 5). Dexamethasone administration had no effect on the plasma leakage in control rats 4 h following saline injection (Figures 4 and 5).

Effect of L-NMMA on plasma leakage

Administration of L-NMMA (12.5–50 mg kg^{-1}, s.c.), 3 h after LPS injection, caused a dose-dependent reduction in radiolabelled albumin leakage in both jejunum and colon, determined 1 h later, as shown in Figure 6. Pretreatment with L-arginine (300 mg kg^{-1}, s.c.) 15 min prior to L-NMMA (50 mg kg^{-1}) abolished ($P<0.001$) this inhibition of albumin leakage in the jejunum and colon (82 ± 8 and 75 ± 7 μl plasma g^{-1} tissue, respectively; $n=8$ for each, not significantly different from LPS alone). L-Arginine (300 mg kg^{-1}) did not itself affect the increases in albumin leakage induced by LPS over this time period ($n=8$ for each; $P>0.05$).

By contrast, under control conditions in the absence of LPS challenge, L-NMMA (50 mg kg^{-1}) did not significantly

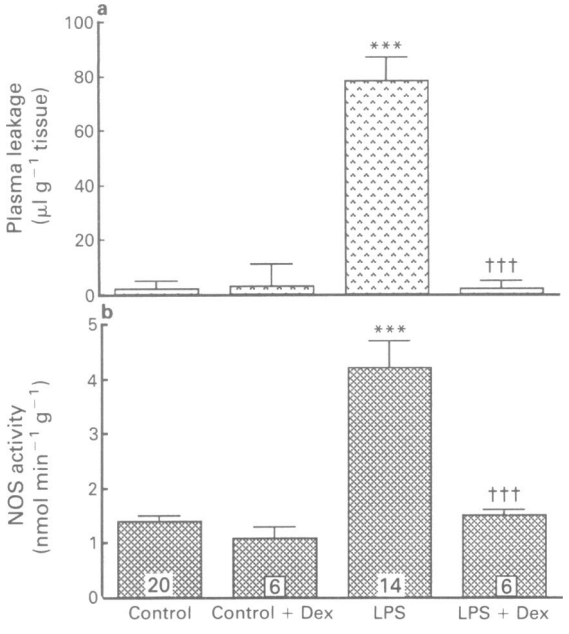

Figure 4 Extravasation of plasma (a) and induction of nitric oxide synthase (NOS) in the rat colon (b) following administration of *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) and the actions of dexamethasone (Dex) pretreatment (1 mg kg^{-1}, s.c., 2 h before LPS challenge). Results, shown as NOS activity (nmol min^{-1} g^{-1} tissue) and leakage of radiolabelled albumin (μl g^{-1} tissue) 4 h following saline (2 ml kg^{-1}, i.v.) or LPS administration with and without dexamethasone pretreatment, are the mean values ± s.e.mean of the number of experiments shown in each column, where significant difference from control values is given as ***$P<0.001$ and inhibition of LPS-induced actions as †††$P<0.001$.

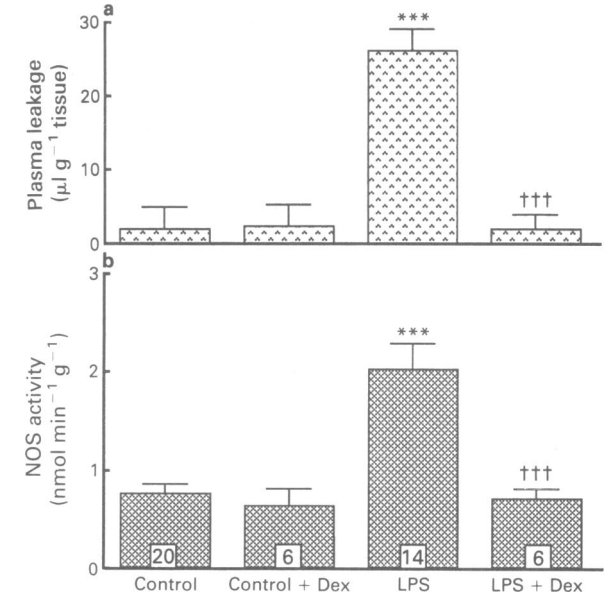

Figure 5 Extravasation of plasma (a) and induction of nitric oxide synthase (NOS) in the rat jejunum (b) following administration of *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) and the actions of dexamethasone (Dex) pretreatment (1 mg kg^{-1}, s.c., 2 h before LPS challenge). Results, shown as NOS activity (nmol min^{-1} g^{-1} tissue) and leakage of radiolabelled albumin (μl g^{-1} tissue) 4 h following saline (2 ml kg^{-1}, i.v.) or LPS administration with and without dexamethasone pretreatment, are the mean values ± s.e.mean of the number of experiments shown in each column, where significant difference from control is given as ***$P<0.001$ and inhibition of LPS-induced actions as †††$P<0.001$.

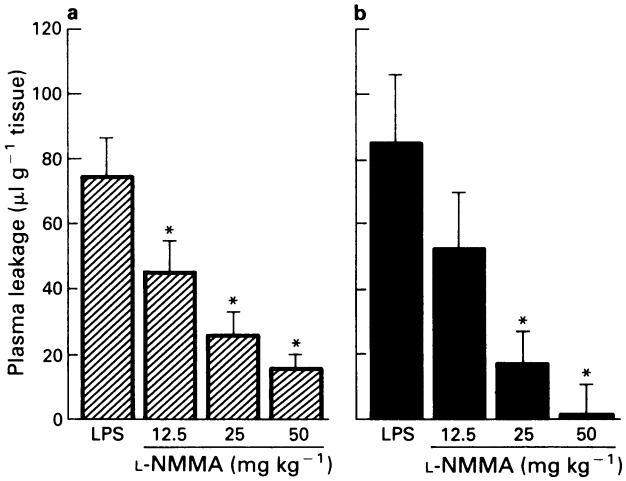

Figure 6 Effects of N^G-monomethyl-L-arginine (L-NMMA, 12.5–50 mg kg^{-1}, s.c.), or isotonic saline (0.4 ml) administered 3 h after challenge with *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) on the increase in leakage of radiolabelled albumin (plasma leakage μl g^{-1} tissue) observed 1 h later (i.e. 4 h after LPS challenge) in the rat colon (a) and jejunum (b). Results are shown as mean values ± s.e.mean of 6–8 experiments in each group, where significant inhibition from LPS group is given as *$P<0.05$.

induce jejunal or colonic albumin leakage determined after 1 h (17 ± 12 and 3 ± 7 μl plasma g^{-1} tissue compared with saline alone, respectively; $n = 6$, $P > 0.05$).

Discussion

In the present study, endotoxin produced *in vivo*, after a lag period of 3 h, a time-dependent increase in vascular leakage of plasma albumin in the rat colon. The change in colonic vascular permeability was preceded by the induction of a calcium-independent NOS activity. Likewise, in the rat jejunum an increase in extravasation of albumin 4 and 5 h after challenge with endotoxin was observed at a time of the induction of the calcium-independent NOS. This temporal relationship is therefore compatible with the concept that excessive NO production by the inducible NOS is involved in the intestinal vascular injury produced by endotoxin after several hours.

The increase in vascular permeability observed in the rat colon several hours after LPS contrasts with our previous study on the acute effects of endotoxin on the colon (Hutcheson *et al.*, 1990). In that study, a high dose of LPS (50 mg kg^{-1}) produced an increase in plasma leakage in the stomach, duodenum, jejunum and ileum after 15 min, whereas the colon was not affected. These regional differences in sensitivity of the intestinal vasculature after acute or more prolonged exposure to endotoxin suggest the involvement of different mediators and mechanisms underlying the microvascular damage following such periods of challenge. Indeed, the intestinal damage induced by acute administration of high doses of endotoxin involves the release of a number of vasoactive mediators including platelet activating factor (PAF) and thromboxane A_2 (Wallace *et al.*, 1987; Whittle *et al.*, 1987; Boughton-Smith *et al.*, 1989). The acute release of these mediators may also account for the transient initial rise in jejunal vascular permeability, seen in the current study, 1 h after endotoxin challenge, whereas no such acute change was observed in colon.

The present study identifies the presence of a calcium-dependent constitutive NOS in the rat jejunal tissue, and confirms its presence in colonic tissue (Salter *et al.*, 1991). Although the presence of an inducible NOS enzyme could not be detected in the colon in a previous study using a spectrophotometric assay, the technique used critically depended on a haemoglobin-free tissue extract being obtained from perfused tissue (Salter *et al.*, 1991). In the current study using the conversion of radiolabelled L-arginine, the increase by endotoxin of NOS activity was more marked in colonic tissue than in the jejunum. Although this activity was predominantly calcium-independent, EGTA did cause a partial reduction in the elevated levels of NOS in colonic tissue at the earlier phases of induction after LPS challenge. This could reflect the contribution of the calcium-dependent constitutive enzyme to the total levels of NOS under these conditions, or to the induction or stimulation of a calcium-dependent isoform, as found previously in the ileum (Salter *et al.*, 1991). In the jejunum, EGTA abolished NOS activity under control conditions yet had no significant effect on the increase in the NOS activity observed 4 h after LPS, which therefore could indicate a reduction in constitutive NOS activity as a consequence of NOS induction, as seen in other tissues (Nishida *et al.*, 1992; de Belder *et al.*, 1993). It will be of interest to determine the cytosolic and particulate distribution of these NOS activities (Forstermann *et al.*, 1991; Mitchell *et al.*, 1991) in further studies and to define the full biochemical nature and immunohistochemical specificity of these NOS isoforms.

The induction by endotoxin and cytokines of a calcium-independent NOS *in vitro* is prevented by dexamethasone and by inhibitors of DNA transcription and translation, indicating that the effects of the corticosteroid are a consequence of inhibition of *de novo* synthesis of NOS (Radomski *et al.*, 1990; Knowles *et al.*, 1990; Di Rosa *et al.*, 1990). Furthermore, the induction of the calcium-independent NOS in the rat ileum by endotoxin administration *in vivo* was also prevented by dexamethasone pretreatment (Salter *et al.*, 1991) as confirmed in the present study on jejunal and colonic tissue. Since the anti-inflammatory corticosteroids can inhibit cytokine synthesis (Snyder & Unanue, 1982; Waage & Baake, 1988; Kern *et al.*, 1988), the actions of dexamethasone seen in the present study could also reflect inhibition of endotoxin-stimulated synthesis of the cytokines involved in the process of NOS induction under these *in vivo* conditions.

The prevention of NOS induction in both colon and jejunum by dexamethasone could be responsible for the concurrent abolition of the vascular permeability changes provoked by LPS in these tissues. Dexamethasone can also inhibit eicosanoid or PAF synthesis, through suppression of phospholipase A_2 activity as a consequence of stimulating lipocortin production (Blackwell *et al.*, 1980; Hirata *et al.*, 1980). However, pretreatment with high doses of dexamethasone caused only a modest reduction in intestinal damage and in the jejunal formation of PAF or thromboxane B_2 following acute endotoxin challenge (Boughton-Smith *et al.*, 1989). Such actions of dexamethasone may therefore not make a major contribution to the effects on vascular permeability seen in the current study.

Adhesion of neutrophils to vascular endothelium has been implicated in intestinal vascular injury (Hernandez *et al.*, 1987; Kubes *et al.*, 1991). Studies on the cat mesentery have demonstrated that acute inhibition of NO biosynthesis augments neutrophil adherence to vascular endothelium and provokes acute changes in intestinal vascular permeability, effects reversed by the NO donor, nitroprusside (Kubes *et al.*, 1991; Kubes & Granger, 1992). Such findings support the role of endogenous constitutive NO, probably located in the endothelium, in the regulation of the integrity of the microvasculature (Hutcheson *et al.*, 1990). The cellular sources of the constitutive and inducible forms of NOS observed in the current study on intestinal tissue are not yet known but the increase following endotoxin administration could reflect induction of the enzyme in vascular and epithelial tissue, or in resident or invading inflammatory cells. How neutrophil adhesion to the vascular endothelium would be affected by the high levels of NO produced by the induced NOS is not clear, but vascular damage under these conditions may be independent of such cellular interactions. The actions of corticosteroids on cell adhesion to the endothelium with the present experimental protocol also warrants attention as a possible contributory mechanism in the prevention of vascular injury by dexamethasone.

The process by which excessive NO production by an inducible NOS could produce increases in vascular permeability may partly involve an increase in blood flow. While an increase in blood flow alone would not itself result in increased vascular permeability, it would augment the actions of other pro-inflammatory mediators released by endotoxin, such as PAF or cytokines, which have a direct injurious action on the microvascular endothelium. Studies in rat skin have demonstrated that intradermal injection of endotoxin induced a time-dependent increase in blood flow that was inhibited by local administration of NOS inhibitors and by pretreatment with a corticosteroid (Warren *et al.*, 1992). Inhibitors of NO synthase can also attenuate the changes in vascular permeability and oedema formation induced by pro-inflammatory agents in rat skin, a process that may involve reduction in local blood flow (Hughes *et al.*, 1990; Ialenti *et al.*, 1992). In the present study, no substantial change in the intravascular volume of the jejunum or colon could be detected following endotoxin challenge. However, determination of intestinal blood flow will be needed to clarify the contribution of microcirculatory blood flow to the overall changes in plasma leakage associated with NO induction.

NO, or a subsequent product may also have a direct

injurious action on endothelial cells to produce the observed changes in intestinal vascular permeability. Induction of NOS in activated macrophages accounts for their cytotoxic action against bacterial and protozoal microorganisms and against tumour cells (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Drapier *et al.*, 1988; Stuehr *et al.*, 1989; Granger *et al.*, 1990; Adams *et al.*, 1990; Liew *et al.*, 1990). Furthermore, *in vitro* findings suggest that endothelial cell cytotoxicity produced by LPS and cytokines is dependent on the induction of NOS activity, and induced NO synthesis is implicated in damage to adenocarcinoma cells (Palmer *et al.*, 1992; O'Connor & Moncada, 1991). NO can interact with the superoxide anion to produce a reactive peroxynitrite radical which can subsequently lead to the production of the highly reactive hydroxyl radical (Beckman *et al.*, 1990). The hydroxyl radical can produce cell damage and cytotoxicity in a variety of cells, including endothelial cells, and has been implicated in intestinal vascular damage produced by ischemia-reperfusion in the intestine (Parks & Granger, 1983; Hernandez *et al.*, 1987).

The involvement of induced NO synthesis in the vascular injury provoked by LPS is supported by the finding that administration of the NO synthase inhibitor, L-NMMA at a time when elevation of NOS activity was just detectable, prevented the subsequent increase in plasma leakage in the jejunum and colon, an action abolished by prior administration of L-arginine. The doses of L-NMMA used did not themselves lead to any change in vascular permeability in the rat jejunum or colon over a 1 h period under control conditions in the absence of endotoxin, which contrasts with studies in the cat intestine with the more potent inhibitor, N^G-nitro-L-arginine methyl ester (Kubes & Granger, 1992). However, it is possible that these doses of L-NMMA used in the present study, which presumably act by inhibiting the activity of the induced NOS, are insufficient to abolish the constitutive NOS activity in the rat intestine, perhaps required for such permeability changes under control conditions and indeed they did not affect resting intestinal or colonic blood flow in control rats (Pique *et al.*, 1992b). Studies with selective inhibitors of the inducible NOS isoform will clarify the role of NO in such endotoxin-induced vascular damage.

The current findings thus suggest that the plasma extravasation, an index of inflammation and microvascular injury in the colonic and jejunal mucosa that follows the prolonged exposure to endotoxin *in vivo*, is temporally associated with the induction of intestinal tissue NOS. Induction of a calcium-independent NOS has also been demonstrated recently in colonic tissue from a rat model of inflammatory bowel disease, while the inducible NOS can be detected in inflamed colonic mucosa for ulcerative colitis patients (Boughton-Smith *et al.*, 1992c,d). Furthermore, elevated levels of luminal nitrate have been observed in a model of ileitis, and enhanced levels of the NO co-product, citrulline has been observed in colonic tissue from colitic patients (Miller *et al.*, 1993; Middleton *et al.*, 1993). Thus, the ability of dexamethasone to prevent both the induction of NOS and the concurrent changes in intestinal vascular permeability, as seen in the present study, may contribute in part to the therapeutic benefit of such corticosteroids in the treatment of inflammatory conditions of the intestine. Moreover, selective inhibitors of the inducible isoforms of NOS may prove of clinical value in such intestinal diseases.

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L-694,247: a potent 5-HT_{1D} receptor agonist

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1 The 5-hydroxytryptamine (5-HT) receptor binding selectivity profile of a novel, potent 5-HT_{1D} receptor agonist, L-694,247 (2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine) was assessed and compared with that of the 5-HT_1-like receptor agonist, sumatriptan.

2 L-694,247 had an affinity (pIC_{50}) of 10.03 at the 5-HT_{1D} binding site and 9.08 at the 5-HT_{1B} binding site (sumatriptan: pIC_{50} values 8.22 and 5.94 respectively). L-694,247 retained good selectivity with respect to the 5-HT_{1A} binding site ($pIC_{50} = 8.64$), the 5-HT_{1C} binding site (6.42), the 5-HT_2 binding site (6.50) and the 5-HT_{1E} binding site (5.66). The pIC_{50} values for sumatriptan at these radioligand binding sites were 6.14, 5.0, <5.0 and 5.64 respectively. Both L-694,247 and sumatriptan were essentially inactive at the 5-HT_3 recognition site.

3 L-694,247, like sumatriptan, displayed a similar efficacy to 5-HT in inhibiting forskolin-stimulated adenylyl cyclase in guinea-pig substantia nigra although L-694,247 ($pEC_{50} = 9.1$) was more potent than sumatriptan (6.2) in this 5-HT_{1D} receptor mediated functional response. L-694,247 ($pEC_{50} = 9.4$) was also more potent than sumatriptan (6.5) in a second 5-HT_{1D} receptor mediated functional response, the inhibition of K^+-evoked [^{3}H]-5-HT release from guinea-pig frontal cortex slices.

4 The excellent agreement observed for L-694,247 between the 5-HT_{1D} radioligand binding affinity and the functional potency confirm that the two functional models (the inhibition of forskolin-stimulated adenylyl cyclase in guinea-pig substantia nigra and the inhibition of K^+-evoked [^{3}H]-5-HT release from guinea-pig frontal cortex) do indeed reflect 5-HT_{1D}-mediated events.

5 L-694,247 is a novel, highly potent 5-HT_{1D}/5-HT_{1B} receptor ligand which should prove useful for the exploration of the physiological role of these receptors in animals.

Keywords: L-694,247; 5-HT_{1D} receptor; affinity; potency; efficacy; selectivity

Introduction

The 5-HT_{1D} recognition site was first described by Heuring & Peroutka (1987) as a binding site in bovine brain that could be specifically labelled with low nanomolar concentrations of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) in the presence of 100 nM 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and 100 nM mesulergine, which blocked out 5-HT_{1A} and 5-HT_{1C} receptors respectively. This site possessed a distinct pharmacological profile including a high affinity for 5-HT and 5-carboxamidotryptamine (5-CT) which is characteristic of 5-HT_1-like receptors. 5-HT_{1D} recognition sites have subsequently been shown to be present in most species studied (Peroutka *et al.*, 1989; Waeber *et al.*, 1989; Beer *et al.*, 1992) including rat and mouse which had previously been thought to be devoid of this receptor subtype (Hartig *et al.*, 1992).

Interest in this area was heightened further with the introduction of the anti-migraine drug sumatriptan. This is a 5-HT_1-like receptor agonist (Humphrey *et al.*, 1988) which is thought to mediate its action either postjunctionally via 5-HT_{1D} receptors located on the vascular smooth muscle, endothelial cells or sympathetic fibres of the dura causing the blood vessels to contract (Feniuk *et al.*, 1989) or via prejunctional receptors coupled to the inhibition of neuropeptide release resulting in a block of neurogenic plasma extravasation (Buzzi *et al.*, 1991).

In this study, the radioligand binding and functional characteristics of a novel 5-HT receptor agonist L-694,247 (2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine) (Figure 1) are described in detail. This compound displays a very high affinity and potency for the 5-HT_{1D} receptor and should prove to be a useful tool in characterizing the physiological role of 5-HT_{1D} receptors in animals.

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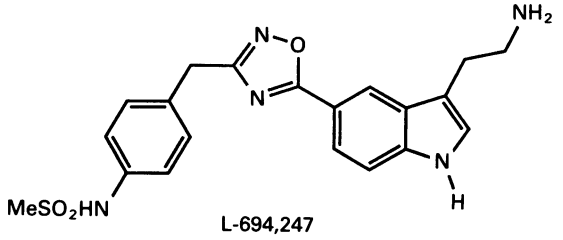
Methods

Radioligand binding

Frozen pig brains were obtained from Imperial Research Laboratories, thawed and the cerebral cortex and caudate dissected on ice. Crude membrane homogenates were prepared from the cerebral cortex for 5-HT_{1A} and 5-HT_{1C} binding assays and from the caudate for 5-HT_{1D} binding assays. The tissue was homogenized in 10–15 volumes of ice cold 50 mM Tris HCl (pH 7.7 at room temperature) with a Kinematica polytron (setting 5, 10 s) and centrifuged at 48,000 *g* at 4°C for 11 min. The resulting supernatant was discarded and the pellet resuspended in the same volume of ice cold Tris HCl buffer before being recentrifuged at 48,000 *g*, 4°C for a further 11 min. The pellet was then resuspended in 10 volumes of 50 mM Tris HCl followed by a 10 min incubation at 37°C to remove any endogenous 5-HT. Finally the tissue was recentrifuged at 48,000 *g*, 4°C for 11 min and the pellet resuspended, in assay buffer (see below), to give the required volume immediately prior to use. The original wet weight:volume ratio for the final membrane



L-694,247

Figure 1 Structure of L-694,247 (2-[5-[3-(4-methylsulphonylamino) benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine).

preparation was 1:100 for the 5-HT$_{1A}$ binding assay, 1:50 for the 5-HT$_{1C}$ binding assay and 1:10 for the 5-HT$_{1D}$ binding assay.

5-HT$_{1E}$ radioligand binding assays were carried out on HEK 293 cells previously transfected with the cDNA encoding the human 5-HT$_{1E}$ receptor (McAllister *et al.*, 1992). These cells, grown to confluence in EMEM medium (Flow Laboratories) containing 10% foetal calf serum, 1% penicillin/streptomycin, 1% glutamine and 0.5 mg ml^{-1} of the selective antibiotic G418, were scraped, harvested and centrifuged at 1,800 *g*, 4°C for 10 min. The resulting pellet was then resuspended in 50 mM Tris HCl (pH 7.7 at room temperature) using a Kinematica polytron (setting 5, 10 s) and centrifuged at 48,000 *g*, 4°C for 11 min. Following resuspension in 50 mM Tris HCl the membranes were incubated at 37°C for 10 min in a shaking water bath and finally recentrifuged at 48,000 *g* for 11 min. The pellet was then resuspended in assay buffer (see below) to give a final assay concentration of 150 μg protein per assay tube.

Crude P2 pellet homogenates were prepared from rat frontal cortex for the 5-HT$_{1B}$ and 5-HT$_2$ binding assays and from rat whole cortex for the 5-HT$_3$ binding assay. Male Sprague-Dawley rats (250–300 g) were stunned, decapitated and the brains dissected on ice. Tissue was promptly transferred to 10–15 volumes of ice cold 0.32 M sucrose. The tissue was then homogenized using ten strokes of a motor-driven Teflon/glass homogeniser (Janke and Kunkel) at 500 r.p.m. The homogenate was centrifuged at 1,000 *g*, 4°C for 10 min and the supernatant recentrifuged at 48,000 *g*, 4°C for 21 min. The supernatant was then discarded and the pellet resuspended in 10–15 volumes of 50 mM Tris HCl, pH 7.7 at room temperature (5-HT$_{1B}$/5-HT$_2$ binding assays), 2.5 mM HEPES, pH 7.4 at room temperature (5-HT$_3$ binding assay) and left to stand for 15 min at 37°C (5-HT$_{1B}$/5-HT$_2$ binding assays), room temperature (5-HT$_3$ binding assay), to remove endogenous 5-HT. Finally, the homogenates were recentrifuged for a further 21 min at 48,000 *g*, 4°C and the resulting pellet stored on ice. Immediately prior to use the pellet was made up to the required volume in assay buffer (see below). The original wet weight:volume ratio for the final membrane preparation was 1 to 50 for the 5-HT$_{1B}$ binding assay, 1 to 100 for the 5-HT$_2$ binding assay and 1 to 30 for the 5-HT$_3$ binding assay.

Membranes, ligand and drugs were prepared, in duplicate, in assay buffer which consisted of 50 mM Tris HCl containing 10 μM pargyline, 0.1% ascorbate, 5.7 mM $CaCl_2$, pH 7.7 at room temperature for the 5-HT$_{1A}$ binding assay (Gozlan *et al.*, 1983), 10 mM Tris HCl containing 10 μM pargyline, 154 mM NaCl, pH 7.7 at room temperature for the 5-HT$_{1B}$ binding assay (Hoyer *et al.*, 1985), 50 mM Tris HCl containing 10 μM pargyline, 0.1% ascorbate, 4.0 mM $CaCl_2$, pH 7.7 at room temperature for the 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_{1E}$ binding assays (Pazos *et al.*, 1984; Heuring & Peroutka, 1987; McAllister *et al.*, 1992) 50 mM Tris HCl containing 10 μM pargyline, 0.1% ascorbate, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4 at room temperature for the 5-HT$_2$ binding assay (Branchek *et al.*, 1990) and 10 mM HEPES containing 10 μM pargyline, 0.1% ascorbate, pH 7.1 at room temperature for the 5-HT$_3$ binding assay (Watling *et al.*, 1988). The test compound was incubated with appropriate radioligand to give a final volume of 1 ml with the exception of the 5-HT$_{1B}$, 5-HT$_2$ and 5-HT$_{1D}$ binding assays which were carried out in a total volume of 300 μl, 2 ml and 250 μl respectively. [^{3}H]-8-OH-DPAT (1.5 nM), [^{125}I]-iodocyanopindolol (150 pM), [^{3}H]-mesulergine (1.5 nM), [^{125}I]-GTI (20 pM), [^{3}H]-5-HT (2.0 nM), [^{3}H]-DOB (0.6 nM) and [^{3}H]-Q-ICS 205-930 (0.5 nM) were used to label 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_2$ and 5-HT$_3$ binding sites respectively. (−)-Isoprenaline (30 μM) was added to the 5-HT$_{1B}$ assay tubes to prevent binding to β-adrenoceptors. 5-HT (10 μM) was used to define non-specific binding in the 5-HT$_{1A}$, 5-HT$_{1C}$ and 5-HT$_{1E}$ binding assays, 5-HT (100 μM) in the 5-HT$_{1B}$ binding assay, 5-HT (1 μM) in the 5-HT$_{1D}$ binding assay, cyproheptadine (1 μM) in the 5-HT$_2$ binding assay and MDL 72222 (10 μM) in the 5-HT$_3$ binding assay. 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_2$ binding assay incubations were carried out in a shaking water bath at 37°C for 20, 20, 30, 30, 30 and 15 min respectively. 5-HT$_3$ binding assay incubations were carried out on ice for 15 min. In all cases the reaction was started by the addition of the membrane suspension and terminated by rapid filtration through Whatman G/FB glass fibre filters (ice cold for the 5-HT$_{1C}$ binding assay), or Whatman G/FC glass fibre filters for the 5-HT$_{1B}$ and 5-HT$_{1D}$ binding assays, using a Brandel cell harvester. Each assay tube was washed twice with 4 ml 50 mM Tris HCl (5 mM HEPES for the 5-HT$_3$ binding assay) and the washings passed through the filters. G/FB filters had previously been soaked in 0.3% polyethylenimine (PEI)/0.5% Triton X to minimize non-specific binding. The filters were then transferred to scintillation vials containing 10 ml Hydrofluor and the radioactivity determined by liquid scintillation spectrometry at 30–40% efficiency.

Inhibition of forskolin-stimulated adenylyl cyclase in guinea-pig substantia nigra

The studies were performed essentially as described by DeVivo & Maayani (1986). Male Duncan Hartley guinea-pigs (300–400 g) were killed by cervical dislocation, the brains removed rapidly and the substantia nigra dissected, pooled, weighed and transferred to 100 volumes of ice cold Tris HCl-sucrose buffer (composition in mM: Tris HCl 20, sucrose 300, dithiothreitol 5, EDTA 5, EGTA 5, pH 7.4 at room temperature). The tissue was then homogenized in a motor driven teflon/glass homogenizer followed by centrifugation at 900 *g* for 10 min at 4°C. The resulting pellet was discarded and the supernatant recentrifuged at 20,000 *g* for 20 min at 4°C to yield a crude P2 pellet. The supernatant was then discarded and the pellet resuspended in 100 volumes of ice cold Tris HCl-EGTA buffer (composition in mM: Tris HCl 10, EGTA 1, pH 8.0 at room temperature) and incubated on ice for 30–60 min. The tissue was then recentrifuged at 20,000 *g* for 20 min at 4°C, the supernatant discarded and the pellet finally suspended in 10 volumes of Tris HCl-EDTA buffer (composition in mM: Tris HCl 50, EDTA 5, pH 7.6 at room temperature) just prior to assay.

The adenylyl cyclase activity was determined by measuring the conversion of α-[^{32}P]-ATP to [^{32}P]-cyclic AMP. All assays were carried out in quadruplicate. The incubation medium consisted of 50 mM Tris HCl (pH 7.6 at room temperature) containing 100 mM NaCl, 30 μM GTP, 50 μM cyclic AMP, 1 mM dithiothreitol, 1 mM ATP, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 3.5 mM creatine phosphate, 0.2 mg ml^{-1} creatine phosphokinase, 0.5–1 μCi α-[^{32}P]-ATP (30 Ci mmol^{-1}) and 1nCi [^{3}H]-cyclic AMP (30–50 Ci mmol^{-1}) in order to estimate column recoveries. A 10 μl aliquot of membrane suspension was incubated, for 10–15 min, at 30°C in a shaking water bath, with or without 10 μM forskolin, in the presence or absence of test compound. The incubation (carried out in a final volume of 50 μl) was initiated by the addition of membrane, following a 5 min preincubation at 30°C and was terminated by the addition of 100 μl SDS (composition in mM: sodium lauryl sulphate 2%. ATP 45, cyclic AMP 1.3, pH 7.5 at room temperature). The α-[^{32}P]-ATP and [^{32}P]-cyclic AMP were separated on a double column chromatography system (Dowex exchange resin AG50W × 4, BioRad, and neutral alumina) (Salomon *et al.*, 1974). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin used as standard.

Inhibition of K$^+$-evoked [^{3}H]-5-HT release from slices of guinea-pig frontal cortex

These studies were carried out essentially as described by Wilkinson *et al.* (1993). Male Duncan Hartley guinea-pigs

(250–400 g) were killed by cervical dislocation, the brains removed rapidly, frontal cerebral cortices dissected and cross-chopped (250 μm) on a McIlwain chopper. The slices were then incubated at 37°C for 15 min in Krebs buffer (composition in mM: NaCl 134, KCl 5, KH_2PO_4 1.25, $NaHCO_3$ 25, $MgSO_4$ 1, glucose 10, $CaCl_2$ 1.3, bubbled with 95% O_2, 5% CO_2) containing 10 μM pargyline and 0.1 μM [^{3}H]-5-HT. The slices were then washed 3 times in Krebs buffer, gently mixed and 50 μl aliquots transferred into individual chambers of a superfusion apparatus and superfused with Krebs buffer at a rate of 0.4 ml min^{-1}. Following a 30 min period of superfusion the slices were then exposed to four 4 min stimulation periods of Krebs buffer containing 30 mM K^+ ions at 42, 78, 114 and 150 min (S1, S2, S3 and S4 respectively). The NaCl concentration of the 30 mM K^+ Krebs buffer was reduced to maintain iso-osmolarity. Test compound was added to the chambers, where appropriate, in increasing concentrations, 4 min prior to periods S2, S3 and S4. Successive 4 min fractions were collected throughout the experiment starting at 30 min after onset of the superfusion. The 5-HT uptake blocker, fluvoxamine (10 μM) was present throughout the superfusion to prevent the re-uptake of 5-HT into the nerve endings. At the end of the experiment the radioactivity in the slices and each fraction was determined by liquid scintillation spectrometry at 30–40% efficiency.

Data analysis

Measurement of affinities in radioligand binding studies Experiments were performed on at least 3 separate occasions in duplicate. Each displacement curve consisted of 10 (5-HT_{1A}, 5-HT_{1D} binding assays) or 9 (all other assays) separate concentrations ranging from 10 μM to 300 pM. In all cases, data represent specific binding (total − non-specific) only. Each inhibition curve was analysed by non-linear, least squares regression analysis using an iterative curve fitting routine (Marquardt-Levenburg method) provided by the data manipulation software RS/1 (BBN Software Products Corporation, Cambridge MA, U.S.A.) All curves were analysed based on the assumption of a one-site model. The affinity values are expressed as pIC_{50} values ($-\log_{10} IC_{50}$) where the IC_{50} is the molar concentration of drug necessary to inhibit specific binding by 50%. The data are expressed as mean ± s.e.mean from n experiments.

Measurement of potencies at the receptor mediating the inhibition of forskolin-stimulated adenylyl cyclase Experiments were performed on 3 separate occasions in quadruplicate. The adenylyl cyclase activity levels were calculated as pmol cyclic AMP min^{-1} mg^{-1} protein and expressed as a percentage of those containing forskolin but no drug. These values were then plotted as concentration-response curves which were analysed using ALLFIT, a least square curve fitting programme (DeLean *et al.*, 1978) and from which E_{max} (maximal effect) and EC_{50} (the molar concentration of drug necessary to inhibit the maximal effect by 50%) values were obtained. The EC_{50} values were then converted to pEC_{50} values ($-\log_{10} EC_{50}$). Results are given as mean ± s.e.mean from 4–18 experiments.

Measurement of potencies in inhibiting K^+-evoked [^{3}H]-5-HT release A time versus fractional release rate was produced for each chamber and the stimulated [^{3}H]-5-HT release for S1, S2, S3, S4 calculated (Wilkinson *et al.*, 1993). S2/S1, S3/S1 and S4/S1 ratios were then determined for control and treated slices. Data from 4 to 5 chambers was averaged to yield $n = 1$ at each test compound concentration and this ratio converted to a percentage of its own control. This allowed a percentage decrease in [^{3}H]-5-HT release to be determined at three test compound concentrations in one experiment. Experiments were plotted as concentration-response curves and the 50% inhibition point (EC_{50}) determined and converted to the pEC_{50} value (see above). Results are given as mean ± s.e.mean from n experiments.

Drugs

The following radioligands were purchased: 8-hydroxy-[^{3}H]-DPAT (201 Ci mmol^{-1}), 5-hydroxy[G-^{3}H]-tryptamine creatinine sulphate (11.1 Ci mmol^{-1}), [^{3}H]-quaternised ICS 205,930 (55 Ci mmol^{-1}), adenosine 5'-[α-^{32}P]-triphosphate (30 Ci mmol^{-1}), [2,8-^{3}H]-adenosine 3',5',-cyclic phosphate (42 Ci mmol^{-1}), [N-6-methyl-^{3}H]-mesulergine (70–85 Ci mmol^{-1}), (Amersham International), serotonin-o-carboxymethyl-glycyl-[^{125}I]-tyrosinamide (2000 Ci mmol^{-1}), (Immunotech), 4-bromo-2,5-dimethoxyphenylisopropylamine-(±)-[propyl-1,2-^{3}H] (10–30 Ci mmol^{-1}), 5-[1,2-^{3}H(N)]-(5-hydroxytryptamine creatinine sulphate) (15–30 Ci mmol^{-1}), (−)-[^{125}I]-iodocyanopindolol (2200 Ci mmol^{-1}), (NEN Research Products).

The following compounds were purchased: 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Co.), ketanserin, mesulergine (Research Biochemicals Inc.). The following compounds were gifts: cyanopindolol (Sandoz), paroxetine (SmithKline Beecham), fluvoxamine (Duphar).

The following compounds were synthesized at MSD: L-694,247, sumatriptan (3-[2-dimethylamino)ethyl]-N-methyl-1H-indole-5 methane sulphonamide), MDL 72222 (1 αH,3α, 5αH-tropan-3yl-3,5 dichlorobenzoate), 5-carboxamidotryptamine, cyproheptadine.

Results

Radioligand binding studies

Specific binding accounted for 33–94% of total binding for all the radioligand binding assays used in this study. Specific binding, for each radioligand binding assay, is expressed as fmol mg^{-1} wet weight tissue and given in Table 1. In all assays, binding to filters comprised less than 10% of the non-specific binding (data not shown). Computer-assisted iterative curve fitting analysis consistently gave monophasic displacement curves at all the recognition sites studied for both L-694,247 and sumatriptan.

L-694,247 displayed very high affinity ($pIC_{50} = 10.0$) for the 5-HT_{1D} site labelled with [^{125}I]-GTI and was some 65 fold more potent in this regard than sumatriptan. It displayed somewhat lower affinity for the 5-HT_{1B} binding site ($pIC_{50} = 9.1$) compared with the corresponding pIC_{50} value of 6.0 obtained with sumatriptan. L-694,247 also yielded a higher affinity at the [^{3}H]-8-OH-DPAT binding site compared to sumatriptan and hence the resulting 5-HT_{1D}/5-HT_{1A} receptor selectivity was slightly lower for L-694,247 (25 ×) compared with that for sumatriptan (120 ×). L-694,247, however, displayed greater 5-HT_{1D} receptor selectivity over the 5-HT_{1C} and 5-HT_{1E} binding sites when compared with sumatriptan. Sumatriptan displayed a negligible affinity at the 5-HT_2 site labelled with [^{3}H]-DOB ($pIC_{50} < 5.0$) whereas L-694,247 yielded a pIC_{50} of 6.5. Finally both compounds were essentially inactive at the 5-HT_3 binding site as assessed with [^{3}H]-Q-ICS 205-930.

Inhibition of forskolin-stimulated adenylyl cyclase in guinea-pig substantia nigra

The addition of 10 μM forskolin to the incubation medium consistently resulted in a 7 to 14 fold increase from a basal activity of 0.63 ± 0.11 fmol min^{-1} mg^{-1} protein to a maximal stimulation of 5.83 ± 1.27 fmol min^{-1} mg^{-1} protein (mean ± s.e.mean, $n = 15$). Both L-694,247 and sumatriptan produced a concentration-dependent inhibition of the forskolin-stimulated adenylyl cyclase activity with maximal inhibitions of 14% and 19% respectively (Figure 2). These maximal effects were not significantly different from that produced

Table 1 The relative affinities of L-694,247 and sumatriptan in seven 5-HT radioligand binding assays

Receptor	*5-HT$_{1D}$*	*5-HT$_{1B}$*	*5-HT$_{1A}$*	*5-HT$_{1C}$*	*5-HT$_{1E}$*	*5-HT$_2$*	*5-HT$_3$*
Radioligand	[^{125}I]-GTI	[^{125}I]-iodocyano-pindolol	[^{3}H]-8-OH-DPAT	[^{3}H]-mesulergine	[^{3}H]-5-HT	[^{3}H]-DOB	[^{3}H]-Q-ICS 205-903
Radioligand specifically bound (fmol mg^{-1} wet wt)	0.04 ± 0.003	1.82 ± 0.07	1.15 ± 0.17	1.38 ± 0.17	13.89 ± 2.84	1.43 ± 0.23	0.79 ± 0.03
L-694,247 (pIC$_{50}$)	10.03 ± 0.16		8.64 ± 0.17	6.42 ± 0.25	5.66 ± 0.13	6.50 ± 0.07	< 5.0
Sumatriptan (pIC$_{50}$)	8.22 ± 0.08	9.08 ± 0.17 5.94 ± 0.02	6.14 ± 0.09	5.0 ± 0.08	5.64 ± 0.06	< 5.0	< 5.0

Data yielded displacement curves which were fitted best by one site models ($P < 0.05$ partial F test) and from which pIC$_{50}$ values were obtained. pIC$_{50}$ values represent $-\log_{10}$ IC$_{50}$ where the IC$_{50}$ is the molar concentration of compound necessary to inhibit binding by 50%. Results are arithmetic means ± s.e.mean of 3–6 experiments. The amount of specifically bound radioligand for each assay is given as fmol mg^{-1} wet weight tissue (arithmetic means ± s.e.mean, $n = 6$–8). For details of experimental conditions see methods and data analysis sections.

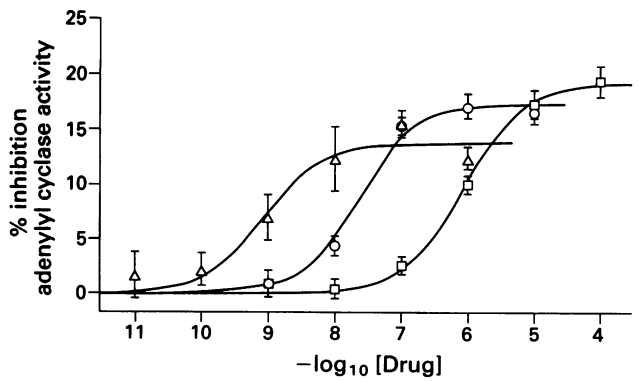


Figure 2 Concentration-response curves for L-694,247 (Δ), 5-hydroxytryptamine (5-HT) (○) and sumatriptan (□) in inhibiting forskolin-stimulated adenylyl cyclase from guinea-pig substantia nigra. Curves were analysed with ALLFIT, a least squares curve fitting programme as described in data analysis. Each point represents mean values from 3 experiments with the s.e.mean. Details of experimental conditions are given in the methods section. The pEC$_{50}$ values for L-694,247, 5-HT and sumatriptan were 9.1, 7.6 and 6.2 respectively. pEC$_{50}$ values represent $-\log_{10}$ EC$_{50}$ where the EC$_{50}$ is the molar concentration of compound required to inhibit the maximal effect by 50%.

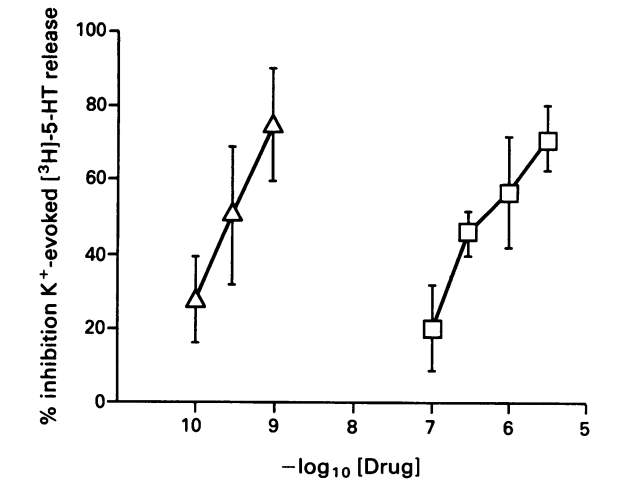


Figure 3 Concentration-response curves for L-694,247 (Δ) and sumatriptan (□) in inhibiting K$^+$-evoked [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) release from guinea-pig frontal cortex. Each point represents mean values from 3–4 experiments with the s.e.mean. Details of experiments are given in the methods and data analysis sections. The pEC$_{50}$ values for L-694,247 and sumatriptan were 9.4 and 6.5 respectively. pEC$_{50}$ values represent $-\log_{10}$ EC$_{50}$ where the EC$_{50}$ is the molar concentration of compound required to inhibit the maximal response by 50%.

by 5-HT (17%), $P < 0.05$ (Tukey test following ANOVA) indicating that both compounds acted as full agonists in this assay system. The pEC$_{50}$ values for L-694,247 and sumatriptan at this putative 5-HT$_{1D}$ receptor in the guinea-pig substantia nigra were 9.1 and 6.2 respectively.

Inhibition of K$^+$-evoked [^{3}H]-5-HT release from guinea-pig frontal cortex

A consistent increase in the release of preloaded [^{3}H]-5-HT from the frontal cortical slices was observed with an elevation in Krebs buffer K$^+$ concentration to 30 mM, S1 = 2.6 ± 0.1% ($n = 9$). The control S2/S1, S3/S1 and S4/S1 ratios were 0.71 ± 0.04, 0.57 ± 0.02 and 0.48 ± 0.02 respectively ($n = 8$–9). Addition of L-694,247 or sumatriptan to the superfusion medium between S1 and S2, S2 and S3 or S3 and S4 resulted in a concentration-dependent decrease in the K$^+$-evoked [^{3}H]-5-HT release (Figure 3). The pEC$_{50}$ values for L-694,247 and sumatriptan in inhibiting K$^+$-evoked release of [^{3}H]-5-HT from slices of the guinea pig frontal cortex were 9.4 and 6.5 respectively.

Discussion

Recent developments in the field of 5-HT$_1$-like receptor research has led to the cloning of a number of receptors belonging to this 5-HT receptor sub-family. To date, five 5-HT$_1$-like human receptors have been cloned, the 5-HT$_{1A}$, 5-HT$_{1D\alpha}$, 5-HT$_{1D\beta}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ receptors (Fargin *et al.*, 1988, Hartig *et al.*, 1992, McAllister *et al.*, 1992, Adham *et al.*, 1993). The development of selective, high affinity ligands for these receptor subtypes is an essential prerequisite for the investigation of the mechanism of action of, and the physiological necessity for, this receptor multiplicity. Interest in the 5-HT$_{1D}$ receptor has been kindled by the anti-migraine drug, sumatriptan, which to date is the most selective and potent 5-HT$_{1D}$ receptor ligand described. In this study a novel 5-HT$_{1D}$ receptor agonist, L-694,247, has been compared with sumatriptan in terms of its 5-HT receptor binding profile and also in two *in vitro* functional models believed to be predictive of activation of central 5-HT$_{1D}$ receptors.

The affinity of L-694,247 for the 5-HT$_{1D}$ radioligand binding site, as assessed by its ability to diplace [^{125}I]-GTI from pig caudate membranes, was some 65 times greater than that seen with sumatriptan. The improved affinity of L-694,247 over sumatriptan at the 5-HT$_{1B}$ binding site was even more marked (1300 ×). As 5-HT$_{1B}$/5-HT$_{1D}$ receptors are species-specific, the 5-HT$_{1B}$ receptor predominating in rat and mouse whilst most other laboratory species possess the 5-HT$_{1D}$ receptor subtype (Hoyer & Middlemiss, 1989), the relatively high affinities of L-694,247 at both receptor subtypes render this compound a powerful tool regardless of the animal under study. L-694,247 and sumatriptan also possess appreciable but lower binding affinities at the 5-HT$_{1A}$ recognition

site. L-694,247 displayed weak affinities for the 5-HT_{1C} and 5-HT_2 binding sites which was not seen with sumatriptan. The L-694,247 selectivity for the 5-HT_{1D} binding site with respect to the 5-HT_{1C} and 5-HT_2 recognition sites was, however, greater than 3,000 fold. Both L-694,247 and sumatriptan displayed similar, weak binding to the human cloned 5-HT_{1E} receptor and were essentially inactive at the 5-HT_3 recognition site.

In this study we have investigated the affinity of L-694,247 in seven 5-HT receptor radioligand binding assays. This, however, is by no means an exhaustive list as determined by the isolation of cDNAs encoding novel 5-HT receptors. For example the 5-HT_{1D} receptor is now known to exist as two subtypes the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors in man and other species (Hartig *et al.*, 1992). Other 5-HT_1-like receptors more recently cloned include the mouse 5-$HT_{1E\beta}$ receptor (Amlaiky *et al.*, 1992), the human 5-HT_{1F} receptor (Adham *et al.*, 1993) and the mouse 5-HT_{5A} receptor which displays a 5-HT_1-like pharmacology including a moderate affinity for sumatriptan (Plassat *et al.*, 1992). Although the present radioligand selectivity profile for L-694,247 does not include these novel receptors such a high potency compound should prove useful in determining the pharmacology of these novel receptor subtypes.

The L-694,247 and sumatriptan 5-HT_{1D} radioligand binding affinities were reflected by their potency seen in stimulating the 5-HT_{1D} receptor in two functional models. Sumatriptan was some 800 fold less potent than L-694,247 in inhibiting forskolin-stimulated adenylyl cyclase activity in guinea-pig substantia nigra and also in inhibiting K^+-evoked [^{3}H]-5-HT release from guinea-pig hippocampal slices. The excellent agreement seen in the potencies for L-694,247 in the two functional models of activation of 5-HT_{1D} receptors, which is also mirrored by its affinity at the 5-HT_{1D} binding site, confirms that the inhibition of forskolin-stimulated adenylyl cyclase in guinea-pig substantia nigra (Waeber *et al.*, 1989) and the inhibition of K^+-evoked [^{3}H]-5-HT release from guinea-pig hippocampus (Hoyer & Middlemiss, 1989) are indeed models of 5-HT_{1D} receptor function.

This study therefore indicates that L-694,247 is an extremely potent 5-HT_{1D} receptor agonist which, in addition, displays high affinity for the rodent 5-HT_{1B} receptor. Additional studies carried out in this laboratory and not described here indicate that L-694,247 possesses limited oral bioavailability in animals and so is unsuitable for development as an oral anti-migraine agent. Nevertheless its properties, in particular its much improved potency over the only other readily available 5-HT_{1D} receptor ligand, sumatriptan, should result in L-694,247 being a useful tool for the exploration of the physiological function of the 5-HT_{1D} receptor.

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Evidence for heterogeneity of endothelin receptor distribution in human coronary artery

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1 The receptors mediating endothelin-evoked contraction of human coronary artery have been investigated in isolated segments of the left anterior descending coronary artery (LAD).

2 Endothelin-1 (ET-1) was 10 times more potent in distal than in proximal segments but the potency ratio between ET-1 and ET-3 (endothelin-3) was similar and close to 100 in any segment of the artery.

3 BQ-123, an ET_A receptor antagonist, competitively antagonized the response to ET-1 of distal segments (pA_2 equal to 7.47). In the proximal segments, part of the contractile response was BQ123 sensitive, but the antagonism was non-competitive. In both groups of segments, the response to ET-3 could be completely blocked by BQ-123.

4 These observations indicate that ET_A receptors mediate the contractile response to ET-1 in distal, pre-resistant coronary arteries, but that other ET receptors are also involved in the contractile response of proximal segments.

Keywords: Endothelin receptors; human coronary artery; ET_A antagonist BQ-123

Introduction

Since their discovery, endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988) and its isopeptides, endothelin-2 (ET-2) and endothelin-3 (ET-3), (Inoue *et al.*, 1989) have generated considerable interest because they elicit very potent vasoconstrictor effects on various animal and human vessels *in vitro* and *in vivo*. For instance, they have been implicated in the pathogenesis of coronary ischaemic syndromes, especially vasospasm and could play a role in myocardial infarction (see Miller *et al.*, 1993). Therefore, several authors have studied the action of endothelin in human isolated coronary arteries (Franco-Cereceda, 1989; Godfraind *et al.*, 1989; Costello *et al.*, 1990; Klöckner & Isenberg, 1991; Dashwood *et al.*, 1991; Chester *et al.*, 1992).

The differential potencies of endothelin isopeptides and the action of BQ-123, a cyclic pentapeptide antagonist, have enabled a functional differentiation of two subtypes of endothelin receptor to be demonstrated (Inoue *et al.*, 1989; Ihara *et al.*, 1991). They are termed ET_A, which has selectivity for ET-1 over ET-3 and mediates contraction, and ET_B for which ET-1 and ET-3 are equipotent. The functional differentiation of receptors has been confirmed by molecular biology since two subtypes have been cloned and sequenced (Arai *et al.*, 1990; Sakurai *et al.*, 1990).

In this study, the functional characteristics of the contractile response of human coronary arteries to ET-1 and ET-3 and the action of BQ-123, a specific ET_A antagonist (Ihara *et al.*, 1992), have been analysed with the aim of characterizing the endothelin receptors involved in the response of human coronary arteries to the peptides. The action of endothelin has been examined on proximal coronary arteries and on distal pre-resistant arteries in view of a previous observation that large and small human coronary arteries may present different sensitivities to contractile agents (Godfraind *et al.*, 1989).

The results show that ET_A receptors mediate the contractile response to ET-1 in distal segments of the left anterior descending coronary artery but suggest that other ET receptor subtypes are also involved in the response of proximal segments.

Methods

Coronary arteries were obtained from 11 patients (mean age, 43.4 ± 2.4; age range 30 to 59 years; 7 males and 4 females). Six underwent heart transplantation and five died from neurological trauma. Explanted hearts were stored at 4°C for up to 12 h. The segments 5, 6 and 8 of the left anterior descending coronary artery were identified according to the schematic anatomical diagram of Figure 1 following the code numbers of the classification of the American Heart Association Committee Report (AHA Committee Report, 1975). Hearts were removed and cleaned of all adherent tissues; thereafter they were used immediately or stored at 4°C in a physiological Krebs solution (composition in mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5) for up to 24 h. Pieces showing any atherosclerotic lesion were discarded, since we have shown that segments close to atherosclerotic plaques have a different reactivity from non-diseased segments (Godfraind & Miller, 1983).

Rings of arteries, about 2 mm wide, were mounted on an isometric myograph connected to a microcomputer. The organ chamber was filled with 25 ml of physiological Krebs solution bubbled with 95% O_2:5% CO_2 and containing N^{ω}-nitro-L-arginine (L-NOARG, 0.1 mM), an inhibitor of NO synthesis (Moore *et al.*, 1990) in order to avoid any influence of spontaneous endothelium derived relaxing factor (EDRF) (NO) release on the contractile response to vasoconstrictor agonists (Eglème *et al.*, 1984). Relaxation evoked by stimu-

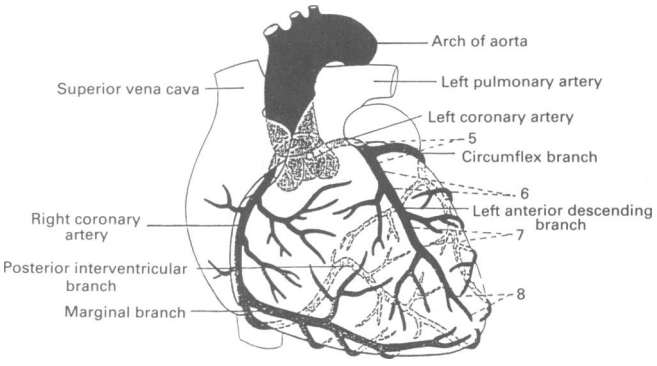

Figure 1 Schematic anatomical diagram of human coronary arteries with indication of the segment numbers.

lated EDRF release is inhibited when NO-synthase is blocked (Chester *et al.*, 1990).

The diameter of the vessels was estimated at a transmural tension equivalent to 100 mmHg. For recording contractions, rings of arteries were set at a normalized internal circumference, estimated to be 0.9 times the circumference at 100 mmHg (Mulvany & Halpern, 1977). The preparations were allowed to equilibrate for 120 min in the physiological solution with L-NOARG. Contraction was evoked by changing the physiological solution with a K-depolarizing solution containing (in mM): NaCl 17, KCl 100, $NaHCO_3$ 25, glucose 11.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and $CaCl_2$ 1.25 (Godfraind & Kaba, 1969).

Cumulative concentration-response relationships were obtained in response to ET-1 and ET-3 and expressed either as a percentage of the response to K-solution or as a percentage of the maximum response to ET-1. The vascular sensitivity to ET-1 and to ET-3 and the effect of the antagonist BQ-123 were investigated as follows: 30 min after the first K-contraction, preparations obtained from the same heart were randomized either for ET-1 and ET-3 comparison or for BQ-123 study. Since the effect of endothelin is slowly reversible, only one cumulative concentration-response relationship was established for each preparation, either untreated or pretreated with various concentrations of BQ-123 in order to characterize any antagonism of ET-1.

Drugs

Endothelins and BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) were purchased from Novabiochem (Switzerland) and L-NOARG (N^{ω}-nitro-L-arginine) from Sigma. The endothelins were reconstituted in 0.1% acetic acid and then diluted in physiological solution. BQ-123 was dissolved in physiological solution.

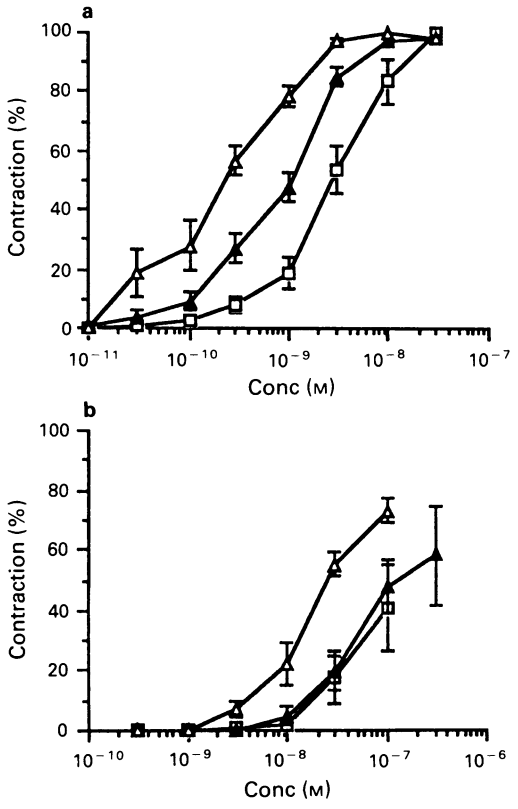

Figure 2 Concentration-response curves (a) for endothelin-1 (ET-1) in human left anterior descending coronary artery. Rings were prepared from (□) segment 5 ($n = 7$), (▲) segment 6 ($n = 20$) and (△) the more distal part of segment 8 ($n = 6$). The data are means with the s.e.mean, (b) for endothelin-3 (ET-3): (□) segment 5 ($n = 3$), (▲) segment 6 ($n = 8$) and (△) segment 8 ($n = 3$).

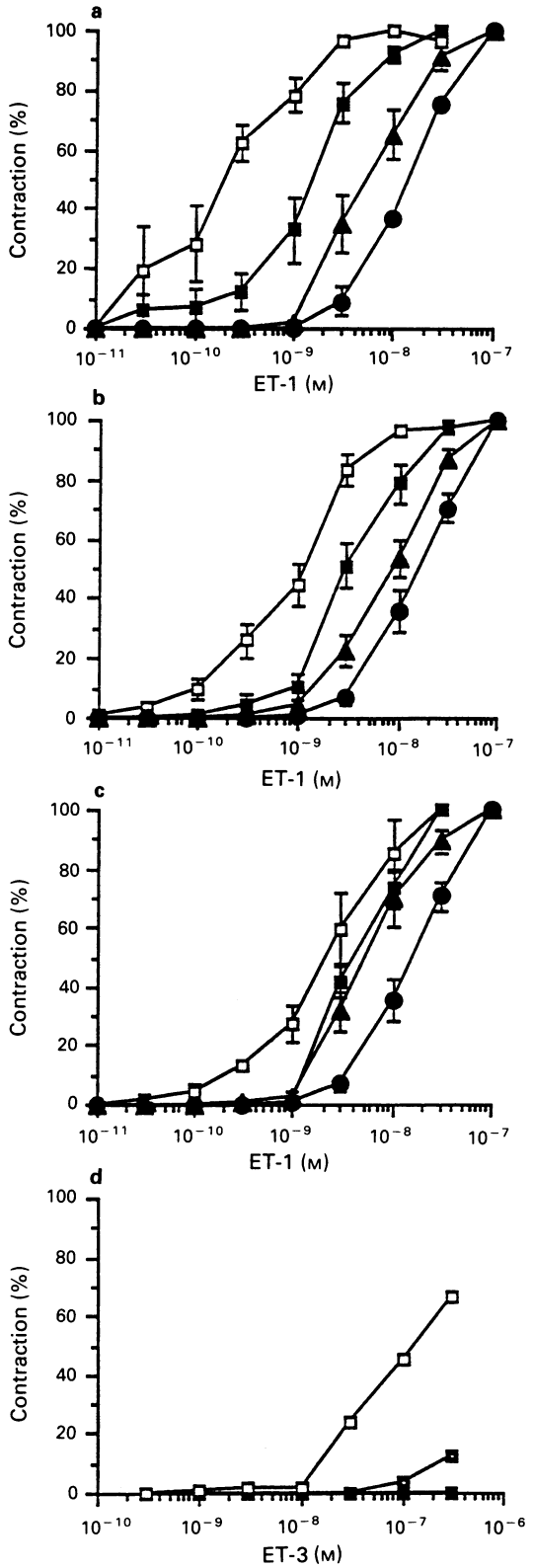

Figure 3 Effect of BQ-123 on endothelin-1 (ET-1) and ET-3-evoked contraction in human left anterior descending coronary artery. (a) Segment 8, mean diameter: 1.67 ± 0.09 mm ($n = 12$); (□) untreated ($n = 3$); (■) pretreated with BQ-123 300 nM ($n = 3$); (▲) pretreated with BQ-123 1 μM ($n = 3$); (●) pretreated with BQ-123 3 μM ($n = 3$). (b) Segment 6, mean diameter: 3.16 ± 0.13 mm ($n = 39$); (□) untreated ($n = 11$); (■) pretreated with BQ-123 300 nM ($n = 8$); (▲) pretreated with BQ-123 1 μM ($n = 10$); (●) pretreated with BQ-123 3 μM ($n = 10$). (c) Segment 5, mean diameter: 4.71 ± 0.09 mm ($n = 19$); (□) untreated ($n = 4$); (■) pretreated with BQ-123 300 nM ($n = 5$); (▲) pretreated with BQ-123 1 μM ($n = 5$); (●) pretreated with BQ-123 3 μM ($n = 5$). (d) Segment 5, mean diameter 4.500 μm; typical experiment showing the action of ET-3 in an untreated preparation (□) and in preparations pretreated with BQ-123 100 nM (□) and 300 nM (■).

Table 1 Potencies of endothelin-1 (ET-1) and ET-3 as contractile agonists in segments 5, 6 and 8 of the human left descending coronary artery

		Segment		
		5	6	8
Peptide	ø (mm):	4.44 ± 0.02	2.90 ± 0.01	1.41 ± 0.01
ET-1				
Max resp % KCl		124.11 ± 15.51	111.27 ± 4.51	105.07 ± 10.27
ED_{50} (nM)		2.68	1.07	0.23
		(1.76–4.07)	(0.86–1.33)	(0.14–0.37)
n		7	20	6
		$P<0.01$	$P<0.01$	
ET-3				
ED_{50} (nM)		158	157	25.2
		(by extrapolation)	(77.4–318)	(16.0–39.7)
n		3	8	3
			$P<0.01$	

ED_{50} values (with 95% confidence limits) were calculated from experiments shown in Figure 2. Maximum responses (± s.e.mean) to endothelins are expressed as a percentage of the contractile response evoked by K-depolarizing solution before any stimulation by the peptides.

Statistical analysis

Values are means (± s.e.mean or with 95% confidence interval). ED_{50} values were estimated by linear regression from log concentration-effect curves. Agonist concentration-ratio (CR) values were calculated by dividing the ED_{50} obtained in the presence of the antagonist by that obtained in its absence (for the same heart) and subjected to Schild analysis (Arunlakshana & Schild, 1959); pA_2 values were estimated by linear regression from log (CR − 1) values. Tests of significance were made by Student's *t* test; comparisons between ED_{50}s were made by analysis of covariance (Armitage & Berry, 1990). *P* values less than 0.05 were considered significant.

Results

Agonist potencies

ET-1 and ET-3 produced sustained and concentration-related contractions of the various segments of the left anterior descending human coronary artery. As shown in Figure 2, in distal segment 8, ET-1 was 5 times more potent than in segment 6 and some 10 times more potent than in segment 5 ($P<0.01$). Data reported in Table 1 show that the maximum responses matched with responses to K-solution were not different in these three segments ($P>0.05$). Although a maximum response could not be achieved with ET-3, due to the fact that concentrations of peptides higher than 3×10^{-7} M were not available, ED_{50} values were estimated assuming that the maximum response to this peptide was not different from that of ET-1 (Table 1). ET-3 activity was similar in segments 5 and 6 and was about 5 times higher in segment 8 than in the proximal ones ($0.05>P>0.01$). In the three segments so far examined, ET-1 was about 100 fold more potent than ET-3.

Preparations from two subjects were pretreated over 30 min not only with L-NOARG 0.1 mM but also with indomethacin 0.1 μM; their contractile response to ET-1 and to ET-3 was not significantly different from the response of other preparations stimulated by ET-1 and ET-3 in the presence of L-NOARG alone.

Antagonist activity

In preparations pretreated with BQ-123, there was a concentration-dependent rightward parallel displacement of the ET-1 concentration-effect curves without change in the maximum response. As Figure 3 illustrates, the shift was the most important in segment 8. Schild analysis showed that in segment 8, the pA_2 value was 7.47 ± 0.27 with a slope of 1.03. In the other segments, the slope was lower than unity and the intercept with the abscissa scale gave an apparent pA_2 value of 6.8 in segment 6 and of 6.4 in segment 5. It is worth noting that in the presence of BQ-123 3 μM, the ED_{50} for ET-1 was not much different in the three segments studied.

As illustrated in Figure 3 for segment 5 and also observed in segment 8 (data not shown), the action of ET-3 was completely blocked after pretreatment with BQ-123 300 nM, a concentration producing a shift to the right of ET-1 concentration-effect curves.

Discussion

The present results show that there is considerable heterogeneity in the response of different segments of the human left anterior descending coronary artery to endothelins and that this heterogeneity is related to the anatomical localization of the segment studied. Since the preparations were treated with L-NOARG which inhibits the production of NO, changes in the sensitivity to vasoconstrictors by either spontaneous or stimulated EDRF release (Egleme *et al.*, 1984; Godfraind *et al.*, 1985) were not involved. In the present experiments, it was observed that indomethacin did not change the ET dose-effect curve in the presence of L-NOARG. However, this does not rule out the possibility that ET could evoke prostacyclin release from endothelin cells in this artery as shown in other vessels (Filep *et al.*, 1991; Emori *et al.*, 1991); other experiments should consider this possibility. Since the drugs used in this study are peptides, the difference in activity between the segments studied could be related to a differential protease content of proximal and distal coronary artery segments. The observation that the activity of ET-1 was not different when the three segments were treated with BQ123 3 μM indicates that a differential protease content would not play a major role, if any. Therefore, the present results suggest that observed differences in sensitivity to endothelins could be related to pharmacologically distinct receptor subtypes in vascular smooth muscle cells located in different anatomically defined segments of the human left descending coronary artery.

Until recently, it was generally thought that the ET_A receptor was the predominant subtype mediating the vasoconstrictor response to endothelins (Ihara *et al.*, 1991; Masaki *et al.*, 1991). However, the diversity of functional endothelin receptors has been demonstrated in both *in vitro* and *in vivo* studies on several vascular beds of various animal species (Bigaud & Pelton, 1992; Harrison *et al.*, 1992; Okamura *et*

al., 1992; Sumner *et al.*, 1992; Cristol *et al.*, 1993; McMurdo *et al.*, 1993) showing that not only ET_A, but also ET_B and another unidentified receptor are involved in vasoconstriction. Those receptors have been functionally identified mainly by their differential sensitivity to ET-1 and ET-3 and to BQ-123. Use of other agents such as the ET_B-selective agonists, sarafotoxin S6C and tetra-Alamine ET-1, have been proposed in order to characterize the receptors (Miller *et al.*, 1993), but have not been used in the present study.

In rabbit jugular vein, a tissue nearly as sensitive to ET-1 as segment 8 of the human left anterior descending coronary artery, Sumner *et al.* (1992) have observed that ET-3 is as potent as ET-1 and that ET-1 contraction is insensitive to BQ-123: they concluded that rabbit jugular vein contraction is mediated by ET_B receptors. In the present study, it was observed in the three segments of human coronary artery used, that the ratio of potency of ET-1 and ET-3 was close to 100 and that the response to ET-1 was antagonized by BQ-123. It is therefore unlikely that ET_B receptors are the only subtype involved in the response of the human coronary artery to endothelins.

Rat thoracic aorta is 20 times less sensitive to ET-1 than segment 8 of the left anterior descending human coronary artery and nearly as sensitive as segment 5. In rat aorta in which Sumner *et al.* (1992) found one type of receptor classified as ET_A, the ratio of potency between ET-1 and ET-3 is close to 80, the pA_2 of BQ-123 is equal to 6.93 and the Schild plot has a slope equal to unity. The present observations made in the distal segment 8 of the left anterior descending human coronary artery were similar as far as the potency ratio between ET-1 and ET-3 is concerned. Furthermore, BQ-123 behaved as a competitive antagonist with a pA_2 value equal to 7.47, close to its affinity for ET_A receptors estimated in radioligand studies (Ihara *et al.*, 1992). This indicates that the contractile response to endothelin of distal segment 8 was probably related to the activation of a homogeneous population of ET_A receptors. However, for segments 5 and 6, in which BQ-123 did not behave as a competitive antagonist, it is likely that more than one receptor subtype was involved in the contractile response to endothelins. Complete blockade of the response to ET-3 by BQ-123 concentrations producing an antagonism easily surmountable by ET-1 could be due to a lower receptor reserve, but this interpretation requires further analysis.

This study provides the first information on the receptors involved in the contractile response of human coronary artery to endothelins but it also indicates that the pharmacological responsiveness of human coronary arteries *in situ* might be compared to *in vitro* responses. Interestingly, the mean diameter of segment 8 measured *in vitro* at a transmural pressure of 100 mmHg was similar to the diameter estimated in healthy human beings by McFadden *et al.* (1992) using quantitative coronary angiography. Furthermore, it was observed in the present study that distal arteries with a mean diameter of 1.4 mm were as sensitive to endothelin as two small resistant coronary arteries of respectively 0.8 and 0.7 mm diameter (data not shown). Therefore distal pre-resistant arteries of segment 8 could be used as a model for the study of the pharmacological properties of endothelins in the resistance vessels of the human coronary vascular bed.

In conclusion, this study demonstrates that the vasoconstriction evoked by endothelins in human coronary artery is mediated via different receptor subtypes, but that ET_A is the only subtype involved in the contraction of the distal pre-resistant coronary segment. This observation may be relevant for the study of the action of endothelin antagonists in the human coronary circulation.

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Formation of sulphidopeptide-leukotrienes by cell-cell interaction causes coronary vasoconstriction in isolated, cell-perfused heart of rabbit

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1 We have studied the transcellular biosynthesis of bioactive leukotrienes (LTs), generated upon blood cell-vascular wall interactions and their functional consequences, in the spontaneously beating, cell-perfused, heart of the rabbit. Rabbit isolated hearts were perfused under recirculating conditions (50 ml) with 5×10^6 cells of unpurified (buffy coat) or purified human neutrophils (PMNL), and challenged with 0.5 μM A23187 for 30 min. Coronary perfusion pressure (CPP), heart rate (HR), left ventricular end-diastolic pressure (LVEDP) and left ventricular pressure (LVP) were monitored continuously. Leukotriene formation was measured by specific enzyme-immunoassay and confirmed by reversed phase h.p.l.c. and u.v. spectral analysis.

2 Basal CPP values averaged 44 ± 1.4 mmHg; A23187 triggered a marked increase in CPP both in the presence of buffy coat cells (+ 100% above basal) and PMNL (+ 270% above basal); the latter change in CPP was accompanied by a rise in LVEDP (+ 138% above basal).

3 The increase in CPP was preceded by a statistically significant rise in $iLTC_4-D_4$ concentration in the circulating buffer. Pretreatment with two structurally unrelated LTD_4 receptor antagonists, LY171883 and SKF104353 (10 μM), fully prevented the increase in CPP and LVEDP. A similar protection was also observed when the rabbit heart was perfused with PMNL that had been pretreated with MK886 (1 μM), a potent inhibitor of leukotriene biosynthesis.

4 The increased coronary tone was accompanied by a marked release of lactate dehydrogenase (LDH), a marker of ischaemic damage; pretreatment of the heart with the LTD_4 receptor antagonists as well as of the PMNL with MK886 resulted in a complete suppression of LDH activity release.

5 Positive identification of LTC_4-D_4 in the perfusates was obtained and a significant correlation observed between the CPP values and $iLTC_4-D_4$ concentrations.

6 This study suggests that challenge of PMNL present within the coronary vasculature, causes a LTD_4-dependent coronary vasoconstriction, favoured by an efficient uptake of PMNL-derived LTA_4 by endothelial cells. The activation of the 5-lipoxygenase pathway in the context of tight interactions between blood cells and coronary vasculature, is suggested to have an important outcome in the alterations of coronary flow and cardiac contractility.

Keywords: Transcellular biosynthesis; PMNL; cardiac ischaemia; MK886; sulphidopeptide leukotrienes; endothelial cells; LY171883; SKF104353

Introduction

The generation of leukotrienes (LTs) exhibits remarkable cellular specificity; both polymorphonuclear leukocytes (PMNL) and eosinophils contain a 5-lipoxygenase to generate the 5-hydroperoxy eicosatetraenoic acid and leukotriene A_4 (LTA_4), but their secondary enzymes, i.e. LTA_4 hydrolase vs leukotriene C_4 (LTC_4) synthase, differ depending on the cell (Lewis & Austen, 1984). Following challenge with the calcium ionophore, A23187 (Borgeat & Samuelsson, 1979), PMNL generate predominantly leukotriene B_4 (LTB_4), a compound with very potent chemoattractant activities. On the other hand eosinophils (Weller *et al.*, 1983) show preferential generation of LTC_4, a potent bronchoconstrictor. Recently another process of biosynthesis of LTs has emerged. It involves the participation of different cell types whereby PMNL (i.e. donor cells) can synthesize the unstable metabolic intermediate LTA_4 which can be metabolized by vicinal cells (i.e. acceptor cells) into LTs B_4 or C_4. Such reaction involves the cooperation of PMNL with erythrocytes, platelets or the endothelial cell (Marcus *et al.*, 1982; McGee & Fitzpatrick, 1986; Feinmark & Cannon, 1986; Maclouf & Murphy, 1988). This process has been termed 'transcellular biosynthesis' and suggests that the cellular environment (i.e. cell-cell interaction) is an important control in the production of eicosanoids (Maclouf *et al.*, 1989). Most *in vitro* studies of transcellular biosynthesis have used cells isolated from blood or cultured endothelial cells as a reflection of what might happen in pathological situations such as in inflammatory reactions or in cardiovascular diseases where cell-cell interactions constitute an important part of this process (Lucchesi & Mullane, 1986). Fewer studies have addressed situations using blood cell-organ interactions (Voelkel *et al.*, 1992), where it seems logical to assume that such reactions do exist in disease. Adhesive reactions between blood and vascular wall cells, mediated by the selectins (Johnston *et al.*, 1990) or the integrins (Hynes, 1987), are likely to play an active role for the 'anchoring' of circulating cells that may allow more efficient reactive substrate uptake between adjacent cells rather than via the extracellular matrix or fluid phase. Furthermore a local synthesis of eicosanoids such as LTs, taking place at sites of adhesion, may alter the organ function.

It is well known that, in addition to their effects on the lungs, sulphidopeptide leukotrienes affect all major components of the cardiovascular system and could play a major

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role in vaso-occlusive processes. These mediators can constrict small and large vessels, modify cardiac and coronary functions, the microcirculation and the manifestations of ischaemia-reperfusion injury (Michelassi *et al.*, 1982; Evers *et al.*, 1985; Feuerstein, 1986; Mullane, 1988). Additionally, they are known to have vasopermeant properties that might have relevance for the extravasation of white cells from the vessel lumen to the tissue.

We took advantage of the rather well characterized capacity of the human PMNL to interact with platelets and endothelial cells for the production of LTC_4 to study the transcellular biosynthesis of bioactive LTs, generated upon blood cell-vascular wall interaction and their functional consequences, in rabbit spontaneously beating, isolated heart. Different pharmacological tools, sulphidopeptide-leukotriene receptor antagonists and synthetase inhibitors, were used in order to elucidate the role of the different cellular species involved.

Methods

Isolated perfused heart preparation

Albino rabbits weighing between 2.5 and 3.0 kg were used. Hearts were isolated and perfused retrogradely at 37°C through the aorta as previously described (Berti *et al.*, 1988). The perfusate contained (mM): NaCl 137, KCl 4, $CaCl_2$ 2, Na_2HPO_4 1.8, glucose 5.5, $MgCl_2$ 0.5 and $NaHCO_3$ 25. The hearts were placed in a temperature-equilibrated plastic housing chamber; silicon tubing and connections were used in the perfusing system (HW55 Ascenso, Milano, Italy). After equilibration at 37°C with a 5% CO_2:95% O_2 gas mixture, the pH of the perfusate was 7.4. The rate of perfusion was maintained at 20 ml min^{-1} with a roller pump (Gilson Minipulse 2, Biolabo, Milano, Italy). A latex balloon was inserted into the left ventricular cavity for measurement of left ventricular pressure (LVP) and dP/dt, recorded with a Hewlett Packard carrier amplifier (mod. 8805B) and recorder (mod. 7754A). The balloon was slowly filled with saline until end diastolic pressure stabilized between 8 and 12 mmHg. All hearts were equilibrated for 30 min at a flow rate of 20 ml min^{-1} to allow extensive rinsing of the vascular bed; the hearts were then perfused in a recirculating system at the same flow of 20 ml min^{-1} with a total volume of 50 ml. Coronary perfusion pressure (CPP), heart rate (HR), left ventricular pressure (LVP) and left ventricular end-diastolic pressure (LVEDP) were monitored continuously.

Cell perfused hearts

Recirculated rabbit hearts were perfused with unpurified (buffy coat) or purified human neutrophils (PMNL) in order to study interactions between blood cells and the heart. These human cells were obtained from blood (40 ml) withdrawn from healthy donors who had taken no medication for at least 1 week; it was collected into a 50 ml polypropylene centrifuge tube containing 5.7 ml of ACD (citric acid × H_2O, 41 mM; Na-citrate × $2H_2O$, 100 mM; glucose, 136 mM) as anticoagulant and aspirin (lysine acetylsalycilate, final concentration 1 mM). After centrifugation for 15 min at 200 *g*, platelet rich plasma (PRP) was removed and residual blood was combined with an equal volume of saline and half a volume of dextran T-500 (6%, w/v, in saline), followed by thorough mixing, and allowed to stand at room temperature for 30 min. The leukocyte-enriched upper phase was centrifuged for 15 min at 280 *g*. The pelleted cells were then subjected to erythrocyte lysis by gently resuspending them in 5 ml of a 0.2%, w/v, NaCl solution and further diluted with 5 ml of a solution of the following composition: 3.98 g NaCl + 0.5 g sucrose in 250 ml of distilled water, at + 4°C. The cell suspension was centrifuged for 15 min at 200 *g* and the pelleted cells (buffy coat cells) resuspended in Tyrode buffer without Ca^{2+} and Mg^{2+} for direct perfusion or in 2 ml of a 1:1 solution of PPP and saline for further purification by a discontinuous Percoll density gradient (42% and 51%, v/v, in PPP) (Haslett *et al.*, 1985). After centrifugation for 10 min at 180 *g*, the lower PMNL containing band was transferred; cells were washed twice with 10–15 ml of a 1:1 solution of PPP in saline. Cells were finally resuspended to appropriate volume in Tyrode solution without Ca^{2+} and Mg^{2+}. This preparation contains more than 95% PMNL, as checked on cytocentrifugates fixed in methanol and stained with Giemsa-losung (Merck-Bracco, Milano, Italy).

When cells had to be pretreated with compound MK886, (3- (1-(4-chlorobenzyl) -3-t-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid), prior to heart perfusion, they were supplied with Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM), preincubated at 37°C for 2 min and then exposed to MK886 (1 μM) for 5 min; cells were subsequently washed twice, resuspended in Tyrode solution without Ca^{2+} and Mg^{2+} and counted.

Sulphidopeptide-leukotriene receptor antagonists, compound LY171883, (1-(2-hydroxy-3-propyl-4(1H-tetrazol-5-yl) butoxy) phenyl)ethanone, and SKF104353, (2(**S**)-hydroxy-3(**R**) -((2-carboxyethyl) thio)-3- (2-(8-phenyloctyl) phenyl)-propanoic acid), were added to the recirculating medium 15 min prior to the addition of blood cells.

Buffy coat cells and PMNL (5×10^6 cells) were supplied with Ca^{2+} (2 mM) and Mg^{2+} (0.5 mM) before infusion into the recirculating medium of rabbit isolated hearts, at a flow rate of 0.6 ml min^{-1} in a volume representing 3%–5% (1–3 ml) of the total perfusate, in order to avoid mechanical obstruction of coronary vasculature. Aliquots (1 ml) of the recirculating medium were collected at different time intervals, centrifuged at 20,000 *g* for 1 min (Minifuge, Heraeus, Milano, Italy) and the supernatants divided into smaller aliquots for storage under argon atmosphere at – 20°C until the enzyme immunoassay (EIA) analysis. Fractions of these aliquots were eventually used for lactate dehydrogenase (LDH) activity determination, using a Sigma commercially available diagnostic kit (Cat. No. DG1340-K).

H.p.l.c. analysis of heart perfusates

The entire heart reservoir (approx. 45 ml) was collected into 1 volume ice-cold methanol, spiked with 25,000 d.p.m. [^{3}H]-LTC_4 and stored at – 20°C. After centrifugation at 3,500 *g* for 15 min, the supernatant was diluted with ½ volume of H_2O and the pH adjusted to 4.5 with formic acid. Sample extraction was quickly carried out using a solid phase cartridge (Supelclean Envi-TM, Supelco, Bellafonte, PA, U.S.A.) and elution performed with 90% aqueous methanol. After evaporation, the dried extract was reconstituted in h.p.l.c. solvent A (0.5 ml) containing 30 ng prostaglandin B_2 (PGB_2); 0.1 ml were added to 10 ml of liquid scintillator (Aqualuma plus, Packard, Milano, Italy) and radioactivity measured in a β-counter (Packard mod. 4000, Milano, Italy). Recovery from the extraction step ranged between 45–80%. The sample (0.4 ml) was injected into an h.p.l.c. gradient pump system (Beckman mod. 126) connected to a diode array u.v. detector (Beckman mod. 168). U.v. absorbance was monitored at 280 nm, and full u.v. spectra (210–340 nm) acquired at a rate of 0.5 Hz. A multilinear gradient from solvent A (40% aqueous methanol, 0.02% acetic acid, pH 5.5 with ammonium hydroxide) to solvent B (100% methanol) was used (from 0% to 35% solvent B, in 6 min; then to 65% over 20 min). The use of [^{3}H]-LTC_4 to monitor recovery was necessary because of differences in the extraction pattern of sulphidopeptide LTs and PGB_2 from the large volumes used. PGB_2 was then used for h.p.l.c. quantitation of sulphidopeptide leukotrienes. Positive identification of LTs was obtained through u.v. spectral analysis of chromatographic peaks eluting at characteristic retention times. Quantitation was performed on positively identified peaks only, using standard

curves of synthetic LTD_4 and LTB_4 (Cascade Biochem., Reading, UK).

Cell incubation

Buffy coat cells and PMNL (5×10^6 ml^{-1}) were allowed to equilibrate for 5 min at 37°C and challenged with A23187 (0.5 μM). Stimulation was terminated after 30 min by addition of 2 ml ice cold methanol containing 30 ng of PGB_2 and the samples stored at − 20°C overnight. Incubates were then centrifuged for 15 min at 3,500 *g*, the supernatant diluted to 15 ml with H_2O and extracted using a solid phase cartridge (Supelclean C18). Ninety percent aqueous methanol eluates were taken to dryness in a SpeedVac evaporating centrifuge (Savant Instruments, Farmingdale, NY, U.S.A.), reconstituted in the h.p.l.c. solvent C and analyzed with a gradient pump system (Beckman mod. 126) connected to a diode array detector (Beckman mod. 168). U.v. absorbance was monitored at 235 nm and 280 nm, and full u.v. spectra (210–340 nm) were acquired at a rate of 0.5 Hz. A multilinear gradient from solvent C (methanol/acetonitrile/water/acetic acid, 10:10:80:0.02, v:v:v:v, pH 5.5 with ammonium hydroxide) to solvent D (50% methanol, 50% acetonitrile) was used. This method allowed easy separation of LTB_4 from both 5S,12S-dihydroxy eicosatetraenoic acid (5S,12S-diHETE) and Δ6-*trans*-LTB_4 epimers. Synthetic standards (10–1000 pmols) were used to quantitate LTB_4 and LTC_4 (Cascade Biochem., Reading, UK).

Enzyme immunoassay (EIA) for LTB_4 and LTC_4–D_4

Quantitative determination of eicosanoids was performed with solid phase EIA by competition using acetylcholinesterase coupled to different eicosanoids as label (Pradelles *et al.*, 1985; Antoine *et al.*, 1991). A microtitration system (Labsystems, Åbå, Finland), including an automatic dispenser (Autodrop), a washer (Multiwash) and a spectrophotometer (Multiskan MC), was used. Briefly, mouse monoclonal anti-rabbit IgG (200 μl of a 10 μg ml^{-1} solution in 0.05 M potassium phosphate buffer, pH 7.4) was distributed into each well of microtitre plates (Nunc 96F with certificate, Denmark); after overnight incubation (at least 18 h) at room temperature, 100 μl of buffer (NaN_3 0.03% w/v, NaCl 0.4 M, EDTA 1 mM, BSA 0.3% w/v, in phosphate buffer 0.1 M, pH 7.4) were added and the plates stored at 4°C for 24 h before use. The plates were then washed with 0.01 M phosphate buffer, pH 7.4, containing 0.05% v/v, Tween 20. The assay was performed in EIA buffer (0.1 M phosphate buffer pH 7.4 containing 0.4 M NaCl, 1 mM EDTA, 0.01% w/v, BSA and 0.07%, w/v, NaN_3), total volume 150 μl. Each component was added in a volume of 50 μl: standard or biological sample, enzymatic tracer and specific antiserum (final dilution of 1:60,000, 1:40,000, respectively for LTB_4 and LTC_4). Anti-LTC_4 antibody showed a cross-reactivity of 80 ± 8% with LTD_4 and therefore the measured response is collectively referred as immunoreactive LTC_4-LTD_4 ($iLTC_4$-LTD_4). After overnight incubation at 4°C, the plates were washed and 200 μl of Ellman's reagent consisting of enzymatic substrate (acetylthiocholine, 7.5×10^{-4} M) and chromogen (5,5′-dithio*bis*(2-nitro-benzoic acid), 5×10^{-4} M) in 0.01 M phosphate buffer was automatically dispensed into each well with the Autodrop; after 3 h, the absorbance at 414 nm was measured. In order to evaluate the concentration of LTB_4 in biological samples, we used a standard curve from 7.8 pg ml^{-1} to 1 ng ml^{-1} and for LTC_4 a standard curve from 15.6 pg ml^{-1} to 2 ng ml^{-1}. Standard curves and biological samples were analyzed with an IBM computer using a linear log-logit transformation.

Statistics

CPP, $iLTC_4$-LTD_4, and LVEDP data, at individual times, were analyzed by analysis of variance (ANOVA) and Dunnett's multiple comparison vs control group test. Linear regression analysis and ANOVA were used to examine the relationship between $iLTC_4$-LTD_4 and CPP.

Results

Effect on CPP

When rabbit isolated hearts were perfused under recirculating conditions at a constant flow of 20 ml min^{-1}, CPP averaged 44 ± 1.4 mmHg and remained constant for the observation period which extended up to 30 min. Similarly HR (beats min^{-1}, 163 ± 12), LVP (86 ± 2 mmHg) and LVEDP (10.2 ± 0.55 mmHg) did not vary throughout the experimental period. Addition of unchallenged buffy-coat cells (5×10^6 cells) to the recirculating medium, did not modify the above parameters during the same period (i.e. 30 min). Challenge of the rabbit heart with A23187, 0.5 μM, did not alter myocardial contractility nor CPP. However, when the ionophore challenge took place in presence of buffy-coat cells, CPP was increased by 100% at 30 min (Figure 1), without changes in strength of contractility or other functional parameters. When purified PMNL (5×10^6 cells) were perfused through the rabbit heart, no changes in the functional parameters were detected. However, challenge with A23187 (0.5 μM)

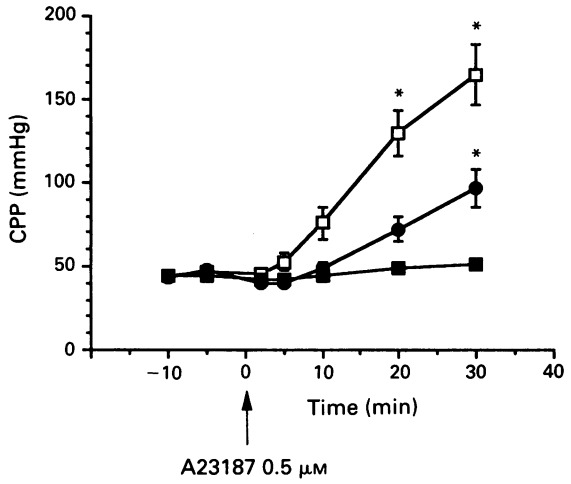

Figure 1 Coronary perfusion pressure (CPP) of rabbit isolated hearts perfused under recirculating conditions with plain buffer (■, $n = 8$), human buffy-coat cells (●, $n = 7$) or PMNL (1×10^5 cells ml^{-1}, □, $n = 10$) and challenged with calcium ionophore A23187 (0.5 μM). Values are mean ± s.e.mean. *$P < 0.05$ compared with control (heart + A23187) group.

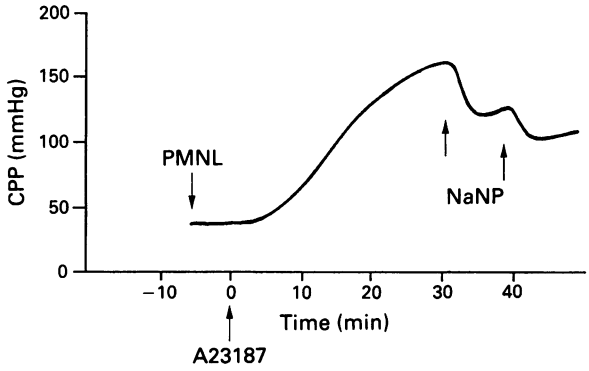

Figure 2 Reversal by sodium nitroprusside (NaNP, 100 μg bolus injections) of increase in coronary perfusion pressure (CPP) evoked by A23187 0.5 μM in PMNL-perfused rabbit isolated heart preparation.

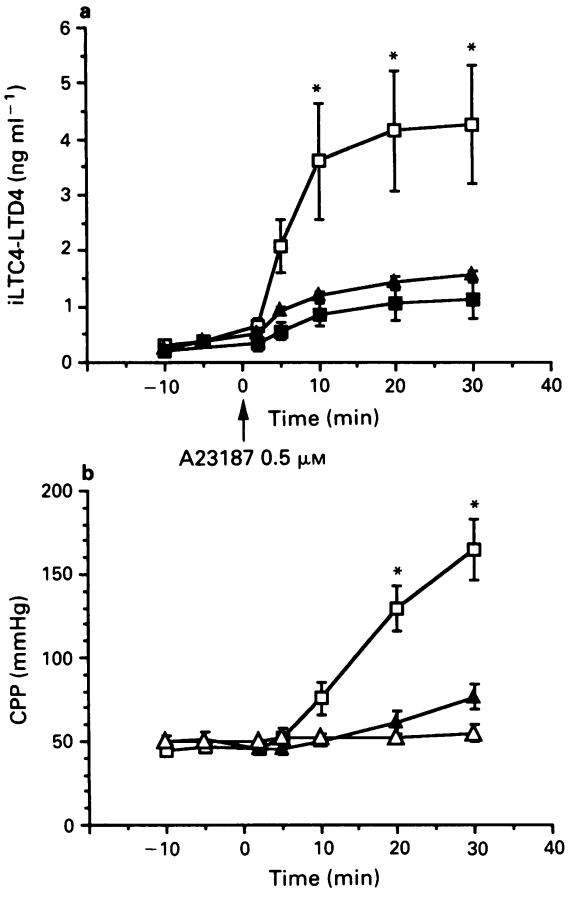

Figure 3 (a) Time-course levels of $iLTC_4$-LTD_4 in the recirculating medium of buffer-perfused rabbit isolated hearts (■, $n = 8$) or PMNL-perfused, rabbit isolated hearts (□, $n = 9$) challenged with 0.5 μM A23187; effect of pretreatment of the PMNL with compound MK886 (1 μM, ▲, $n = 3$). (b) Coronary perfusion pressure (CPP) of rabbit isolated hearts perfused under recirculating conditions with human PMNL (10^5 cells ml^{-1}, △, $n = 3$) and after challenge with calcium ionophore A23187 (0.5 μM, □, $n = 10$); effect of pretreatment of PMNL with compound MK886 (1 μM, ▲, $n = 3$). Values are mean ± s.e.mean. $^*P < 0.05$ compared to control (heart + A23187) group.

induced a more severe increase in CPP (Figure 1), with a time course profile showing an earlier onset of coronary constriction, as compared to perfusion with buffy-coat cells; at 30 min the resistance to perfusion pressure had increased by 280%. Bolus injection of 100 μg nitroprusside, i.a., reduced by approx. 35% the increase in CPP evoked by A23187 in presence of PMNL; a second bolus injection of the vasodilator caused a further 25% drop in vascular tone (Figure 2).

Leukotriene production

Aliquots of the recirculating medium, withdrawn at different time intervals, were analyzed for presence of sulphidopeptide leukotrienes by EIA; preliminary experiments had indicated that the rabbit heart possesses a significant metabolic capacity to convert LTC_4 to LTD_4. Radioactive tracer experiments ([^{3}H]-LTC_4, 350,000 d.p.m.) as well as addition of exogenous LTC_4 (2 μg, bolus injection), resulted in almost complete conversion to LTD_4 (75 ± 8%) after 30 min of perfusion. Detectable concentrations of $iLTC_4$-D_4 in the circulating medium were observed in absence (0.29 ± 0.04 ng ml^{-1}, $n = 40$) as well as 5 min following addition of blood cells (0.37 ± 0.06 ng ml^{-1}, $n = 20$). A spontaneous two fold increase of $iLTC_4$-D_4 was observed when PMNL were present, after 30 min, in the absence of A23187. Challenge of rabbit hearts with the Ca-ionophore (0.5 μM, without recirculating blood cells), was followed by a slight increase in LT formation (Figure 3a), which did not affect heart contractility or CPP. When PMNL were present, A23187 elicited a tenfold increase in $iLTC_4$-D_4 concentrations in the recirculating medium (Figure 3a).

H.p.l.c. analysis of the final volume of the circulating perfusate (approximatively 45 ml), gave results that were fully consistent with the EIA quantitation of LTs at 30 min after challenge and allowed positive identification of LTD_4 and LTB_4 by on-line u.v. spectrum analysis. A significant correlation was observed between the CPP values and $iLTC_4$-D_4 concentrations in PMNL experiments ($r^2 = 0.639$, $P < 0.0001$). LTB_4 was detectable in perfusates from hearts challenged with A23187 both in the absence or presence of blood cells. Nevertheless the amounts detected in the latter experimental conditions, were always lower than those expected, given the biosynthetic capacity of buffy-coat cells or PMNL, as assessed in the cell incubation experiments (Table 1); moreover challenge of PMNL preparations in suspension, showed the expected releases of LTA_4 as indicated by presence of its non-enzymatic degradation products (Δ6-*trans*-LTB_4, 12-epi-Δ6-*trans*-LTB_4, 5S6SdiHETE and 5S6RdiHETE) in the h.p.-l.c. chromatographic analysis.

In contrast, the amount of sulphidopeptide LTs found in heart perfusates at 30 min showed a marked increase over the combined amount of LTs formed, independently, by the same number of isolated cells in suspension and by the heart itself (Table 1).

Effect of sulphidopeptide-leukotriene receptor antagonists and biosynthesis inhibitors

Pretreatment of PMNL only with MK886 (1 μM), resulted in an almost complete suppression of the rise in $iLTC_4$-D_4 observed using untreated PMNL (Figure 3a); this effect was accompanied by a highly significant protection against the increase in coronary tone (Figure 3b).

Pretreatment of the heart with two structurally unrelated LTD_4 end-organ antagonists, LY171883 (10 μM) (Fleish *et al.*, 1985) and SKF104353 (10 μM) (Mong *et al.*, 1987), fully prevented the rise in CPP (Figure 4).

Effect on LVEDP

Left ventricular end-diastolic pressure (LVEDP) was also affected by A23187 challenge in presence of PMNL (basal: 9.8 ± 0.6 mmHg, 30 min after A23187: 23.4 ± 6.2 mmHg, $n = 9$) (Figure 5), indicating onset of an ischaemic process;

Table 1 Formation of leukotrienes

	Heart perfusates no PMNL	*Heart perfusates with PMNL*	*Net contribution by presence of PMNL*	*Isolated PMNL*
LTB_4	17 ± 7	118 ± 28	101	285 ± 35
LTC_4-D_4	75 ± 13	358 ± 71	283	60 ± 9

Values are expressed as pmol, mean ± s.e.
Challenge was A-23187, 0.5 μM, 30 min.
The same number of PMNL (5×10^6 cells) was used in isolated cell experiments and in heart perfusions. Leukotrienes were assayed by r.p. h.p.l.c.

this functional change was fully prevented by pretreatment of the heart with LY171883 or by PMNL exposure to MK886 (Figure 5).

Effect on LDH activity release

The release of lactate dehydrogenase activity (LDH) into the effluent was determined and found markedly elevated only in hearts that had been perfused with PMNL and stimulated with A23187. Pretreatment with the LTD_4 receptor antagonist LY171883 or MK886 abolished LDH activity release (Table 2).

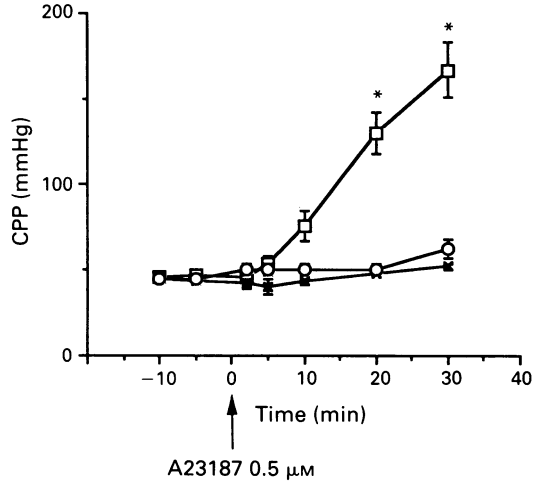

Figure 4 Coronary perfusion pressure (CPP) of rabbit isolated hearts perfused under recirculating conditions with human PMNL challenged with calcium ionophore A23187 (0.5 μM) (1×10^5 cells ml^{-1}, $n = 10$, □); effect of pretreatment of the heart with the LTD_4 receptor antagonists LY171883 (10 μM, ×, $n = 3$) and SKF104353 (10 μM, ○, $n = 3$). Values are mean ± s.e.mean. $^*P < 0.05$ vs PMNL group.

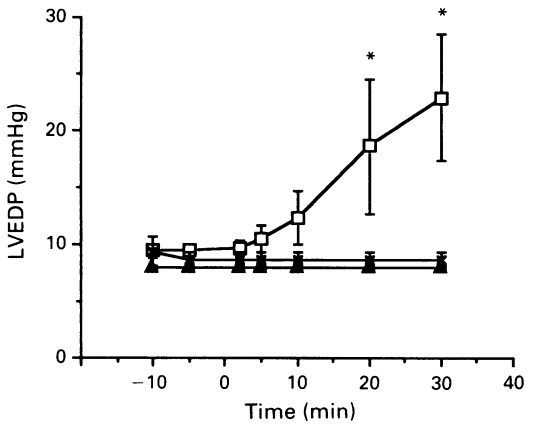

Figure 5 Left ventricular end-diastolic pressure (LVEDP) of rabbit isolated hearts perfused under recirculating conditions with human PMNL after challenge with A23187 (0.5 μM, □, $n = 10$); effect of pretreatment of the heart with the LTD_4 receptor antagonist, LY 171883 (10 μM, ×, $n = 3$), or of pretreatment of the PMNL with MK886 (1 μM, ▲, $n = 3$). Values are mean ± s.e.mean. $^*P < 0.05$ vs control (heart + A23187) group.

Dynamics of circulating cells

Cytocentrifugation of aliquots from the perfusate of hearts recirculated with PMNL, before ionophore stimulation, showed the expected presence of human PMNL; after challenge with A23187 it was not possible to identify any circulating cell in the reperfusion medium, suggesting a quantitative attachment of leukocytes to the coronary vascular bed. This was confirmed by optical microscopy of preparations from PMNL-perfused rabbit hearts.

Discussion

The present results clearly show that challenge of recirculating human PMNL with A23187, in a spontaneously beating, perfused rabbit heart, causes a marked and progressive increase in coronary perfusion pressure, indicating a constriction of the smooth muscle of this vasculature; in fact, the elevated coronary tone was reversed by nitroprusside, a non-specific vasodilator. The vasoconstriction observed in our experimental conditions did not occur in the absence of white cells and it was already significant when unpurified buffy-coat cell preparations were used but became stronger when PMNL preparations were recirculated and challenged with the Ca^{2+}-ionophore, A23187.

Our results indicate that, at least in the rabbit heart, a slight increase in LVEDP takes place only after a significant sulphidopeptide LT-dependent coronary vasoconstriction has occurred. The greater increase in CPP seen with PMNL, as compared to an equal number of buffy coat cells, related the functional changes specifically to the number of PMNL present in the recirculating system and not to the total number of cells. The low PMNL concentration used in our experiments was deliberately selected to avoid the potential problem of clumping caused by PMNL aggregation resulting from the effect of the calcium ionophore and allowed a better evaluation of the effect of released soluble mediators. The use of human blood cells in perfusing rabbit heart, which might raise the problem of interspecies compatibility, was selected because preliminary experiments had shown that rabbit blood cells did not provide the expected profile of sulphidopeptide leukotriene formation due to transcellular biosynthesis. The aim of the study concerned the evaluation of the effect of locally-generated LTs due to tight interactions between PMNL and endothelial cells; electron as well as optical microscopy evidence confirmed adhesion and extravasation of added PMNL (unpublished observations).

Biochemical evidence supports unequivocally the presence of sulphidopeptide LTs in the recirculating medium, with preferential formation of LTD_4. This lipid mediator can constrict coronary vessels both *in vitro* and *in vivo* (Roth & Lefer, 1983; Piper & Samhoun, 1987). Moreover the present results indicate that the rabbit heart possesses an efficient metabolism that can convert LTC_4 into LTD_4 which seems to

Table 2 Release of lactate dehydrogenase (LDH) activity

	Heart + PMNL	*Heart + PMNL + A23187*	*Heart + LY171883 10 μM + PMNL + A23187*	*Heart + PMNL pretreated with MK886 1 μM + A23187*
LDH activity	36.1 ± 12.6	190.7 ± 46.3*	26.7 ± 14.2	18.7 ± 6.2
n	3	4	3	3

Measurements were performed on aliquots of heart perfusates, 30 min after challenge.
Values are expressed as B/B units ml^{-1}, mean ± s.e.
$^*P < 0.05$ (vs all other groups)

represent the main metabolite of this specific pathway in this system.

The increase in coronary vascular resistance and the alterations in heart mechanics observed in the presence of PMNL, i.e. increase in LVEDP, suggest the onset of an ischaemic process. This was also confirmed by the increased presence in the perfusate of lactate dehydrogenase activity, a common diagnostic aid in myocardial ischaemia. These functional and metabolic changes of the heart were fully prevented by LY171883 and SKF104353, two structurally unrelated LTD_4 receptor antagonists, indicating a pivotal role of sulphidopeptide LTs in the alteration of coronary vascular tone. This is confirmed by the observation that a similar degree of protection was also observed when PMNL that had been pretreated with MK886 were perfused through the coronary vascular bed and challenged with A23187. MK886 prevents leukotriene A_4 formation in intact polymorphonuclear leukocytes (Gillard *et al.*, 1989) by dose-dependent inhibition of the translocation of 5-LO to the membrane and its inhibitory effect cannot readily be removed by elimination of the free drug from the incubation medium.

In this respect it is interesting to note that the amount of $iLTC_4$-D_4 detectable in the recirculating medium when MK 886-treated PMNL were used, was significantly lower than that released when untreated PMNL were perfused and was barely distinguishable from the amount contributed by the heart alone. These findings support the concept that the increase in coronary vascular resistance, observed following PMNL activation, is a consequence of transcellular metabolism between PMNL, which act as donors of LTA_4, and endothelial cells, their proximal acceptor cells.

Evidence for LT synthesis via transcellular metabolism between PMNL and endothelial cells has already been documented in isolated cell preparations (Feinmark & Cannon, 1987) as well as in a more complex model of pulmonary leukostasis in the isolated, perfused lung of the rabbit (Grimminger *et al.*, 1990), but it has never been addressed for cardiac functions. Indeed, ionophore challenge of the same PMNL preparations in suspension, clearly showed release of LTA_4 reflected by the presence of significant amounts of non-enzymatic products of LTA_4 metabolism (Δ6-*trans*-LTB_4, 12-epi Δ6-*trans*-LTB_4, 5.6 diHETEs) together with the normal arachidonate metabolic profile of these cells. The difference in sulphidopeptide LT production, observed between challenge of isolated PMNL versus PMNL perfused hearts, is probably the result of an efficient uptake of LTA_4 by the endothelial cells, favoured by adhesion of PMNL to the vascular walls. The conversion of LTA_4 into LTC_4-D_4 would result in elevated local concentrations of mediators at effector sites, e.g. smooth muscle cells adjacent to the endothelial cell lining.

Taken together, our results underline an important role for peptido-LTs in the cardiovascular system as a consequence of transcellular biosynthesis, involving the transfer of PMNL-derived LTA_4 and its conversion, by vascular or perivascular acceptor cells, to sulphidopeptide LTs. The activation of the 5-lipoxygenase biosynthetic pathway in the context of a tight interaction between circulating blood cells and coronary vasculature, indicates an important outcome in the alteration of coronary flow. This event may become critical in those pathological conditions associated with vaso-occlusive processes leading to myocardial infarction or in conditions consistent with deficiency of endothelium-derived relaxing factor generation, where hyperreactivity of vascular smooth muscle and enhanced leukocyte adhesion occur.

The recent observation of increased urinary LT excretion in patients with cardiac ischaemia (Carry *et al.*, 1992), as well as the observation that LY171883 has a protective effect in ischaemia-reperfusion injury to the myocardium (Hock *et al.*, 1992), support this hypothesis.

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Vascular responses to endothelin-1 following inhibition of nitric oxide synthesis in the conscious rat

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1 The objectives of the present experiments were to assess the role of endogenous nitric oxide (NO) in mediating and/or modulating the effects of endothelin-1 (ET-1) on blood pressure and microvascular permeability in conscious rats.

2 Intravenous administration of the NO synthesis inhibitors, N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) at a dose (25 mg kg^{-1} or 2 mg kg^{-1}, respectively) which evoked maximum increase in mean arterial blood pressure (MABP) significantly attenuated (by about 40%) the vasodepressor response and potentiated (by 100–180%) the pressor response to ET-1 (1 nmol kg^{-1}, i.v.) compared to the effects of ET-1 in animals where the peripheral vasoconstrictor effects of L-arginine analogues were mimicked by an infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}). Similar inhibition of the depressor and potentiation of the pressor actions of ET-1 were observed when the MABP which had been elevated by L-NMMA or L-NAME was titrated to normotensive levels with hydralazine or diazoxide before injection of ET-1.

3 L-NAME (2 mg kg^{-1}) increased the vascular permeability of the large airways, stomach, duodenum, pancreas, liver, kidney and spleen (up to 280%) as measured by the extravasation of Evans blue dye. The permeability of pulmonary parenchyma, skeletal muscle and skin was not affected significantly by L-NAME treatment. Elevation of MABP by noradrenaline infusion did not evoke protein extravasation in the vascular beds studied with the exception of the lung. In the large airways, tissue Evans blue content was similar following noradrenaline infusion and L-NAME.

4 Both the pressor and permeability effects of L-NAME (2 mg kg^{-1}) were effectively reversed by L-arginine (300 mg kg^{-1}) but not by D-arginine (300 mg kg^{-1}). The D-enantiomer of L-NAME, D-NAME (2 mg kg^{-1}) had no effect on the parameters studied.

5 Protein extravasation was significantly enhanced by ET-1 (1 nmol kg^{-1}) in the upper and lower bronchi, stomach, duodenum, kidney and spleen (up to 285%). This was potentiated by L-NAME (2 mg kg^{-1}), resulting in marked increases in tissue Evans blue accumulation (up to 550%) in these tissues. The effects of L-NAME and ET-1 were additive in the trachea, duodenum, pancreas and liver. Combined administration of L-NAME plus ET-1 significantly increased protein extravasation in the pulmonary parenchyma, where neither L-NAME nor ET-1 alone caused significant increases.

6 Noradrenaline infusion (620–820 ng kg^{-1} min^{-1}) potentiated the permeability action of ET-1 (1 nmol kg^{-1}) in the pulmonary circulation, whereas it did not modify ET-1-induced protein extravasation in the other vascular beds.

7 These results indicate that endogenous NO mediates, in part, the vasodepressor effect and attenuates the vasopressor action of ET-1 and modulates the effects of ET-1 on vascular permeability. These findings confirm the role of NO in the maintenance of blood pressure and suggest an important role for NO in the regulation of microvascular permeability.

Keywords: Endothelin-1; protein extravasation; vascular permeability; blood pressure; nitric oxide; L-arginine analogues

Introduction

Endothelial cells are capable of producing a variety of substances, including endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988) and endothelium-derived relaxing factor/nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) that play a role in the regulation of vascular tone, local blood flow and permeability. Once released, these substances may act on the same cells to modulate the synthesis and/or release of other substances. Thus, ET-1-induced NO release (De Nucci *et al.*, 1988) and inhibition of ET-1 release by NO have been described (Boulanger & Lüscher, 1990; Saijonmaa *et al.*, 1990).

The discovery that L-arginine is the physiological precursor for the formation of NO (Palmer *et al.*, 1988a,b; Sakuma *et al.*, 1988) led to the development and use of N^G-substituted analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988) and N^G-nitro-L-arginine methyl ester (L-NAME) (Moore *et al.*, 1990) as inhibitors of endothelial NO synthesis. The findings that intravenous injection of L-arginine analogues evoke large increases in arterial blood pressure in experimental animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990a; Hecker *et al.*, 1990; Vargas *et al.*, 1991) are considered to indicate that the resultant decrease in endothelial NO production is responsible for the elevation of blood pressure. The studies investigating the role of NO in mediating the vasodilator and vasodepressor responses to ET-1 resulted in conflicting results (cf. Fozard & Part, 1992) since both significant inhibition of the depressor action of ET-1 by L-NMMA (Whittle *et al.*, 1989) and failure of L-NMMA to attenuate the depressor response to ET-1 (Gardiner *et al.*, 1989) have been described in anaesthetized and conscious rats, respectively. Furthermore, L-NAME, a more potent inhibitor of NO synthesis than L-NMMA (Moore *et al.*, 1990; Rees *et al.*, 1990), was found to attenuate the

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vasodilator action of ET-1 in the conscious rat (Gardiner *et al.*, 1990b).

Recent evidence suggest that both ET-1 and NO could also affect vascular permeability. Administration of ET-1 evoked haemoconcentration in dogs (Goetz *et al.*, 1988) and rats (López-Farré *et al.*, 1989; Filep *et al.*, 1991), led to oedema formation in the forearm of man (Dahlöf *et al.*, 1990) and enhanced protein extravasation in the large airways, heart, stomach, duodenum, kidney and spleen in rats (Filep *et al.*, 1991; 1992; Zimmerman *et al.*, 1992). On the other hand, NO may inhibit vascular permeability to macromolecules, since L-NAME has recently been reported to increase protein extravasation in the cat mesenteric circulation (Kubes & Granger, 1992) and rat coronary circulation (Filep *et al.*, 1993).

The objective of the present experiments was to study whether or not inhibition of endogenous NO synthesis could modulate the effects of ET-1 on blood pressure and microvascular permeability in conscious rats. The experimental design incorporated animals where mean arterial blood pressure was adjusted to similar levels as observed following administration of L-arginine analogues.

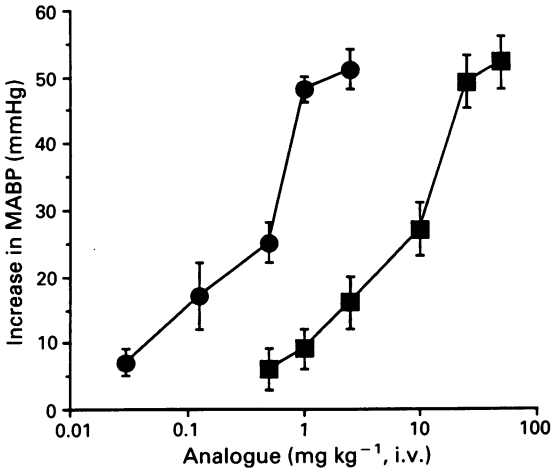


Figure 1 Peak pressor responses to intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and N^G-monomethyl-L-arginine (L-NMMA, ■) in the conscious rat. Basal mean arterial blood pressure was 110 ± 1 mmHg ($n = 12$). Values are means ± s.e.means of 4–8 experiments.

Methods

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 220–310 g. The animals were kept in individual metabolic cages and were prepared as described previously (Filep *et al.*, 1987). Briefly, under anaesthesia (ketamine, 75 mg kg^{-1} and sodium pentobarbitone, 15 mg kg^{-1}) catheters were implanted into the abdominal aorta and vena cava through the central tail artery and left femoral vein, respectively. The venous catheter was led subcutaneously to the root of the tail. The catheters emerging from the tail were protected by an acrylic cuff-metal spiral device and were fed through the top of the metabolic cage. The animals were allowed to recover completely for at least 4 days following the surgical procedures. During the experiments the rats could move freely and had free access to food and water. Mean arterial blood pressure (MABP) was continuously monitored by an electromanometer using a Statham P23 dB pressure transducer.

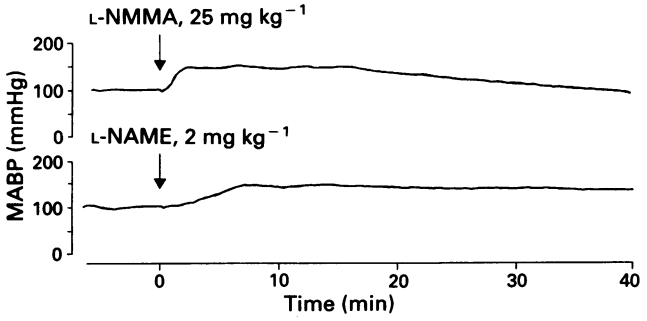


Figure 2 Time-course of changes in mean arterial blood pressure (MABP) following intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}) and N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}) in conscious rats. These traces are representative for 8 and 4 experiments, respectively.

Experimental protocols

Initial experiments were designed to compare the pressor potency of L-arginine analogues. A 4 to 6 point dose-response curve was generated in each animal. On any one day each animal received only L-NAME or L-NMMA at 40–60 min intervals. In the second series of studies, the blood pressure responses to ET-1 (1 nmol kg^{-1}) were compared in the absence and presence of L-arginine analogues. The animals were pretreated with L-NAME (2 mg kg^{-1}) or L-NMMA (25 mg kg^{-1}) for 10 and 2 min, respectively before injection of ET-1. On any one day each animal received only one injection of ET-1. In order to compare directly the blood pressure responses to ET-1, in some animals MABP was either elevated by infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) to levels observed following L-NAME-treatment or L-NAME-induced elevation in MABP was titrated to normotensive levels with diazoxide (90 μmol kg^{-1}) or hydralazine (1.2–1.5 μmol kg^{-1}) before injection of ET-1. Both diazoxide and hydralazine cause direct (endothelium-independent) relaxation of the arterial smooth muscle. In another set of experiments, the ability of L-arginine analogues to inhibit the hypotensive effect of acetylcholine, which causes endothelium-dependent vasorelaxations *in vitro*, was studied in control rats and in animals receiving L-arginine analogues, noradrenaline infusion, L-NAME plus diazoxide or L-NAME plus hydralazine. At least 3 days were allowed to elapse between different experiments on the same rat.

In subsequent experiments protein extravasation was quantitated by measuring the extravasation of Evans blue dye, which binds to plasma albumin (Rawson, 1943). In this series of experiments, Evans blue dye (20 mg kg^{-1}, 25 mg ml^{-1} in 0.9% NaCl) was injected intravenously together with vehicle or ET-1 (1 nmol kg^{-1}) into animals pretreated with L-NAME (2 mg kg^{-1}) or during noradrenaline infusion (620–820 ng kg^{-1} min^{-1}). An additional group of animals received L-arginine (300 mg kg^{-1}, i.v.) or D-arginine (300 mg kg^{-1}, i.v.) 5 min after injection of L-NAME (2 mg kg^{-1}). Four animals were treated with N^G-nitro-D-arginine methyl ester (D-NAME, 2 mg kg^{-1}) for 10 min before injection of Evans blue dye. Ten min after injection of Evans blue dye, the animals were anaesthetized (sodium pentobarbitone, 50 mg kg^{-1}) and were perfused with 50 ml 0.9% NaCl through a catheter inserted into the abdominal aorta to remove the excess of intravascular dye. Then portions of the trachea, upper bronchi (airways extending from the bifurcation of the trachea to its entry to parenchyma), lower bronchi (defined as major airways surrounded by pulmonary parenchyma that can be easily dissected without magnification), pulmonary parenchyma, liver, spleen, pancreas, kidney, stomach, duodenum, skeletal muscle (right quadriceps) and dorsal skin were excised and weighed. Tissue Evans blue dye content was measured by spectrophotometry following extraction with formamide as described previously (Filep *et al.*, 1991). Evans

Table 1 Effects of L-arginine and D-arginine on N^G-nitro-L-arginine methyl ester (L-NAME)-induced changes in arterial blood pressurre and protein extravasation in conscious rats

	Vehicle ($n = 6$)	*L-NAME* ($n = 6$)	*D-Arginine plus L-NAME* ($n = 4$)	*L-Arginine plus L-NAME* ($n = 4$)	*D-NAME* ($n = 4$)
Basal MABP (mmHg)	106 ± 3	105 ± 4	104 ± 5	104 ± 3	107 ± 2
Peak ΔMABP (mmHg)	2 ± 2	43 ± 3**	39 ± 4*	3 ± 2†	2 ± 1†
Evans blue dye (μg mg^{-1} tissue weight)					
Trachea	62 ± 6	108 ± 12*	88 ± 7*	55 ± 6†	60 ± 5†
Upper Bronchi	52 ± 3	104 ± 9**	89 ± 14*	46 ± 8†	45 ± 7†
Lower Bronchi	49 ± 5	112 ± 10**	104 ± 12*	53 ± 7†	51 ± 6†
Pulmonary parenchyma	81 ± 7	110 ± 8	106 ± 13	97 ± 10	81 ± 18
Liver	53 ± 5	111 ± 10**	102 ± 11*	54 ± 18†	43 ± 8†
Spleen	118 ± 8	195 ± 23*	160 ± 21	90 ± 5†	111 ± 7†
Pancreas	38 ± 5	100 ± 11*	90 ± 15*	32 ± 6†	33 ± 7†
Kidney	57 ± 9	136 ± 20*	107 ± 14*	53 ± 13†	51 ± 7†
Stomach	71 ± 5	155 ± 25*	126 ± 16*	61 ± 8†	69 ± 13†
Duodenum	90 ± 9	252 ± 21**	205 ± 41*	86 ± 13†	99 ± 13†

Values are means ± s.e.mean. The animals were given L-arginine (300 mg kg^{-1}) or D-arginine (300 mg kg^{-1}) 5 min after injection of L-NAME (2 mg kg^{-1}). Ten min after administration of L-NAME, D-NAME (2 mg kg^{-1}) or their vehicle (0.9% NaCl, control), Evans blue dye (20 mg kg^{-1}) was injected i.v. The rats were killed 10 min after injection of the dye.
*$P<0.05$; **$P<0.01$ compared to controls; †$P<0.05$ compared to L-NAME-treated animals by Dunn's multiple contrast hypothesis test.

blue content of each sample was expressed as μg dye g^{-1} dry tissue weight to avoid underestimation of changes due to plasma fluid extravasation.

Drugs and chemicals

ET-1 was synthesized in our laboratories by solid-phase methodology. The purity of the preparation was greater than 97%. ET-1 was dissolved in distilled water and stored at − 20°C. On the day of the experiments an aliquot was removed and diluted further in 0.9% NaCl. Evans blue dye, L-arginine hydrochloride, D-arginine hydrochloride, noradrenaline hydrochloride, acetylcholine chloride, diazoxide, hydralazine hydrochloride and L-NAME were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-NMMA was obtained from Calbiochem, San Diego, CA, U.S.A.; D-NAME was purchased from Research Biochemicals International, Natick, MA, U.S.A. All drugs were dissolved in 0.9% NaCl immediately before use.

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical evaluation of the data was performed by Dunn's multiple contrast hypothesis test (Dunn, 1964) when various treatments were compared to the same control or by the Wilcoxon signed rank test and Mann-Whitney U test for paired and unpaired observations, respectively. A $P<0.05$ level was considered significant for all tests.

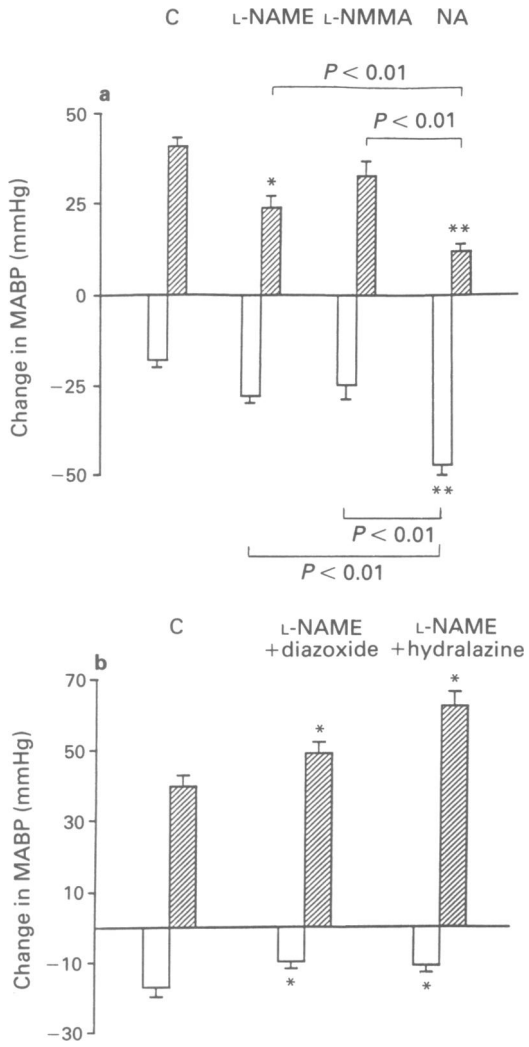
Results

Effects of L-arginine analogues on blood pressure responses to ET-1

Intravenous injection of L-NMMA and L-NAME produced dose-dependent increases in MABP in conscious rats (Figure 1). The maximum increase in MABP that could be evoked by these L-arginine analogues was similar (ΔMABP were 52 ± 4 mmHg and 51 ± 3 mmHg, respectively). Estimated ED_{50} values for L-NMMA and L-NAME were 7.8 and 0.5 mg kg^{-1}, respectively, indicating that L-NAME was approximately 16 times more potent than L-NMMA. The time course of the pressor action of the L-arginine analogues differed considerably (Figure 2). L-NMMA produced an immediate increase in MABP with a peak effect occurring within 1–3 min after the injection. In contrast, the pressor effect of L-NAME developed slowly, reaching the maximum change in MABP between 5 and 9 min (Figure 2). The duration of the pressor responses to L-NMMA was shorter than those of L-NAME; MABP returned to preinjection levels by 10–45 min depending on the dose of L-NMMA. The nitro analogue, L-NAME, however, evoked prolonged increases in MABP. For example, MABP remained stable at 145 mmHg for 50–60 min following administration of 2 mg kg^{-1} L-NAME (Figure 2). L-NAME (2 mg kg^{-1})-induced increase in MABP was effectively reversed by L-arginine (300 mg kg^{-1}), but not by D-arginine (300 mg kg^{-1}) (Table 1). At the dose of 2 mg kg^{-1}, D-NAME did not affect MABP (Table 1).

In subsequent experiments, L-NMMA and L-NAME were used at doses that evoked maximum increase in MABP, i.e. at 25 mg kg^{-1} and 2 mg kg^{-1}, respectively. As well established, ET-1 (1 nmol kg^{-1}) produced a sustained pressor effect preceded by a transient depressor action (Figure 3). Elevation of MABP by L-NAME, but not L-NMMA, significantly decreased the pressor response to ET-1, whereas the hypotensive response to ET-1 was not affected significantly (Figure 3a). In some animals, MABP was either elevated by an infusion of noradrenaline to levels comparable to those seen after injection of L-arginine analogues or was titrated to control levels by hydralazine or diazoxide and then the responses to ET-1 were tested. L-NMMA and L-NAME caused on average 40 and 47% inhibition of the depressor response to ET-1 and a 2 and 2.8 fold increase in the pressor action of ET-1 compared to the effects of ET-1 in animals receiving noradrenaline infusion (Figure 3a). Similarly, an approximately 40% inhibition of the depressor action and a 1.2–1.5 fold increase in the pressor effect of ET-1 were detected in animals treated with L-NAME plus hydralazine or L-NAME plus diazoxide compared to the effects of ET-1 in control (normotensive) animals (Figure 3b). These changes were statistically significant.

In another series of experiments, the ability of L-arginine analogues to inhibit the hypotensive effect of acetylcholine was tested. When the effects of acetylcholine in L-NMMA or L-NAME-treated animals and during noradrenaline infusion (i.e. when MABP values were similar before injection of acetylcholine) were compared, 40% inhibition of the depressor response to acetylcholine by L-arginine analogues was detected (Figure 4a). Similarly, the hypotensive effects of



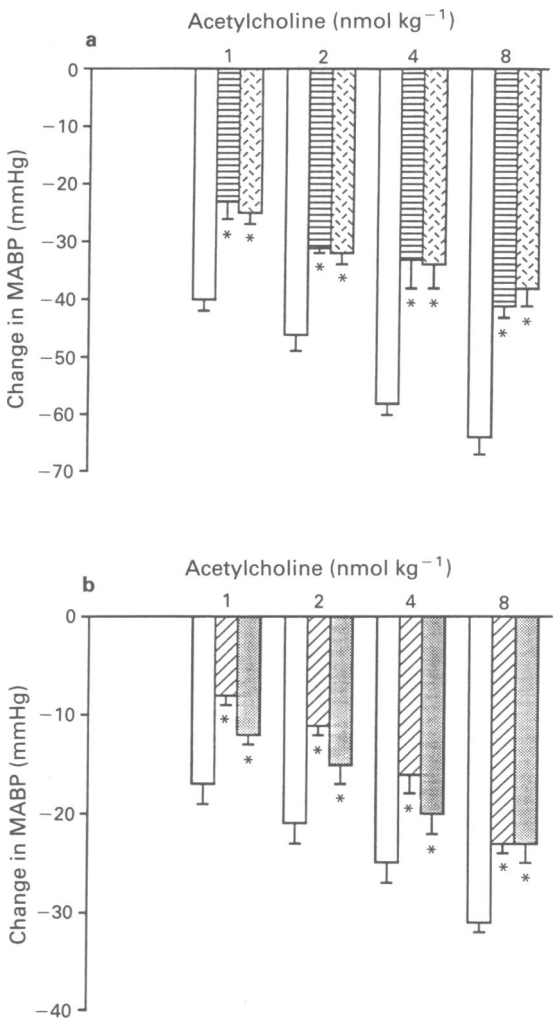


Figure 3 Endothelin-1-induced maximum decrease (open columns) and peak increase (hatched columns) in mean arterial blood pressure (MABP) in conscious rats. (a) The animals were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}) for 10 min, N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}) for 2 min, noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) for 10 min or 0.9% NaCl (control, C) before bolus i.v. injection of endothelin-1 (ET-1, 1 nmol kg^{-1}). MABP were 110 ± 3 mmHg ($n = 8$), 152 ± 5 mmHg ($n = 8$), 149 ± 4 mmHg ($n = 6$) and 152 ± 3 mmHg ($n = 6$) in control, L-NAME, L-NMMA and noradrenaline-treated animals, respectively, before injection of ET-1. (b) Following injection of L-NAME (2 mg kg^{-1}), the elevated MABP was restored to normotensive levels with diazoxide (90 μmol kg^{-1}) or hydralazine (1.2–1.5 μmol kg^{-1}) before bolus i.v. injection of ET-1 (1 nmol kg^{-1}). MABP were 110 ± 3 mmHg ($n = 6$), 107 ± 2 mmHg ($n = 6$) and 109 ± 5 mmHg ($n = 5$) in control animals receiving vehicle of L-NAME and diazoxide (C) and in rats treated with L-NAME plus diazoxide or L-NAME plus hydralazine, respectively, before injection of ET-1. Values are means with s.e.mean. $*P < 0.05$; $**P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

Figure 4 Hypotensive responses to acetylcholine in conscious rats following treatment with N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA). (a) In control animals, mean arterial blood pressure (MABP) was elevated by an infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) (open columns) to levels observed following injection of L-NAME (2 mg kg^{-1}) (▤) and L-NMMA (25 mg kg^{-1}) (▩) before i.v. bolus injections of acetylcholine. MABP values were 156 ± 5 mmHg ($n = 10$), 159 ± 4 mmHg ($n = 8$) and 151 ± 8 mmHg ($n = 6$) in noradrenaline, L-NAME and L-NMMA-treated animals, respectively, before injection of the first dose of acetylcholine. (b) In animals treated with L-NAME (2 mg kg^{-1}), the elevated MABP was restored to normotensive levels with diazoxide (90 μmol kg^{-1}) (▨) or hydralazine (1.2–1.5 μmol kg^{-1}) (▩). Control animals (open columns) were given vehicle of L-NAME and diazoxide. MABP values were 111 ± 2 mmHg ($n = 6$), 107 ± 1 mmHg ($n = 4$) and 111 ± 2 mmHg ($n = 6$) in control animals and following L-NAME plus diazoxide and L-NAME plus hydralazine, respectively, before injection of the first dose of acetylcholine. Values are means with s.e.mean. $*P < 0.05$ compared to the control group by Dunn's multiple contrast hypothesis test.

acetylcholine were reduced by 30–50% in animals treated with L-NAME plus hydralazine or L-NAME plus diazoxide relative to the hypotensive effects of acetylcholine in control (untreated) animals (Figure 4b).

Effects of L-NAME on endothelin-1-induced protein extravasation

In agreement with our previous studies, injection of 1 nmol kg^{-1} ET-1 increased tissue Evans blue content in the upper and lower bronchi (Figure 5), stomach, duodenum, kidney and spleen (Figure 6), whereas no significant changes were detected in the trachea and pulmonary parenchyma (Figure 5), pancreas, liver, skeletal muscle and skin (Figures 6 and 7).

Treatment of the animals with L-NAME (2 mg kg^{-1}) resulted in enhanced tissue Evans blue accumulation in all vascular beds studied. The highest increases (up to 260–280%) were detected in the duodenum and pancreas (Figure 6). Tissue Evans blue content increased by 139, 129, 121, 118, 109, 74 and 65% in the kidney, upper and lower bronchi, stomach, liver, trachea and spleen, respectively

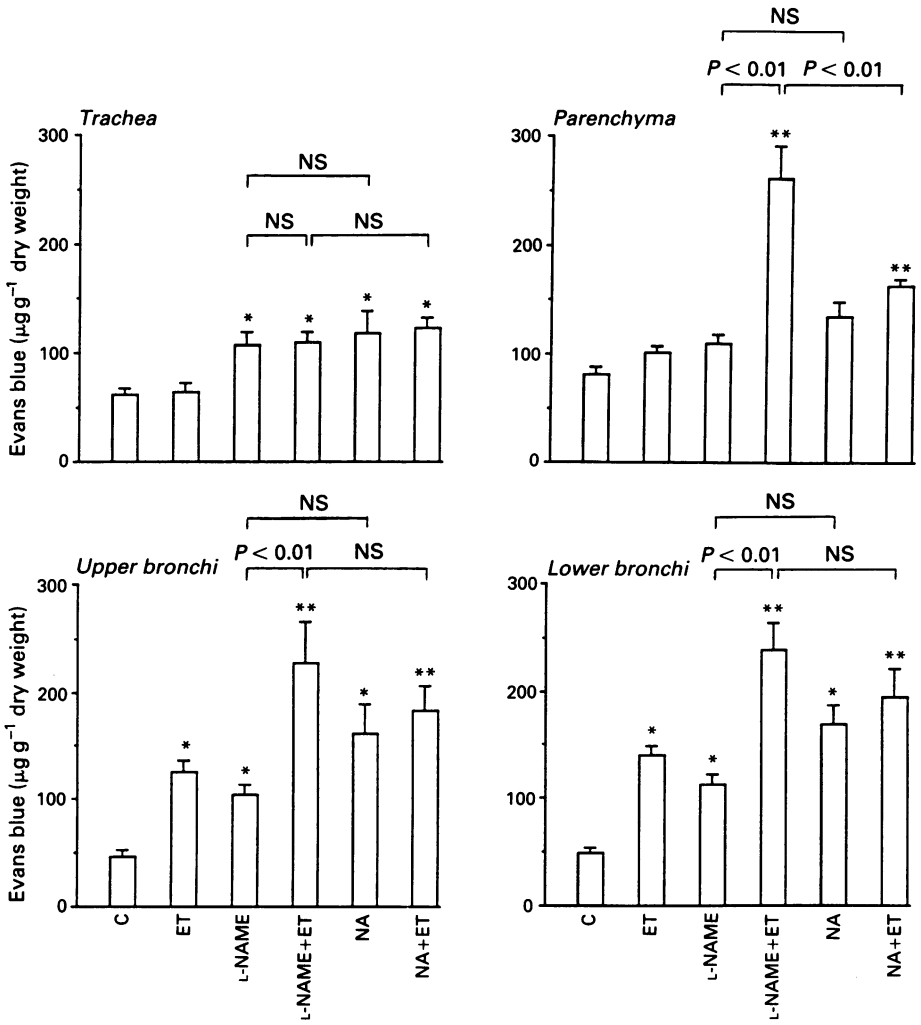


Figure 5 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat airways. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P<0.05$; $^{**}P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test). NS, not significant.

(Figures 5 and 6). No significant changes were detected in the pulmonary parenchyma, skeletal muscle and skin (Figures 5 and 7). Administration of L-arginine, but not D-arginine (300 mg kg^{-1}) prevented the L-NAME-induced increases in protein extravasation (Table 1). Neither L-arginine nor D-arginine alone affected protein extravasation (data not shown). D-NAME (2 mg kg^{-1}) had no significant effect on Evans blue accumulation in the vascular beds studied (Table 1). Elevation of MABP by infusion of noradrenaline to levels seen following L-NAME did not affect Evans blue accumulation in all organs studied with the exception of the lung (Figures 5, 6 and 7). Noradrenaline infusion and L-NAME treatment evoked similar increases in Evans blue content in the large airways and pulmonary parenchyma (Figure 5).

When ET-1 was administered to rats treated with L-NAME, protein extravasation was markedly enhanced. L-NAME potentiated (up to 550%) the permeability effect of ET-1 in the upper and lower bronchi, stomach and kidney (Figures 5 and 6). A significant increase in protein extravasation was observed in the pulmonary parenchyma following L-NAME plus ET-1, where neither ET-1 nor L-NAME alone affected significantly Evans blue content (Figure 5). In the trachea, duodenum, pancreas and liver, the effects of ET-1 and L-NAME were additive (Figures 5 and 6). No significant changes were detected in the skeletal muscle and skin (Figure 7). Noradrenaline infusion did not modify ET-1-induced Evans blue accumulation in the organs studied with the exception of the lung (Figures 6 and 7). In the upper and lower bronchi, combined administration of noradrenaline plus ET-1 markedly enhanced Evans blue accumulation, albeit tissue Evans blue content was somewhat lower than that seen following L-NAME plus ET-1 (Figure 5). Although combined administration of noradrenaline and ET-1 resulted in a 100% increase in protein extravasation in the parenchyma, tissue Evans blue content was significantly lower than that observed following L-NAME plus ET-1 (Figure 5).

Discussion

The present results show that treatment of the animals with L-arginine analogues attenuates the depressor and potentiates the pressor response to ET-1 and markedly enhances protein extravasation elicited by ET-1 in the airways, kidney and gastrointestinal tract. The findings that the pressor and permeability responses to L-NAME can be reversed by L-arginine, but not D-arginine and the failure of D-NAME to affect blood pressure and protein extravasation are consistent with the notion that the effects of L-NAME result from inhibitory actions on NO synthesis.

In confirmation of earlier observations (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990a;

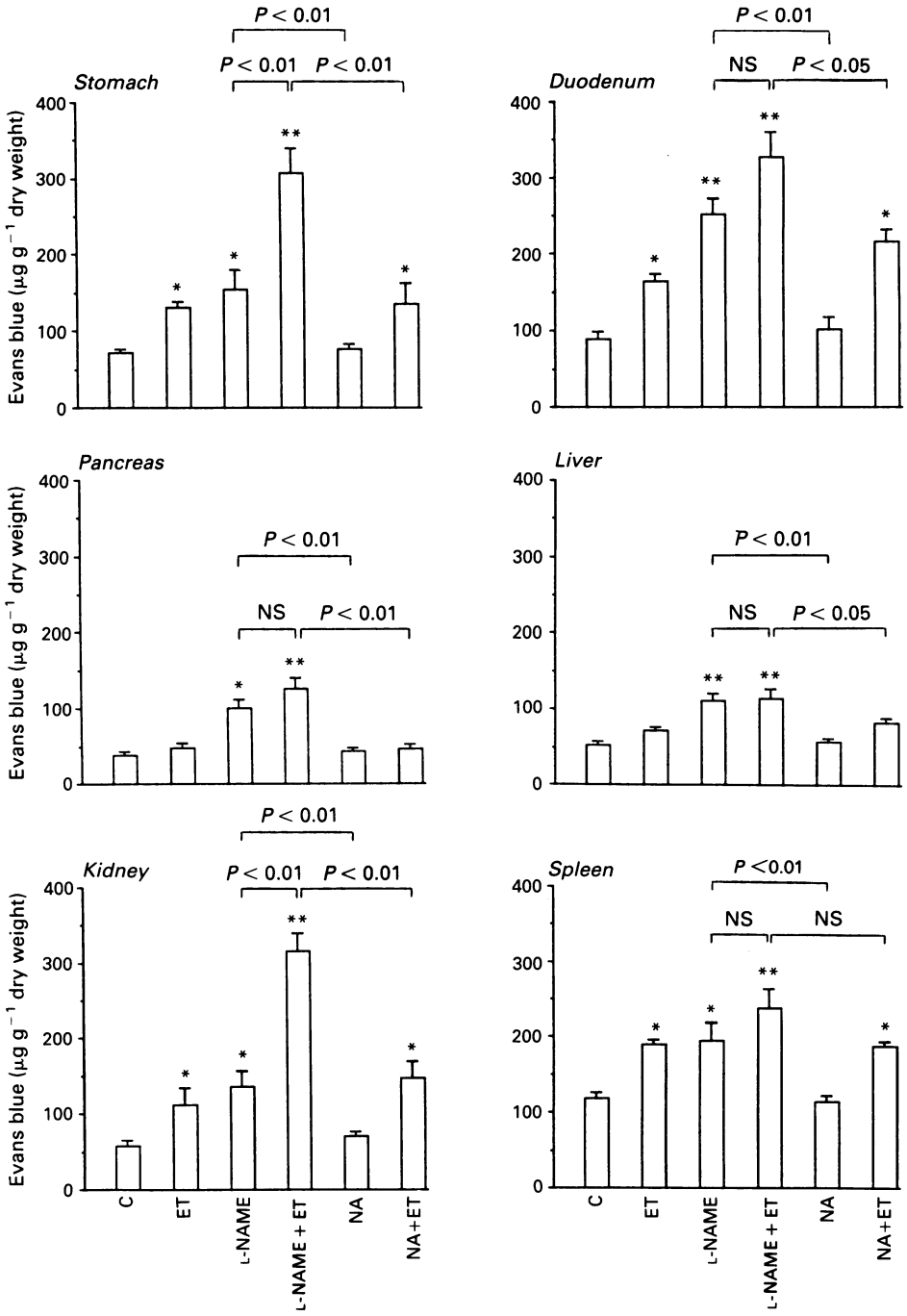


Figure 6 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat stomach, duodenum, pancreas, liver, kidney and spleen. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The animals were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P < 0.05$; $^{**}P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test). NS, not significant.

Hecker *et al.*, 1990; Vargas *et al.*, 1991), the present study also shows a profound increase in MABP following administration of L-NMMA and L-NAME. L-NAME appeared to be approximately 16 times more potent than L-NMMA. The pressor action of L-NMMA was rapid in onset and shorter in duration relative to L-NAME. This might be attributed to rapid metabolization of L-NMMA to L-citrulline and L-arginine by endothelial cells (Hecker *et al.*, 1990). On the other hand, the pressor action of L-NAME developed slowly, but the elevation in MABP remained stable for more than 40 min.

Since an elevation of basal MABP may augment the apparent vasodepressor activity of various substances, such effects of L-arginine analogues on basal MABP may lead to an underestimation of the degree of inhibition of vasodepressor responses. In order to circumvent MABP differences between control and L-NMMA or L-NAME-treated animals, MABP was either elevated in the control group by an infusion of noradrenaline to comparable levels as seen after NO blockade or the elevated MABP was titrated to control (normotensive) levels by diazoxide or hydralazine before injection of ET-1. Under these paradigms, significant attenuation, but not complete inhibition, of ET-1-induced hypotension could be demonstrated. Similar attenuation of the hypotensive effect of ET-1 has been reported in the presence of L-NAME when glyceryl trinitrate (an endo-

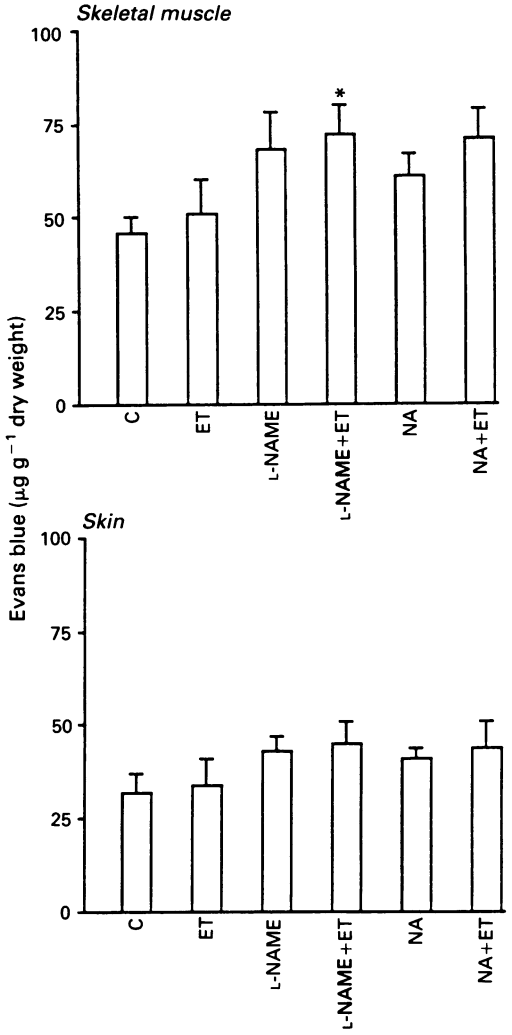


Figure 7 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat skeletal muscle and dorsal skin. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The animals were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P < 0.05$ (compared to control by Dunn's multiple contrast hypothesis test).

thelium-independent vasodilator) was used as a reference (Gardiner *et al.*, 1990b). The present study also shows that NO blockade significantly potentiated the pressor response to ET-1 under conditions when the confounding influence of varying basal MABP was avoided. In contrast, L-NMMA-treatment did not lead to a consistent appearance of a vasopressor response to ET-1 in the anaesthetized rat (Whittle *et al.*, 1989). It is not known at present whether this apparent discrepancy might be attributed to the fact that in these latter experiments ET-1 was used at doses which did not evoke a pressor action or to the transient action of L-NMMA (see above). The present findings provide further support to the hypothesis that the vasopressor action of ET-1 is attenuated by NO (De Nucci *et al.*, 1988).

Although *in vitro* experiments suggest unambiguously that NO mediates the vasorelaxant action of acetylcholine (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), studies which sought to correlate the *in vivo* hypotensive action of acetylcholine with NO have been controversial. L-NMMA has been reported to attenuate (Vargas *et al.*, 1991) or to inhibit (Whittle *et al.*, 1989) the hypotensive response to acetylcholine, whereas others did not detect any inhibition (Aisaka *et al.*, 1989; Van Gelderen *et al.*, 1991). Furthermore, L-NAME did not affect the decrease in MABP elicited by acetylcholine relative to glyceryl trinitrate (Gardiner *et al.*, 1990b). However, the different preinjection levels of MABP might also have affected the responses to glyceryl trinitrate. In the present study, attenuation of the hypotensive effects of acetylcholine by both L-NMMA and L-NAME was observed when the effects of acetylcholine were compared either at elevated MABP (i.e. following L-NMMA or L-NAME treatment versus noradrenaline infusion) or at normal MABP (i.e. control animals vs. animals receiving L-NAME plus diazoxide or hydralazine). Since the degree of inhibition was similar under these conditions and never exceeded 50%, it is feasible that a substantial part of the hypotensive response to acetylcholine is independent of NO.

Based on its potency and long duration of action, L-NAME was chosen for the vascular permeability studies. As anticipated, ET-1 (1 nmol kg^{-1}) did evoke significant increases in tissue Evans blue content in the upper and lower bronchi, stomach, duodenum, kidney and spleen. These effects of ET-1 were markedly potentiated by L-NAME (2 mg kg^{-1}), despite the fact that L-NAME by itself also promoted Evans blue accumulation in these tissues. Furthermore, a significant increase in protein extravasation was detected in the pulmonary parenchyma following L-NAME plus ET-1, where both L-NAME and ET-1 alone evoked only a slight, statistically non-significant increase in Evans blue content. The present data suggest that the effects of L-NAME on permeability are not simply a consequence of changes in systemic blood pressure as noradrenaline infusion neither mimicked the effects of L-NAME nor potentiated Evans blue accumulation elicited by ET-1 with the exception of the lung. These findings are consistent with the notion that changes in systemic blood pressure and/or an elevation in capillary hydrostatic pressure are not major determinants of protein extravasation (Grega *et al.*, 1986). Indeed, mediator-stimulated protein extravasation can be primarily attributed to an increase in the hydraulic conductivity of the microvascular membrane secondary to formation of interendothelial cell gaps in the venules (Grega *et al.*, 1986). In the pulmonary circulation, capillary filtration rate increases dramatically when left atrial pressure exceeds 40 cmH_2O (Nicolaysen *et al.*, 1979; Rippe *et al.*, 1984). During the acute phase of generalized vasoconstriction elicited by noradrenaline or L-NAME, there may be significant increases in left atrial end diastolic pressure. Rapid increases in pulmonary perfusion pressure have been reported to cause structural changes (disruption and widening of the endothelial junctions) in the capillaries leading to increases in permeability (Tsukimoto *et al.*, 1990). Large doses of catecholamines (Theodore & Rabin, 1976) or stimulation of sympathetic nerves (Hakim *et al.*, 1981; Sakakibara *et al.*, 1992) produce severe pulmonary oedema with protein-rich oedema fluid. However, the findings that tissue Evans blue content in the pulmonary parenchyma was significantly higher following administration of L-NAME plus ET-1 than following noradrenaline plus ET-1, suggest that an increase in pulmonary pressure might not be the sole mechanism responsible for the permeability enhancing effect of L-NAME. L-NAME treatment did not result in an increase in protein extravasation in the skeletal muscle and skin. One possible explanation is that oedema formation could probably be masked by arterial vasoconstriction elicited by L-NAME. Alternatively, the different susceptibility of different vascular beds to L-NAME may either reflect differences in local NO production or differences in the activity of NO in different regions.

Our results also show that the potentiating effect of NO synthesis inhibition upon ET-1-induced protein extravasation is not due to the haemodynamic effect of L-NAME alone. Furthermore, the enhanced vascular permeability by L-NAME cannot be attributed to an increase in capillary surface area for protein filtration, since vasoconstrictors like

L-NAME generally cause derecruitment of capillaries (Granger *et al.*, 1989). It is likely, however, that inhibition of NO synthesis would lead to endothelial dysfunction (Lieberthal *et al.*, 1989; Hutcheson *et al.*, 1990; Lefer & Ma, 1991). Since NO may scavenge small amounts of superoxide released by endothelial cells (Zweier *et al.*, 1988) and inhibit adhesion of platelets and neutrophil granulocytes to the vascular endothelium (Radomski *et al.*, 1987; Kubes *et al.*, 1991), decreased NO production would result in local accumulation of free radicals and/or adhesion of platelets and neutrophil granulocytes to endothelial cells, which, in turn, would lead to an increase in microvascular permeability (Del Maestro *et al.*, 1981; Kubes & Granger, 1992).

The present observations may have relevance in pathological conditions where plasma and local tissue levels of ET-1 are elevated and endothelial NO production is decreased. In addition to vascular spasm, ET-1-induced plasma extravasation with local oedema formation would induce or predispose to tissue damage. When NO synthesis is decreased, the effects of ET-1 appear to become more dominant, leading to an imbalance of endothelium-dependent regulation of microvascular protein permeability. The findings that NO donors can decrease intestinal tissue damage and attenuate endothelial dysfunction in splanchnic artery occlusion-induced shock (Aoki *et al.*, 1990) lend further support to this hypothesis.

In conclusion, the present data demonstrate that endogenous NO mediates, in part, the vasodepressor effect and attenuates the vasopressor action of ET-1 and counteracts the vascular permeability effect of ET-1. These findings suggest an important role for NO in the regulation of vascular tone and microvascular permeability in conscious rats.

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Attenuation by phentolamine of hypoxia and levcromakalim-induced abbreviation of the cardiac action potential

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1 The effects of phentolamine (5–30 μM) and glibenclamide (10 μM) on action potential characteristics were examined in guinea-pig papillary muscle exposed to either hypoxia or levcromakalim (20 μM).

2 The hypoxia-induced abbreviation of action potential duration (APD) and effective refractory period (ERP) were attenuated but not abolished by glibenclamide (10 μM). Hypoxia reduced APD by 24 ± 2 vs 65 ± 4% in glibenclamide- and vehicle-treated tissue, respectively.

3 Phentolamine (10–30 μM) was less effective than glibenclamide in attenuating the hypoxic shortening of APD since APD was reduced by 38 ± 10, 51 ± 6% vs 65 ± 4% in 10 and 30 μM phentolamine and vehicle-treated muscle, respectively.

4 Phentolamine, at concentrations of 10 and 30 μM, also reduced the upstroke velocity of the action potential and at 5 μM it increased the APD from 193 ± 9 to 221 ± 12 ms.

5 Glibenclamide completely abolished and phentolamine (30 μM) significantly attenuated levcromakalim-induced changes in duration and ERP. Levcromakalim reduced APD by 71 ± 2 and 55 ± 2% in control and phentolamine pretreated muscle, respectively.

6 It is concluded that phentolamine may block K_{ATP} channels at concentrations that also block sodium channels.

Keywords: Phentolamine; glibenclamide; K_{ATP} channels; hypoxia; guinea-pig papillary muscle

Introduction

The ability of phentolamine to confer protection against ischaemia-induced ventricular arrhythmias has been demonstrated both in experimental animal models (Sheridan *et al.*, 1980; Srivastava *et al.*, 1980; Daugherty *et al.*, 1986; Sheridan, 1986) and in man (Gould *et al.*, 1975). The precise mechanism(s) underlying this antiarrhythmic action, however, remains unclear and open to debate. Direct cardiac α-adrenoceptor blockade has been invoked by some as the basis of this antiarrhythmic activity (Sheridan *et al.*, 1980; Sheridan, 1986). This has, however, been questioned by others who point to the reported failure to demonstrate protection against ischaemic arrhythmias with several other α-adrenoceptor antagonists and to establish a direct relationship between the potency of α-adrenoceptor blockade and antiarrhythmic efficacy of phentolamine and other cardioprotective α-adrenoceptor antagonists (Bolli *et al.*, 1984; Daughtery *et al.*, 1986). Since phentolamine also exhibits a 'membrane stabilising' or fast Na channel blocking activity in cardiac tissues (Rosen *et al.*, 1971; Ledda & Marchetti, 1971; Northover, 1983), it has been suggested that this Class I property (Vaughan Williams, 1980) may consititute the basis of its antiarrhythmic actions (Bralet *et al.*, 1985; Daugherty *et al.*, 1986).

More recently, it has been shown that phentolamine and certain other imidazoline α-adrenoceptor antagonists increase insulin release by blocking ATP-sensitive K channels in pancreatic β-cells and that this effect is unrelated to α-adrenoceptor blockade (Plant & Henquin, 1990; Dunne, 1991; Jonas *et al.*, 1992). ATP-sensitive K channels occur in the heart and several recent studies suggest that their blockade with known specific channel blockers, such as glibenclamide and tolbutamide, can confer protection against experimental ischaemic arrhythmias (Wollenben *et al.*, 1989; Kantor *et al.*, 1990), presumably via consequent attenuation of ischaemia-induced enhanced K^+ efflux, membrane depolarization and action potential shortening (Kantor *et al.*, 1990; Hicks & Cobbe, 1991). We hypothesised, therefore, that if phentolamine blocked ATP-sensitive K channels, this would constitute another possible underlying mechanism for its antiarrhythmic actions.

The aim of this study, therefore, was to determine whether phentolamine blocks cardiac ATP-sensitive K channels at antiarrhythmic concentrations. We have, therefore, compared the effects of phentolamine with those of glibenclamide, a specific blocker of cardiac ATP-sensitive K channels, on hypoxia/zero glucose- and levcromakalim-induced electrophysiological changes in guinea-pig isolated papillary muscles. Levcromakalim is a specific opener of cardiac ATP-sensitive K channels (Sanguinetti *et al.*, 1988) and hypoxia/zero glucose is also known to open these channels in the heart (Sanguinetti *et al.*, 1988; Berndorf *et al.*, 1991).

Methods

Action potential recording

Guinea-pigs were killed, the hearts removed and arrested in ice cold physiological salt solution (PSS). Left papillary muscles were excised and placed on the silastic base of the recording chamber. The muscles were superfused at a rate of 10 ml min^{-1} with normal PSS equilibrated with 95% O_2:5% CO_2 and maintained at 36 ± 1°C. The composition of the normal PSS was (in mM): NaCl 118.4, $NaHCO_3$ 24.9, KH_2PO_4 1.19, $MgSO_4$ 1.19, KCl 3.4, $CaCl_2$ 2.5 and glucose 11.1.

The preparations were stimulated at a frequency of 1 Hz by rectangular pulses, 1 ms in duration and twice threshold voltage, delivered through a bipolar platinum electrode. Transmembrane action potentials were recorded by conven-

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tional microelectrode techniques. The action potentials were recorded on-line to the hard disk of a computer for later analysis using a custom-made software package (CMAP Programme; John Dempster, University of Strathclyde). The variables measured were as follows: resting membrane potential (RMP); action potential amplitude (APA); the maximum rate of depolarization of phase 0 (MRD); and the action potential duration at 50 and 90% of repolarization (APD_{50} and APD_{90}). The effective refractory period (ERP) was measured by the extra stimulus method. The time interval between the pacing and the extra stimulus was increased until a propagated action potential was produced and this time interval was taken as the ERP.

Experimental protocol

Hypoxia and removal of glucose Following an equilibration period of about 1 h in normal PSS, 5-7 action potentials were measured before and at, 5, 15, 30, 45 and 60 min after the introduction of hypoxia/zero glucose. Under hypoxic conditions, the PSS was similar to that described above except that the glucose was absent and the solution was gassed with 95% N_2:5% CO_2 to yield a Po_2 in the organ bath of 63 ± 4 mmHg. This compares with a Po_2 of 636 ± 14 mmHg in normal PSS.

Effects of levcromakalim In the presence of normal PSS, action potentials and ERP were recorded at the time points described above before and after superfusion with levcromakalim (20 μM) for 60 min.

Effects of glibenclamide and phentolamine Action potentials and ERP were measured before and after 30 min of superfusion with glibenclamide (10 μM) or phentolamine (5, 10 or 30 μM) followed by exposure to drug together with hypoxia/zero glucose or levcromakalim for a further 60 min. This dose of glibenclamide was selected because it has been shown to block cromakalim-induced effects in cardiac tissue (Sanguinetti *et al.*, 1988). In control experiments the effects of phentolamine (10 μM) alone over a 90 min period in normal PSS were also measured.

Analysis of data

The measurements from each set of 5–7 multiple impalements were meaned and the mean values used to represent the data from each muscle. Data are given as mean values ± s.e.mean or mean percentage change from control values ± s.e.mean and are derived from n experiments. ANOVA for repeated measurements were performed and followed, where appropriate, by a modified Student's t test. A value of $P<0.05$ was considered to be statistically significant.

Drugs

Stock solutions of glibenclamide (Hoechst, UK), levcromakalim (SmithKline Beecham Pharmaceuticals) and phentolamine (Ciba-Geigy) were made up daily by dissolving in a 80% dimethylsulphoxide (DMSO) 20% PSS solvent solution. Drugs were diluted to the appropriate final bath concentration in PSS.

Results

Hypoxia/zero glucose studies

Effects of hypoxia/zero glucose on action potential characteristics Table 1 shows the effects of a 60 min period of hypoxia/zero glucose on paced guinea-pig papillary muscle action potential characteristics. Hypoxia/zero glucose caused a marked time-dependent shortening of APD_{50}, APD_{90} (Figure 1) and ERP (Figure 2). The changes in ERP paralleled those in APD_{90}. Over this time period of exposure to hypoxia/zero glucose there were no significant changes in MRD (Figure 3), although there was a slight membrane depolarization and an associated reduction in action potential amplitude (Table 1).

Effects of glibenclamide and phentolamine in the presence of hypoxia/zero glucose Glibenclamide (10 μM) attenuated but did not abolish the changes in APD_{50} (data not shown), APD_{90} (Figure 1) and ERP (Figure 2) induced by hypoxia/zero glucose in guinea-pig papillary muscle. At 30 min post-hypoxia/zero glucose the percentage reduction in APD_{90} in glibenclamide-treated tissue was significantly less ($24 \pm 2\%$) than that in untreated preparations ($65 \pm 4\%$). Glibenclamide had no statistically significant effect on MRD either before or during the period of hypoxia (Figure 3). Under normoxic conditions, glibenclamide caused a slight membrane hyperpolarization (-86.7 ± 0.4 vs -88.4 ± 0.6 mV) and an increase in action potential amplitude (113.3 ± 2 vs 116.1 ± 1 mV).

In a few experiments, the effect of glibenclamide was tested on preparations paced at 3 Hz. Introduction of hypoxia/zero glucose in papillary muscle paced at 3 Hz caused a reduction in APD_{90} from 95 ± 12 to 51 ± 6 ms (at 10 min post-hypoxia) and in glibenclamide (10 μM) pretreated preparations from 111 ± 11 to 88 ± 10 ms ($n = 3$). The % reductions in APD_{90} under these conditions were 45 ± 4 and 21 ± 2 in control and glibenclamide treated muscles, respectively.

Phentolamine, in concentrations of 10 and 30 but not 5 μM, also attenuated the changes in APD_{50} (data not shown), APD_{90} (Figure 1) and ERP (Figure 2) induced by hypoxia/zero glucose in guinea-pig papillary muscle. This

Table 1 Effects of hypoxia/zero glucose on paced guinea-pig papillary muscle action potential characteristics

		Time post-hypoxia/zero glucose (min)				
	Control	5	15	30	45	60
RMP (mV)	-87.0 ± 0.9	-85.8 ± 0.7	-85.6 ± 0.6	-85.3 ± 0.3*	-84.8 ± 0.5*	-85.1 ± 0.5*
MRD (Vs^{-1})	225.4 ± 14.5	212.3 ± 14.9	217.3 ± 13.6	227.0 ± 8.1	222.6 ± 15.2	248.0 ± 22.2
APA (mV)	114.9 ± 1.2	109.6 ± 1.4*	108.4 ± 1.6*	108.7 ± 1.2*	106.0 ± 1.8*	103.4 ± 1.4*
APD_{50} (ms)	149.4 ± 4.5	91.2 ± 10.0*	43.6 ± 5.5*	36.9 ± 3.9*	21.9 ± 2.4*	10.1 ± 1.4*
APD_{90} (ms)	182.1 ± 4.6	122.9 ± 10.8*	68.7 ± 7.1*	62.7 ± 6.0*	45.6 ± 4.7*	27.9 ± 3.7*
ERP (ms)	170 ± 3	–	69 ± 7*	61 ± 7*	46 ± 5*	34 ± 4*

$n = 7$; *$P<0.05$ significantly different from appropriate control value. Abbreviations are RMP: resting membrane potential; MRD: maximum rate of depolarization of phase 0; APA: action potential amplitude; APD_{50} and APD_{90}: action potential duration measured at 50 and 90% repolarization respectively; ERP: effective refractory period.

protective effect of phentolamine was more marked with 10 than 30 μM. At 30 min post-hypoxia, the % reductions in APD_{90} in phentolamine-treated muscles were 38 ± 10 and 51 ± 6% for 10 and 30 μM, respectively, compared with 65 ± 4% in untreated tissues. MRD was significantly reduced by phentolamine (30 μM) under both normoxic and hypoxic conditions, whereas with 10 μM significant reductions in MRD were only noted at certain time points during the hypoxic period (Figure 3). Phentolamine did not modify resting membrane potential either before or during hypoxia/zero glucose.

Both APD_{50} and APD_{90} were slightly increased by phentolamine prior to exposure to hypoxia/zero glucose. This effect was most marked at the lowest concentration studied, i.e. 5 μM. At this concentration, phentolamine increased APD_{90} from 193 ± 9 ms to 221 ± 12 ms after 30 min of superfusion. Increasing the duration of superfusion to 90 min, i.e. comparable to that in the hypoxia/zero glucose experiments, did not cause a further prolongation of APD_{90} under normoxic conditions. APD_{90} was 174 ± 10 and 189 ± 12 ms before and after 90 min of superfusion with 10 μM phentolamine. The vehicle for the drugs had no effect on any of the action potential variables.

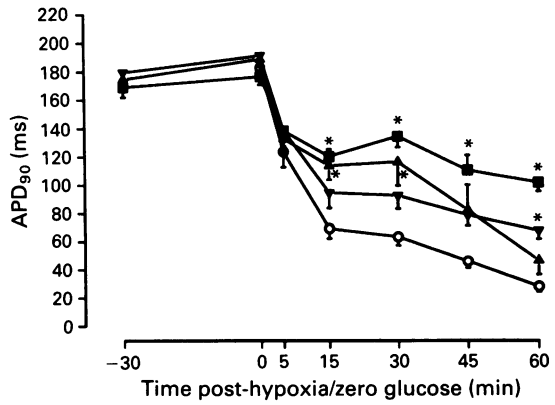

Figure 1 The effects of hypoxia/zero glucose over a 60 min period on the action potential duration at 90% of repolarization (APD_{90}, ms) in control (○) guinea-pig papillary muscle and in muscle exposed to glibenclamide (10 μM, ■) or phentolamine (10 μM, ▲; 30 μM, ▼). *$P<0.05$ indicates a significant difference from control preparations. $n = 5-7$.

Levcromakalim studies

Effects of levcromakalim on action potential characteristics Levcromakalim (20 μM) caused marked time-dependent reductions in APD_{50} (Table 2), APD_{90} (Figure 4) and ERP (Figure 5) which were comparable in magnitude to those seen in muscle exposed to hypoxia/zero glucose. Resting membrane potential was not modified by levcromakalim whereas action potential amplitude was slightly reduced. MRD was

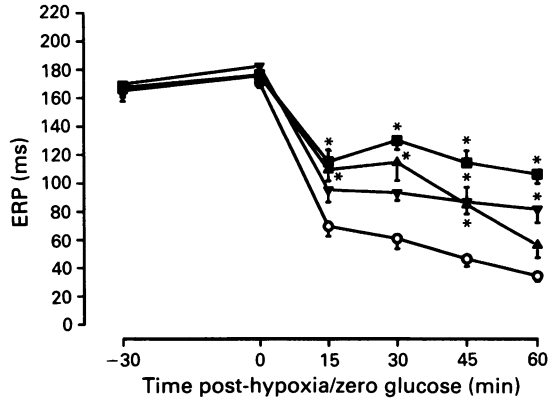

Figure 2 The effects of hypoxia/zero glucose over a 60 min period on the effective refractory period (ERP, ms) in control (○) guinea-pig papillary muscle and in muscle exposed to glibenclamide (10 μM, ■) or phentolamine (10 μM, ▲; 30 μM, ▼). *$P<0.05$ indicates a significant difference from control preparations. $n = 5-7$.

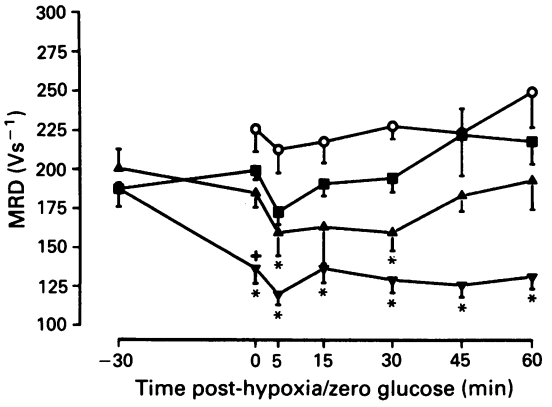

Figure 3 The effects of hypoxia/zero glucose on the maximum rate of depolarization of phase 0 of the action potential upstroke (MRD, Vs^{-1}) in control (○) guinea-pig papillary muscle and in muscle exposed to glibenclamide (10 μM, ■) or phentolamine (10 μM, ▲; 30 μM, ▼). †*$P<0.05$ indicates a significant difference from drug free (− 30 min) and control preparations respectively. $n = 5-7$.

Table 2 Effects of levcromakalim (20 μM) on paced guinea-pig papillary muscle action potential characteristics following a 60 min exposure period

		Time post-levcromakalim (20 μM) (min)				
	Control	5	15	30	45	60
RMP (mV)	− 86.9 ± 1.9	− 84.1 ± 1.7	− 84.2 ± 1.2	− 83.4 ± 0.5	− 85.0 ± 1.3	− 85.7 ± 0.2
MRD (Vs^{-1})	167.7 ± 10.1	182.6 ± 13.8	210.9 ± 9.4*	200.8 ± 8.6	196.2 ± 6.5	234.5 ± 9.6*
APA (mV)	114.6 ± 1.5	107.2 ± 1.5*	105.6 ± 2.5*	103.7 ± 2.4*	104.3 ± 2.8*	106.1 ± 2.3*
APD_{50} (ms)	154.0 ± 13.6	87.8 ± 13.9*	51.9 ± 7.2*	35.8 ± 4.8*	27.8 ± 4.5*	23.8 ± 3.5*
APD_{90} (ms)	190.7 ± 14.8	121.0 ± 15.0*	76.9 ± 8.3*	55.8 ± 5.3*	46.7 ± 4.5*	36.3 ± 2.4*
ERP (ms)	181 ± 14	-	85 ± 10*	61 ± 4*	50 ± 4*	41 ± 4*

$n = 5$; *$P<0.05$ significantly different from appropriate control value. Abbreviations are RMP: resting membrane potential; MRD: maximum rate of depolarization of phase 0; APA: action potential amplitude; APD_{50} and APD_{90}: action potential duration measured at 50 and 90% repolarization respectively; ERP: effective refractory period.

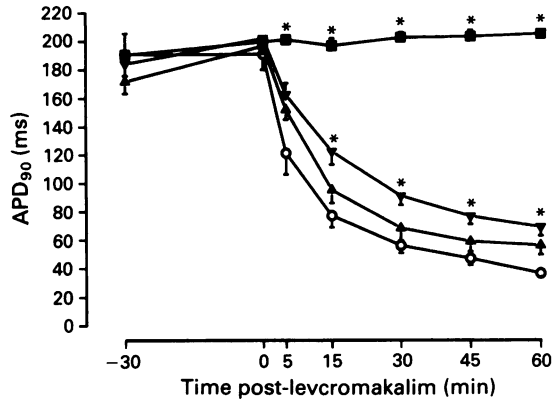

Figure 4 The effects of levcromakalim (20 μM) over a 60 min exposure period on the action potential duration at 90% of repolarization (APD_{90}, ms) in control guinea-pig papillary muscle (○) and in muscle exposed to glibenclamide (10 μM, ■) or phentolamine (10 μM, ▲; 30 μM, ▼). $*P<0.05$ indicates a significant difference from control preparations. $n = 5-7$.

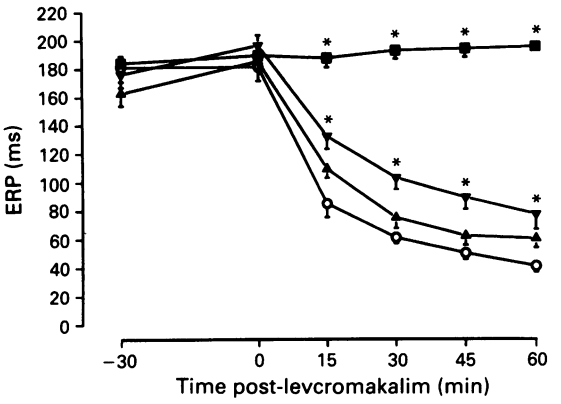

Figure 5 The effects of levcromakalim (20 μM) over a 60 min exposure period on the effective refractory period (ERP, ms) in control (○) guinea-pig papillary muscle and in muscle exposed to glibenclamide (10 μM, ■) or phentolamine (10 μM, ▲; 30 μM, ▼). $*P<0.05$ indicates a significant difference from control preparations. $n = 5-7$.

slightly increased by levcromakalim but this effect was inconsistent and was not statistically significant at all time points studied (Table 2).

Effects of glibenclamide and phentolamine in the presence of levcromakalim Glibenclamide (10 μM) completely abolished the shortening of the action potential duration and ERP induced by levcromakalim (Figures 4 and 5) without significantly modifying MRD or resting membrane potential. This complete abolition of levcromakalim-induced shortening of APD_{90} contrasts with the attenuation, but not abolition, of that induced by hypoxia/zero glucose, i.e. -24 ± 2 vs $-65 \pm 4\%$ change in APD in glibenclamide- and vehicle-treated tissue, respectively (compare Figures 1 and 4).

Phentolamine at 30 but not 10 μM significantly attenuated the levcromakalim-induced abbreviation of APD_{90} and ERP (Figures 4 and 5) but this effect was much less marked than that observed with glibenclamide. As in the muscles subsequently exposed to hypoxia/zero glucose, phentolamine slightly increased action potential duration and reduced MRD prior to superfusion with levcromakalim.

Discussion

This study has demonstrated that phentolamine can partially attenuate the hypoxia/zero glucose and levcromakalim-induced shortening of the action potential duration and effective refractory period in guinea-pig papillary muscle. The ability of phentolamine to attenuate the hypoxic abbreviation of the action potential was more pronounced at a concentration of 10 μM than at 30 μM. In contrast, phentolamine caused a small but concentration-dependent attenuation of the levcromakalim-induced shortening of the cardiac potential. Since levcromakalim is thought to shorten the action potential by opening K_{ATP} channels, these results suggest that phentolamine may block cardiac K_{ATP} channels. Phentolamine was much less effective in this regard than glibenclamide which at a concentration of 10 μM, completely prevented the levcromakalim-induced abbreviation of the cardiac potential. It also appears that phentolamine may be less effective in blocking cardiac K_{ATP} channels than those in pancreatic β cells since the studies by Plant & Henquin (1990) and Jonas *et al.* (1992), show that concentrations of phentolamine of 10–20 μM caused a marked inhibition of K_{ATP} channels in that tissue. Furthermore, in smooth muscle (Murray *et al.*, 1989; McPherson *et al.*, 1990) phentolamine blocks cromakalim-induced responses at concentrations of 10 and 30 μM.

It should also be noted that the effects of phentolamine (10 μM) were time-dependent. At this concentration but not at 30 μM, the ability of phentolamine to attenuate the hypoxic-abbreviation of the action potential was most pronounced over the first 30 min of hypoxia and this protection was lost by 60 min of hypoxia. However, this time-dependency was not apparent when the action potential was abbreviated by levcromakalim.

Phentolamine also exhibited other electrophysiological effects in guinea-pig papillary muscle. In line with previous reports (Rosen *et al.*, 1971; Northover, 1983), phentolamine was shown to block fast inward sodium channels as indicated by its ability to reduce the maximum rate of depolarization of phase zero of the action potential. This effect was apparent at 10 μM during hypoxia and at 30 μM of phentolamine both under normoxic and hypoxic conditions.

Phentolamine was also shown to prolong slightly the duration of the cardiac action potential under normoxic conditions. This effect was most pronounced at the lowest concentration studied, i.e. 5 μM, a concentration that did not confer protection against the hypoxic abbreviation of the action potential, suggesting that this Class III effect is not responsible for the attenuation of the effects of hypoxia or levcromakalim. This is further substantiated by the finding that during hypoxia or levcromakalim treatment the attenuation of the action potential shortening by higher concentrations of phentolamine was more marked than could be accounted for by their ability to prolong the action potential under normal conditions.

The ability of phentolamine to protect against ischaemia reperfusion-induced arrhythmias in isolated perfused hearts is apparent at concentrations of 10 μM (Bralet *et al.*, 1985; Daugherty *et al.*, 1986; Gwilt *et al.*, 1992). At this concentration, phentolamine did attenuate the hypoxic/zero glucose abbreviation of the cardiac action potential, suggesting that this action may at least, in part, account for its antiarrhythmic action. However, at this concentration, phentolamine also blocked sodium channels, an action which may also confer antiarrhythmic activity.

Glibenclamide in a concentration of 10 μM completely prevented the abbreviation of the action potential induced by levcromakalim. This is in line with previous studies using K_{ATP} channel openers such as cromakalim (Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) or pinacidil (Nakaya *et al.*, 1991). However, glibenclamide at 10 μM only attenuated the action potential shortening induced by hypoxia/zero glucose, which is similar to the effect shown by Nakaya *et al.* (1991).

Such results have led to the suggestion that opening of K_{ATP} channels may not be solely responsible for the action potential shortening caused by hypoxia or ischaemia. Interestingly, in our other studies on guinea-pig isolated perfused hearts paced at 4 Hz, glibenclamide at a concentration of 3 μM completely prevented the abbreviation of the effective refractory period induced by a reduction in coronary flow (Tweedie *et al.*, 1992). This raised the possibility that the opening of K_{ATP} channels during hypoxia may have contributed to a lesser extent in the papillary muscle preparation than in perfused hearts because of the lower stimulation frequency used. However, when the preparations were paced at 3 Hz, glibenclamide had no greater ability to attenuate the hypoxia/zero glucose shortening of the action potential than at 1 Hz. The other main difference between the papillary muscle preparation and the perfused hearts subjected to low flow is that accumulation of metabolites and H^+ will occur in the latter but not the former. It is known, for instance, that the K_{ATP} channel blocking properties of glibenclamide are potentiated by low pH (Findlay, 1992) and this may account for the greater effect of glibenclamide in perfused hearts as compared with papillary muscle.

In conclusion, phentolamine at concentrations of 10 and 30 μM did attenuate the abbreviation of the cardiac action potential induced by hypoxia and zero glucose or by levcromakalim. This effect of phentolamine was less marked than that of the K_{ATP} channel blocking drug, glibenclamide. Phentolamine may, therefore, block K_{ATP} channels in cardiac tissue but confirmation of such an action would require more direct studies such as patch clamp analysis of K_{ATP} channel activity.

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Bradykinin initiates cytokine-mediated inflammatory hyperalgesia

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1 The hyperalgesic activities in rats of bradykinin, carrageenin and lipopolysaccharide (LPS) were investigated in a model of mechanical hyperalgesia.

2 Bradykinin and carrageenin evoked dose-dependent hyperalgesia with maximum responses of similar magnitude to responses to LPS (1 and 5 μg).

3 Hoe 140, an antagonist of BK_2 receptors, inhibited in a dose-dependent manner hyperalgesic responses to bradykinin, carrageenin and LPS (1 μg) but not responses to LPS (5 μg), prostaglandin E_2, dopamine, tumour necrosis factor α (TNFα), IL-1, IL-6 and IL-8.

4 Responses to bradykinin and LPS (1 and 5 μg) were inhibited by the cyclo-oxygenase inhibitor, indomethacin and by the β-adrenoceptor antagonist, atenolol. The effects of indomethacin and atenolol were additive: their combination abolished responses to bradykinin and LPS (1 μg) and markedly attenuated the response to LPS (5 μg).

5 Antiserum neutralizing endogenous TNFα abolished the response to bradykinin whereas antisera neutralizing endogenous IL-1β, IL-6 and IL-8 each partially inhibited the response. The combination of antisera neutralizing endogenous IL-1β + IL-8 or IL-6 + IL-8 abolished the response to bradykinin.

6 Antisera neutralizing endogenous TNFα, IL-1β, IL-6 and IL-8 each partially inhibited responses to LPS (1 and 5 μg). Increasing the dose of antiserum to TNFα or giving a combination of antisera to IL-1β + IL-8 or IL-6 + IL-8 further inhibited responses to LPS (1 and 5 μg).

7 These data show that bradykinin can initiate the cascade of cytokine release that mediates hyperalgesic responses to carrageenin and endotoxin (1 μg). The lack of effect of Hoe 140 on hyperalgesic responses to LPS (5 μg) suggests that the release of hyperalgesic cytokines can be initiated independently of bradykinin BK_2 receptors.

Keywords: Bradykinin; inflammatory hyperalgesia; tumour necrosis factor; interleukin-1; interleukin-6; interleukin-8

Introduction

Bradykinin has two roles in the development of inflammatory pain: activation and sensitization of pain receptors (nociceptors). Activation of nociceptors causes immediate, overt pain whereas sensitization of nociceptors is responsible for the development of inflammatory hyperalgesia. Bradykinin was established as a pain mediator because it produced immediate, overt pain in man (Armstrong *et al.*, 1957; Sicuteri *et al.*, 1965; Ferreira, 1972; Whalley *et al.*, 1987). This immediate effect of bradykinin has been the subject of numerous behavioural and electro-physiological studies (Dray *et al.*, 1988; Lang *et al.*, 1990; Handwerker & Reeh, 1991) and appears to result from the activation of the high threshold nociceptors associated with C fibres. In contrast, little attention has been given to the delayed and long-lasting hyperalgesic effect of bradykinin. This effect was inhibited in rat paws by a bradykinin receptor antagonist and by the cyclo-oxygenase inhibitor, indomethacin (Steranka *et al.*, 1987; 1988; Taiwo & Levine, 1988).

Bradykinin is believed to participate in various models of inflammatory hyperalgesia (Steranka *et al.*, 1988; Costello & Hargreaves, 1989; Fujiyoshi *et al.*, 1989; Chau *et al.*, 1991) and recently a specific BK_2 receptor antagonist, Hoe 140, was shown to inhibit the delayed hyperalgesic effect of bradykinin. Hoe 140 produced analgesia in experimental models of inflammatory pain, particularly in carrageenin induced hyperalgesia (Beresford & Birch, 1992).

Previously we showed that in a rat paw pressure test, carrageenin-evoked hyperalgesia resulted from the combined effect of the release of cyclo-oxyenase products and sympathomimetic amines (Nakamura & Ferriera, 1987). More recently we showed that in this model, a cascade of cytokine release preceded the release of the cyclo-oxygenase products and sympathomimetics (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; 1992). The proposed sequence of events was that carrageenin stimulated the release of TNFα, which: (i) induced IL-1β and IL-6, which stimulated the production of cyclo-oxygenase products and (ii) induced IL-8 which stimulated production of sympathomimetics (Cunha *et al.*, 1992).

Given the capacity of the bradykinin antagonist Hoe 140 to inhibit carrageenin-evoked inflammatory hyperalgesia and a recent report that bradykinin evoked the release of TNFα and IL-1 from macrophage monolayers (Tiffany & Burch, 1989) we investigated the possibility that bradykinin was involved in the cytokine-mediated hyperalgesic pathways activated by carrageenin and lipopolysaccharide (LPS).

(A preliminary account of this work was given at the 14th European Workshop on Inflammation/British Inflammation Research Association joint meeting, London, July 1992).

Methods

Nociceptive test

A constant pressure of 20 mmHg was applied to the hind paws of rats and discontinued when they presented a typical freezing reaction (reaction time). This reaction was characterized by a reduction of escape movements: animals usually made several attempts to escape from the position imposed by the experimental situation. These were followed by alterations in respiratory frequency with the onset of a typical

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shivering reaction. The intensity of hyperalgesia was quantified as the variation in reaction time (Δ reaction time) obtained by subtracting values measured 3 h after administration of hyperalgesic substances from (control) reaction times (measured before injection at zero time, Ferreira *et al.*, 1978).

Experimental protocol

Hyperalgesia was induced by bradykinin (0.01–1 μg), carrageenin (30–300 μg), LPS (0.5–5 μg), PGE_2 (100 ng), dopamine (10 μg), TNFα (2.5 pg), IL-1β (0.1 iu), IL-6 (1.0 ng), and IL-8 (0.1 ng), injected in 100 μl into the hind paws of rats (intraplantar, i.pl.). Hoe 140 (0.1–10 mg kg^{-1}) was given s.c. and indomethacin (100 μg) and atenolol (25 μg) were injected in 100 μl into the (same) hind paws, 30 min before the hyperalgesic substances. Antisera neutralising rat TNFα, IL-1β, IL-6 and IL-8 and mixtures of antisera were injected in 50–150 μl into the (same) hind paws, 30 min before the hyperalgesic agents. Results are presented as means with s.e.mean of groups of 5 animals. The statistical tests used are indicated in the text. Formal statistical tests are not reported for differences where means differed by three times the larger s.e.mean.

Materials

Drugs IL-1β, IL-6, IL-8 (72 amino acids) and TNFα were NIBSC preparations coded 86/680, 88/514, 89/520 and 87/650, respectively. Indomethacin was a gift from Merck, Sharpe & Dohme Ltd (Hoddesdon, Herts). Carrageenin was a gift from the FMC Corporation (Philadelphia, U.S.A.). Atenolol, bradykinin, lipopolysaccharide from *E. coli* 0111: B4 (LPS) and dopamine were purchased from Sigma (St. Louis, U.S.A.). PGE_2 was a gift from the Upjohn Co (U.S.A.) and Hoe 140 ([D-Arg^1, Thi^6, (1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl)^8, ((3as,7as)-octahydroindol-2-ylcarbonyl)^9]bradykinin) was a gift from Hoechst AG (Frankfurt, Germany).

Antisera Sheep anti-rat IL-1β; rabbit anti-rat IL-6, a generous gift from Professor J. Gauldie (McMaster University, Hamilton, Ontario, Canada); sheep anti-human IL-8 serum and sheep anti-murine TNFα serum were kindly provided by Dr R. Thorpe and Dr T. Meager, Division of Immunobiology, NIBSC.

Animals Male Wistar rats, 130–180 g, housed in temperature controlled-rooms (22–25°C) with water and food *ad libitum* until use.

Results

Time course of hyperalgesic responses to bradykinin

Injection of bradykinin into one hind paw of rats (0.01–1.0 μg/paw, i.pl.) evoked dose-dependent hyperalgesia in injected paws, which began within 30 min of injection, reached a plateau within 2 h and was maximum at 3 h after injection (Figure 1a). Responses had begun to decline at 4 h and had returned to pre-injection values within 24 h.

Inhibition by Hoe 140 of the hyperalgesic responses to bradykinin

Hoe 140 (0.1–10 mg kg^{-1}) given s.c., 30 min before bradykinin (500 ng/paw), dose-dependently inhibited bradykinin evoked hyperalgesia (Figure 1b). Maximum inhibition was achieved with 1 and 10 mg kg^{-1} Hoe 140, there being no significant difference (ANOVA, $P > 0.05$) in responses to bradykinin in the presence of these two doses of antagonist. In contrast to its capacity to abolish the effect of bradykinin,

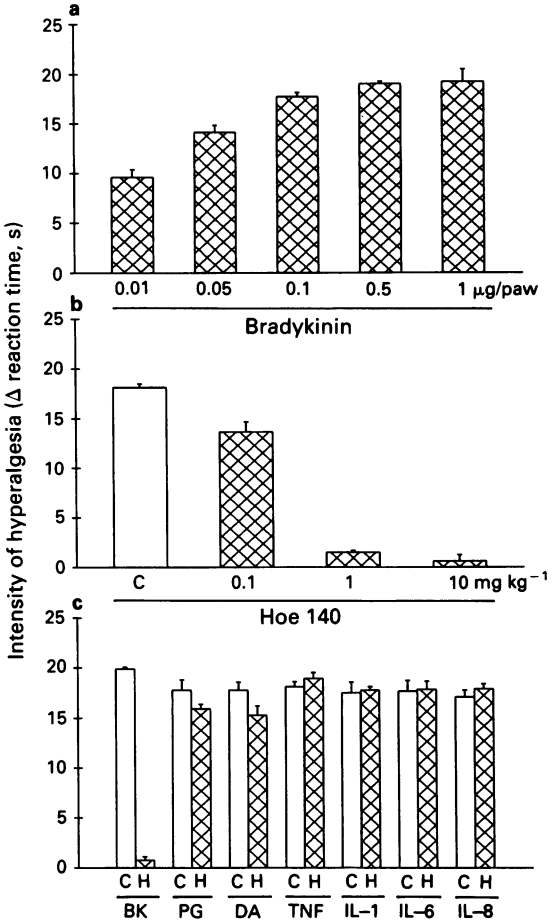

Figure 1 Antagonism by Hoe 140 of the hyperalgesic effects of bradykinin. Responses were measured 3 h after injection (in 100 μl, i.pl.) of bradykinin and other hyperalgesic substances. Hoe 140 was given s.c. 30 min before hyperalgesic substances. Panel (a) shows the intensity of hyperalgesia in injected paws in response to bradykinin (0.01–1.0 μg). Panel (b) shows the effect of saline (C, open column) and Hoe 140 (0.1–10 mg kg^{-1}, cross-hatched columns) on responses to bradykinin (500 ng). Panel (c) shows the effect of saline (C, open columns) and Hoe 140 (H, 1 mg kg^{-1}, cross-hatched columns) on hyperalgesic responses to bradykinin (BK, 500 ng), and prostaglandin E_2 (PG, 100 ng), dopamine (DA, 10 μg), TNFα (TNF, 2.5 μg), IL-1β (IL-1, 0.1 i.u.), IL-6 (1.0 ng) and IL-8 (0.1 ng). Mean ± s.e.mean in groups of 5 rats are shown.

Hoe 140 (1 mg kg^{-1}, s.c.) did not inhibit hyperalgesic responses to prostaglandin E_2 (100 ng), dopamine (10 μg), TNFα (2.5 μg), IL-1β (0.1 iu), IL-6 (1.0 ng) and IL-8 (0.1 ng, Figure 1c). The doses of these agents were the smallest that evoked maximum responses.

Inhibition by Hoe 140 of the hyperalgesic responses to carrageenin and LPS

Hoe 140 (0.1–10 mg kg^{-1}) injected s.c., 30 min before carrageenin (100 μg/paw) and LPS (1.0 μg/paw), dose-dependently inhibited hyperalgesic responses to these substances (Figure 2a) but not responses to a larger dose of LPS (5 μg/paw, Figure 2a and 2c). There was no significant difference between the antinociceptive effects of 1 and 10 mg kg^{-1} Hoe 140 (ANOVA, $P > 0.05$). Hoe 140 at 1 mg kg^{-1} (s.c.) also inhibited hyperalgesic responses to carrageenin (30 and 300 μg/paw, Figure 2b) and LPS (0.5 and 2 μg/paw, Figure 2c). (The volumes of paws injected with carrageenin alone (100 μg) were 700 ± 57 μl, compared with 430 ± 72 μl after Hoe 140 at 0.1 mg kg^{-1}, 316 ± 49 μl after Hoe 140 at 1.0 mg kg^{-1} and 330 ± 56 μl after Hoe 140 at 10 mg kg^{-1}. Bradykinin and LPS did not cause oedema at the doses tested).

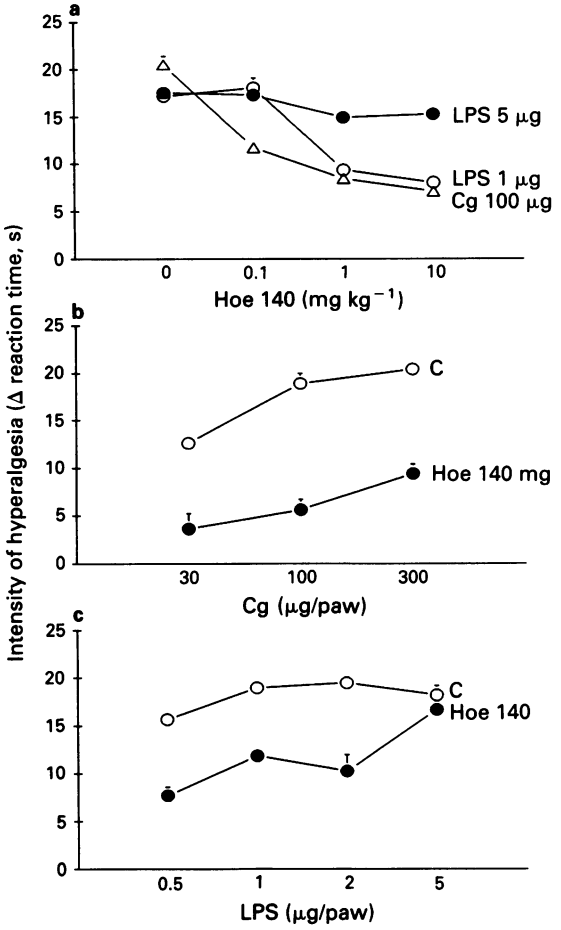

Figure 2 The effect of Hoe 140 on hyperalgesic responses to carrageenin and lipopolysaccharide (LPS). Responses were measured 3 h after injection (in 100 μl, i.pl.) of carrageenin and LPS. Hoe 140 was given s.c. 30 min before carrageenin and LPS. Panel (a) shows the effect of Hoe 140 (0.1–10 mg kg^{-1}) on responses to carrageenin (100 μg, Δ), LPS (1 μg, O) and LPS (5 μg, ●). Panels (b) and (c) show the effects of saline (C, O) and Hoe 140 (Hoe 1 mg kg^{-1}, s.c., ●) on hyperalgesic responses to carrageenin (30–300 μg) and LPS (0.5–5 μg), respectively. Mean ± s.e.mean (frequently within the dimensions of the symbols) in groups of 5 rats are shown.

Inhibition by atenolol and indomethacin of the hyperalgesic responses to bradykinin and LPS

Local pretreatment with indomethacin (100 μg/paw) or atenolol (25 μg/paw), 30 min before the injection of bradykinin (500 ng/paw) or LPS (1 and 5 μg/paw) into the same paw, inhibited the hyperalgesic responses to these substances (Figure 3). The effects of indomethacin and atenolol were additive: their combination markedly attenuated responses to bradykinin and LPS (1 and 5 μg).

Inhibition by anti-cytokine sera of the hyperalgesic responses to bradykinin

Hyperalgesia evoked by bradykinin (500 ng/paw) was inhibited by local pretreatment with anti-cytokine sera, injected into the same paw, 30 min before the bradykinin (Figure 4a). Responses to bradykinin were effectively abolished (i.e., Δ reaction times were reduced by ⩾ 85%) by pretreatment with antiserum neutralising TNFα (50 μl) and inhibited by antisera neutralising IL-1β (50 μl), IL-6 (50 μl) and IL-8 (50 μl). The effects of anti-IL-1β + anti-IL-6 sera (50 μl of each) were not additive whereas the effects of anti-IL-1β + anti-IL-8 sera

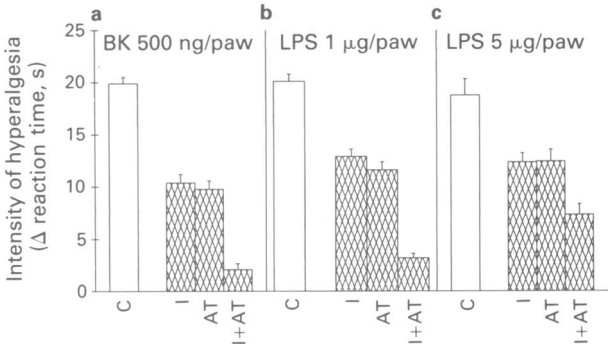

Figure 3 Inhibition by atenolol and indomethacin of the hyperalgesic responses to bradykinin and lipopolysaccharide (LPS). Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) bradykinin (BK, 500 ng), (b) LPS (1 μg), and (c) LPS (5 μg). Pretreatments were given (in 100 μl, i.pl.) 30 min before bradykinin or LPS. C = saline; I = indomethacin, 100 μg; AT = atenolol, 25 μg. Mean ± s.e.mean in groups of 5 rats are shown.

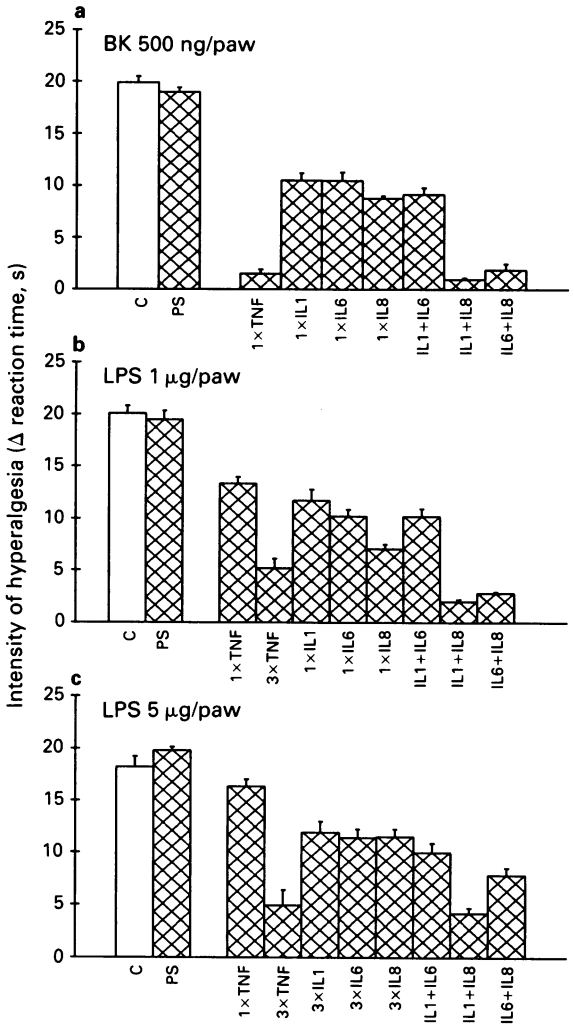

Figure 4 Inhibition of the hyperalgesic responses to bradykinin and lipopolysaccharide (LPS) by anti-cytokine sera. Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) bradykinin (BK, 500 ng), (b) LPS (1 μg), and (c) LPS (5 μg). Antisera were injected (in a total volume of 150 μl) 30 min before bradykinin or LPS. C = saline; PS = preimmune serum; 1 × TNF = 50 μl anti-TNFα serum; 3 × TNF = 150 μl anti-TNFα serum; 1 × IL1 = 50 μl anti-IL-1β serum; 3 × IL1 = 150 μl anti-IL-1β serum; 1 × IL6 = 50 μl anti-IL-6 serum; 3 × IL6 = 150 μl anti-IL-6 serum; 1 × IL8 = 50 μl anti-IL-8 serum; 3 × IL8 = 150 μl anti-IL-8 serum; 1 + 6 = 50 μl anti-IL-1β + 50 μl anti-IL-6 sera; 1 + 8 = 50 μl anti-IL-1β + 50 μl anti-IL-8 sera; 6 + 8 = 50 μl anti-IL-6 + 50 μl anti-IL-8 sera. Mean ± s.e.mean in groups of 5 rats are shown.

(50 μl of each) or anti-IL-6 + anti-IL-8 sera (50 μl of each) were additive: these combinations effectively abolished the response to bradykinin.

Inhibition by anti-cytokine sera of the hyperalgesic responses to LPS

The pattern of inhibition by the anti-cytokine sera of responses to LPS (1 μg) was similar to that of responses to bradykinin except that a larger dose of anti-TNFα serum (150 μl) was required to attenuate markedly responses to LPS (1 μg, Figure 4b). Hyperalgesic responses to LPS (5 μg) were also markedly attenuated by the larger dose (150 μl) but not by the smaller dose (50 μl) of anti-TNFα serum (Figure 4c). Responses to LPS (5 μg) were inhibited by antisera neutralising IL-1β (150 μl), IL-6 (150 μl) and IL-8 (150 μl). The effects of anti-IL-1β + anti-IL-6 sera (50 μl of each) were not additive whereas the effects of anti-IL-1β + anti-IL-8 sera (50 μl of each) or anti-IL-6 + anti-IL-8 sera (50 μl of each) were additive, especially the former (Figure 4c).

Discussion

Bradykinin has been shown to cause a dose-dependent hyperalgesia that is inhibited in a dose-dependent manner by the BK_2 receptor antagonist Hoe 140 (Heapy *et al.*, 1991). The lack of effect of Hoe 140 on responses to PGE_2, dopamine, TNFα IL-1, IL-6 and IL-8 confirmed the specificity of Hoe 140 reported in other systems (Heapy *et al.*, 1991; Beresford & Birch, 1992) and provided evidence that BK_2 receptors are not involved in the mediation of responses to these other hyperalgesic stimuli.

Previously it was shown that the inflammatory agent, carrageenin, induced production of TNFα, which activated a cascade of cytokine release (Cunha *et al.*, 1992). IL-8, induced by TNFα, stimulated the release of hyperalgesic sympathomimetics (sympathetic hyperalgesia), whereas IL-1β and IL-6, induced by TNFα, stimulated the release of hyperalgesic cyclo-oxygenase products (inflammatory hyperalgesia). An early and crucial role for TNFα was proposed because a single injection of this cytokine mimicked the response to carrageenin by inducing production of IL-1β, IL-6 and IL-8, and a single injection of antiserum neutralizing endogenous TNFα abolished the response to carrageenin (Cunha *et al.*, 1992).

In the present study, bradykinin-evoked hyperalgesia was abolished by pretreatment with antiserum neutralizing endogenous TNFα whereas TNFα-evoked hyperalgesia was not inhibited by Hoe 140, suggesting that bradykinin induced TNFα. Consistent with this hypothesis was the finding that drugs and antisera shown previously to inhibit responses to TNFα (Cunha *et al.*, 1992) also inhibited responses to bradykinin with a pattern of inhibition similar to that shown previously for TNFα. Also consistent with the hypothesis is a report that bradykinin induced production of TNF and IL-1 in cultures of murine macrophage cell lines (Tiffany & Burch, 1989).

Recent studies showing that IL-1β but not TNFα could induce BK_1 receptor-mediated hyperalgesia in rats in thermal (Perkins & Kelly, 1993) and mechanical (Davies & Perkins, 1993) models more severe than that used in the present study suggest roles for both types of bradykinin receptor in a complex network of hyperalgesic mediators in which bradykinin can induce cytokines and *vice versa*. It has been suggested that BK_2 receptors may play a more significant part in the earlier stages of inflammatory pain, with BK_1 receptors maintaining the hyperalgesic state during inflammation and injury (Dray & Perkins, 1993). Knowledge of the precise location of the two receptor types (e.g., on nociceptive neurones, macrophages, vascular smooth muscle and synovial cells) and of the conditions required for their expression should improve our understanding of the kinin/cytokine pathways.

The pattern of inhibition of hyperalgesic responses to LPS (1 and 5 μg) by indomethacin, atenolol, and the anti-cytokine sera was similar to that shown previously for carrageenin (Cunha *et al.*, 1991; 1992), suggesting that LPS, like carrageenin, evoked hyperalgesia by activating the cytokine cascade. However, the finding that Hoe 140 inhibited hyperalgesic responses to the smaller dose of LPS (1 μg) but not responses to the larger dose (5 μg) shows that the cytokine cascade can be activated independently of BK_2 receptors if the hyperalgesic stimulus is of sufficient magnitude. Subsequent studies should investigate a possible role for BK_1 receptors in the development of hyperalgesia in response to stimuli such as large doses of LPS.

There is a substantial literature showing that carrageenin and LPS activate the plasma kinin system (Rothschild & Gascon, 1966; Damas & Remacle-Volon, 1992) and that LPS is a potent, 'direct' activator of cytokine production, especially from cells of the monocyte/macrophage lineage. Therefore, the contribution of bradykinin to the development of inflammatory hyperalgesia in certain pathological processes may well be overshadowed by either 'direct' stimulation of cytokine production by stimuli such as LPS or by other intermediates in hyperalgesic pathways. Consequently, the usefulness of bradykinin antagonists as analgesics will depend upon the relative contribution of bradykinin to the release of cytokines and other hyperalgesic mediators. Bradykinin antagonists are most likely to be useful analgesics in conditions such as mumps, pancreatitis, intense burns and gram-negative infections in which kinin systems are activated.

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Inhibitory actions of diphenyleneiodonium on endothelium-dependent vasodilatations *in vitro* and *in vivo*

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1 This study examined the *in vitro* and *in vivo* inhibitory effects of diphenyleneiodonium (DPI), a novel inhibitor of nitric oxide (NO) synthase, on endothelium-dependent vasodilatations.

2 DPI (3×10^{-8}–3×10^{-6} M) concentration-dependently inhibited acetylcholine (ACh)-induced relaxation in preconstricted rat thoracic aortic rings, with an IC_{50} of 1.8×10^{-7} M and a maximal inhibition of nearly 100%. DPI (3×10^{-6} M) also completely inhibited the relaxation induced by the calcium ionophore, A23187 but not by sodium nitroprusside (SNP). The inhibitory effect of DPI (3×10^{-7} M) on ACh-induced relaxation was prevented by pretreatment with NADPH (5×10^{-3} M) and FAD (5×10^{-4} M) but not L-arginine (L-Arg, 2×10^{-3} M). Pretreatment with NADPH did not alter the inhibitory effect of N^G-nitro-L-arginine on ACh-induced relaxation.

3 The inhibitory effect of DPI on ACh-induced relaxation in the aortae lasted >4 h after washout. In contrast to pretreatment, post-treatment (1 h later) with NADPH (5×10^{-3} M) reversed only slightly the inhibitory effect of DPI.

4 In conscious rats, DPI (10^{-5} mol kg^{-1}) inhibited the depressor response to i.v. infused ACh, but not SNP. However, it caused only a transient pressor response which was previously shown to be due completely to sympathetic activation.

5 Thus, DPI is an efficacious and 'irreversible' inhibitor of endothelium-dependent vasodilatation *in vivo* and *in vitro*. The mechanism of the inhibition may involve antagonism of the effects of FAD and NADPH, co-factors of NO synthase. However, unlike the N^G-substituted arginine analogues (another class of NO synthase inhibitors), DPI-induced suppression of endothelium-dependent vasodilatation *in vivo* does not lead to a sustained rise in blood pressure.

Keywords: Diphenyleneiodonium (DPI); nitric oxide synthase inhibitor; endothelium-dependent relaxation; blood pressure; acetylcholine (ACh); A23187; sodium nitroprusside (SNP); FAD

Introduction

A group of iodonium compounds have been reported to be a new class of nitric oxide (NO) synthase inhibitors in the macrophage (Stuehr *et al.*, 1991b; Kwon *et al.*, 1991; Keller *et al.*, 1992). These compounds include diphenyleneiodonium (DPI), iodoniumdiphenyl and di-2-thienyliodonium, all of which have chemical structures distinct from those of N^G-substituted arginine (Arg) analogues. DPI was initially found to be a potent hypoglycaemic agent (Stewart & Hanley, 1969; Gatley & Martin, 1979) which, by inhibiting gluconeogenesis from lactate and aspartate, suppressed the oxidation of NADH-linked substances (Holland *et al.*, 1973). It was later shown that DPI, iodoniumdiphenyl and di-2-thienyliodonium suppressed the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), probably *via* inhibition of a flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989; O'Donnell *et al.*, 1993).

The pharmacology of N^G-substituted Arg analogues, which include N^G-monomethyl-L-Arg (L-NMMA), N^G-nitro-L-Arg (L-NOARG), N^G-nitro-L-Arg methyl ester (L-NAME), L-iminoethyl-ornithine (L-NIO) and N^G-amino-L-Arg (L-NAA) (see Moncada *et al.*, 1991), has been extensively studied. These compounds cause sustained inhibition of endothelium-dependent relaxation *in vitro* and produce prolonged pressor responses in whole animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; 1990; Wang & Pang, 1990; Wang *et al.*, 1991; 1992; 1993; Pang & Wang, 1993). The pressor effects of these compounds have been attributed to the inhibition of the L-Arg/NO pathway and endothelium-dependent vasodilatation *in situ* (Aisaka *et al.*, 1989; Rees *et al.*, 1989; see Moncada *et al.*, 1991). Accordingly, DPI being an inhibitor of NO synthase, would be expected to cause a pressor response in whole animals. Indeed, i.v. injections of DPI produced transient pressor responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). However, unlike that of the N^G-substituted Arg analogues (Wang & Pang, 1991), the pressor effect of DPI was attenuated or abolished by blockers of the sympathetic nervous system (Wang & Pang, 1993a,b).

The reasons for the differences in the causative factor and time course of the pressor responses elicited by these two classes of NO synthase inhibitors are unclear. One possibility is that DPI does not produce prolonged inhibition of endothelium-dependent vasodilatations *in vitro* and/or *in vivo*. Another possibility is that inhibition of endothelium-dependent vasodilatation by DPI is not the cause of the elevation of blood pressure. Hence, the first aim of this study was to find out if DPI causes prolonged *in vitro* and *in vivo* inhibition of endothelium-dependent vasodilatations, as DPI has been shown to cause prolonged inhibition of NO biosynthesis in macrophages (Stuehr *et al.*, 1991b). This was assessed by studying the effects of DPI on relaxations induced by the endothelium-dependent vasodilators, acetylcholine (ACh) and A23187 (calcium ionophore), and the endothelium-independent vasodilator sodium nitroprusside (SNP) in preconstricted rat aorta. In addition, the effects of DPI on depressor responses to ACh and SNP were studied in conscious, unrestrained rats. The second aim was to determine if slower onset pressor responses (other than the initial transient rise in blood pressure) followed the administration of DPI. The third aim was to examine if the NO synthase co-factors NADPH and FAD, as well as the NO synthase substrate L-Arg, reversed the inhibitory effect of DPI on endothelium-dependent relaxation.

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Methods

Male Sprague-Dawley rats (350–420 g) were used in this study.

Isolated aortic rings

The rats were killed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cleared of connective tissue. Four ring segments of 0.5 cm length were prepared from one aorta and suspended in random order in separate organ baths. Each ring was connected to a Grass FT-03-C force-displacement transducer for isometric recording with a preload of 1 g. The rings were equilibrated for 1 h (with 3 washouts) in Krebs solution (pH 7.4) at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. The Krebs solution had the following composition (mM): NaCl 118, glucose 11, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgCl_26H_2O$ 1.2.

The rings were first incubated with vehicle or drugs (see later) followed by phenylephrine (PE, 10^{-6} M, EC_{90}). After 15–20 min, at the steady-state phase of the contractile response to PE, a cumulative concentration-response curve to ACh, A23187 (calcium ionophore) or SNP was obtained. Each drug concentration was left in the bath until a plateau response was reached. The time taken to complete each concentration-response curve was approximately 20 min. In groups where more than one concentration-response curve of ACh was constructed, the preparations were washed three times within 30 min and given another 30 min to recover completely from the effects of the previous applications of PE and ACh. Afterwards, PE was again added followed by the construction of ACh concentration-response curves.

Conscious rats

The rats were anaesthetized with halothane (4% in air for induction and 1.2% in air for surgery). Polyethylene cannulae (PE50) were inserted into the left iliac artery for the measurement of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) and into the left iliac vein for the administration of drugs. The cannulae were filled with heparinized (25 iu ml^{-1}) normal saline, tunnelled s.c. along the back and exteriorized at the back of the neck. The rats were put into small cages allowing free movement and given >6 h recovery from the effects of surgery and halothane before use.

Drugs

The following drugs were purchased from Sigma Chemical Co. (MO, U.S.A.): acetylcholine (ACh) chloride, A23187, phenylphrine (PE) hydrochloride, flavin adenine dinucleotide (FAD) disodium, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), N^{ω}-nitro-L-arginine (L-NOARG) and L-arginine (L-Arg) hydrochloride. Diphenyleneiodonium (DPI) sulphate and sodium nitroprusside (SNP) were obtained from Colour Your Enzyme Ltd. (Ontario, Canada) and Fisher Scientific Co. (N.J., U.S.A.), respectively. All drugs were dissolved in normal saline (0.9% NaCl) except for DPI and A23187 which were dissolved in 5% glucose solution and 10% dimethyl sulphoxide, respectively.

Experimental protocols

Each experiment included 6–8 aortic rings or 6 conscious, unrestrained rats.

Protocol 1: Effects of DPI on ACh-, A23187- and SNP-induced relaxations in the aorta Six groups of aortic rings were incubated with the vehicle or DPI (3×10^{-8}–3×10^{-6} M) followed by PE 10 min later. After another 10–15 min, concentration-response curves to ACh (10^{-8}–3×10^{-5} M) were obtained. Only one concentration of DPI was studied in each group. Another two groups were treated with the vehicle or DPI (3×10^{-6} M) followed by the application of PE and construction of concentration-response curve to A23187 (10^{-9}–10^{-6} M). The last two groups were treated in the same way as the previous two groups except that SNP (3×10^{-10}–10^{-7} M) was used in place of A23187.

Protocol 2: Effects of pretreatment with NADPH, FAD or L-Arg on the inhibitory effect of DPI Twelve groups of aortic rings were treated with vehicle + vehicle, NADPH (1.5×10^{-3} M) + vehicle, NADPH (5×10^{-3} M) + vehicle, FAD (5×10^{-6} M) + vehicle, FAD (5×10^{-4} M) + vehicle and L-Arg (2×10^{-3} M) + vehicle, vehicle + DPI, NADPH (1.5×10^{-3} M) + DPI, NADPH (5×10^{-3} M) + DPI, FAD (5×10^{-6} M) + DPI, FAD (5×10^{-4} M) + DPI, and L-Arg (2×10^{-3} M) + DPI, with 3×10^{-7} M DPI added in all cases. Another two groups of aortic rings were treated with vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M). The first treatments were given 10 min prior to the second treatments. Afterward, the rings were preconstricted with PE and relaxed with ACh as described in Protocol 1.

Protocol 3: Time course and reversibility of the inhibitory effect of DPI on relaxation response of ACh The time course of the inhibitory effect of DPI was studied in 3 groups of aortic rings. After completing the first concentration-response curve to ACh in PE-preconstricted rings in the presence of vehicle or DPI (3×10^{-7} or 3×10^{-6} M), the preparations were washed out without further addition of drug or vehicle. Second, third and fourth ACh curves were constructed in preconstricted rings at 1.5, 4 and 9 h after the preparations were washed out. In another two groups of aortic rings, the effect of post-treatment with NADPH on the inhibitory effect of DPI was studied. These rings were incubated with DPI (3×10^{-7} M) or vehicle for 1 h, followed by the application of NADPH (5×10^{-3} M) and the construction of ACh concentration-response curves in PE-preconstricted conditions.

Protocol 4: Effect of DPI on resting MAP and depressor response to ACh and SNP In one group of rats, DPI (10^{-5} mol kg^{-1}) was injected (i.v. bolus) and blood pressure was continuously monitored for 2 h.

Two groups of rats were injected (i.v. bolus) with the vehicle or DPI (10^{-5} mol kg^{-1}) 20 min prior to i.v. infusions of ACh (6×10^{-8}–1.8×10^{-6} mol kg^{-1} min^{-1}, each dose for 4 min) and SNP (3×10^{-8}–4.8×10^{-7} mol kg^{-1} min^{-1}, each dose for 4 min). The sequence of ACh and SNP administrations was reversed in half of the studies with 20 min recovery after the completion of the first dose-response curve. The time taken to complete the experiment was approximately 2 h after i.v. injection of DPI or the vehicle.

Calculation and statistical analysis

IC_{50} and E_{max} were calculated from individual concentration-response curves (see Wang & Pang, 1993a). All results are expressed as mean ± standard error (s.e.mean) except for the points where the error bars were smaller than the symbols (see figures). The results were analysed by the analysis of variance/co-variance followed by Duncan's multiple range test with $P<0.05$ selected as the criterion for statistical significance.

Results

Effects of DPI on ACh, A23187- and SNP-induced relaxations

All five concentrations of DPI (3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M) slightly potentiated PE-induced contraction

from the baseline value of 0.99 ± 0.10 g to 1.09 ± 0.15, 1.27 ± 0.11, 1.32 ± 0.10, 1.31 ± 0.10 and 1.18 ± 0.14 g, respectively. However, only the effects of the third and fourth concentrations of DPI were statistically significant.

In the vehicle-treated group, ACh relaxed the preconstricted aorta concentration-dependently with maximum relaxation of approximately 60% (Figure 1a). DPI inhibited concentration-dependently the ACh-induced relaxation. At 3×10^{-5} M ACh, the IC_{50} of DPI was 1.8×10^{-7} M with a maximum inhibition of 96% (Figure 1b).

In another two vehicle groups, A23187 and SNP also relaxed concentration-dependently the preconstricted aortae, with maximal relaxations of approximately 60 and 100%, respectively. DPI (3×10^{-6} M) completely inhibited A23187-induced relaxation (Figure 2a) but did not affect the relaxation response of SNP (Figure 2b).

Influences of NADPH, FAD and L-Arg on the inhibitory effects of DPI and, the effect of L-NOARG on ACh-induced relaxation

Baseline contractions elicited by PE in the presence of vehicle or DPI (3×10^{-7} M) were 1.29 ± 0.07 and 1.67 ± 0.12 g, respectively. Treatment with NADPH (1.5 and 5×10^{-3} M), FAD (5×10^{-4} and 5×10^{-6} M) and L-Arg (2×10^{-3} M) did not significantly affect PE-induced contractions in the presence of either the vehicle (1.04 ± 0.06, 1.04 ± 0.11, 1.16 ± 0.06, 1.33 ± 0.11, 1.23 ± 0.08 g, respectively) or DPI (1.43 ± 0.10, 1.37 ± 0.14, 1.59 ± 0.06, 1.52 ± 0.13, 1.44 ± 0.04 g, respectively).

DPI inhibited ACh-induced relaxations (Figure 3a). Treatment with L-Arg did not affect either the ACh-induced

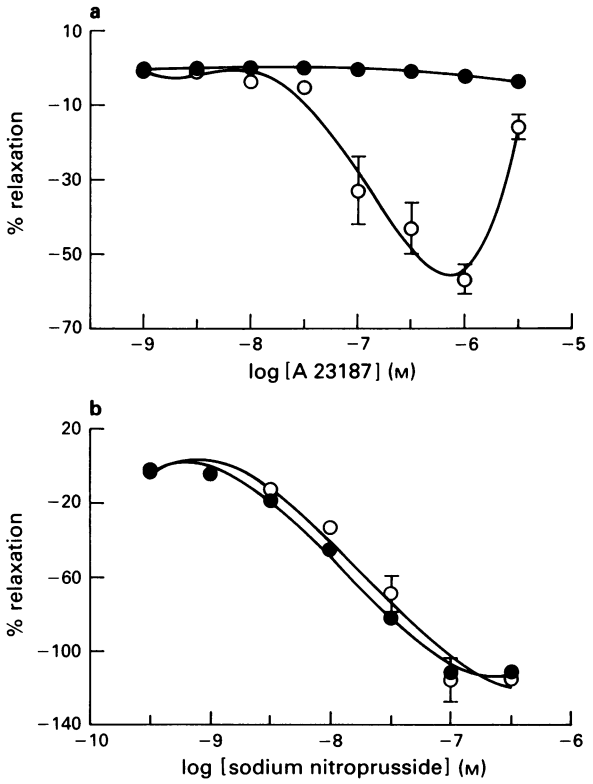

Figure 2 Effects (mean ± s.e.mean) of vehicle (○) or diphenyleneiodonium (DPI, 3×10^{-6} M, ●) on A23187 (a) and sodium nitroprusside (b) induced relaxations in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

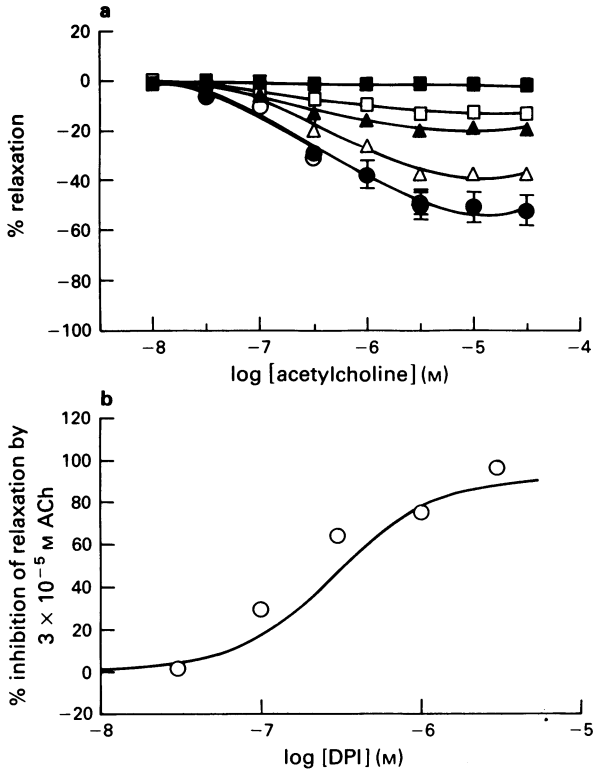

Figure 1 (a) Concentration-response (mean ± s.e.mean) curves of diphenyleneiodonium (DPI) on acetylcholine-induced relaxation in phenylephrine (10^{-6} M)-preconstricted aortic rings ($n = 7$ each group). Concentrations of DPI were as follows: vehicle (○); 3×10^{-8} M (●); 10^{-7} M (△); 3×10^{-7} M (▲); 10^{-6} M (□); 3×10^{-6} M (■). (b) Percentage inhibition by DPI of 3×10^{-5} M acetylcholine-induced relaxation in the aorta. The data were derived from the mean values of (a).

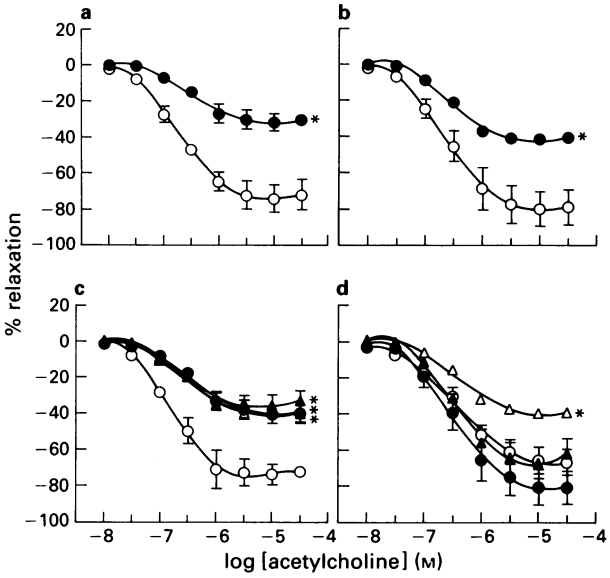

Figure 3 Effects (mean ± s.e.mean) of vehicle, NADPH, FAD and L-arginine on acetylcholine-induced relaxation and on the inhibitory influence of diphenyleneiodonium (DPI, 3×10^{-7} M) on relaxation in phenylephrine (10^{-6} M), preconstricted aortic rings ($n = 6$ each group). The first treatments were performed 10 min before the second treatments. (a) Vehicle + vehicle (○); vehicle + DPI (●). (b) L-Arginine (2×10^{-3} M) + vehicle (○); L-arginine (2×10^{-3} M) + DPI (●). (c) FAD (5×10^{-6} M) + vehicle (○); FAD (5×10^{-4} M) + vehicle (●); FAD (5×10^{-6} M) + DPI (△); FAD (5×10^{-4} M) + DPI (▲). (d) NADPH (1.5×10^{-3} M) + vehicle (○); NADPH (5×10^{-3} M) + vehicle (●); NADPH (1.5×10^{-3} M) + DPI (△); NADPH (5×10^{-3} M) + DPI (▲). *Significant difference from control curve ($P < 0.05$).

relaxation or the inhibitory effect of DPI on ACh-induced relaxation (Figure 3b). Although the lower concentration (5×10^{-6} M) of FAD also did not alter either ACh-induced relaxation or the inhibitory effect of DPI on ACh, the higher concentration (5×10^{-4} M) of FAD suppressed the relaxant effect of ACh and prevented further inhibition by DPI on ACh-induced relaxation (Figure 3c). Although neither concentration of NADPH significantly affected ACh-induced relaxation, the higher (5×10^{-3} M) but not the lower (1.5×10^{-3} M) concentration completely prevented the inhibitory effect of DPI (Figure 3d). The effectiveness of pretreatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as the ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−67%) to that in the absence of NADPH (−32%), was 209%.

PE caused contractions of 1.46 ± 0.12 and 1.67 ± 0.13 g in the presence of vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M), respectively. Compared to the pooled vehicle control derived from Figures 1a and 3a, L-NOARG markedly inhibited ACh-induced relaxation. Pretreatment with NADPH did not affect the inhibitory effect of L-NOARG (Figure 4).

Time course and reversiblity of the inhibitory effect of DPI on ACh-induced relaxation

The PE-induced contractions in the presence of vehicle or DPI did not change with the passage of time (data not shown). The ACh-induced maximal relaxation was not altered until at least 4 h after washout. Maximal relaxation at 9 h was $-48 \pm 7\%$, which was significantly less than that ($-69 \pm 6\%$) at 0 h (Figure 5). DPI at 3×10^{-7} and 3×10^{-6} M inhibited ACh-induced relaxation by approximately 50 and 100%, respectively (Figure 5a). The inhibitory effect of DPI remained at least 4 h after washout (Figure 5b,c). At 9 h after washout, the relaxations of DPI-pretreated rings were still less, though insignificantly, than those of vehicle-pretreated rings (Figure 5d).

Maximum relaxation to ACh after 1 h exposure to 3×10^{-7} M DPI ($-38.3 \pm 3.1\%$, Figure 6) was similar to that after a 10 min exposure to DPI ($-32.2 \pm 4.8\%$, Figure 3a). Post-treatment (1 h later) with NADPH (5×10^{-3} M) slightly but significantly, suppressed the inhibitory effect of DPI. The effectiveness of post-treatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as a ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−51%) to that in the absence of NADPH (−38%), was 134% (Figure 6).

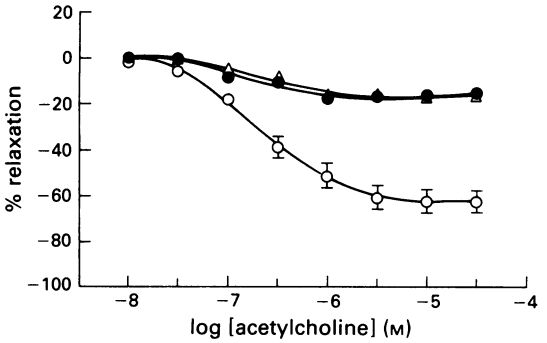

Figure 4 Effect (mean ± s.e.mean) of pretreatments (10 min earlier) of NADPH (5×10^{-3} M) on the inhibitory effect of N^G-nitro-L-arginine (L-NOARG, 10^{-6} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 8$ each group except for the pooled control rings where $n = 13$). Vehicle (○); vehicle + L-NOARG (●); NADPH + L-NOARG (△). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

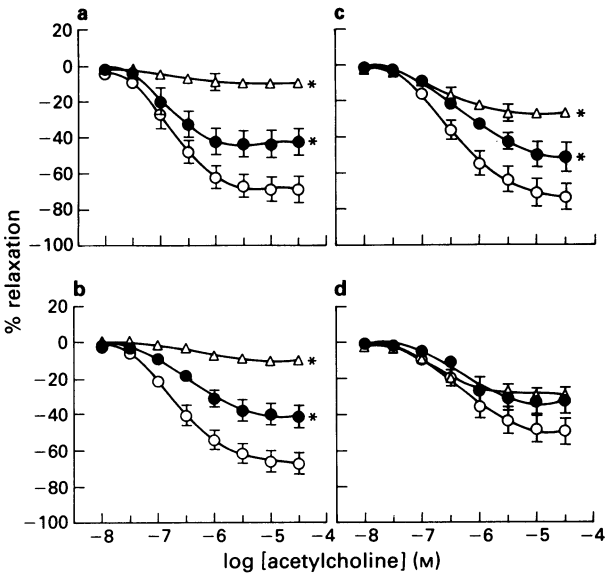

Figure 5 The time course of the effects (mean ± s.e.mean) of vehicle (○), diphenyleneiodonium (DPI, 3×10^{-7} M, ●) and DPI (3×10^{-6} M, △) on acetylcholine-induced relaxations in phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). (a), (b), (c) and (d) represent responses at 0, 1.5, 4 and 9 h after washout without further addition of the vehicle or DPI. *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

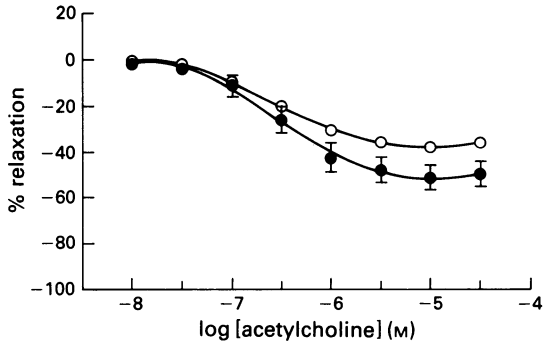

Figure 6 Effects (mean ± s.e.mean) of post-treatment (1 h later) with vehicle (○) or NADPH (5×10^{-3} M, ●) on the inhibitory effect of diphenyleneiodonium (DPI, 3×10^{-7} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

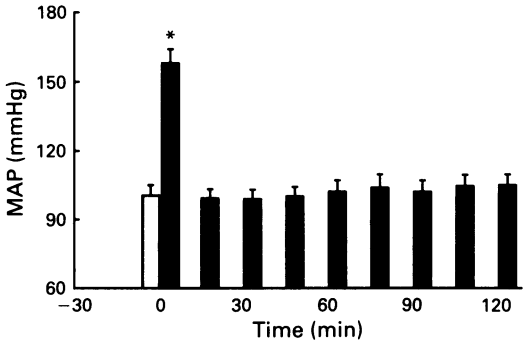

Figure 7 Effect (mean ± s.e.mean) of i.v. bolus injections of diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}) on mean arterial pressure (MAP) in conscious rats ($n = 6$). Open and solid columns represent pre- and post-administration with DPI. *Significant difference from pre-administration with DPI ($P < 0.05$).

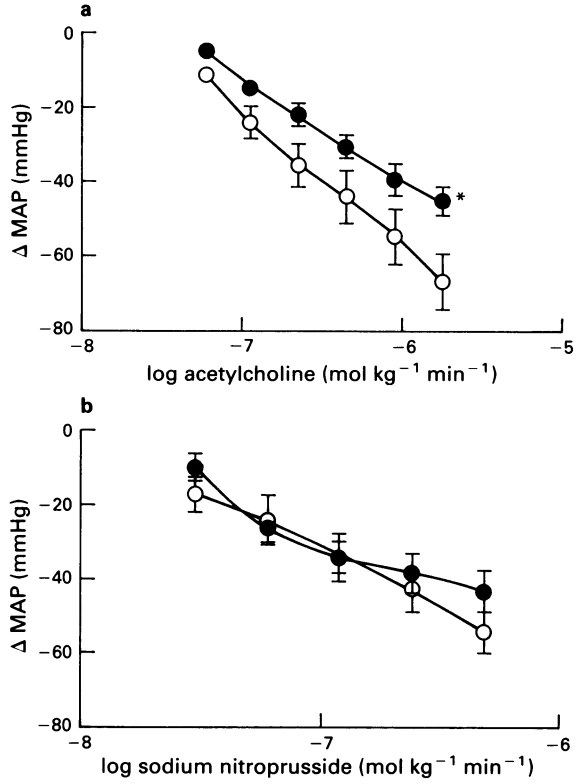

Figure 8 Dose-response curves (mean ± s.e.mean) of i.v. infusions of acetylcholine (a) and sodium nitroprusside (b) on mean arterial pressure (MAP) in conscious rats ($n = 6$ each group) pretreated with i.v. bolus injection of vehicle (○) or diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}, ●). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

Effects of DPI on resting blood pressure and depressor responses to ACh and SNP

Intravenous bolus injections of DPI (10^{-5} mol kg^{-1}) in conscious rats caused immediate and transient increases in MAP which were similar to the responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). MAP returned to the baseline level approximately 4 min after the injection of DPI and remained there during the 2 h observation period (Figure 7).

Baseline MAPs of rats before and 20 min after treatment with vehicle were 109 ± 2 and 112 ± 3 mmHg, respectively, which were similar to those of DPI-treated rats (10^{-5} mol kg^{-1}, i.v. bolus) (118 ± 5 and 114 ± 5 mmHg). Intravenous infusions of ACh and SNP caused dose-dependent depressor responses. Pretreatment with DPI significantly attenuated the depressor responses to ACh but not to SNP (Figure 8).

Discussion

Our *in vitro* results show that DPI selectivity inhibits endothelium-dependent relaxation induced by receptor-mediated (ACh) or non-receptor-mediated (A23187) mechanisms. The results are consistent with the report that DPI inhibits ACh-induced relaxation in the rabbit aorta (Stuehr *et al.*, 1991b) and further suggest there is no species difference for the actions of DPI. DPI also attenuates ACh- but not SNP-induced decreases in MAP in conscious rats, and ACh- but not SNP-induced vasodilatation in the perfused rat hindquarter preparation (unpublished observation). The results suggest that DPI inhibits endothelium-dependent vasodilatations in both conductance and resistance vessels. Therefore, the *in vitro* inhibitory effects of DPI on endothelium-dependent vasodilatations are similar to those of the N^G-substituted Arg analogues. These results are in accordance with the hypothesis that inhibition of NO synthesis causes the suppression of endothelium-dependent vasodilatation.

It has been known since 1973 that DPI suppresses the oxidation of NADH-like substrates thereby inhibiting mitochondrial oxidation (Holland *et al.*, 1973). It was later shown that DPI inhibits NADPH-dependent oxidase of neutrophils and macrophages (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), and macrophage NO synthase (Stuehr *et al.*, 1991b), by specifically binding to and inhibiting the action of a plasma membrane polypeptide which may be a component of flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989). This suggests that flavin is the site of attack by DPI and that a protein is associated with FAD (O'Donnell *et al.*, 1993). Isoenzymes of NO synthase are known to be flavoproteins which contain FAD as a cofactor in the macrophage (Stuehr *et al.*, 1980; 1990; 1991a; Hevel *et al.*, 1991; White & Marletta, 1992), neutrophil (Yui *et al.*, 1991), brain (Mayer *et al.*, 1991; Lowenstein *et al.*, 1992; Bredt *et al.*, 1991; 1992; Hiki *et al.*, 1992) and liver (Evans *et al.*, 1992). There is, however, no functional documentation of a role for FAD as a cofactor of NO synthase in endothelial cells. Our *in vitro* results demonstrate that FAD interferes with both ACh-induced relaxation and the inhibitory effect of DPI on ACh-induced relaxation. The latter result, which is consistent with Stuehr *et al.*'s observation (1991b) that FAD antagonizes the inhibitory effect of DPI on macrophage NO synthesis, suggests that FAD and DPI may inhibit endothelial NO synthesis by a mechanism similar to that in macrophages. The former result is puzzling, since as a cofactor, FAD should facilitate rather than interfere with endothelium-dependent relaxation. FAD was indeed reported to facilitate macrophage NO synthesis (Stuehr *et al.*, 1990; Hevel *et al.*, 1991). The mechanism by which FAD inhibits ACh-induced relaxation is not clear at the moment, however, the effect may not be specific as FAD also inhibits SNP-induced relaxation (unpublished observation).

Our *in vitro* results also show that NADPH interferes with the inhibitory effect of DPI on ACh-induced relaxation. The antagonism of DPI by NADPH was specific since the same concentration of NADPH did not alter the inhibitory effect of L-NOARG. The inhibitory effect of DPI was also not affected by L-Arg, at a concentration previously found to reverse the inhibitory effects of L-NOARG and L-NAME on endothelium-dependent relaxations in aortic rings (Wang *et al.*, 1992; 1993). Our results with NADPH are consistent with those which show that both the constitutive (e.g. brain and endothelial) and inducible (e.g. macrophage and smooth muscle) NO synthases are dependent on NADPH as an essential cofactor (Mayer *et al.*, 1989; Stuehr *et al.*, 1989; 1990; 1991a; see McCall & Vallance, 1992). Regarding the nature of the interaction between NADPH and FAD, it has been suggested that NADPH suppresses the binding of DPI to the flavoprotein in neutrophil oxidase by preventing the attachment of DPI to a site in close proximity to the NADPH-binding site (Cross & Jones, 1986). It is very likely that NADPH may interfere with the action of DPI on endothelial NO synthase by the same mechanism.

Pretreatment with DPI was found to inhibit ACh-induced relaxation in aortic rings for at least 4 h after washout and to suppress ACh-induced vasodilatation for at least 2 h after intravenous bolus injection. Therefore, our *in vitro* and *in vivo* results are supportive of a prolonged inhibitory effect of DPI on endothelium-dependent vasodilatations. DPI has been reported to inhibit irreversibly macrophage NO synthase (Stuehr *et al.*, 1991b); the mechanism may involve the formation of a covalent bond with components of a flavoprotein (Ragan & Bloxham, 1977; O'Donnell *et al.*, 1993). However, our results show that post-treatment (1 h later) with NADPH still attenuates the effect of DPI, although the response is significantly less than that following pretreatment

(10 min earlier). These results may imply that fresh synthesis of NO occurs in endothelial cells.

It is well-known that all N^G-substituted Arg analogues which inhibit endothelium-dependent relaxation *in vitro* cause long-lasting pressor effects in whole animals. The pressor response of N^G-substituted Arg analogues is not blocked by the impairment of the central nervous system (Tabrizchi & Triggle, 1992; Wang & Pang, 1993a), sympathetic nervous system (Wang & Pang, 1991), renin-angiotensin system (Wang & Pang, 1991), or prostaglandin system (Rees *et al.*, 1989; Wang & Pang, unpublished data, 1993), but is inhibited by L-Arg (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Wang & Pang, 1990; Wang *et al.*, 1991b; 1992). These observations have been accepted as evidence of a role of NO in the regulation of blood pressure (Rees *et al.*, 1989; Aisaka *et al.*, 1989; see Moncada *et al.*, 1991). As an 'irreversible' inhibitor of NO synthase, DPI should also cause a prolonged pressor response. However, unlike the N^G-substituted Arg analogues, intravenous bolus injections of DPI produced only immediate and transient increases in MAP. The pressor response of DPI was blocked by procedures which impair the activities of the central or sympathetic nervous systems, namely, pithing, spinal cord transection and the administration of tetrodotoxin, reserpine, guanethidine, phentolamine or prazosin (Wang & Pang, 1993a). Moreover, the pressor response to DPI, but not to L-NOARG, was accompanied by elevations of plasma noradrenaline and adrenaline (Wang & Pang, 1993a,b). These results show that the transient pressor response of DPI, unlike that of the N^G-substituted Arg analogues, is solely dependent on the activation of the sympathetic nervous system, i.e., DPI does not elicit a NO-dependent sustained rise in blood pressure as do the other NO synthase inhibitors.

Although one may postulate that the lack of effect of DPI is due to inadequate accumulation of drug *in situ* to inhibit NO synthesis, this is unlikely. DPI was shown to distribute rapidly and adequately to all organs or tissues (Gatley & Martin, 1979); moreover, its peak hypoglycaemic effect was reached at 1.5 h (Holland *et al.*, 1973) or 4 h (Gatley & Martin, 1979) after intraperitoneal injections, suggesting a long duration of action. Our present results also show that DPI inhibits irreversibly endothelium-dependent relaxation *in vitro* for more than 4 h, and partially inhibits ACh-induced vasodilatation *in vivo* even at 2 h after intravenous injection. It should be noted that a lack of complete inhibition of ACh-induced relaxation is a typical observation with NO synthase inhibitors since N^G-substituted Arg analogues, at maximal pressor doses, also cause partial inhibition of ACh-induced vasodilatation – this suggests that either the depressor/vasodilatation response of ACh *in vivo* is only partially due to the release of NO (Rees *et al.*, 1990; Wang *et al.*, 1992) or it is entirely independent of the biosynthesis and/or release of NO (Pang & Wang, 1993).

Although it is generally accepted that endogenous NO modulates vascular tone and blood pressure and that N^G-substituted Arg analogues produce pressor response by inhibition of endothelial NO synthesis and endothelium-dependent vasodilatations *in situ* (see Moncada *et al.*, 1991), our data with DPI suggest otherwise, i.e. inhibition of NO synthesis and endothelium-dependent vasodilatations do not always cause vasoconstriction *in vivo*. This hypothesis is supported by the recent publications which show that methylene blue does not produce a pressor response (Loeb & Longnecker, 1992; Pang & Wang, 1993) although it inhibits endothelium-dependent vasodilatation *in vitro* (Pang & Wang, 1993) and *in vivo* (Loeb & Longnecker, 1992). L-NOARG was also shown to cause much longer inhibition of endothelium-dependent vasodilatation than elevation of blood pressure in conscious rabbits, suggesting that the suppression of NO synthesis alone does not result in hypertension (Cocks *et al.*, 1992). Therefore, the hypothesis that endogenous NO modulates vascular tone and Arg analogues produce pressor response by inhibition of endothelial NO biosynthesis may need re-examination.

In summary, DPI efficaciously and 'irreversibly' inhibits endothelium-dependent vasodilatation *in vitro* and *in vivo* by a mechanism involving the suppression of the actions of FAD and NADPH. Unlike the N^G-substituted Arg analogues, DPI does not cause NO-mediated sustained pressor response. Instead, DPI causes immediate and transient pressor responses which are solely due to the activation of the sympathetic nervous system (Wang & Pang, 1993a,b). These results suggest that inhibition of NO synthesis *in situ* does not necessarily cause a pressor response.

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Muscarinic regulation of cytosolic free calcium in canine tracheal smooth muscle cells: Ca^{2+} requirement for phospholipase C activation

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1 The relationship between muscarinic receptor-mediated phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown and the increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) has been examined in canine cultured tracheal smooth muscle cells (TSMCs).

2 Addition of acetylcholine (ACh) and carbachol led to a 2–3 fold increase in $[Ca^{2+}]_i$ over the resting level as determined by fura-2, with half-maximal stimulation (EC_{50}) obtained at concentrations of 97 and 340 nM, respectively. Addition of the partial agonist, bethanechol, showed a smaller increase in PIP_2 turnover and $[Ca^{2+}]_i$ than did ACh or carbachol.

3 Addition of ACh or carbachol to TSMCs that had been prelabelled with [^{3}H]-inositol led to the rapid (5–15 s) release of inositol mono, bis and trisphosphates IP_1, IP_2 and IP_3. The time course of IP_3 accumulation is correlated with the time course of the peak rise in $[Ca^{2+}]_i$.

4 Inclusion of EGTA lowered the resting $[Ca^{2+}]_i$ and markedly reduced the extent of the agonist-induced rise in $[Ca^{2+}]_i$. When assayed under conditions similar to those used for the $[Ca^{2+}]_i$ measurements, EGTA reduced the muscarinic agonist-stimulated inositol phosphates (IPs) accumulation. Conversely, ionomycin could stimulate IPs accumulation and elevate $[Ca^{2+}]_i$. The addition of Ca^{2+} (2.7–617 nM) to digitonin-permeabilized TSMCs directly stimulated IPs accumulation.

5 Both Ca^{2+} and guanosine-5′-O-(3-thiotriphosphate) (GTPγS) stimulated the formation of IPs in digitonin-permeabilized TSMCs prelabelled with [^{3}H]-inositol. A further calcium-dependent increase in IPs accumulation was obtained by inclusion of either GTPγS or carbachol. The combined presence of carbachol and GTPγS elicited a synergistic effect on IPs accumulation, with half-maximal stimulation observed at approximately 8 nM free Ca^{2+}.

6 These results indicate that (i) the magnitude of the initial rise in $[Ca^{2+}]_i$ is directly related to the production of IPs and (ii) the phospholipase C-mediated PIP_2 breakdown in TSMCs is sensitive to regulation by physiologically relevant concentrations of free Ca^{2+} ($[Ca^{2+}]_f$).

Keywords: Inositol phosphates; tracheal smooth muscle cells; calcium signal; muscarinic agonists; muscarinic receptors

Introduction

The contractile response of tracheal smooth muscle to cholinoceptor agonists is mediated through muscarinic receptors (Barnes, 1992). Stimulation of muscarinic receptors results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) through activation of phospholipase C (PLC), thus generating two intracellular messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Yang *et al.*, 1991a,b; Coburn & Baron, 1990; Baron *et al.*, 1984; Grandordy *et al.*, 1986; van Amsterdam *et al.*, 1989; Rosenberg *et al.*, 1991). IP_3 causes an increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Hashimoto *et al.*, 1985), and DAG activates protein kinase C (Coburn & Baron, 1990). Since the agonist-induced activation of PLC has been shown in certain tissues to be dependent on guanine nucleotides, a guanine nucleotide binding protein (G protein) may be involved in the transduction of extracellular signals from the receptor binding sites to PLC (Litosch & Fain, 1985; Fain, 1990). A stable analogue of guanosine triphosphate (GTP), guanosine 5′-O-(3-thiotriphosphate) (GTPγS), has been previously shown to increase IPs accumulation in permeabilized tracheal smooth muscles of rabbits (Rosenberg *et al.*, 1991) and cows (van Amsterdam *et al.*, 1989). Furthermore, a G protein α subunit purified from bovine brain has been shown to stimulate selectively PIP_2 hydrolysis by a partially purified PLC (Smrcka *et al.*, 1991). However, potentiation of GTPγS of agonist-induced IPs accumulation at various free Ca^{2+} concentrations has not been demonstrated in canine tracheal smooth muscle cells (TSMCs).

In some tissues, an increase in $[Ca^{2+}]_i$ may regulate intracellular metabolism involving PLC activation (Best, 1986; Eberhard & Holz, 1991). We previously reported that muscarinic receptor activation by carbachol caused an increase in $[Ca^{2+}]_i$ in canine TSMCs (Yang, 1992). This increase could be due to the release of Ca^{2+} from internal stores or to the entry of Ca^{2+} from outside the cells (Yang, 1992). The presence of extracellular Ca^{2+} was found to be required for the generation of an initial increase, followed by a plateau in $[Ca^{2+}]_i$ with carbachol-stimulation of TSMCs. Furthermore, in the absence of extracellular Ca^{2+}, carbachol only caused an immediate and transient increase in $[Ca^{2+}]_i$, the magnitude of which was less than that observed in Ca^{2+}-containing buffer (Yang, 1992). No sustained elevation of $[Ca^{2+}]_i$ was observed. In permeabilized chromaffin cells (Eberhard & Holz, 1987) and human SK-N-SH neuroblastoma cells (Fisher *et al.*, 1989), the formation of IPs appeared to be induced by micromolar Ca^{2+} as well as by activators of G proteins (Fisher *et al.*, 1989; Eberhard & Holz, 1991). Therefore, the question arises as to whether an influx of external Ca^{2+} links the muscarinic receptors to the cellular responses mentioned above.

In the present study, we demonstrate that exposure of TSMCs to muscarinic agonists could produce a pronounced increase in $[Ca^{2+}]_i$, that is closely related to the magnitude of IPs accumulation elicited by respective agonists. Both Ca^{2+} signalling and IPs accumulation in intact cells are markedly

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influenced by inclusion of the Ca^{2+} chelator, EGTA. In digitonin-permeabilized cells, the increase of PLC activity due to addition of either a muscarinic agonist or GTPγS, seemed to be regulated by nanomolar concentrations of free Ca^{2+}. Therefore, in TSMCS, the enzymatic machinery underlying generation of the $[Ca^{2+}]_i$ signal may be sensitive to changes in $[Ca^{2+}]_i$ within the physiological range.

Methods

Animals

Mongrel dogs, 10–20 kg, purchased from a local supplier, were used throughout this study. Dogs were housed indoors in the animal facilities with automatically controlled temperature and light cycle and were fed standard laboratory chow and tap water *ad libitum*. Dogs of either sex were anaesthetized with pentobarbitone (30 mg kg^{-1}, intravenously) 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 methods described previously (Yang, 1990; Yang *et al.*, 1991a,b). The trachea was cut longitudinally through the cartilage rings and the smooth muscle was dissected. The muscle was minced and transferred to dissociation medium containing 0.2% collagenase I, 0.01% DNase I, 0.01% elastase IV, and antibiotics in a 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 residue 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% foetal bovine serum (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 a concentration of 2×10^5 cells ml^{-1}. The cell suspension (2 ml/well) was plated onto 12-well culture plates or 6-well culture plates containing glass coverslips coated with collagen. Culture medium was changed after 24 h and then every 3 days. After 5-days in culture, cells were moved 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 and used for measurements 3 to 5 days later.

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 to light chain myosin (Gown *et al.*, 1985). Over 95% of the cells were smooth muscle cells.

Accumulation of inositol phosphates

Effects of muscarinic agonists on the hydrolysis of PIP_2 were 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-^{3}H]-inositol at 37°C for 2 days. TSMCs were washed two times with, 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 the addition of a muscarinic agonist incubation was continued for another 60 min. When antagonists were used, they were applied 10 min before the addition of the agonist. Reactions were terminated by addition of 5% perchloric acid followed by sonication and centrifugation at 3000 *g* for 15 min.

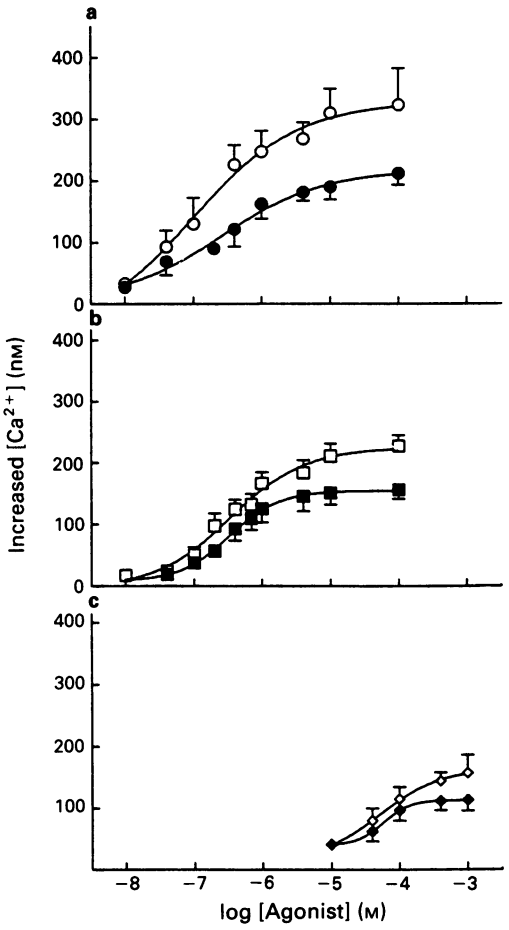

Figure 1 Dependence of the rise in $[Ca^{2+}]_i$ on acetylcholine (a), carbachol (b), and bethanechol (c) concentrations. Confluent cultures of canine TSMCs on glass coverslips were loaded with 5 μM fura-2/AM and fluorescent measurement of $[Ca^{2+}]_i$ was carried out in a dual excitation wavelength spectrofluorometer, with excitation at 340 and 380 nm. The dose-dependent curves of the muscarinic agonists ACh, carbachol, and bethanechol-induced initial and sustained $[Ca^{2+}]_i$ were calculated based on six separated experiments. The results are expressed as mean ± s.e.mean as the increase above the basal levels. Open symbols represent the initial peaks and closed symbols are the plateau phases.

The perchloric acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied to 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*-[^{3}H]-inositol and glycerophosphoinositol, respectively. The fraction of total IPs was eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^{3}H]-IPs was determined in a radiospectrometer (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Permeabilized cells

After a prelabelling period, TSMCs were washed two times with PBS and then permeabilized in KGEH buffer containing (mM): K^+ glutamate 139, ATP 2, $MgCl_2$ 4, LiCl 10, EGTA 2, HEPES 20 (pH 7.4) and digitonin 10 μM, as described by Eberhard & Holz (1987). Cells were incubated for 5 min at 37°C, then washed two times with KGEH buffer without digitonin. Cells were then incubated in KGEH with 2 mM EGTA for 15 min at 37°C, before $CaCl_2$ was added to obtain the required free Ca^{2+} concentrations ($[Ca^{2+}]_f$). The actual

$[Ca^{2+}]_f$ was measured by adding 10 μM fura-2 (K^+-salt). Carbachol or ACh (100 μM) was added and incubated for 60 min at 37°C. Reactions were terminated by adding 5% perchloric acid. The procedures for extraction, separation, and quantification of IPs were the same as those used for intact cells.

Measurement of intracellular Ca^{2+} level

$[Ca^{2+}]_i$ was measured in monolayers with the calcium-sensitive dye fura-2/AM as described by Grynkiewicz *et al.* (1985). Upon confluence, the cells were cultured in DMEM/F-12 with 1% FBS 1 day before measurements were taken. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μM fura-2/AM and were incubated at 37°C for 45 min. At the end of the loading period, the cells were washed twice with the physiological buffer solution (PBS) containing (mM): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10 and glucose 10, pH 7.4, and then incubated in PBS for a further 30 min to complete dye de-esterification. The coverslip was inserted into a quartz cuvette at an angle of approximately 45° to the excitation beam and placed in the thermostatted holder of a SLM 8000C spectrofluorometer (SLM Aminco, Urbana, IL, U.S.A.). Continuous stirring was achieved with a magnetic stirrer.

Table 1 The maximum increase in inositol phosphates (IPs) accumulation induced by the various muscarinic agonists

Agonist	*IPs accumulation* (d.p.m. × 10^{-4})
Control	1.03 ± 0.05
Acetylcholine	11.48 ± 0.17
Carbachol	10.75 ± 0.96
Oxotremorine	4.00 ± 0.61*
Bethanechol	4.79 ± 0.35*
Arecoline	4.26 ± 0.26*

[^{3}H]-inositol labelled TSMCs, were incubated for 60 min with either acetylcholine (100 μM), carbachol (100 μM), oxotremorine (1 mM), bethanechol (1 mM) or arecoline (1 mM). IPs were separated and quantitated. Results are expressed as mean ± s.e.mean from three separate experiments.
*$P<0.001$ as compared with cells stimulated by acetylcholine.

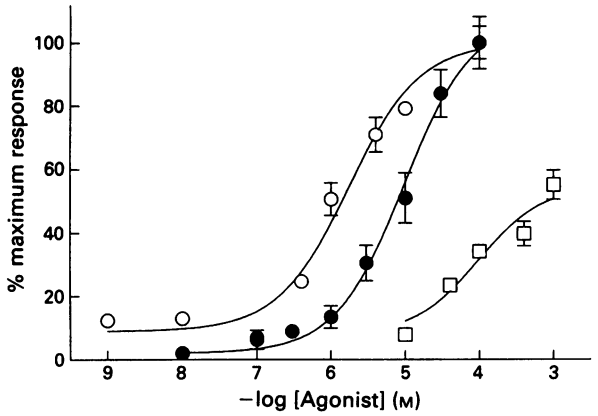

Figure 2 Concentration-dependence of muscarinic agonist-evoked inositol phosphates (IPs) accumulation in TSMCs. Muscarinic agonists, acetylcholine ACh (○), carbachol (●) or bethanechol (□) were added to [^{3}H]-inositol labelled TSMCs and incubated for 60 min. The reactions were terminated by addition of 5% PCA, and IPs were separated and quantitated. Results are expressed as the percentage of maximal response to ACh (116500 ± 900 d.p.m./well) from at least three separate experiments.

Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) of the fluoresence at the two wavelengths were computed and used to calculate changes in $[Ca^{2+}]_i$. The ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by lysing the cells with ionomycin (10 μM) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in a Ca^{2+}-free PBS, respectively. Values obtained were 14.09, 0.96, and 22.07 for R_{max}, R_{min}, and β, respectively. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz *et al.*, 1985).

Analysis of data

Data were expressed as the mean ± s.e.mean of at least four experiments with statistical comparisons based on Student's two-tailed t test at a $P<0.05$ 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 obtained from Boehringer Mannheim (GmbH, Germany). Fura-2/AM was from Molecular Probes Inc (Eugene, U.S.A.). *Myo*-[^{3}H]-inositol (18 Ci mmol^{-1}) was obtained from Amersham (Buckinghamshire, U.K.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.).

Results

Relationship between muscarinic agonist concentration and rise in $[Ca^{2+}]_i$

The muscarinic agonists, acetylcholine (ACh), carbachol, and bethanechol, were found to cause a dose-dependent elevation of $[Ca^{2+}]_i$ levels, as measured directly in cultured TSMCs loaded with fura-2 (Figure 1). The resting free $[Ca^{2+}]_i$ in fura-2 loaded TSMCs was 190 ± 24 nM ($n = 39$). In the presence of extracellular Ca^{2+} (1.8 mM), agonist addition to the fura-2-loaded cells resulted in a rapid increase in $[Ca^{2+}]_i$ and was followed by a decline to a steady value which was significantly higher than the resting $[Ca^{2+}]_i$. The biphasic responses became less pronounced as the agonist concentration was reduced. ACh (10 μM), carbachol (100 μM) and bethanechol (1 mM) caused a maximum increase in the $[Ca^{2+}]_i$, reaching a peak of 457 ± 41, 406 ± 33, and 363 ± 35 nM, respectively, ($n = 8$, Figure 1). The initial rise in $[Ca^{2+}]_i$ was very rapid, reaching the peak within approximately 5 s, was transient and declined to a plateau of 350 ± 33, 339 ± 25, and 296 ± 29 nM ($n = 8$) for ACh, carbachol, and bethanechol, respectively, in approximately 30 s. The half-maximal effects (EC_{50}) of ACh, carbachol, and bethanechol to induce the initial peak in $[Ca^{2+}]_i$ were 97 ± 5 nM, 340 ± 53 nM, and 53.5 ± 7.8 μM, compared with values of 264 ± 48 nM, 330 ± 65 nM, and 57.6 ± 9.0 μM for the plateau phases, respectively. The plateau persisted until the agonist was displaced by the addition of atropine, when it declined to a level substantially higher than that of the resting levels (data not shown).

Comparison of changes in IPs accumulation and $[Ca^{2+}]_i$ induced by muscarinic agonists

The increase of IPs accumulation appeared to be induced to different extents by the muscarinic agonists ACh, carbachol, oxotremorine, bethanechol, and arecoline in TSMCs as shown in Table 1. Bethanechol, oxotremorine and arecoline

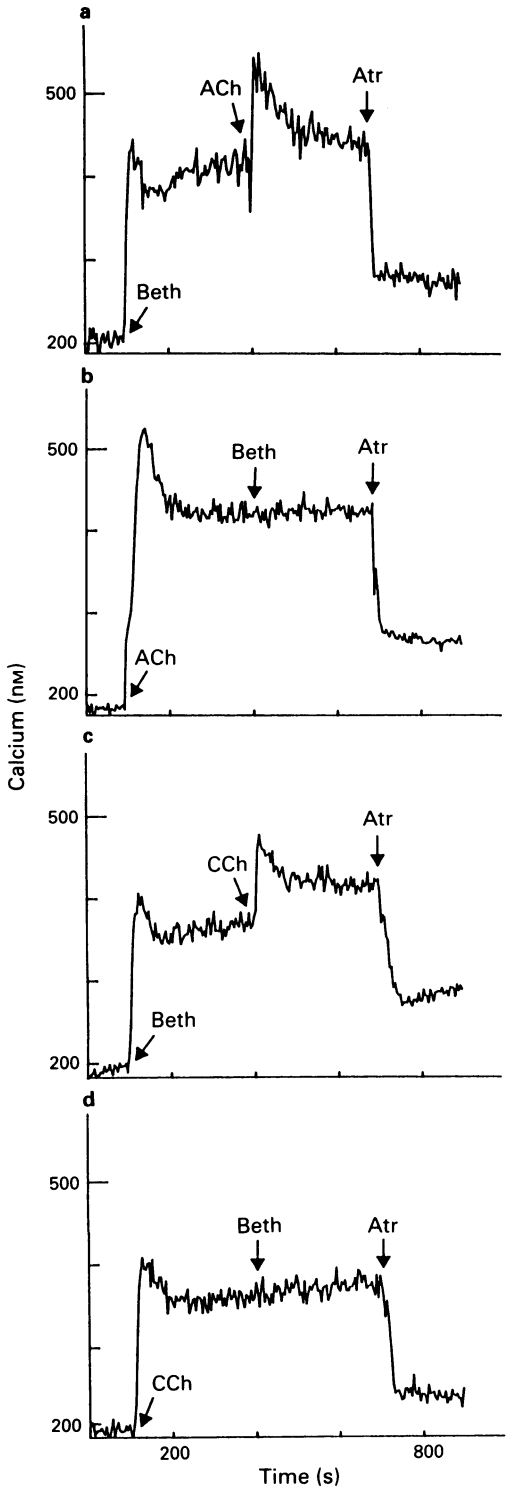

Figure 3 Full and partial muscarinic agonists mobilize the same metabolic pool of Ca^{2+}. (a), To fura-2-loaded TSMCs were added 1 mM bethanechol (Beth), followed by 100 μM acetylcholine (ACh). Note that the addition of ACh after an initial exposure of the cells to bethanechol elicited a further rise in $[Ca^{2+}]_i$. The initial peak $[Ca^{2+}]_i$ values obtained with bethanechol and ACh were 464 and 474 nM, respectively. The corresponding plateau $[Ca^{2+}]_i$ values were 358 and 427 nM. (b) When the cells were first challenged with ACh, the subsequent addition of bethanechol had no effect on $[Ca^{2+}]_i$. (c and d), Parallel experiments were performed using carbachol (CCh) under the same conditions as those of (a) and (b). (c) The addition of carbachol (100 μM) after an initial exposure to bethanechol (1 mM) elicited a further increase in $[Ca^{2+}]_i$. The initial peak $[Ca^{2+}]_i$ values obtained with bethanechol and carbachol were 389 and 419 nM, respectively. The respective plateau $[Ca^{2+}]_i$ values were 292 and 387 nM. (d) When TSMCs were first exposed to carbachol, the subsequent addition of bethanechol had no effect on $[Ca^{2+}]_i$. The traces are from one of at least five experiments that gave similar results. Atr, 10 μM atropine.

at maximally effective concentrations induced less increase in IPs than maximal concentrations of ACh and carbachol ($P<0.001$). The increase of IPs accumulation induced by various concentrations of ACh, carbachol and bethanechol in TSMCs was dose-dependent as shown in Figure 2. Among these agonists, bethanechol at a maximally effective concentration caused the least increase in IPs accumulation. The order of potency for muscarinic agonists with respect to EC_{50} values in TSMCs was: ACh (1.8 μM) > carbachol (7.6 μM) > bethanechol (47 μM). Addition of agonists such as ACh and carbachol, known to stimulate maximally PIP_2 hydrolysis, resulted in an increase of $[Ca^{2+}]_i$ (Table 2). In contrast, partial agonists of PIP_2 hydrolysis such as bethanechol, oxotremorine and arecoline, were less effective ($P<0.01$, as compared with that of $[Ca^{2+}]_i$ stimulated by ACh; Table 2). Once an increase of $[Ca^{2+}]_i$ had been elicited by addition of either a full or partial agonist, the subsequent addition of the same agonist did not result in a further rise in $[Ca^{2+}]_i$ (data not shown). However, if TSMCs were first exposed to bethanechol, a partial agonist, the subsequent addition of either ACh or carbachol did result in a further rise of $[Ca^{2+}]_i$ (Figure 3a and c). If ACh or carbachol was applied first, the subsequent addition of bethanechol failed to increase $[Ca^{2+}]_i$ further (Figure 3b and d). These results indicated that both full and partial muscarinic agonists mobilize the same cellular Ca^{2+} pools but to different extents.

Effect of EGTA on calcium signalling

Reduction of extracellular Ca^{2+} to approximately 60 nM $[Ca^{2+}]_i$ by addition of 4 mM EGTA resulted in three distinct consequences (Figure 4). First, the resting $[Ca^{2+}]_i$ was reduced from 190 ± 24 to 140 ± 20 nM ($n = 20$) within 5 min for fura-2-loaded TSMCs, indicating that extracellular EGTA may deplete the cytoplasmic Ca^{2+}, as previously noted for aortic smooth muscle cells (Capponi *et al.*, 1985). Second, the magnitude of the initial rise of $[Ca^{2+}]_i$ by addition of either ACh or carbachol was reduced to 200 ± 14 and 203 ± 24 nM ($n = 10$, Figure 4b and e) from normal values of approximately 457 ± 41 and 396 ± 33 nM (Figure 4a and d), respectively. As a third consequence, the plateau phase of $[Ca^{2+}]_i$ was abolished and the peak declined to the resting levels within 50 s after the application of the agonist (Figure 4b and e). Subsequent addition of ionomycin (50 nM) to these cells did evoke a further increase of $[Ca^{2+}]_i$, presumably due to the discharge of other intracellular stores of Ca^{2+} (Figure 4b and e). Upon the addition of Ca^{2+} (4 mM) to EGTA-treated TSMCs, the plateau phase of muscarinic stimulated increase in $[Ca^{2+}]_i$ appeared to be quantitatively increased, while the magnitude of the initial peak to cholinoceptor agonists was not restored (Figure 4c and f). When TSMCs were incubated in Ca^{2+}-containing buffer and exposed to 50nM ionomycin, there was a rapid increase of $[Ca^{2+}]_i$ (853 ± 160 nM, $n = 12$), followed by a steady plateau $[Ca^{2+}]_i$ which was sustained for several minutes (Figure 5). In the presence of 4 mM EGTA, the addition of ionomycin induced a rapid but only transient increase of $[Ca^{2+}]_i$ (703 ± 127 nM, $n = 14$). Addition of ACh or carbachol did not induce any further detectable increase in $[Ca^{2+}]_i$ no matter whether TSMCs were exposed to ionomycin in the absence or presence of extracellular Ca^{2+} (Figure 5). The data obtained from Figures 4 and 5 indicate that, whereas ionomycin and full muscarinic agonists (ACh and carbachol) both release intracellular pools of Ca^{2+} in TSMCs, the ionophore is capable of producing approximately twice the $[Ca^{2+}]_i$ response to ACh or carbachol.

IPs accumulation in intact and permeabilized TSMCs: dependence on calcium availability

When TSMCs were prelabelled with [^{3}H]-inositol for 2 days and then exposed to ACh or carbachol, a rapid release (5–30 s) of IP_3, IP_2 and IP_1 occurred (Figure 6). Although

Table 2 The maximum increase in $[Ca^{2+}]_i$ induced by various muscarinic agonists

Agonist	*Basal* (nM)	*Initial* (nM)	*Plateau* (nM)	n
Acetylcholine	178 ± 46	501 ± 43 (323 ± 32)	390 ± 30 (212 ± 19)	7
Carbachol	202 ± 13	430 ± 36 (228 ± 18)*	378 ± 34 (176 ± 25)	10
Oxotremorine	213 ± 14	408 ± 12 (195 ± 21)*	346 ± 15 (133 ± 17)*	6
Bethanechol	233 ± 37	410 ± 23 (157 ± 29)*	369 ± 26 (113 ± 18)*	8
Arecoline	195 ± 24	381 ± 42 (186 ± 31)*	261 ± 33 (66 ± 10)*	5

To fura-2-loaded TSMCs was added either acetylcholine (100 μM), carbachol (100 μM), bethanechol (100 μM), oxotremorine (1 mM) or arecoline (1 mM). Both initial peak and sustained plateau $[Ca^{2+}]_i$ values were monitored. The net increases in $[Ca^{2+}]_i$ above the basal level are shown in parentheses. Results shown are means ± s.e.mean for at least five separate experiments.
*$P < 0.01$, as compared with acetylcholine-induced responses.

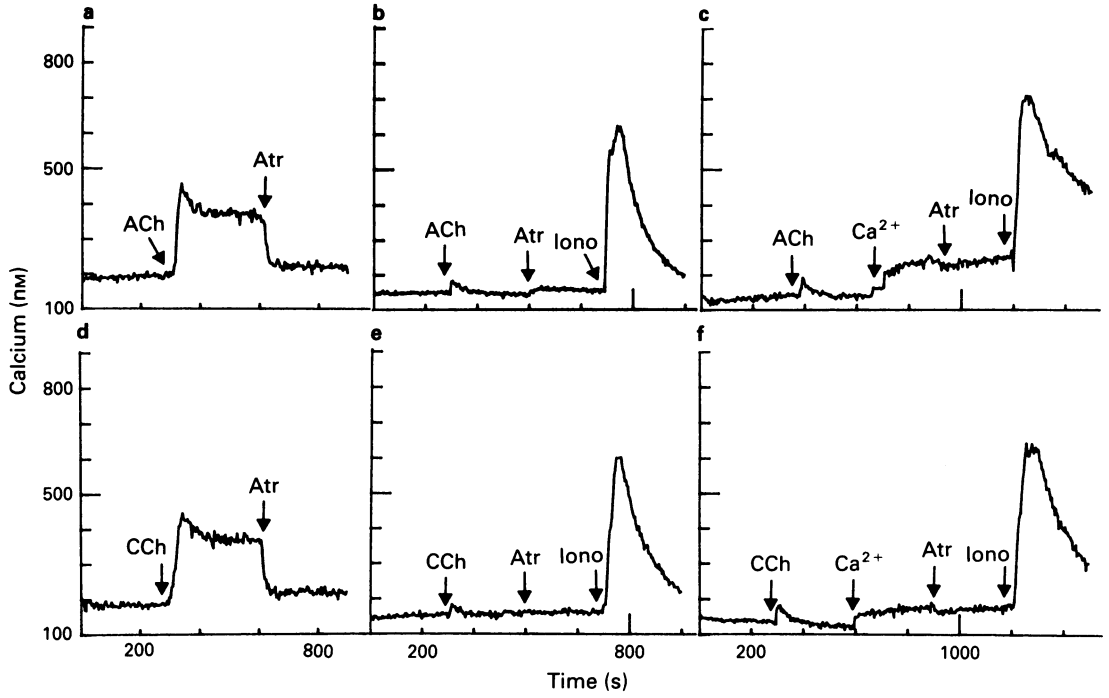

Figure 4 Effect of EGTA on $[Ca^{2+}]_i$ levels induced by muscarinic agonist addition. (a), Fura-2-loaded TMSCs were exposed to acetylcholine (ACh) (100 μM). The initial peak and plateau $[Ca^{2+}]_i$ values were 433 and 332 nM, respectively, whereas the resting $[Ca^{2+}]_i$ was 201 nM. (b) EGTA (4 mM) was added to TSMCs before the addition of ACh (extracellular $[Ca^{2+}]_f$ about 50 nM). The initial peak $[Ca^{2+}]_i$ (190 nM) is less than in (a); $[Ca^{2+}]_i$ returns to the resting level (129 nM) within 1 min. The addition of 10 μM ionomycin (Iono) elicits a further rise in $[Ca^{2+}]_i$ to 646 nM. In (c) TSMCs were treated as in (b) but after the return of $[Ca^{2+}]_i$ to the resting value, 4 mM $CaCl_2$ (Ca^{2+}) was added and the $[Ca^{2+}]_i$ increased to 224 nM. The subsequent addition of 10 μM atropine (Atr) reduced the $[Ca^{2+}]_i$ from 224 to 178 nM. (d–f) Parallel experiments were carried out using carbachol (CCh) as a muscarinic stimulant. (d), Fura-2-loaded TSMCs were exposed to carbachol (100 μM). The initial peak and plateau $[Ca^{2+}]_i$ value were 413 and 387 nM, respectively, whereas the resting $[Ca^{2+}]_i$ was 189 nM. (e) To TSMCs was added 4 mM EGTA (extracellular $[Ca^{2+}]_f$ about 50 nM) before the addition of carbachol. The initial peak $[Ca^{2+}]_i$ obtained (171 nM) is less than in (d) and $[Ca^{2+}]_i$ returns to the resting level (117 nM) within 1 min. The addition of 10 μM ionomycin (Iono) elicits a further rise in $[Ca^{2+}]_i$ to 484 nM. In (f) TSMCs were treated as in (e) but after the return of $[Ca^{2+}]_i$ to the resting value, 4 mM $CaCl_2$ (Ca^{2+}) was added and the $[Ca^{2+}]_i$ raised to 241 nM. Subsequent addition of 10 μM atropine (Atr) reduced the $[Ca^{2+}]_i$ from 241 to 208 nM.

IP_3 was often the most rapidly released IP following the addition of agonist, the appearance of IP_3 and IP_2 were kinetically indistinguishable. Because total IPs accumulation was linear with time in the presence of 10 mM LiCl for at least 60 min, a 60 min incubation period was routinely employed in subsequent experiments. Without adding ACh or carbachol, only a limited amount of IPs was released from the prelabelled TSMCs during a 60 min incubation (data not shown). Addition of ACh (100 μM), carbachol (100 μM), oxotremorine (1 mM), bethanechol (1 mM), and arecoline (1 mM) increased the IPs accumulation by 13.8, 12.5, 3.8, 5.7 and 5.9 fold, respectively, over the control. Ionomycin appeared to increase IPs accumulation 4 fold (Figure 7). However, treatment with 4 mM EGTA was found to block most of the increased IPs accumulation induced by the muscarinic agonists and the ionophore. Only very small increases in IPs accumulation were detected (Figure 7).

To assess more directly the role played by physiologically relevant Ca^{2+} concentrations in the regulation of the PLC activity, prelabelled TSMCs were first permeabilized in digitonin-containing KGEH buffer and then exposed to Ca^{2+}-EGTA buffer solutions. IPs accumulation was measured after 60 min incubation. Elevation of $[Ca^{2+}]_f$ from 2.7 to 617 nM resulted in a 1.6 fold increase of IPs accumulation, with an

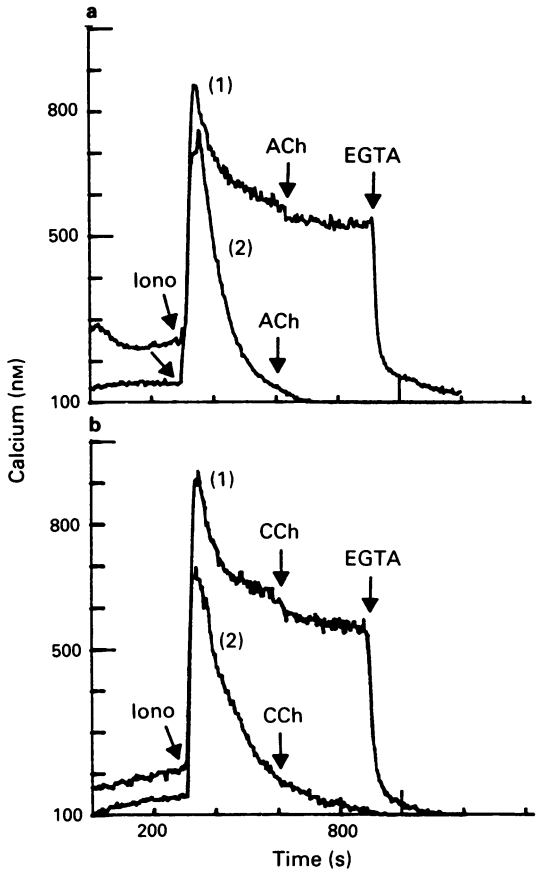

Figure 5 The increase in $[Ca^{2+}]_i$ induced by addition of ionomycin (Iono) in the presence or absence of extracellular Ca^{2+}. (a) (1) Addition of 10 μM ionomycin to TSMCs incubated in Ca^{2+}-containing buffer resulted in a rise in $[Ca^{2+}]_i$ that was sustained for several min. Addition of ACh (100 μM) did not cause any further rise in $[Ca^{2+}]_i$. (2) TSMCs were incubated with Ca^{2+}-containing buffer and 4 mM EGTA for 5 min before the addition of ionomycin. Note that the transient rise in $[Ca^{2+}]_i$ and ACh failed to induce a further increase in $[Ca^{2+}]_i$. (b) Parallel experiments were performed using carbachol (CCh) as an agonist. (1) Addition of 10 μM ionomycin to TSMCs incubated in Ca^{2+}-containing buffer, resulted in a rise in $[Ca^{2+}]_i$, that was sustained for several min. Addition of carbachol (100 μM) did not cause any further rise in $[Ca^{2+}]_i$. (2) TSMCs were incubated with Ca^{2+}-containing buffer and 4 mM EGTA for 5 min before the addition of ionomycin. Note that the transient rise in $[Ca^{2+}]_i$ and carbachol failed to induce any further increase in $[Ca^{2+}]_i$.

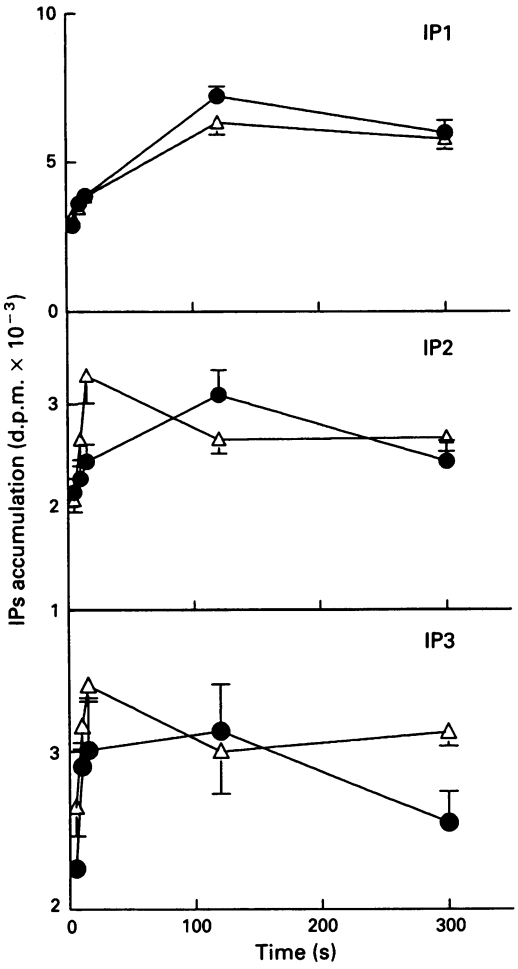

Figure 6 Rapid release of inositol phosphates from TSMCs in the presence of muscarinic agonist. Cells that had been prelabelled for 2 days with [^{3}H]-inositol were washed with KHS, and then incubated in the presence of either 100 μM acetylcholine (ACh, ●), or 100 μM carbachol (Δ). Reactions were terminated by the addition of 5% PCA and inositol phosphates were extracted and separated. Results shown are the means ± s.e.mean of triplicate determinations in three separate experiments. In the experiments shown, labelled IP_3 appeared before IP_2 which was kinetically indistinguishable from IP_3 but was always detected within 5–10 s after the addition of agonists.

EC_{50} of 35 nM ($n = 3$) (Figure 8). Addition of 50 μM guanosine 5'-O-(3-thiotriphosphate) (GTPγS) further potentiated this Ca^{2+}-dependent accumulation of IPs with an EC_{50} for Ca^{2+} of approximately 11 nM (Figure 8). The effect of carbachol was also potentiated by the inclusion of 50 μM GTPγS. Under these conditions, the $[Ca^{2+}]_f$ required to elicit a 50% increase in IPs accumulation was reduced from 23 to 8 nM. When carbachol and GTPγS were simultaneously present, the stimulated accumulation of IPs was 343 ± 59% of control ($n = 3$), a value that compares favourably with the degree of stimulation obtained in intact cells. Thus, GTPγS and carbachol together elicited a synergistic increase in IPs accumulation. Both the effect of carbachol alone and the synergistic increase in IPs accumulation from permeabilized TSMCs could be blocked by the addition of atropine (Table 3). Taken together, these results indicate that stimulation of muscarinic receptors caused IPs accumulation in TSMCs through PLC activation which in turn was regulated by changes of physiologically relevant $[Ca^{2+}]_f$.

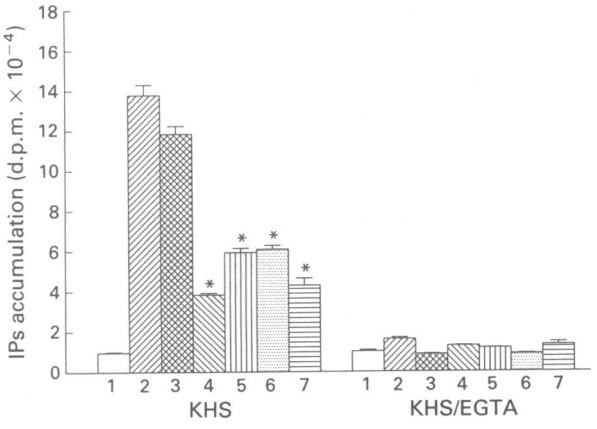

Figure 7 Calcium-dependence of phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown in intact TSMCs. Cells were prelabelled with [^{3}H]-inositol for 2 days. Labelled cells were incubated in either KHS or KHS containing 4 mM EGTA. Then acetylcholine (100 μM, 2), carbachol (100 μM, 3), oxotremorine (1 mM, 4), bethanechol (1 mM, 5), arecoline (1 mM, 6), or ionomycin (10 μM, 7), was added to appropriate wells (1 = basal). Reaction was terminated after a 60 min incubation and inositol phosphates were separated and quantitated. Results are expressed as mean ± s.e.mean from three separate experiments. *$P < 0.01$, as compared with ACh.

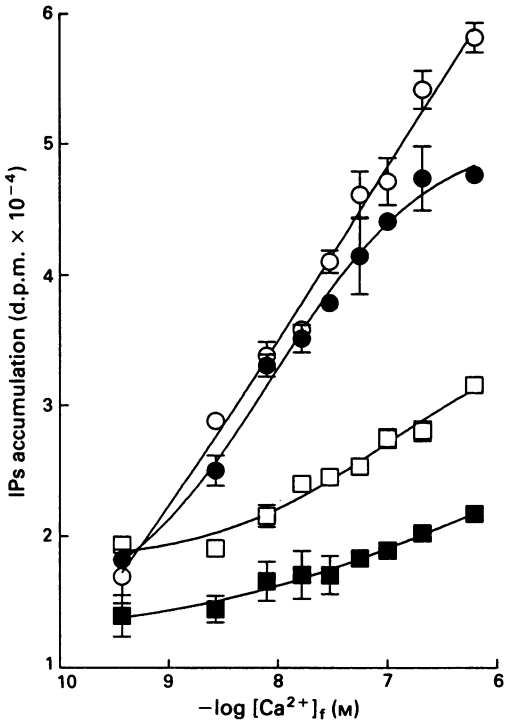

Figure 8 Increases in $[Ca^{2+}]_f$ release inositol phosphates (IPs) from digitonin-permeabilized TSMCs. [^{3}H]-inositol-prelabelled cells were permeabilized and incubated in KGEH buffer with added Ca^{2+} to achieve the required $[Ca^{2+}]_f$ (as measured by the addition of fura-2). Cells were incubated for 60 min in the absence or presence of either carbachol (□), GTPγS (●), or carbachol plus GTPγS (○). The EC_{50} values of $[Ca^{2+}]_f$ for basal (■), carbachol (100 μM), GTPγS (50 μM), and carbachol plus GTPγS were 35, 27, 11 and 8 nM, respectively. Results are expressed as means ± s.e.mean from three separate experiments determined in triplicate. $[Ca^{2+}]_f$ values of 0.37, 2.69, 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, 0.13, 0.25, 0.38, 0.50, 0.63, 0.75, 0.88 and 1, respectively, with EGTA maintained at a concentration of 2 mM.

Table 3 Effect of atropine on carbachol- and GTPγS-induced inositol phosphates (IPs) accumulation in digitonin-permeabilized TSMCs

Treatment	*IPs accumulation* (d.p.m. × 10^{-4})
Basal	3.18 ± 0.27
Carbachol	5.18 ± 0.18
Atropine	3.09 ± 0.36
Atropine + carbachol	3.10 ± 0.05*
GTPγS	6.64 ± 0.36
GTPγS + carbachol	9.81 ± 0.06
Atropine + GTPγS + carbachol	6.82 ± 0.09

Permeabilized TSMCs were incubated for 15 min in KGEH buffer and sufficient Ca^{2+} was added to provide a $[Ca^{2+}]_f$ of 616.8 nM, in the presence of either atropine (10 μM), carbachol (100 μM) or GTPγS (50 μM). The basal level represents the release of IPs obtained in the absence of agents. Results are expressed as mean ± s.e.mean from three separate experiments determined in triplicates.
*$P < 0.01$ as compared with carbachol-induced response.

Discussion

It well established that muscarinic receptor-mediated increase of $[Ca^{2+}]_i$ is due to both Ca^{2+} release from internal stores and an influx from external sources (Berridge & Irvine, 1989). The present study demonstrated that IPs accumulation is regulated by Ca^{2+}, GTPγS and muscarinic agonists in digitonin-permeabilized TSMCs. These results suggest that: (1) both full and partial muscarinic agonists stimulate PIP_2 hydrolysis and increase $[Ca^{2+}]_i$ in TSMCs to different extents; (2) a GTP-binding protein participates in Ca^{2+}-induced IPs accumulation by PLC; and (3) Ca^{2+} and GPTγS have a synergistic effect on IPs accumulation. The results obtained with fura-2 loaded TSMCs indicate that muscarinic agonists evoke a rapid increase in $[Ca^{2+}]_i$ to a peak value, followed by a slow decline and sustained $[Ca^{2+}]_i$. The increase is maintained as long as the muscarinic receptors are occupied by the agonist. These data are consistent with those obtained in swine tracheal smooth muscle (Shieh *et al.*, 1991) and in vascular smooth muscle (Sato *et al.*, 1988). Both the initial peak and the plateau phase were abolished by the muscarinic antagonist, atropine (Yang, 1992); thus these changes in $[Ca^{2+}]_i$ were due to the interaction of muscarinic agonists with muscarinic receptors.

The rapid initial increase in $[Ca^{2+}]_i$ due to the activation of muscarinic receptors by agonists has been shown to be mediated through the release of IP_3 and the subsequent mobilization of Ca^{2+} from internal stores (Berridge & Irvine, 1989). In the present study, the results obtained from the TMSCs indicate that the time course for IP_3 accumulation is correlated with the time of the peak rise in $[Ca^{2+}]_i$. Both partial and full muscarinic agonists for PIP_2 breakdown, mobilize Ca^{2+} from the same metabolic pools. The partial muscarinic agonists bethanechol, oxotremorine and arecoline produced relatively smaller increases in $[Ca^{2+}]_i$ than did the full agonists, ACh and carbachol. These results are consistent with previous studies demonstrating that muscarinic receptor agonists stimulate accumulation of different levels of IPs in tracheal smooth muscle (van Amsterdam *et al.*, 1989) and therefore increase $[Ca^{2+}]_i$ to different extents.

In agreement with others (Chew & Brown, 1986; Merrit & Rink, 1987; McDonough *et al.*, 1988; Yang, 1992), both the initial increase and the plateau of $[Ca^{2+}]_i$ were dependent on the availability of extracellular Ca^{2+}. Addition of EGTA abolished the plateau phase of $[Ca^{2+}]_i$. Although the rapid increase of $[Ca^{2+}]_i$ following muscarinic receptor activation in other types of cells was found not to be completely inhibited by a reduction of extracellular Ca^{2+} (Chew & Brown, 1986; Merrit & Rink, 1987; McDonough *et al.*, 1988; Yang, 1992) in the studies described here EGTA markedly reduced the initial $[Ca^{2+}]_i$ values in TSMCs. It is conceivable that the influx of extracellular Ca^{2+} plays an important role in the generation of the initial increase in $[Ca^{2+}]_i$.

It is also plausible that PLC activity is very sensitive to changes in $[Ca^{2+}]_i$ induced by addition of EGTA (Best, 1986). Based on our results, two lines of evidence support this suggestion. First, addition of EGTA reduced muscarinic-stimulated IPs accumulation. Second, the basal, and carbachol and GTPγS-mediated IPs accumulation in digitonin-permeabilized TSMCs was regulated by physiologically relevant $[Ca^{2+}]_f$ concentrations. In the presence of carbachol and GTPγS, a $[Ca^{2+}]_f$ of less than 10 nM was required for half-maximal activation of PIP_2 breakdown. Therefore, it is evident that a small reduction of $[Ca^{2+}]_i$ significantly retards PLC activation.

Further evidence for the role of Ca^{2+} availability in PIP_2 hydrolysis was obtained from experiments with ionomycin. The Ca^{2+} ionophore ionomycin is a pharmacological tool with which to evoke changes in $[Ca^{2+}]_i$ whch bypass the receptor-operated mechanisms. Addition of ionomycin to intact TSMCs induced a 4 fold increase of IPs accumulation and a 4–5 fold increase in $[Ca^{2+}]_i$. Although the extent of ionomycin-stimulated IPs accumulation was only 30% of that induced by ACh, it is possible that the increase in $[Ca^{2+}]_i$ that follows muscarinic receptor activation makes some further contribution to PIP_2 hydrolysis due to the requirement of Ca^{2+} for maximal activation of PLC. This suggests that although the elevation of $[Ca^{2+}]_i$ influences PLC activity in TSMCs, the primary regulation is due to muscarinic activation of PLC.

One of the most notable features of stimulated PIP_2 hydrolysis in TSMCs is that muscarinic receptor-effector coupling in permeabilized cells is similar to that observed in intact cells in the presence of GTPγS. In the presence of carbachol and GTPγS, PIP_2 breakdown in permeabilized TSMCs appeared to be the same as that obtained in intact cells stimulated by carbachol alone. Since GPTγS 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; Merrit *et al.*, 1986; Jones *et al.*, 1988). Although carbachol could enhance IPs accumulation without the addition of GTPγS, this ability presumably reflects the presence of residual endogenous guanine nucleotides still retained within the permeabilized cells. However, the combination of carbachol and GTPγS had a synergistic effect on IPs accumulation. GTPγS may play a role in the breakdown of PIP_2 that reduces the requirement of $[Ca^{2+}]_f$ for activation of PLC activity (Bradford & Rubin, 1986; Smith *et al.*, 1986). The availability of $[Ca^{2+}]_f$ (> 30 nM) is required for stimulation of PIP_2 hydrolysis. A half-maximal (EC_{50}) increase of IPs accumulation induced by carbachol alone occurred at a $[Ca^{2+}]_f$ greater than 47 nM. In the presence of GTPγS, only 11 nM $[Ca^{2+}]_f$ was required for carbachol to induce a half-maximal increase of IPs accumulation. Therefore, guanine nucleotides appear to sensitize the transduction process such that a muscarinic agonist can effectively initiate PIP_2 hydrolysis at a $[Ca^{2+}]_f$ that is normally encountered in quiescent cells.

In conclusion, our results demonstrate that muscarinic receptor activation in TSMCs causes a rapid increase in $[Ca^{2+}]_i$ related to the extent of PIP_2 hydrolysis and followed by a sustained plateau $[Ca^{2+}]_i$. PLC activity in TSMCs appeared to be sensitive to small changes of $[Ca^{2+}]_i$. Consequently, PIP_2 hydrolysis, can be modulated by either a decrease or an increase of $[Ca^{2+}]_i$. Meanwhile, the results obtained from permeabilized TSMCs indicate that guanine nucleotides sensitize the transduction process, thereby enhancing carbachol-stimulated IPs accumulation at a $[Ca^{2+}]_f$ found in the cytoplasm of quiescent cells.

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Photodynamic action of aluminium phthalocyanine tetrasulphonate ($AlPcS_4$) on smooth muscle: effects of thiols and a cyclic GMP analogue

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1 The smooth muscle system of the guinea-pig taenia caeci has been used *in vitro* to characterize the photodynamic action of aluminium phthalocyanine tetrasulphonate ($AlPcS_4$) in the presence or absence of the thiol reductants L-cysteine (Cys), N-acetyl-L-cysteine (NAC), DL-dithiothreitol (DTT) or reduced glutathione (GSH).

2 In all photodynamic experiments the muscle was exposed to $AlPcS_4$ (10^{-5} M) for 30 min, followed by a 30 min washout period before photon irradiation at 32,000 lux ($\lambda > 570$ nm) for 30 min. Photodynamic contractions were measured relative to the contractile response to carbachol (5×10^{-5} M) and relaxation responses were determined in muscle precontracted with either carbachol 5×10^{-5} M or KCl 23.5 mM.

3 Photon-activation of $AlPcS_4$-sensitized smooth muscle evoked a triphasic response: an initial transient contraction and subsequent relaxation followed by a secondary sustained contraction. Cys 10 mM, NAC 10 mM and DTT 5 mM had no effect on the initial photodynamic contraction but significantly decreased the magnitude of the sustained contraction from mean values of 98% to 18%, 95% to 72% and 93% to 6% of the standard carbachol contraction (5×10^{-5} M), respectively; GSH 10 mM was without significant effect on either the initial or sustained contraction.

4 In the absence of extracellular calcium the $AlPcS_4$-sensitized smooth muscle did not respond to photon activation but re-introduction of calcium after cessation of illumination produced a sustained contraction which was markedly inhibited by Cys 10 mM.

5 In precontracted $AlPcS_4$-treated muscle preparations photon activation produced a triphasic relaxation response, i.e. a rapid relaxation followed by a transient contraction and a secondary more sustained relaxation. The sustained phase of photodynamic relaxation was potentiated significantly by Cys 10 mM, NAC 10 mM, DTT 5 mM and GSH 10 mM, the relaxation being approximately doubled in magnitude from mean values of 34% to 68%, 30% to 73%, 34% to 68%, and 48% to 77%, respectively, relative to the standard carbachol (5×10^{-5} M) response.

6 The cyclic GMP analogue, 8-(4-chlorophenylthio)-guanosine-3':5'-cyclic monophosphate (8-PCPT-cGMP) (2×10^{-4} M) alone caused a triphasic relaxation response similar to that produced by photon activation of an $AlPcS_4$-sensitized precontracted preparation in the presence of thiol reductants. The pattern of 8-PCPT-cGMP-induced relaxation was similar in muscle precontracted with carbachol 5×10^{-5} M or KCl 23.5 mM.

7 It is concluded that the rapid generation of reactive intermediates by photon-activation of bound $AlPcS_4$ leads to membrane permeabilization, calcium entry and muscle contraction. These effects may be opposed by a direct stimulatory action of singlet oxygen on guanylate cyclase which is enhanced by the action of thiol reagents and mimicked by the cyclic GMP analogue, 8-PCPT-cGMP.

Keywords: Photodynamic drug action; singlet oxygen; smooth muscle; guinea-pig taenia caeci (coli); aluminium phthalocyanine tetrasulphonate; thiols; guanylate cyclase; cyclic GMP analogue

Introduction

Photodynamic drugs when photon-activated are believed to exert their effects primarily via the local generation of highly-reactive singlet oxygen (Matthews & Cui, 1990a,b). It is, however, often difficult to assess the nature and extent of the biological effects produced by photodynamic agents. As a model system the guinea-pig taenia caeci (coli) has many advantages for analysing the mechanisms of photodynamic drug action since it is composed of a relatively transparent syncytium of smooth muscle cells which readily take up the photosensitizer, respond rapidly to receptor activation or changes in membrane permeability, and produce a well-defined calcium-dependent photodynamic contraction (Matthews & Mesler, 1984b).

In this study the new and highly-effective photosensitizer aluminium phthalocyanine tetrasulphonate ($AlPcS_4$) has been used to investigate the molecular mechanisms of photodynamic drug action on the smooth muscle cells of the taenia caeci and the way in which these actions can be enhanced or inhibited. Photon absorption by a sensitizer molecule converts it from the ground state (S) to the lowest excited singlet state (^1S). Intersystem crossing with spin inversion yields the less energetic but longer-lived triplet excited state (^3S). In the presence of molecular oxygen, energy transfer from ^3S to ground state oxygen (O_2) leads to the formation of highly reactive single oxygen ($^1O_2$), (Girotti, 1990; Foote, 1991). With an absorbance maximum of 675 nm to ensure effective tissue penetration, a good quantum yield of the triplet state, and extended triplet lifetime, $AlPcS_4$ is a potent photodynamic drug the activation and effects of which can be controlled precisely by the wavelength, intensity, and duration of the incident photon irradiation. The free radicals and singlet oxygen generated may react at a number of membrane and subcellular sites although the more immediate effects of $AlPcS_4$ activation are likely to be exerted in the proximity of the cell membrane to which much of the $AlPcS_4$

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is confined by its high net negative charge (Rosenthal, 1991). Membrane lipid peroxidation, membrane permeabilization and the activation of membrane receptor-transduction pathways may all contribute to photodynamic drug action (Matthews & Cui, 1990a,b); guanylate cyclase is also known to be readily activated by oxidation (Waldman & Murad, 1987). Some of these effects may be counteracted by thiol containing reductants. Different thiol reagents have therefore been employed to investigate the involvement of thiol - disulphide oxidation and reduction reactions in photodynamic drug action. In order to assess the extent to which guanylate cyclase and guanosine 3':5'-cyclic monophosphate (cyclic GMP) production may participate in the photodynamic process, the cyclic GMP analogue, 8-(4-chlorophenylthio)-guanosine-3':5'-cyclic monophosphate (8-PCT-cGMP) was used as a potent selective activator of cyclic GMP-dependent protein kinase. In this membrane permeant analogue the lipophilicity of the molecule has been markedly enhanced by replacing the hydrogen in position 8 of the heterocyclic base with the *para*-chlorothio moiety (Francis *et al.*, 1988), so promoting cellular uptake. The results of some of this work were communicated to the Cambridge meeting of the British Pharmacological Society in January, 1993 (Matthews *et al.*, 1993).

Methods

Superfusion of taenia caeci

Male guinea-pigs (200–400 g) were killed by cranial percussion and exsanguination. Strips of taenia muscle were dissected from the caecum, divided into four lengths of approximately 1 cm and superfused at 32°C in a system described in detail by Matthews & Mesler (1984b). Isotonic recordings of muscle contraction were made via a Harvard transducer – amplifier system and Kipp and Zohnen BD 40 chart recorders. A modified Krebs-Henseleit solution was employed of the following composition (mM): NaCl 118, $CaCl_2$ 2.56, KCl 4.7, $MgCl_2$ 1.13, NaH_2PO_4 1.15, $NaHCO_3$ 25, and D-glucose 11.2. The solution was bubbled with 95% O_2 and 5% CO_2 to give a final pH of 7.4. When, in some experiments, the KCl concentration was increased to 23.5 mM the NaCl concentra-

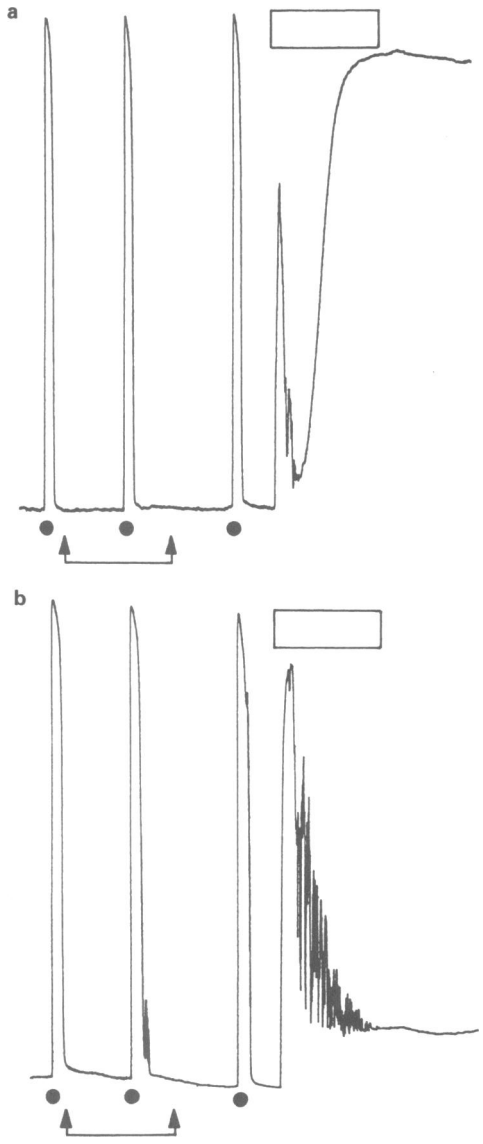

Figure 1 Photon-activation of $AlPcS_4$-sensitized taenia coli. Photodynamic contractile responses (a) in the absence, and (b) in the presence of DL-dithiothreitol (DTT) 5 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to $AlPcS_4$, 10^{-5} M for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (b) DTT, 5 mM was present from the withdrawal of $AlPcS_4$ until the end of the experiment.

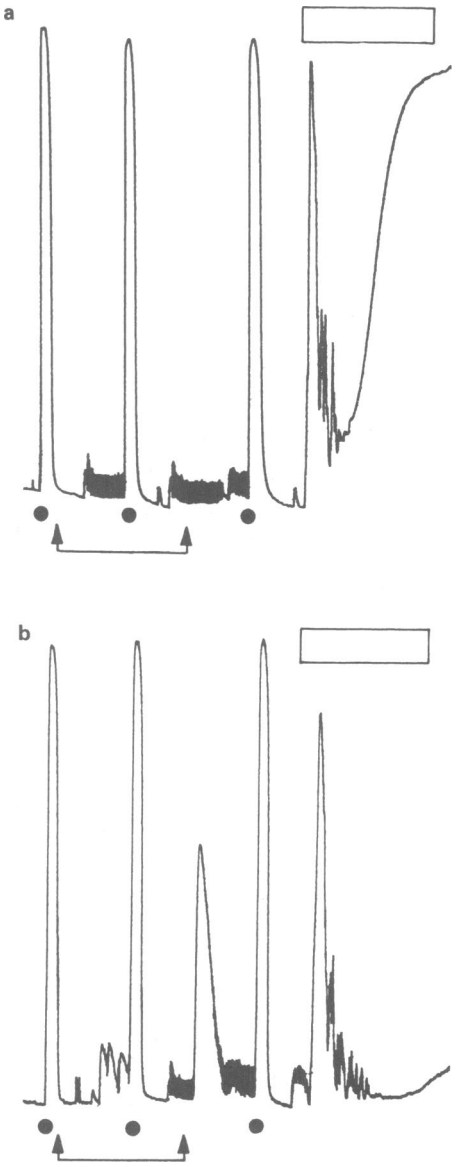

Figure 2 Photon-activation of $AlPcS_4$-sensitized taenia coli. Photodynamic contractile responses (a) in the absence, and (b) in the presence of L-cysteine, 10 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to $AlPcS_4$, 10^{-5} M for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (b) L-cysteine, 10 mM, was present from the withdrawal of $AlPcS_4$ until the end of the experiment.

tion was decreased correspondingly to 99.2 mM to maintain isotonicity. In other experiments, when $CaCl_2$ was omitted ETGA 1 mM was added.

Light intensity

All experiments were carried out in a light-sealed cabinet. Photon irradiation of the tissue was via a fibre-optic probe from a Schott KL150 quartz-halogen light source equipped with a KG1 heat filter. An additional Schott OG-570 sharp-cut glass filter with transmission >570 nm was also used to restrict the spectral output to the range required for activation of $AlPcS_4$ (peak absorbance of 675 nm). Light intensities were measured with a Minolta Illuminance meter T-IH.

Data analysis

Results are expressed as the mean ± s.e.mean and statistical significance was evaluated by Student's *t* test. Photodynamic contraction or relaxation responses are expressed as a percentage of the sustained response to carbachol 5×10^{-5} M. This use of a standardized response to carbachol as a reference allowed for any variation in the magnitude of muscle contraction due to differences in tissue length for each preparation.

Drugs used

Carbachol, L-cysteine, N-acetyl-L-cysteine and glutathione (reduced form) were from Sigma U.K. Chloroaluminium phthalocyanine tetrasulphonate was obtained from Porphyrin Products, Logan, Utah, U.S.A.

Results

$AlPcS_4$-sensitized photodynamic contraction of taenia caeci: effect of thiol reductants

In order to photosensitize the superfused muscle it was first exposed to $AlPcS_4$ (10^{-5} M) for 30 min followed by washout of the drug for a further 30 min. It is clear that the muscle had taken up and retained the $AlPcS_4$ because when illuminated at 32,000 lux ($\lambda > 570$ nm) for 30 min the muscle showed a light-induced contraction (Figure 1a). This effect was not seen in control preparations which had not been exposed to $AlPcS_4$. Thus neither illumination nor $AlPcS_4$ treatment alone had any effect on muscle tone, nor were the contractile responses to repetitive doses of carbachol (5×10^{-5} M) affected by $AlPcS_4$ in the absence of light (Figure 1).

The response of the phthalocyanine-sensitized muscle to light activation was triphasic, i.e. (i) an initial brief contrac-

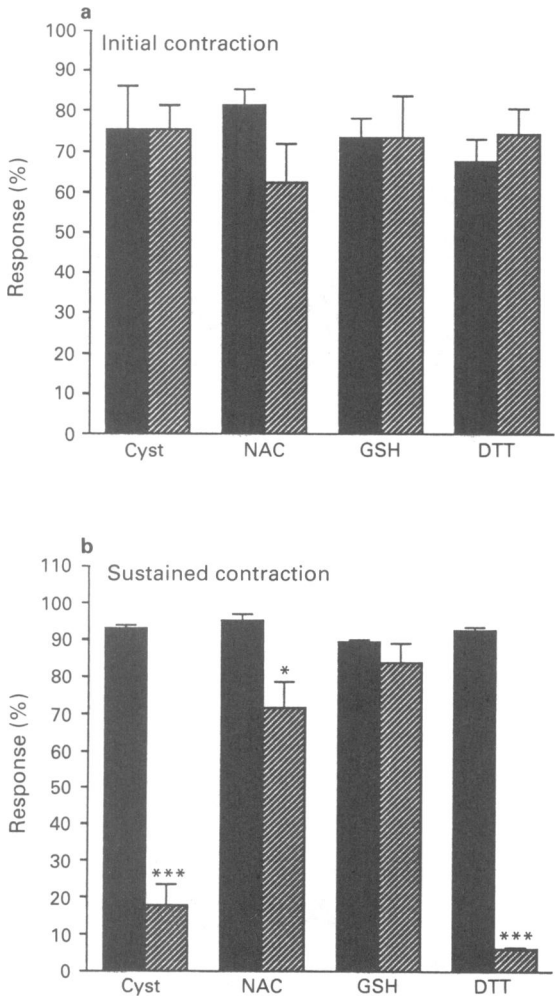

Figure 3 Effects of thiols on (a) the initial, and (b) the sustained photodynamic contraction of $AlPcS_4$-sensitized taenia coli. Experimental protocol as in Figures 1 and 2: Cyst, L-cysteine 10 mM; NAC, N-acetyl-L-cysteine 10 mM; GSH, glutathione 10 mM; DTT, dithiothreitol 5 mM. Responses in the absence (solid columns) and presence (hatched colums) of thiol. $^*P<0.05$; $^{***}P<0.001$; $n=4$ or 5.

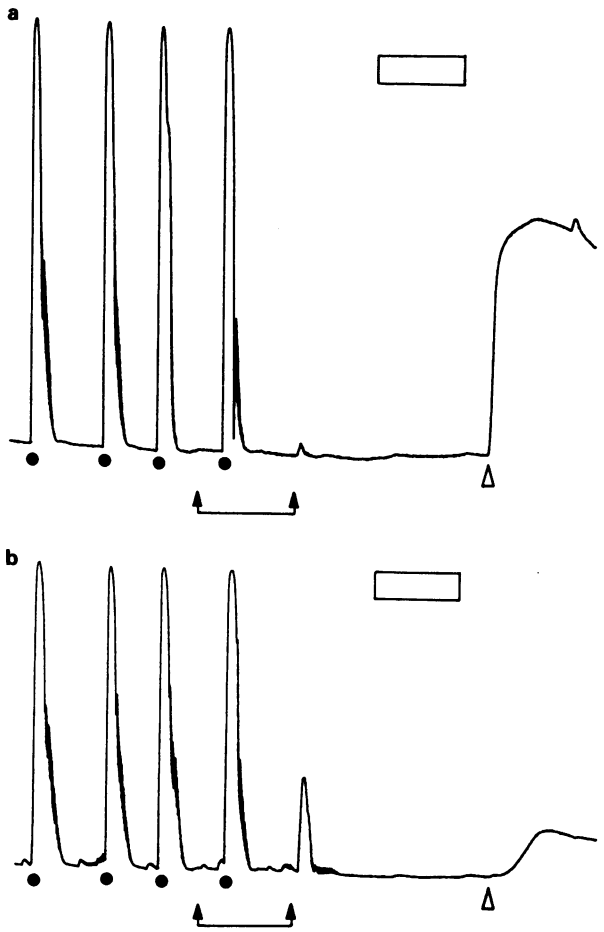

Figure 4 Calcium-dependence of the photodynamic contraction in $AlPcS_4$-sensitized smooth muscle. Contractions to carbachol, 5×10^{-5} M, for 10 s (●) and exposure to $AlPcS_4$, 10^{-5} M, for 30 min (between the arrows). Illumination at 32,000 lux for 30 min (open rectangles). In (a) and (b) extracellular calcium was removed and EGTA, 1 mM, added from the time of $AlPcS_4$ withdrawal until normal $[Ca^{2+}]_o$ was restored at Δ. In (b) L-cysteine, 10 mM, was present from the time of $AlPcS_4$ withdrawal until the end of the experiment.

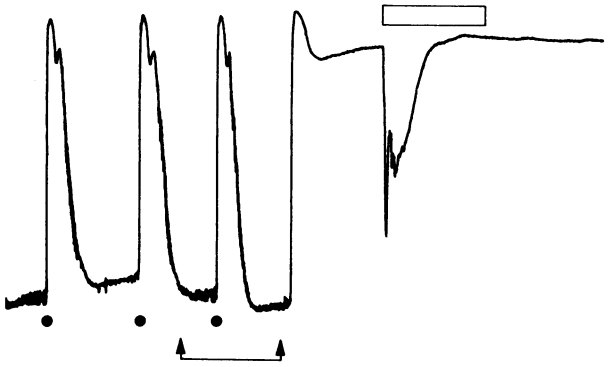

Figure 5 Photodynamic relaxation response produced in muscle precontracted with carbachol. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to $AlPcS_4$, 10^{-5} M, for 30 min (between the arrows). The smooth muscle cells were exposed continuously to carbachol, 5×10^{-5} M, from the withdrawal of $AlPcS_4$ until the end of the experiment. Illumination at 32,000 lux for 30 min (open rectangle).

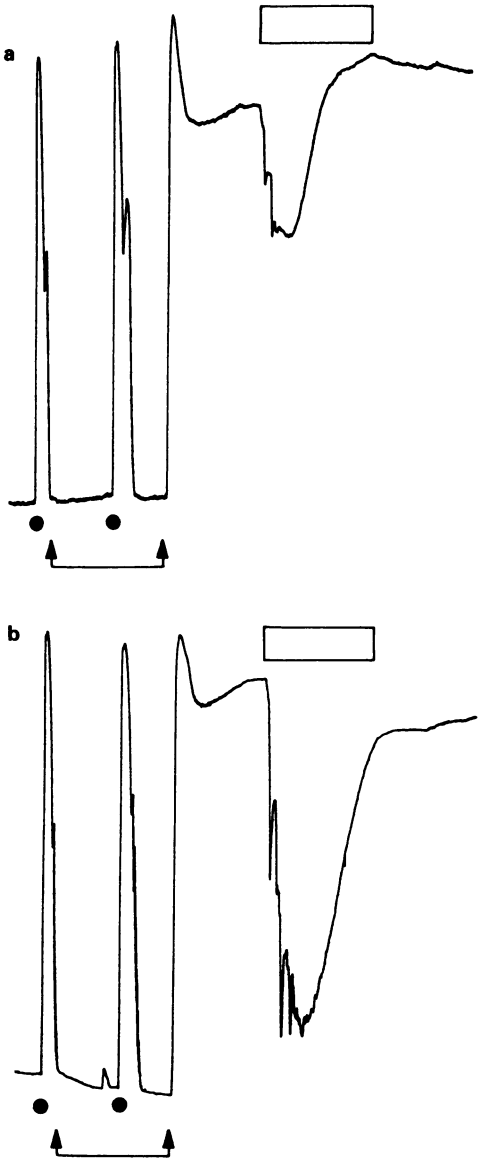

Figure 6 Photodynamic relaxation of smooth muscle in (a) the absence, and (b) the presence of L-cysteine, 10 mM. Contractions to carbachol, 5×10^{-5} M for 10 s (●) and exposure to $AlPcS_4$, 10^{-5} M, for 30 min (between the arrows). Other details as for Figure 3. In (b) the smooth muscle cells were exposed to L-cysteine, 10 mM, in addition to carbachol, 5×10^{-5} M, from the withdrawal of $AlPcS_4$ until the end of the experiment.

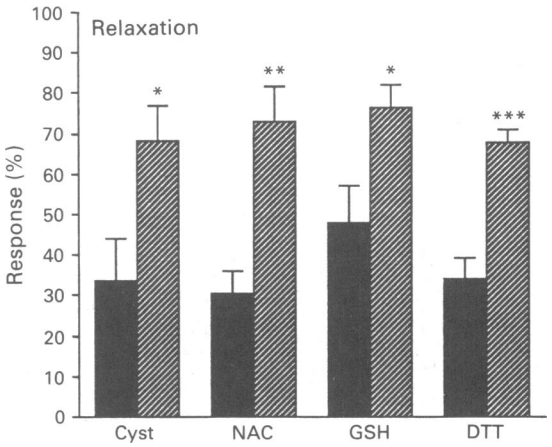

Figure 7 Effects of thiols on the photodynamic relaxation of $AlPcS_4$-sensitized taenia coli. Experimental protocol as in Figures 5 and 6: Cyst, L-cysteine, 10 mM; NAC, N-acetyl-L-cysteine, 10 mM; GSH, glutathione, 10 mM; DTT, dithiothreitol, 5 mM. Responses in the absence (solid columns) and presence (hatched columns) of thiol. $*P<0.05$, $**P<0.001$, $***P<0.001$; $n=4$ or 5.

tion, (ii) a subsequent relaxation, followed by (iii) a secondary sustained contraction (Figure 1a). The third phase of the response, i.e. the sustained contraction, was found to be blocked selectively by DL-dithiothreitol (5 mM) (Figure 1b), by L-cysteine (10 mM), and by other thiols, leaving the initial contractile response unaffected (Figures 1 and 2). Thus in a series of experiments neither L-cysteine (10 mM), N-acetyl-L-cysteine (10 mM), GSH (10 mM), nor dithiothreitol (5 mM) affected the initial contraction induced by photo-activation of $AlPcS_4$ (Figure 3a). In contrast, when compared to the standard carbachol contraction (5×10^{-5} M), there was a major inhibition of the sustained light-induced contraction by L-cysteine and by DTT, and a smaller but significant inhibition with N-acetyl-L-cysteine. (Figure 3b). Interestingly, GSH (10 mM) was without effect (Figure 3b). None of the thiols had any effect on the standard contraction to carbachol (5×10^{-5} M); see Figures 1 and 2.

In a previous investigation (Matthews & Mesler, 1984b) it was suggested that one action of photodynamic drugs may be to increase membrane permeability to calcium and other ions. It is evident from the present study that the photon-induced contraction of the taenia sensitized with $AlPcS_4$ is calcium-dependent since it was abolished when extracellular calcium was removed (Figure 4a). However, as soon as extracellular calcium was re-introduced the muscle contracted, indicating that a photodynamic effect, possibly on membrane permeability, had been produced but could not be expressed until extracellular calcium was restored. It is also of interest to note that the contraction seen on the re-introduction of calcium was sustained and monophasic. Furthermore this calcium-dependent contraction was markedly inhibited by the presence of L-cysteine (Figure 4b). In some experiments (Figures 2b and 4b) a small transient contraction of variable magnitude was seen on the addition of cysteine and this may be attributable to a brief opening of calcium release channels in the sarcoplasmic reticulum.

$AlPcS_4$-sensitized photodynamic relaxation of taenia caeci: effect of thiol reductants

An additional photodynamic effect was seen in response to light if, after exposure to the phthalocyanine compound, the smooth muscle of the taenia was maintained in a contracted state by continuous exposure to carbachol (5×10^{-5} M). When under these conditions, the muscle was illuminated (32,000 lux, $\lambda > 570$ nm), a triphasic relaxation response was

seen, i.e. (i) a rapid relaxation, followed by (ii) a transient contraction, preceding (iii) a secondary more sustained relaxation. The time course of this effect is clearly demonstrated in Figure 5. In comparative experiments the triphasic relaxation response was enhanced in the presence of L-cysteine (Figure 6) and by other thiols. L-Cysteine, N-acetyl-L-cysteine, GSH, and DTT, all significantly increased the magnitude of the photodynamic relaxation relative to the sustained carbachol response, approximately doubling it, although GSH was again the least effective agent (Figure 7). The duration of the photodynamic relaxation was also extended considerably in the presence of L-cysteine, N-acetyl-L-cysteine, and DTT, i.e. from 20 ± 4 min, 15 ± 1 min, and 16 ± 2 min, respectively, to $>>30$ min; $n = 3$ to 5.

Effects of 8-PCPT-cGMP on smooth muscle contraction

Underlying the relaxation response in many smooth muscle cells, including the taenia coli (Katsuki *et al.*, 1977) is the activation of guanylate cyclase and the formation of cyclic GMP. It is also known that guanylate cyclase can be activated by oxidation (Waldman & Murad, 1987). The potent membrane-permeant cyclic GMP analogue, 8-PCPT-cGMP which has recently become available was therefore used to mimic the effect of cyclic GMP generation in muscle pre-contracted with carbachol. There was a reversible and dose-dependent response to the cyclic GMP analogue. Relatively little action was seen with 20 μM but a 10 fold increase to 200 μM for 30 min not only induced a marked relaxation (Figure 8) but one reproducing almost exactly that evoked by photodynamic drug action (see Figures 5 and 6). Furthermore, the effect of the cyclic GMP analogue was still seen whether the muscle was pre-contracted by carbachol acting via a receptor-operated pathway or more directly by depolarization with a five fold increase in $[K^+]_o$ from 4.7 mM to 23.5 mM (Figure 9).

The ability of 8-PCPT-cGMP (200 μM) to affect the photodynamic contractile response was also investigated. Illumination (32,000 lux, $\lambda > 570$ nm), in the presence of the cyclic GMP analogue produced no change in the magnitude of the initial contractile response of $AlPcS_4$-sensitized muscle but both the rate of rise and final plateau level of the sustained contraction was markedly reduced when compared to the preparation not treated with 8-PCPT-cGMP (Figure 10).

Discussion

The characteristic features of photodynamic drug action on smooth muscle have been defined here in an attempt to identify the molecular mechanisms by which these drugs exert their effects at the cellular and subcellular level. The major factor underlying photodynamic drug action is likely to be the generation of singlet oxygen which is highly reactive chemically but has an extremely short half-life of microseconds. The results establish that on an $AlPcS_4$-sensitized photodynamic contraction in the taenia caeci the thiol reductants L-cysteine, N-acetyl-L-cysteine, DTT and GSH have a similar action with GSH being the least potent agent. Furthermore there is a common potentiating effect of the thiols on the photodynamic relaxation response in the precon-

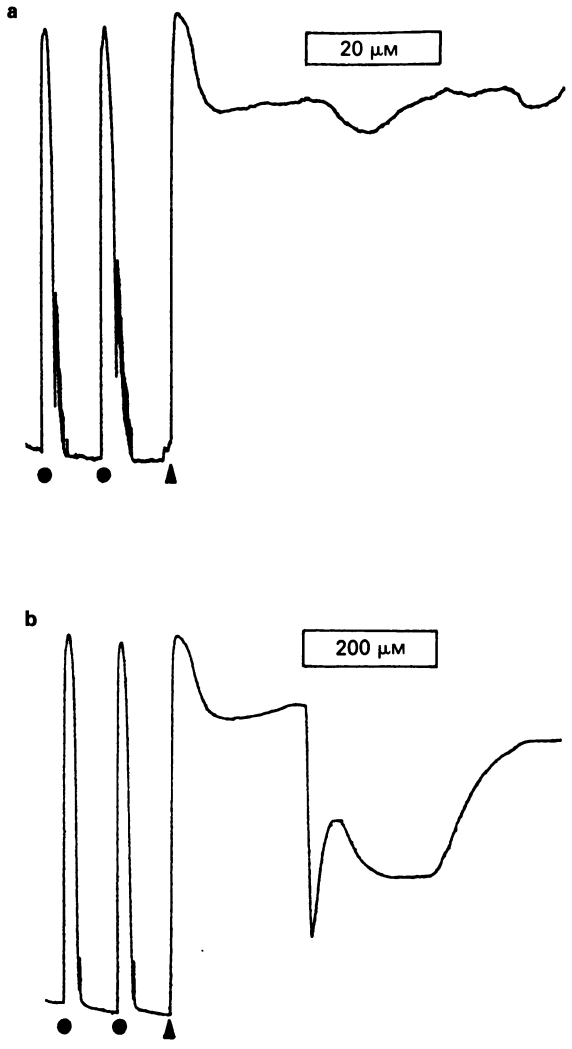

Figure 8 Effects of 8-PCPT-cGMP on smooth muscle precontracted with carbachol. Contractions were evoked with carbachol, 5×10^{-5} M, for 10 s (●) or by continuous exposure (▲). 8-PCPT-cGMP was present for 30 min (open rectangle) at the concentrations shown.

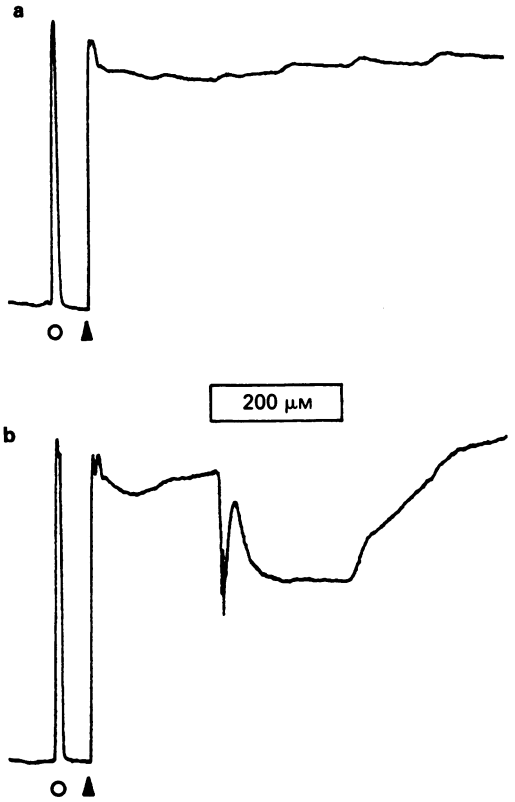

Figure 9 Effects of 8-PCPT-cGMP on smooth muscle contracted with potassium chloride. Contractions were evoked in response to KCl, 23.5 nM for 30 s (○) or continuously (▲). 8-PCPT-cGMP was present for 30 min (open rectangle).

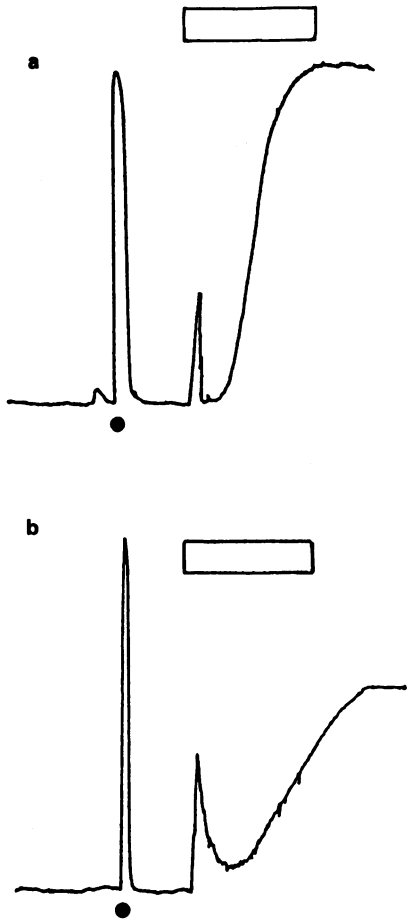

Figure 10 Photodynamic contractile responses (a) in the absence, and (b) in the presence of 8-PCPT-cGMP, 200 μM. Contractions to carbachol, 5×10^{-5} M, for 10 s (●). In both (a) and (b) the smooth muscle cells had been exposed previously to $AlPcS_4$, 10^{-5} M, for 30 min followed by washout period of 30 min before illumination at 32,000 lux for 30 min (open rectangle). In (b) the muscle was also exposed to 8-PCPT-cGMP immediately following the carbachol response until the end of the experiment.

tracted photosensitized muscle. In addition cyclic GMP analogues cause a relaxation of the smooth muscle cells which mimics the photodynamic relaxation response.

In the absence of thiols, light activation of $AlPcS_4$-sensitized muscle produced a typical triphasic response, an initial transient contraction and subsequent relaxation followed by a sustained contraction. Both contractile responses were blocked by the removal of extracellular calcium but the thiol reductants blocked only the sustained secondary contraction. The initial transient contraction was seen within a few seconds of illumination and, as in other cells (Matthews & Ciu, 1990a; Al-Laith *et al.*, 1993a,b), may be due to a rapid, direct stimulatory effect of photon-activated $AlPcS_4$ or singlet oxygen upon the membrane transduction-intracellular signalling system involving the G-protein-IP_3 dependent release of calcium from intracellular stores and subsequent contraction. The intracellular calcium stores are labile and dependent upon extracellular calcium, becoming depleted after the smooth muscle cells have been in a calcium-free EGTA solution for 5 or 6 min (Brading & Sneddon, 1980).

The inhibition of the secondary contractile photodynamic response can be attributed to two effects exerted by the thiols i.e. (i) a potentiation of the relaxation observed after the initial contraction, since all four thiols were able to markedly increase the photorelaxation effect in the precontracted taenia, or (ii) inhibition of the singlet oxygen-dependent membrane permeabilization and subsequent massive influx of extracellular calcium thought to be responsible for a sustained photodynamic contraction (Matthews & Mesler, 1984a,b). The differing abilities of the reductants to potentiate the magnitude and duration of the relaxation or to inhibit singlet oxygen generation or action sufficiently to diminsh the secondary sustained contraction are probably due to differences in membrane uptake or reductant potency. L-Cysteine is a natural amino acid, neutral at physiological pH, and shown to be actively transported into cells (Bannai, 1984). Although N-acetyl-L-cysteine may have some reducing activity itself (Tang *et al.*, 1991) it possesses a bulky acetyl substituent which may prevent or hinder its uptake on the L-cysteine transporter; alternatively, the smooth muscle cells may lack the deacetylation enzyme required to convert the molecule to L-cysteine and so account for its lesser action, DTT has a low redox potential and is a potent thiol reductant. It was

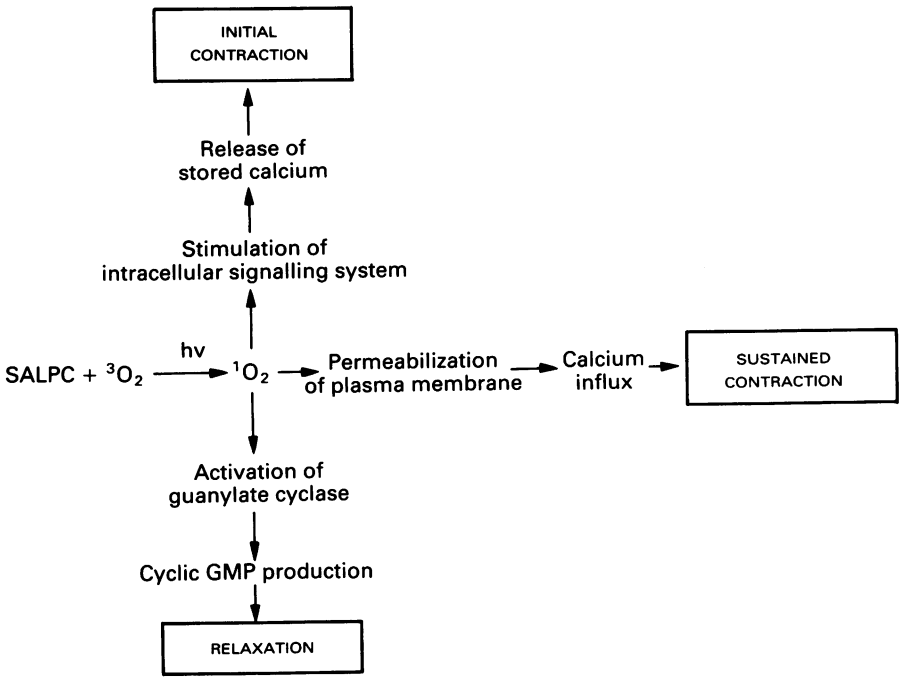

Figure 11 Diagram summarizing the photodynamic effects of $AlPcS_4$ on the smooth muscle cells of the taenia caeci.

therefore, not surprisingly, the most effective of the thiols tested. In contrast, the relative lack of action of GSH may be due to the fact that little exogenous GSH crosses cell membranes as the intact tripeptide (Butterworth *et al.*, 1992) or it is possible that the reduced glutathione is oxidized by oxygenation of superfusion solutions so decreasing its activity. Oxidation may also decrease the activity of L-cysteine (Bannai, 1984), but not DTT, and hence account for the greater effect of the DTT. All thiol reductants can diminish local singlet oxygen concentrations in proportion to their free radical scavenging capacity; they may also delay the process of membrane permeabilization by recycling some of the membrane lipid oxidized by singlet oxygen (Buettner & Hall, 1987). Together these various mechanisms are clearly effective in minimizing the photodynamic process of membrane permeabilization because the restoration of calcium to a previously calcium-free but cysteine-treated muscle produced a subsequent contraction of much slower onset and smaller magnitude than that in an L-cysteine-free preparation (see Figure 4).

To investigate the photodynamic relaxation response, $AlPcS_4$-sensitized muscle was pre-contracted either with carbachol or KCl; in both experimental conditions a photodynamic relaxation was seen which was enhanced by L-cysteine. This makes it unlikely that the relaxation is due simply to permeabilization of nerve endings in the muscle and the release of inhibitory transmitters because the high K^+ concentration will stimulate and persistently depolarize any neurones in the muscle, as well as the muscle itself. A major nerve-mediated component of the relaxation response can therefore be largely discounted. It now seems more probable that an effect of singlet oxygen on guanylate cyclase increases cyclic GMP production. Oxidizing agents have been shown to activate guanylate cyclase directly and dithiothreitol and other reducing agents potentiate activation of the enzyme (Waldman & Murad, 1987). Thiol-disulphide oxidation and reduction thus forms an important mechanism for regulating guanylate cyclase and modulating singlet oxygen action. This conclusion is substantiated by the responses of the precontracted muscle to the membrane permeant cyclic GMP analogue, 8-PCPT-cGMP which closely mimicked those evoked by photodynamic activation of $AlPcS_4$. Specific protein kinases activated by cyclic GMP may not only cause sequentially, phosphorylation and inactivation of myosin light chain kinase, dephosphorylation of the myosin light chain, and smooth muscle relaxation (Ignarro, 1991) but also decrease $[Ca^{2+}]_i$ by (i) reducing Ca^{2+} influx, (ii) increasing Ca^{2+} efflux, and (iii) promoting subcellular sequestration of Ca^{2+} (Francis *et al.*, 1988). All these effects will contribute to the photodynamic relaxation response and can be enhanced in the presence of thiol reductants. Such effects would be expected also to diminish or delay the onset of the sustained photodynamic contraction as does the cyclic GMP analogue itself.

In conclusion, photo-activation of cell-bound $AlPcS_4$ raises it to the higher energy triplet state which in turn generates highly-reactive but short-lived singlet oxygen (Figure 11). This, in the smooth muscle cell of the taenia caeci produces an initial contraction not directly affected by thiol reagents. The initial contraction is followed by a relaxation due to oxidative activation of guanylate cyclase and the production of cyclic GMP and is the major effect seen in precontracted muscle; it is enhanced by thiol reagents. Finally, permeabilization of the cell membrane and calcium influx leads to a sustained contraction and this may be diminished by thiol reductants either by directly opposing the photo-oxidation of membrane proteolipid structure, or by enhancing the generation of cylic GMP, and some of these effects can be mimicked by cyclic GMP analogues. More experiments are therefore now clearly required to assess fully the photodynamic action of $AlPcS_4$ on purified guanylate cyclase *in vitro*.

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Characterization of the adenosine receptor mediating contraction in rat colonic muscularis mucosae

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1 The objective of this study was to characterize the adenosine receptor mediating contraction in rat isolated colonic muscularis mucosae (RCMM).

2 Sequential additions of the adenosine receptor agonist 5'-*N*-ethylcarboxamidoadenosine (NECA; 0.01–10 μM) elicited reproducible, concentration-related contractions in RCMM. The effects of NECA were mimicked by the adenosine A_1 receptor-selective agonists cyclopentyladenosine (CPA), **R**-phenylisopropyladenosine (**R**-PIA) and N-[1S, *trans*)2-hydroxycyclopentyl] adenosine (GR79236) and by S-PIA (the stereoisomer of **R**-PIA). The adenosine A_2 agonists N-[(2-methylphenyl)methyl] adenosine (metrifudil) and 2-[*p*-(2-carboxyethyl)phenethylamine]-5'-*N*-ethylcarboxamidoadenosine (CGS21680) also produced contractions in RCMM but were 54 and 165 times less potent respectively than NECA. The rank order of agonist potency for contraction of RCMM was CPA ≥ GR79236 = **R**-PIA ≥ NECA> >S-PIA = metrifudil > CGS21680, which is identical to that reported for the inhibition of spontaneous rate in rat isolated right atria and inhibition of lipolysis in rat isolated adipocytes by these same agonists.

3 **R**-PIA, S-PIA and metrifudil behaved as partial agonists in RCMM.

4 The adenosine A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) inhibited the contractions produced by all the adenosine agonists tested, with pK_B values between 9.2 and 9.5. The non-selective adenosine antagonist 8-phenyltheophylline (8-PT) antagonized the effects of NECA but also markedly potentiated (by 93.0 ± 10.2% at 3 μM) the maximum contractile response to NECA in RCMM. Neither 8-PT (3 μM) nor DPCPX (0.1 μM) had any effect on the contractions produced by carbachol.

5 The contractile responses to NECA in RCMM were not affected by atropine (1 μM), tetrodotoxin (0.3 μM) or the P_2 antagonist, suramin (100 μM).

6 The present study confirms that contractions to adenosine agonists in the RCMM are mediated via adenosine A_1 receptors.

Keywords: Adenosine A_1 receptors; rat colonic muscularis mucosae; contraction

Introduction

Based on whether adenosine inhibits or stimulates the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in brain cells, adenosine receptors have been divided into two types, namely A_1 and A_2 respectively (Van Calker *et al.*, 1979). Although it is now known that adenosine receptors can be coupled to second messengers other than cyclic AMP, this receptor classification is still used. More recently, it has been suggested that the A_2 receptor can be further divided into A_{2a} and A_{2b} (Bruns *et al.*, 1986), whilst a putative adenosine A_3 receptor has been cloned recently (Zhou *et al.*, 1992).

In general, stimulation of adenosine A_1 receptors is associated with an inhibition of functional responses. For example, stimulation of adenosine A_1 receptors produces an inhibition of electrically-induced twitch responses in guinea-pig ileum (Paton, 1981), an inhibition of lipolysis in rat adipocyte (Londos *et al.*, 1980) and inhibition of spontaneous rate in rat atria (Kurahashi & Paton, 1986). In contrast, it has been reported recently that adenosine elicited contractions in the rat isolated colonic muscularis mucosae (RCMM; Bailey & Hourani, 1990; Bailey *et al.*, 1992) which were mimicked by the adenosine A_1 receptor-selective agonist cyclopentyladenosine (CPA; Londos *et al.*, 1980) and inhibited by very low concentrations (nM) of the A_1 receptor-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Bruns *et al.*, 1987; Haleen *et al.*, 1987). These data are in keeping with the proposal that contraction in RCMM is mediated via adenosine A_1 receptors (Bailey *et al.*, 1992).

The purpose of the present study was firstly, to extend the findings of Bailey *et al.* (1992) in RCMM using a wider range of adenosine receptor agonists and secondly, to characterize further the receptor mediating the contractile effect by use of purine receptor blocking drugs. The data are compared with those obtained for the same agonists in two well characterized adenosine A_1 receptor preparations, namely inhibition of noradrenaline-induced lipolysis in rat isolated adipocytes and the inhibition of spontaneous rate in rat isolated right atrium (Londos *et al.*, 1980; Kurahashi & Paton, 1986; Gurden *et al.*, 1993).

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Methods

Rat colonic muscularis mucosae (RCMM)

Female Wistar rats (100–200 g) were killed by cervical dislocation, the abdomen was opened and a 2 cm section of distal colon removed. A glass rod (diameter 5 mm) was passed inside the lumen of the colonic section and the outer longitudinal and circular muscle layers were carefully cut through with a scalpel blade and then removed by gentle rubbing with moist cotton wool. The remaining tube of colonic muscularis mucosae (RCMM) was then suspended along its longitudinal axis in an organ bath containing a modified Krebs-Henseleit solution at 32°C gassed with 95% O_2/5% CO_2. The tissues were suspended under an initial tension of 1 g and left to equilibrate for at least 1 h. The ionic composition of the Krebs-Henseleit solution (in mM) was: NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 1.3 and glucose 11.1.

Effect of agonists and antagonists

The RCMM were contracted by the addition of a single low concentration of 5'-*N*-ethylcarboxamidoadenosine (NECA, 0.01 μM). When the peak contractile response was obtained, the tissues were washed with fresh Krebs-Henseleit solution and allowed to recover before adding a higher concentration of NECA. This process was repeated to obtain full sequential concentration-effect curves to NECA.

In agonist studies, two sequential concentration-effect curves to NECA were constructed followed by a further curve to either NECA (control tissues), or a test agonist. In antagonist studies, two control concentration-effect curves to an agonist were constructed followed by a curve in the presence of the antagonist (30 min preincubation).

In some experiments, the effects of NECA or CGS21680 on RCMM pre-contracted with a submaximal concentration of carbachol (1 μM) in the absence or presence of DPCPX (0.1 μM) were determined.

The specificity of the adenosine antagonists 8-PT and DPCPX was determined against carbachol-induced contractions. Control cumulative concentration-effect curves to carbachol were constructed, followed by test curves in the presence of the antagonist (30 min preincubation).

Analysis of results

The contractile responses in RCMM are expressed as a percentage (arithmetic mean ± s.e.mean) of the maximum response obtained in the second (control) concentration-effect curve. Equi-effective molar concentration-ratios (EMCR) and EC_{50} values for agonists were calculated graphically at the 50% response level of the control curve and expressed as geometric means (with 95% confidence limits) where *n* is the number of experiments. Rightward shifts in agonist curves (concentration-ratios (CR)) by antagonists were also measured from the 50% response level and are expressed as geometric means (with 95% confidence limits). In both agonist and antagonist studies the EC_{50} value from the 'test' curve was compared directly to the EC_{50} value for the second control curve.

The negative logarithm of the apparent dissociation constant for an antagonist (pK_B) was estimated by calculation of the arithmetic mean (with 95% confidence limits) of the individual results for each concentration of antagonist from the equation: [pK_B = log (concentration-ratio − 1) − log (antagonist concentration)].

Drugs

Commercially obtained drugs were: atropine sulphate (Sigma), carbachol chloride (BDH) and tetrodotoxin (Calbiochem) which were dissolved in distilled water; N^6-(**R**-phenylisopropyladenosine) (**R**-PIA) and N^6-(S-phenylisopropyladenosine) (**S**-PIA) were obtained from Boehringer Mannheim and were dissolved in 0.1 N HCl; and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Research Biochemicals Inc.) was dissolved in dimethylsulphoxide. 2-[*p*-(2-Carboxyethyl) phenethyl amine]-5'-N-ethylcarboxamidoadenosine (CGS21680) and 8-phenyltheophylline (8-PT) were from Research Biochemicals Inc. and were dissolved in 0.1 N NaOH.

Compounds synthesized by Glaxo Group Research Ltd were: N^6-cyclopentyladenosine (CPA), N-[1S,*trans*)-2-hydroxycyclopentyl] adenosine (GR79236), 5'-*N*-ethylcarboxamidoadenosine (NECA) and N-[(2-methylphenyl)methyl] adenosine (metrifudil) which were dissolved in 0.1 N HCl. Suramin was kindly supplied by Bayer UK Ltd and was dissolved in distilled water.

All compounds were initially prepared as stock concentrations of 10 mM.

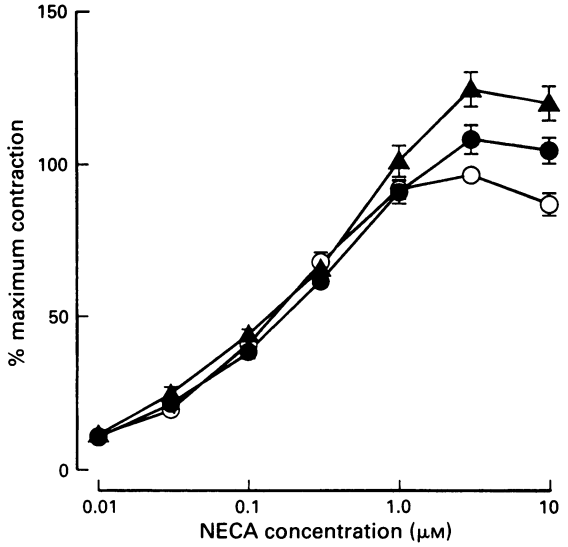

Figure 1 The effect of repeated 5'-*N*-ethylcarboxamidoadenosine (NECA)-induced contractile concentration-effect curves in rat isolated colonic muscularis mucosae: (○) = curve 1, (●) = curve 2 and (▲) = curve 3. Each point is the mean (± s.e.mean) of 12 observations calculated as a percentage of the maximum contraction to NECA obtained in curve 1.

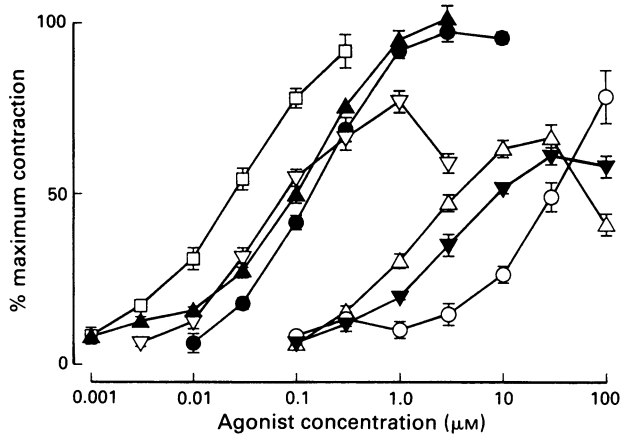

Figure 2 The contractile effects of a range of adenosine agonists in rat isolated colonic muscularis mucosae: (●) NECA ($n = 17$); (□) CPA ($n = 9$); (▽) **R**-PIA ($n = 11$); (▲) GR79236 ($n = 4$); (△) **S**-PIA ($n = 8$); (▼) metrifudil ($n = 3$) and (○) CGS21680 ($n = 4$). Each point represents the mean (± s.e.mean) calculated as a percentage of the maximum contraction obtained in the second curve to NECA. For abbreviations, see text.

Results

Effect of adenosine receptor agonists

NECA (0.01–10 μM) produced concentration-dependent contractions in RCMM (Figure 1). The response to each concentration of NECA produced its maximum effect within 3 min. Following the construction of an initial concentration-effect curve to NECA, the second and third curves to the agonist were very reproducible with mean EC_{50} values of 0.18 (0.14–0.23) and 0.14 (0.11–0.16) μM respectively. The maximum contractions (at 3 μM) for the second and third curve were 0.35 ± 0.04 g and 0.37 ± 0.05 g respectively.

The contractions produced by NECA in the RCMM were mimicked by CPA, GR79236, **R**- and **S**-PIA, metrifudil and CGS21680 (Figure 2). CPA (0.001–0.3 μM) and GR79236 (0.001–3.0 μM) were potent and full agonists when compared to NECA in the RCMM. However, **R**-PIA (0.003–3 μM),

S-PIA (0.1–100 μM) and metrifudil (0.1–100 μM) behaved as partial agonists with maximum responses of only 76.7 ± 3.0, 66.1 ± 3.8 and 61.0 ± 2.5% respectively of that produced by NECA. Since CGS21680 (0.1–100 μM) was so weak, it was not clear if the true maximum response to this agonist was achieved but, at 100 μM, 78.0 ± 7.9% of the maximum response to NECA was obtained. The EMCR values (where NECA = 1) for these agonists at producing contraction in RCMM are shown in Table 1. The rank order of agonist potency in RCMM was: CPA ≥ GR79236 = R-PIA ≥ NECA >> S-PIA = metrifudil > CGS21680.

A submaximal concentration of carbachol (1 μM) produced a maintained contraction of 1.06 ± 0.24 g ($n = 6$) in RCMM. Addition of NECA (10 μM) or CGS21680 (100 μM) produced a further contraction of 0.47 ± 0.131 ($n = 3$) and 0.19 ± 0.04 g ($n = 3$) respectively. DPCPX (0.1 μM) completely abolished the further contractions to NECA or CGS21680 in carbachol contracted RCMM. Under these conditions, no relaxation to these agonists was observed.

Table 1 Relative potencies of adenosine receptor agonists in rat colonic muscularis mucosae

Agonist	*EMCR*	n
NECA	1.0 [EC_{50} = 162 (141–186) nM]	17
CPA	0.19 (0.13–0.27)	9
R-PIA	0.49 (0.34–0.71)	9
GR79236	0.57 (0.40–0.82)	9
S-PIA	40 (33–48)	8
Metrifudil	54 (46–65)	3
CGS21680	165 (102–266)	4

Equi-effective molar concentration ratios (EMCR) together with the EC_{50} value of NECA, are expressed as geometric mean values, with 95% confidence limits in parentheses and where n = number of preparations. For abbreviations, see text.

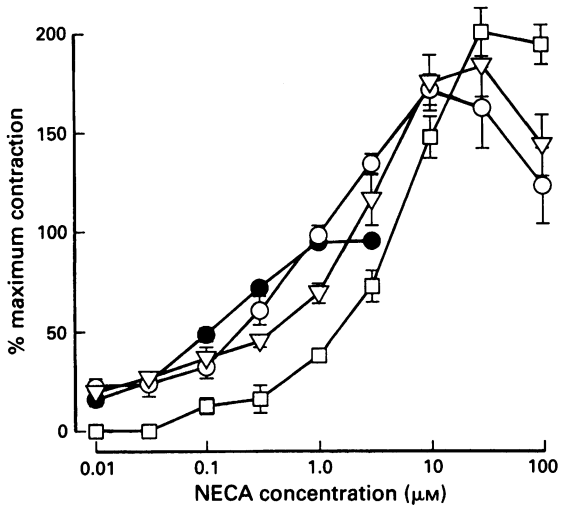

Figure 3 The effect of 8-phenyltheophylline (8-PT) on concentration-effect curves to 5'-*N*-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (●) control NECA, $n = 15$; (○) NECA + 8-PT (0.3 μM, $n = 6$); (▽) NECA + 8-PT (1 μM, $n = 4$); (□) NECA + 8-PT (3 μM, $n = 5$). Each point represents the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

Effect of antagonists

The non-selective adenosine A_1/A_2 receptor antagonist, 8-PT, produced concentration-dependent and rightward shifts of the NECA concentration-effect curves (Figure 3), mean CR values for 8-PT of 2.6 (1.7–3.8) and 11.3 (5.3–24.2) being obtained at 1 ($n = 4$) and 3 μM ($n = 6$) respectively. Interestingly, treatment with 8-PT also produced marked and significant increases in the NECA maximum, an increase of 93.0 ± 10.2% ($P < 0.001$) being observed in the presence of 8-PT at 3 μM.

The adenosine A_1 receptor-selective antagonist, DPCPX (1–10 nM), also produced concentration-dependent and rightward shifts of NECA concentration-effect curves in RCMM (Figure 4), although at 3 and 10 nM significant ($P < 0.05$) reductions in the maximum contractile responses were observed. Mean apparent pK_B values for DPCPX versus NECA of between 9.16 and 9.25 (Table 2) were estimated from these data. DPCPX (1 nM) also antagonized contrac-

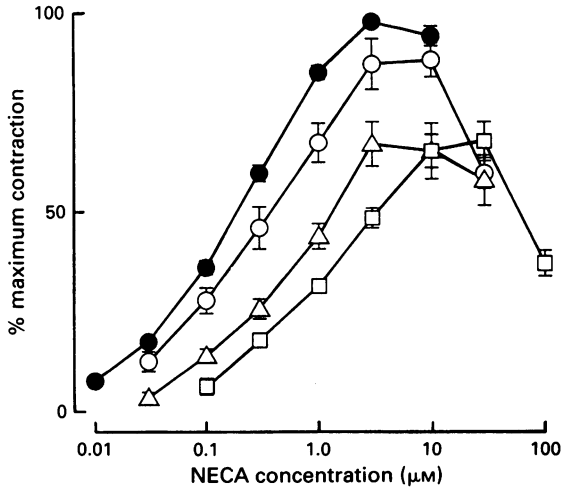

Figure 4 The effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on concentration-effect curves to 5'-*N*-ethylcarboxamidoadenosine (NECA) in rat isolated colonic muscularis mucosae: (●) control NECA, $n = 16$; (○) NECA + DPCPX (1 nM, $n = 5$); (△) NECA + DPCPX (3 nM, $n = 5$); (□) NECA + DPCPX (10 nM, $n = 6$). Each point is the mean (± s.e.mean) calculated as a percentage of the control NECA maximum.

Table 2 Antagonist effect of 8-cyclopentyl-1,3- dipropylxanthine (DPCPX) in rat colonic muscularis mucosae (RCMM)

Agonist	*[DPCPX]* nM (*n*)	*CR*	*pK_B*
NECA	1 (5)	2.67 (1.90–5.20)	9.16 (8.66–9.67)
	3 (5)	5.44 (3.81–7.75)	9.17 (8.98–9.36)
	10 (6)	18.77 (11.64–30.28)	9.25 (9.03–9.47)
CPA	1 (4)	4.45 (2.47–8.00)	9.53 (9.21–9.85)
R-PIA	1 (4)	3.15 (1.16–8.61)	9.28 (8.60–9.96)
GR79236	1 (4)	2.86 (1.99–4.10)	9.26 (9.00–9.52)
S-PIA	1 (4)	3.99 (1.93–8.23)	9.46 (9.02–9.89)
Metrifudil	1 (6)	3.68 (2.03–6.71)	9.40 (9.06–9.74)
CGS21680	1 (6)	3.64 (1.62–8.17)	9.54 (9.26–9.81)

Effective concentration ratios (CR) expressed as geometric means and apparent pK_B values for DPCPX against adenosine receptor agonists in RCMM expressed as arithmetic mean values, with 95% confidence limits in parentheses and where n = number of preparations. For abbreviations, see text.

Table 3 A comparison between rat colonic muscularis mucosae (RCMM), rat atria and rat adipocytes

Agonist	*EMCR* (NECA = 1) †*RCMM*	**Rat atria*	**Rat adipocytes*
NECA	1.0 [EC_{50} = 162(141–186) nM]	1.0 [EC_{50} = 93(72–119) nM]	1.0 [EC_{50} = 20(17–24) nM]
CPA	0.19	0.21	0.21
R-PIA	0.49	0.70	0.54
GR79236	0.57	0.77	0.34
S-PIA	40	25	32
Metrifudil	54	59	56
CGS21680	165	382	253

†This study.
*Reproduced from Gurden *et al.* (1993).
Equi-effective molar concentration ratios (EMCR) calculated from EC_{50} values relative to NECA. A comparison between the relative potencies of agonist-induced contraction in RCMM, inhibition of rate in rat right atria and inhibition of lipolysis in rat adipocytes. For abbreviations, see text.

tions induced by all the other purine agonists tested in the RCMM with pK_B values ranging between 9.26 and 9.54 (Table 2).

Carbachol (0.01–30 μM) produced concentration-dependent and reproducible contractions in RCMM with mean EC_{50} values of 1.99 (1.10–3.45) and 2.60 (1.78–3.85) μM for consecutive curves ($n = 4$). Neither DPCPX (0.1 μM) nor 8-PT (3 μM) produced any significant effect on the responses to carbachol with mean CR values ($n = 4$) of 0.97 (0.74–1.28) and 1.74 (1.19–2.54) respectively compared to the control curve and no change in the carbachol maximum response (98.7 ± 3.0 and 108.3 ± 2.0% of the control curve).

The contractions of the RCMM induced by NECA (0.01–10 μM) were not inhibited by atropine (1 μM), tetrodotoxin (0.3 μM) or suramin (100 μM), which produced mean CR values of 1.16 (0.79–1.70), 0.83 (0.49–1.40) and 1.07 (0.82–1.40) respectively with no change in the maximum response to NECA.

Discussion

The present study has shown that adenosine receptor agonists produce concentration-dependent contractions in the rat isolated colonic muscularis mucosae (RCMM). The contractile responses appeared to result from a direct effect of the agonists on the smooth muscle cells and did not involve cholinergic or neuronal stimulation, since the effects of NECA were not affected by pretreatment with atropine or tetrodotoxin. It has been reported that the RCMM contains both P_1 (adenosine receptors) and P_2 receptors (Bailey & Hourani, 1990). The latter study together with the present work suggests that the contractile effects of adenosine agonists are not due to P_2 receptor stimulation, since the P_2 receptor-selective antagonist suramin (Hoyle *et al.*, 1990) produced no inhibitory effect. Based on the potent antagonism of responses to CPA, NECA and adenosine by DPCPX, it was proposed that the receptor mediating contraction in RCMM was of the adenosine A_1 receptor subtype (Bailey *et al.*, 1992). The present study has further characterized the pharmacological properties of this receptor.

The contractions to NECA in RCMM were reproducible, although the maximum response did increase slightly during consecutive curves. The contractile responses to NECA were mimicked by all of the adenosine analogues tested, with CPA, R-PIA and GR79236, three agonists with selectivity for adenosine A_1 receptors (Londos *et al.*, 1980; Gurden *et al.*, 1993) being more potent than NECA. S-PIA, the stereoisomer of R-PIA, and metrifudil were less potent than NECA while the A_{2a} agonist, CGS21680 (Hutchison *et al.*, 1989) was the least potent compound tested. The EC_{50} value for NECA in producing contraction in RCMM was similar to that reported to produce an inhibition of spontaneous rate in rat isolated right atria but was approximately 8 fold greater than that for the inhibition of lipolysis in rat isolated adipocytes (Table 3). However, the relative potencies of the agonists (Table 3) were remarkably similar and the rank order of agonist potency (CPA ≥ GR79236 = R-PIA ≥ NECA>>S-PIA = metrifudil > CGS21680) was identical in each of these isolated preparations. Interestingly, R-PIA, S-PIA and metrifudil were partial agonists relative to NECA in RCMM, a profile which has not been reported for these agonists in other tissues containing adenosine A_1 receptors. These data may simply reflect a poorer receptor coupling in RCMM, or possibly indicates that there may be a subtle difference between the receptors in RCMM and those in rat atria and rat adipocytes. However, the results with the agonists are generally consistent with contraction in RCMM being mediated via adenosine A_1 receptors.

That the contraction in RCMM is mediated via adenosine A_1 receptors was further substantiated by the effect of DPCPX, an A_1-selective antagonist (Bruns *et al.*, 1987; Haleen *et al.*, 1987). DPCPX produced concentration-dependent, rightward displacements of the NECA concentration-effect curves and mean pK_B values of approximately 9.2 were calculated. In this study, DPCPX at 3 and 10 nM produced a significant reduction in the NECA maximum and thus did not appear to behave as a truly competitive antagonist in the RCMM. However, DPCPX was agonist-independent producing a similar antagonist potency (pK_B 9.2–9.5) irrespective of the adenosine agonist used. The affinity values for DPCPX in RCMM are consistent with those obtained in other preparations containing adenosine A_1 receptors (Bruns *et al.*, 1987; Haleen *et al.*, 1987; Collis *et al.*, 1989). In addition, this antagonism was specific for adenosine since contractions to carbachol were not affected by a high concentration of DPCPX (0.1 μM). These data indicate that the RCMM contains a population of adenosine A_1 receptors which mediate contraction. Furthermore, since NECA and CGS21680 had no effect in carbachol pre-contracted preparations pre-treated with DPCPX, it is unlikely that adenosine A_2 receptors mediating relaxation are present in RCMM. This is in contrast to the longitudinal muscle of the rat colon where relaxant adenosine A_2 receptors have been reported (Bailey & Hourani, 1992).

Treatment with the non-selective adenosine receptor antagonist, 8-PT (Collis *et al.*, 1985) yielded interesting results in RCMM. 8-PT produced concentration-dependent rightward shifts of the NECA curves with CR values consistent with adenosine-receptor antagonism (i.e. pK_B 6.4–6.6, Collis *et al.*, 1985). Interestingly, 8-PT treatment also produced very large potentiations in the maximum response to NECA, an effect not previously reported in other adenosine receptor containing preparations. In contrast to the results with NECA, 8-PT had no effect on contractions to carbachol in RCMM suggesting that potentiation was specific to adenosine receptor-mediated contraction. The mechanism by

which 8-PT enhanced the contraction to NECA in RCMM remains to be determined.

In conclusion, we have extended the work of Bailey and colleagues (1990, 1992) in order to characterize further the adenosine-mediated contraction in RCMM. The present study has demonstrated an identical rank order of agonist potency in RCMM, rat right atria and rat adipocytes. These findings, together with comparable pK_B estimates for the inhibition by DPCPX of all agonists tested, confirm that contraction in RCMM is mediated via adenosine A_1 receptors. Since certain agonists were apparently partial with respect to NECA in RCMM, these receptors may be less well-coupled than in the other adenosine A_1 receptor containing preparations. Furthermore, we have observed an unexpected, and as yet unexplained, potentiation of the contractile effects following treatment with the antagonist, 8-PT.

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Stimulation of lumbar sympathetic nerves evokes contractions of cat colon circular muscle mediated by ATP and noradrenaline

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1 The action of the lumbar sympathetic nerves to cat colon was studied *in vitro* using isolated muscle strips with attached lumbar colonic nerves (LCN) orientated in the axis of circular muscle layer. Electrical stimulation of LCN caused frequency-dependent increases in resting tension and in amplitude of spontaneous contractions. Contractile responses were abolished by tetrodotoxin (3 μM) and by guanethidine (30 μM), indicating that they were neurogenic, involving the release of neurotransmitter from sympathetic fibres.

2 Propranolol (1–9 μM), a β-adrenoceptor antagonist, caused a concentration-dependent potentiation of LCN-evoked contractile responses. Propranolol (3 μM) potentiated contractile responses to exogenously applied noradrenaline but not to phenylephrine.

3 Phentolamine (1–9 μM), an α-adrenoceptor antagonist, and prazosin (1–9 μM), an α_1-adrenoceptor antagonist, caused a concentration-dependent reduction of amplitude but did not abolish LCN-evoked contractile responses. Prazosin (3 μM) or phentolamine (3 μM) antagonized contractile responses to noradrenaline and phenylephrine.

4 Desensitization of purinoceptors with the P_{2x}-receptor agonist, α,β-methylene ATP, caused a decrease in amplitude of LCN-evoked contractile responses and abolished contractile responses to ATP. In muscle strips where α_1-adrenoceptors were blocked with prazosin (3 μM) and P_2-purinoceptors were desensitized with α,β-methylene ATP, the amplitude of contractile responses was reduced by 82–100%.

5 The P_{2X}-purinoceptor antagonists, arylazido amino propyl adenosine triphosphate ($ANAPP_3$) and suramin, affected LCN-evoked contractile responses. $ANAPP_3$ (50–100 μM) caused a concentration-dependent reduction in the amplitude of contractile response. Suramin (100 μM) caused a small reduction in amplitude of contractile responses but potentiated their amplitude at a concentration of 500 μM.

6 $ANAPP_3$ (100 μM) irreversibly inhibited contractions to α,β-methylene ATP or ATP. Suramin (100–500 μM) inhibited contractions to α,β-methylene ATP (0.5–1 μM) or low concentrations of ATP (10–50 μM) but potentiated contractions at higher concentrations. $ANAPP_3$ (100 μM) and suramin (100, 500 μM) had no affect on contractile responses to noradrenaline.

7 Clonidine (0.05–1 μM), a selective α_2-adrenoceptor agonist, caused a concentration-dependent reduction in amplitude of LCN-evoked contractile responses, at 10 Hz, while yohimbine (0.1–1 μM), a selective α_2-adrenoceptor antagonist, increased them. At 1 μM, both compounds affected LCN-evoked contractions at all frequencies. This suggests that prejunctional α_2-receptors are involved in autoinhibition at sympathetic terminals.

8 In summary, LCN-evoked contractile responses involve the corelease of noradrenaline and ATP or a related purine nucleotide from sympathetic fibres. It is likely that the neurogenic responses are mediated through excitatory postjunctional α_1-adrenoceptors, excitatory suramin-sensitive and suramin-insensitive P_{2X}-purinoceptors and inhibitory β-adrenoceptors. Also, autoinhibitory prejunctional α_2-adrenoceptors regulate the LCN excitatory pathway to cat colon circular muscle.

Keywords: α_1-adrenoceptors; P_{2X}-purinoceptors; lumbar sympathetic nerves; contractile responses; cat colon

Introduction

Lumbar prevertebral (inferior mesenteric ganglia) and paravertebral ganglia are the origin of sympathetic postganglionic fibres which travel to the colon in lumbar colonic nerves (LCN) (de Groat & Krier, 1979; Baron *et al.*, 1985). Their action on colonic smooth muscle of non-sphincteric regions has been defined in two ways. Acute transection of them enhances basal intraluminal pressures and the amplitude of spontaneous contractions, indicating a tonic inhibitory action (de Groat & Krier, 1979). Conversely, electrical stimulation of LCN decreases basal intraluminal pressure and amplitude of cholinergic (pelvic nerve-evoked) contractions (Gillespie & McKenna, 1961; de Groat & Krier, 1976; Gillespie & Khoyi, 1977). These inhibitory effects on colonic smooth muscle are mediated through prejunctional α- and postjunctional β-adrenoceptors (Ek & Lungren, 1982; Ek, 1985). Thus, lumbar sympathetic nerves regulate cholinergic contractions of non-sphincteric colon smooth muscle by inhibitory adrenoceptors.

There are postjunctional excitatory α-adrenoceptors, in addition to inhibitory adrenoceptors in non-sphincteric circular smooth muscle of cat (Anurus & Christensen, 1981) and dog colon (Zang *et al.*, 1992) and in circular and longitudinal muscle of rat (Gagnon & Belisle, 1970) and human (Gagnon *et al.*, 1972) colon. While it is known that these excitatory postjunctional receptors respond to α-adrenoceptor agonists with contractions, it is unknown whether they are activated through the release of neurotransmitter from lumbar sympathetic fibres, as has been shown for thoracic sympathetic fibres to guinea-pig stomach circular muscle (Nakayota *et al.*, 1970). Furthermore, the identity of the neurotransmitter(s) released from lumbar sympathetic fibres

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which mediate contractions of colon circular muscle is as yet not known. It could involve the corelease of adenosine 5'-triphosphate (ATP) or a related nucleotide and noradrenaline, as has been shown in vas deferens (Sneddon *et al.*, 1982; Meldrum & Burnstock, 1983; Sneddon & Westfall, 1984; Burnstock & Sneddon, 1985; von Kugelgen *et al.*, 1989) and in arteries and submucosal arterioles (von Kugelgen & Starke, 1985; 1991; Burnstock & Warland, 1987; Evans & Surprenant, 1992).

The aim of the present study was to characterize the mechanical responses of cat colon circular muscle *in vitro* during electrical stimulation of LCN. The involvement of α_1- and α_2-adrenoceptors and β-adrenoceptors in the nerve-mediated contractions was studied by the use of selective antagonists. The involvement of purinoceptors in the nerve-mediated contractions was studied by desensitization of P_2-purinoceptors with the P_{2X}-receptor agonist, α,β-methylene ATP (Burnstock & Kennedy, 1985; Kennedy, 1990) and by antagonists of the P_2-purinoceptor, suramin (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990) and arylazido amino propionyl adenosine triphosphate ($ANAPP_3$) (Hogaboom *et al.*, 1980; Fedan *et al.*, 1982). Also, the inhibitory effects of antagonists on nerve-mediated contractile responses were compared to their actions on contractile responses to agonists.

Methods

Male or female cats, weighing 2.5–4.5 kg were anaesthetized with sodium pentobarbitone (50–70 mg kg^{-1}, i.p.). After exsanguination, bilateral lumbar colonic nerves were excised with an attached segment (4–5 cm) of the mid colon 4 cm caudad to the ileocecal sphincter and placed in ice-cold Krebs solution. The colon was cut along the antimesenteric border and the mucosa removed. Muscle strips (about 10 mm long and 5 mm wide) with attached branches of the LCN were cut in the long axis of circular muscle fibres. Two matching circular smooth muscle strips without lumbar colonic nerves were excised from the same region. All muscle strips contained both muscle layers and the myenteric plexus. Isolated preparations were mounted vertically in the circular axis in 50 ml organ baths to characterize the mechanical responses to LCN stimulation and in 10 ml baths to characterize the responses to exogenously applied agonists. The baths contained a modified Krebs solution of the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The Krebs solution was maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2.

Muscle recordings and stimulation procedures

Spontaneous and neurally-evoked isometric contractions of the circular smooth muscle layer were recorded. Muscle strips were equilibrated for 60–90 min under an optimal resting tension equivalent to a load of 5 g. Optimal tension was determined by repeatedly stretching the muscle strips and adding acetylcholine (10 μM). When contractions to acetylcholine ceased to increase with increasing stretch, that tension was considered optimal. Muscle strips which changed their tone after equilibration were discarded.

Bipolar platinum electrodes were positioned on branches of lumbar colonic nerves (LCN) to stimulate sympathetic fibres. Rectangular pulses from a Grass S88 stimulator were passed through a current amplifier. Frequency-response curves were constructed utilizing trains of pulses (2.0–20 Hz, 0.5 ms pulse duration, for 45 s at supramaximal intensities) applied to LCN. The time interval between stimulus trains was 5–10 min. These stimulation parameters caused reproducible mechanical responses.

Experimental protocols

Effects on LCN-evoked contractile responses To study the noradrenergic component of the contractile response, frequency-response curves were constructed in the absence and in the presence of a β-adrenoceptor antagonist, propranolol, an α-adrenoceptor antagonist, phentolamine, an α_1-adrenoceptor antagonist, prazosin, an α_2-adrenoceptor antagonist, yohimbine, or an α_2-agonist, clonidine.

To study the purinergic component of the contractile response, frequency-response curves were constructed in the absence and in the presence of the P_{2X}-purinoceptor antagonists, suramin and photolyzed $ANAPP_3$, or after desensitization of P_{2X}-purinoceptors by α,β-methylene ATP. Suramin was added to the bath solution as single doses approximately 30 min before construction of the second frequency-response curve. $ANAPP_3$ was added to the bath solution in the dark and incubated with the muscle strips for a period of 5 min. The tissues were next irradiated with a light source for a 20–25 min period.

Desensitization of P_2-purinoceptors involved an initial application of α,β-methylene ATP (5 μM). It caused a transient increase in tone and in amplitude of spontaneous contractions. Subsequent applications of α,β-methylene ATP (5 μM) had no effect. Muscle strips were continuously exposed to α,β-methylene ATP (5 μM) for the duration of the experiments.

In some experiments, nicotinic receptors were either blocked by hexamethonium or desensitized by continuous

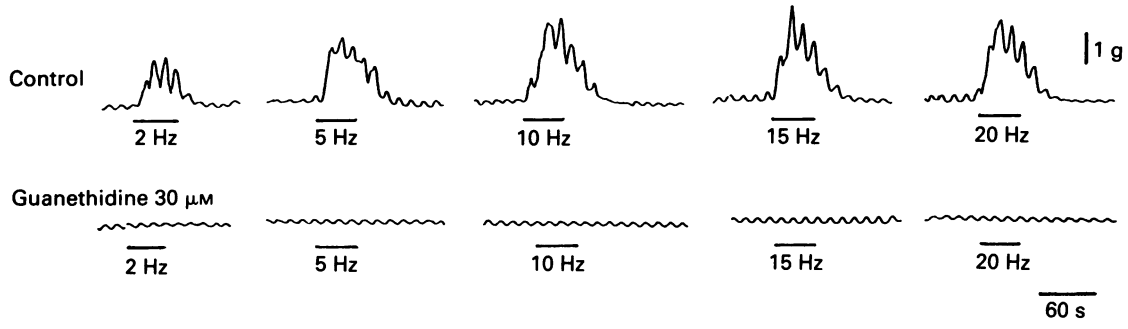

Figure 1 Cat mid-colon circular muscle strip *in vitro*: effects of guanethidine on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage for 45 s at indicated frequencies). Time between stimulus trains was 10 min. Upper traces are responses in the absence of guanethidine. Lower traces are responses 30 min after guanethidine. Horizontal bars show time of electrical stimulation.

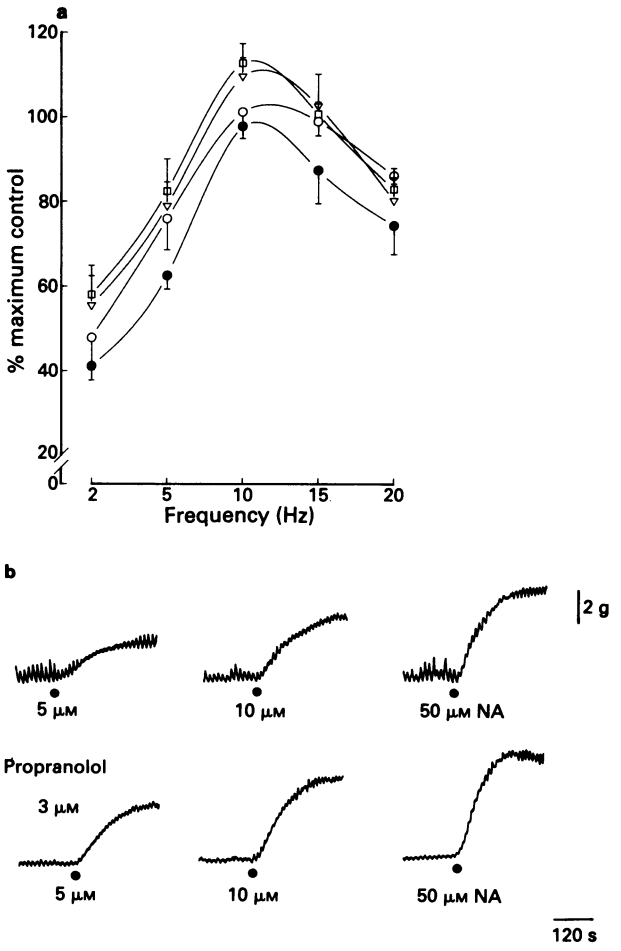

Figure 2 Cat mid-colon circular muscle strip *in vitro*: (a) Effects of propranolol on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage, for 45 s at indicated frequencies). Contractile responses in the absence (●, $n = 3$) and in the presence of propranolol (1 μM, ○, $n = 5$), (3 μM, ▽, $n = 4$), (9 μM, □, $n = 4$). Ordinate scale, contractile responses expressed as a percentage relative to the maximal contraction obtained in the absence of propranolol. Values are mean ± s.e.mean. Effects of propranolol (3 μM, 9 μM) on contractile responses were statistically significant ($P < 0.05$) at frequencies of 2, 5 and 10 Hz but at 1 μM were not significant at all frequencies. (b) Contractile responses to noradrenaline (NA) in the absence (upper traces) and presence (lower traces) of propranolol (3 μM).

exposure of muscle strips to 1,1-dimethyl-4-phenyl-piperazinium (DMPP). The initial application of DMPP (50 μM) caused an increase in resting tension and in amplitude of spontaneous contractions that declined to baseline after 2–3 min. Additional applications of DMPP (100 μM) did not elicit contractile responses.

Effects on spontaneous mechanical activity The contractile responses to exogenous noradrenaline and an α_1-adrenoceptor agonist, phenylephrine were studied in the absence and presence of prazosin (3 μM) or phentolamine (3 μM) and after desensitization of P_2-purinoceptors by α,β-methylene ATP. Also, concentration-response curves to noradrenaline were constructed in the absence and in the presence of the P_{2X}-purinoceptor antagonists, suramin and $ANAPP_3$.

Non-cumulative concentration-responses curves to ATP and α,β-methylene ATP were constructed in the absence and presence of the P_{2X}-purinoceptor antagonists, suramin and photolyzed $ANAPP_3$. ATP and α,β-methylene ATP were consecutively applied at intervals of 30 min to avoid tachyphylaxis. Only one agonist and one antagonist was applied per muscle strip.

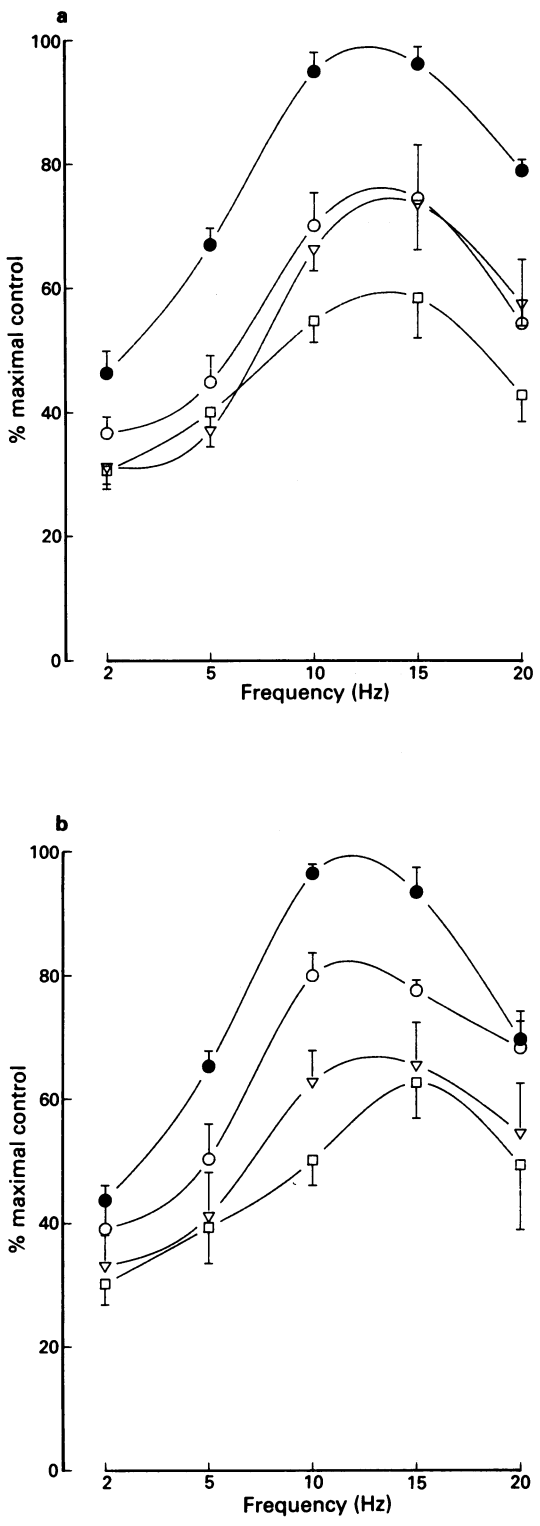

Figure 3 Cat mid-colon circular muscle strip *in vitro*: effects of phentolamine (a) and prazosin (b) on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage, for 45 s at indicated frequencies). Time between stimulus trains was 5 min. Contractile responses in the absence of phentolamine (a) (●, $n = 14$) or prazosin (b) (●, $n = 15$). (a) Contractile responses in the presence of phentolamine (1 μM, ○, $n = 5$), (3 μM, ▽, $n = 6$), (9 μM, □, $n = 4$). (b) Contractile responses in the presence of prazosin (1 μM, ○, $n = 6$), (3 μM, ▽, $n = 6$), (9 μM, □, $n = 4$). Ordinate scale, contractile responses expressed as a percentage relative to the maximal contraction obtained in the absence of phentolamine or prazosin. Effects of phentolamine (1, 3, 9 μM) and prazosin (3, 9 μM) were significant ($P < 0.05$) at all frequencies. Effects of prazosin (1 μM) were significant ($P < 0.05$) at frequencies of 5 to 15 Hz. Values are mean ± s.e.mean.

Data analysis

The amplitudes of LCN-evoked contractile responses were measured and expressed either as tension in g or as a percentage of the maximal contractile response in the absence of antagonists or before desensitization. The amplitude of contractions to agonists in the absence and presence of antagonists were measured and expressed either as tension in g or as a percentage of the maximal response. The EC_{50} values, slopes and correlation coefficients for agonist response in the absence and presence of antagonists were calculated from linear regression of the relation between $-\log_{10}$ of the concentration of agonist and the % maximum response. Data obtained from responses between 20% and 80% of the maximum were used to construct the linear regression-lines. The correlation coefficients of the linear regression lines ranged between 0.90 and 0.98. Data are presented as mean ± s.e.mean. The significance of differences was assessed by a Student's t test for paired observations and P values of less than 0.05 were considered significant. The number of observations (n) are number of muscle strips.

Drugs

Drugs used were: 1,1-dimethyl-4-phenyl-piperazinium (DMPP), atropine sulphate, guanethidine sulphate, hexamethonium bromide, prazosin hydrochloride, phentolamine mesylate, yohimbine hydrochloride, suramin hexasodium salt, tetrodotoxin (TTX), (−)-noradrenaline bitartrate, (−)-phenylephrine hydrochloride, clonidine hydrochloride, adenosine 5′-triphosphate disodium salt (ATP), α,β-methylene adenosine 5′-triphosphate lithium salt (α,β-methylene ATP), arylazido amino propionyl adenosine triphosphate ($ANAPP_3$). All drugs except suramin hexasodium salt (a gift from Center for Disease Control; Atlanta, Georgia, U.S.A.) and $ANAPP_3$ (a gift from Dr R. Theobald, Kirksville College of Osteopathic Medicine, Kirksville, MO, U.S.A.) were obtained from the Sigma Chemical Co. Drugs were dissolved in distilled water and diluted to final concentrations in Krebs solution. Drug concentrations reported refer to final bath values.

Results

Contractile responses to LCN stimulation

Cat colon circular muscle strips had low resting tensions (range, 0.5–1.5 g) and exhibited rhythmic (frequency range, 4–5 min^{-1}) and nonrhythmic spontaneous contractions. Electrical stimulation of branches of LCN caused frequency-dependent increases in resting tension and amplitude of spontaneous contractions (Figure 1). They occurred in muscle strips that were pretreated with hexamethonium (10 μM, $n = 5$), atropine (1 μM, $n = 4$) or when nicotinic receptors were desensitized with DMPP (5 μM, $n = 5$) (data not shown). In contrast, LCN-evoked contractions were completely blocked at all frequencies by tetrodotoxin (0.3 μM, $n = 4$), by sectioning the LCN between the stimulating electrode and the muscle strip ($n = 12$) and by guanethidine (30 μM, $n = 15$) (Figure 1). This indicates that the contractile responses were neurogenic, did not involve activaiton of nicotinic or muscarinic acetylcholine receptors, but involve the release of neurotransmitter(s) from sympathetic fibres contained in branches of LCN.

Effects of adrenoceptor antagonists

Effects of a β-adrenoceptor antagonist The β-adrenoceptor antagonist, propranolol (1–9 μM), did not change resting tension and the amplitude of spontaneous contractions, but caused a concentration-dependent potentiation of LCN-evoked contractile responses at all frequencies (Figure 2a). The amplitude of LCN-evoked contractile responses at 10 Hz was potentiated by 1, 3 and 9 μM propranolol by 2.9 ± 0.8% ($n = 4$), 11.8 ± 2.9% ($n = 5$) and 15.0 ± 3.7% ($n = 4$), respectively.

Noradrenaline (5–50 μM, $n = 5$) or phenylephrine (10–100 μM, $n = 5$) caused concentration-dependent contractile responses. Propranolol (3 μM) potentiated the amplitude of contractile responses to noradrenaline (Figure 2b) but not to phenylephrine (data not shown). The amplitude of the contractile responses to noradrenaline (50 μM) significantly increased ($P < 0.05$) from 3.9 ± 0.25 g to 5.4 ± 0.32 g ($n = 5$).

Effects of α-adrenoceptor antagonists The α-adrenoceptor antagonist, phentolamine (1–9 μM), or the α_1-adrenoceptor antagonist, prazosin (1–9 μM) did not change resting tension and the amplitude of spontaneous contractions. They both caused a concentration-dependent reduction of LCN-evoked contractile responses, at all frequencies, but did not abolish them (Figure 3a,b). Phentolamine at 1, 3 and 9 μM, inhibited contractile responses at 10 Hz by 25.0 ± 5.3% ($n = 4$), 29.0 ± 3.3% ($n = 6$) and 40.2 ± 5.3% ($n = 4$), respectively. Prazosin at 1, 3 and 9 μM, inhibited contractile responses by 16.5 ± 3.7% ($n = 5$), 34.0 ± 5.1% ($n = 6$) and 46.3 ± 4.1% ($n = 4$), respectively. The α-adrenoceptor antagonist resistant component was further abolished by guanethidine (30 μM, $n = 12$) (data not shown), indicating it was mediated by the release of a neurotransmitter from sympathetic fibres.

Prazosin (3 μM) antagonized contractile responses to exogenously applied noradrenaline (5–50 μM, $n = 4$) and phenylephrine (10–100 μM, $n = 4$) (data not shown), suggesting a postjunctional effect mediated through α_1-adrenoceptors.

Effects of P_2-purinoceptor desensitization with α,β-methylene ATP

The component of the LCN-evoked contractile response which was resistant to α-adrenoceptor antagonists was nearly abolished by desensitization of P_2-purinoceptors with α,β-methylene ATP, at all frequencies (Figure 4a,b). In the presence of both prazosin (3 μM) and α,β-methylene ATP (5 μM), the amplitude of contractile responses evoked at 2 Hz was either abolished ($n = 4$) or inhibited by 90.5 ± 4.2% ($n = 3$). At a stimulus frequency of 15 Hz, where responses were maximal, their amplitude was reduced by 84.5 ± 2.3% ($n = 7$). The remainder of the contractile responses was abolished by guanethidine (30 μM, $n = 7$) (data not shown).

In the absence of prazosin, desensitization of P_2-purinoceptors with α,β-methylene ATP caused reduction in the amplitude of LCN-evoked contractile responses at all frequencies (Figure 4c,d). When contractile responses were maximal, (stimulus frequency of 10 Hz) α,β-methylene ATP inhibited them by 30 ± 3.4% ($n = 6$). Subsequent administration of prazosin (3 μM) in the presence of α,β-methylene ATP inhibited the amplitude of maximal contractile responses by 82.4 ± 2.5% (Figure 4c,d). The remainder of the contractile response was abolished by guanethidine (3 μM, $n = 6$) (data not shown).

Desensitization of P_2-purinoceptors with α,β-methylene ATP (5 μM) did not affect contractile responses to noradrenaline (5–50 μM, $n = 5$) but abolished the responses to ATP (10–100 μM, $n = 4$) (data not shown). Also, the contractile responses to ATP (10–100 μM, $n = 4$) were not affected when α_1-adrenoceptors were blocked by prazosin.

Effects of P_2-purinoceptor antagonists

Effects of suramin on contractile responses to LCN stimulation Suramin (50, 100 μM), a reversible antagonist of P_{2X}-purinoceptors in mouse and guinea-pig vas deferens (Dunn & Blakeley, 1988; Leff *et al.*, 1990; von Kugelgen *et al.*, 1990), guinea-pig urinary bladder (Hoyle *et al.*, 1990) and P_{2Y}-purinoceptors in guinea-pig taenia coli (Den Hertog *et al.*,

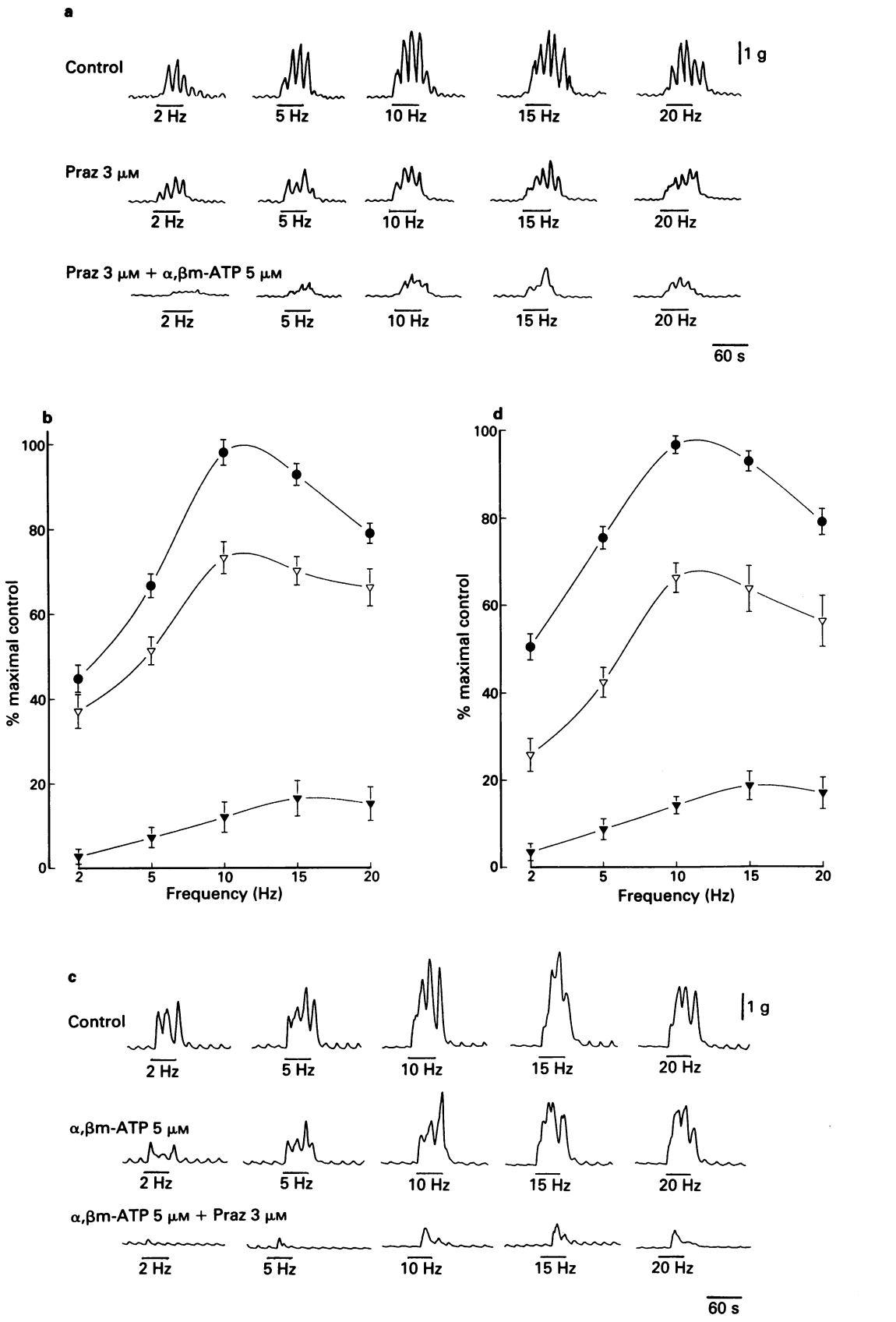

Figure 4 Cat mid-colon circular muscle strip *in vitro*: (a,b) effects of prazosin (Praz) or prazosin and α,β-methylene ATP (α,β-mATP) on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage, for 45 s at indicated frequencies). (c,d) Effects of α,β-methylene ATP or α,β-methylene ATP and prazosin on LCN-evoked contractile responses. Time between stimulus trains was 5 min. (b) Contractile responses in the absence (●, $n = 7$) and in the presence of prazosin (3 μM, ▽, $n = 7$) or in the presence of prazosin (3 μM) and α,β-methylene ATP (5 μM, ▼, $n = 7$). (c) Contractile responses in the absence (●, $n = 6$) and in the presence of α,β-methylene ATP (5 μM, ▽, $n = 6$) or in the presence of α,β-methylene ATP (5 μM) and prazosin (3 μM, ▼, $n = 6$). (b,d) Effects of prazosin or α,βm-ATP alone or in combination were significant ($P < 0.05$) at all frequencies. Ordinate scale, responses expressed as a percentage relative to the maximum contraction obtained in the absence of prazosin and α,β-methylene ATP. Values are mean ± s.e.mean. (a,c) Horizontal bars show time of electrical stimulation.

1989; Hoyle *et al.*, 1990) had no effect on resting tension and amplitude and frequency of spontaneous contractions. At a concentration of 50 μM, it also had no effect on contractile responses to LCN stimulation at all frequencies. At a concentration of 100 μM it significantly ($P<0.05$, $n=7$) inhibited contractile responses at 5 and 10 Hz by 15.2 ± 2.0 and 24 ± 1.8, respectively. In contrast, suramin (500 μM) significantly ($P<0.05$, $n=5$) potentiated LCN-evoked contractions at 2, 5 and 10 Hz (Figure 5a), increased resting tension and the amplitudes of spontaneous contractions.

Effects of suramin on contractile responses to ATP and α,β-methylene ATP The exogenous administration of ATP (10–1 000 μM) and α,β-methylene ATP (0.05–10 μM) caused concentration-dependent reversible contractions of circular muscle strips with EC_{50} values of 87.1 ± 7.8 μM ($n=9$) and 0.393 ± 0.067 μM ($n=9$), respectively (Figure 6). Suramin (100, 500 μM) reduced the amplitude of contractions to lower concentrations of ATP (10, 50 μM) but potentiated responses to higher concentrations (500, 1 000 μM) (Figure 6a). The EC_{50} values for ATP in the presence of suramin could not be calculated because the concentration-response curve did not attain a maximum when ATP (1 000 μM) was tested. The effects of ATP at concentrations greater than 1 000 μM were considered non-specific.

Suramin (100, 500 μM) also caused a concentration-dependent reduction in amplitude of contractions to exogenously applied α,β-methylene ATP (0.05–1 μM) (Figure 6b), and shifted the concentration-response curves to the right. At higher concentrations of α,β-methylene ATP (10, 100 μM), suramin potentiated contractile responses. The EC_{50} values for α,β-methylene ATP in the presence of suramin at 100 and 500 μM were 1.67 ± 0.32 μM ($n=5$) and 6.11 ± 0.43 ($n=4$), respectively and were significantly different ($P<0.05$) from the control value. The slopes of the concentration-response curves to α,β-methylene ATP in the absence and in the presence of 100 and 500 μM of suramin were 42.8 ± 11.8 ($n=9$), 43.6 ± 3.1 ($n=5$) and 40.5 ± 15.2 ($n=4$), respectively. These values were not significantly different, indicating a parallel shift to the right of the concentration-response curves. This suggests that suramin competitively antagonized the effects of α,β-methylene ATP. In contrast, suramin had no effect on concentration-response curves to noradrenaline (Figure 6c). These data suggest that α,β-methylene ATP (0.5–10 μM) and low concentrations of ATP (10–50 μM) cause contractions of colon circular muscle through suramin-sensitive postjunctional P_{2X}-purinoceptors. Similar effects of suramin have been reported on contractions to α,β-methylene ATP and ATP in mouse vas deferens (von Kugelgen *et al.*, 1990).

Effect of $ANAPP_3$ on LCN evoked contractile responses $ANAPP_3$, an antagonist of the contractile responses mediated through P_{2X}-purinoceptors (Hogbaum *et al.*, 1980; Fedan *et al.*, 1982; Burnstock & Kennedy, 1985), caused concentration-dependent irreversible reductions in amplitude of LCN-evoked contractile responses at all frequencies, but did not abolish them (Figure 5b). $ANAPP_3$, at concentrations of 50 and 100 μM, significantly ($P<0.05$) inhibited contractile responses at 10 Hz by 27.5 ± 4.2% ($n=4$) and 41.5 ± 3.6% ($n=6$), respectively. The remainder of the contractile responses were further reduced by prazosin (3 μM) ($n=3$) or abolished by guanethidine (30 μM) ($n=3$) (data not shown).

Effects of $ANAPP_3$ on contractile responses to ATP and α,β-methylene ATP $ANAPP_3$ (100 μM) caused a reduction in amplitude of contractile responses to ATP (10–1,000 μM). It shifted the concentration-response curves to the right without altering the concentration that caused the maximal contraction (Figure 7a). The EC_{50} values for ATP in the absence and in the presence of $ANAPP_3$ were 62.3 ± 8.9 μM ($n=6$) and 216.8 ± 62.1 ($n=6$), respectively, and were significantly ($P<0.05$) different from each other. The slopes of the linear

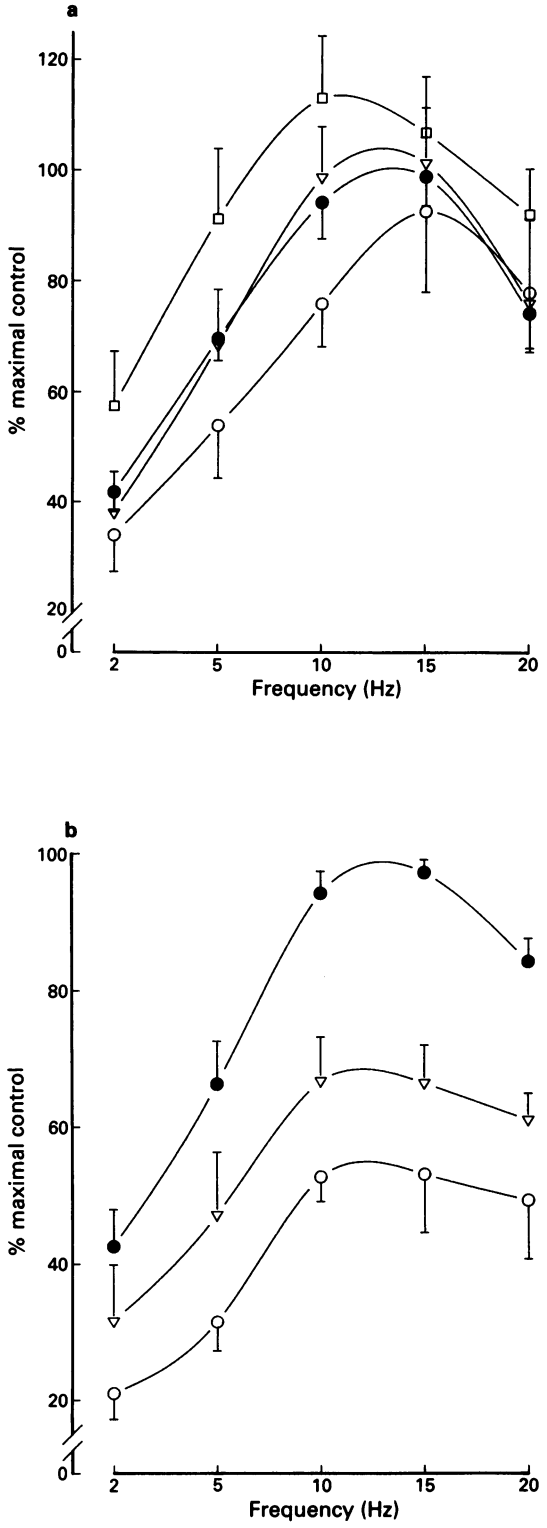

Figure 5 Cat mid-colon circular muscle strip *in vitro*: effects of suramin (a) and arylazido aminopropyl adenosine triphosphate ($ANAPP_3$) (b) on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage, for 45 s at indicated frequencies). Contractile responses in the absence of suramin (●, $n=17$) and $ANAPP_3$ (●, $n=10$). (a) Responses in the presence of suramin (50 μM, ▽, $n=5$), (100 μM, ○, $n=7$), (500 μM, □, $n=5$). (b) $ANAPP_3$ (50 μM, ▽, $n=5$), (100 μM, ○, $n=6$). Ordinate scale, responses expressed as a percentage relative to the maximum contraction obtained in the absence of suramin or $ANAPP_3$. Suramin (100 μM) significantly ($P<0.05$) inhibited contractions at frequencies of 2–15 Hz. $ANAPP_3$, at 50 μM, significantly ($P<0.05$) inhibited contractions at frequencies of 5–20 Hz and at 100 μM, all frequencies. Values are mean ± s.e.mean.

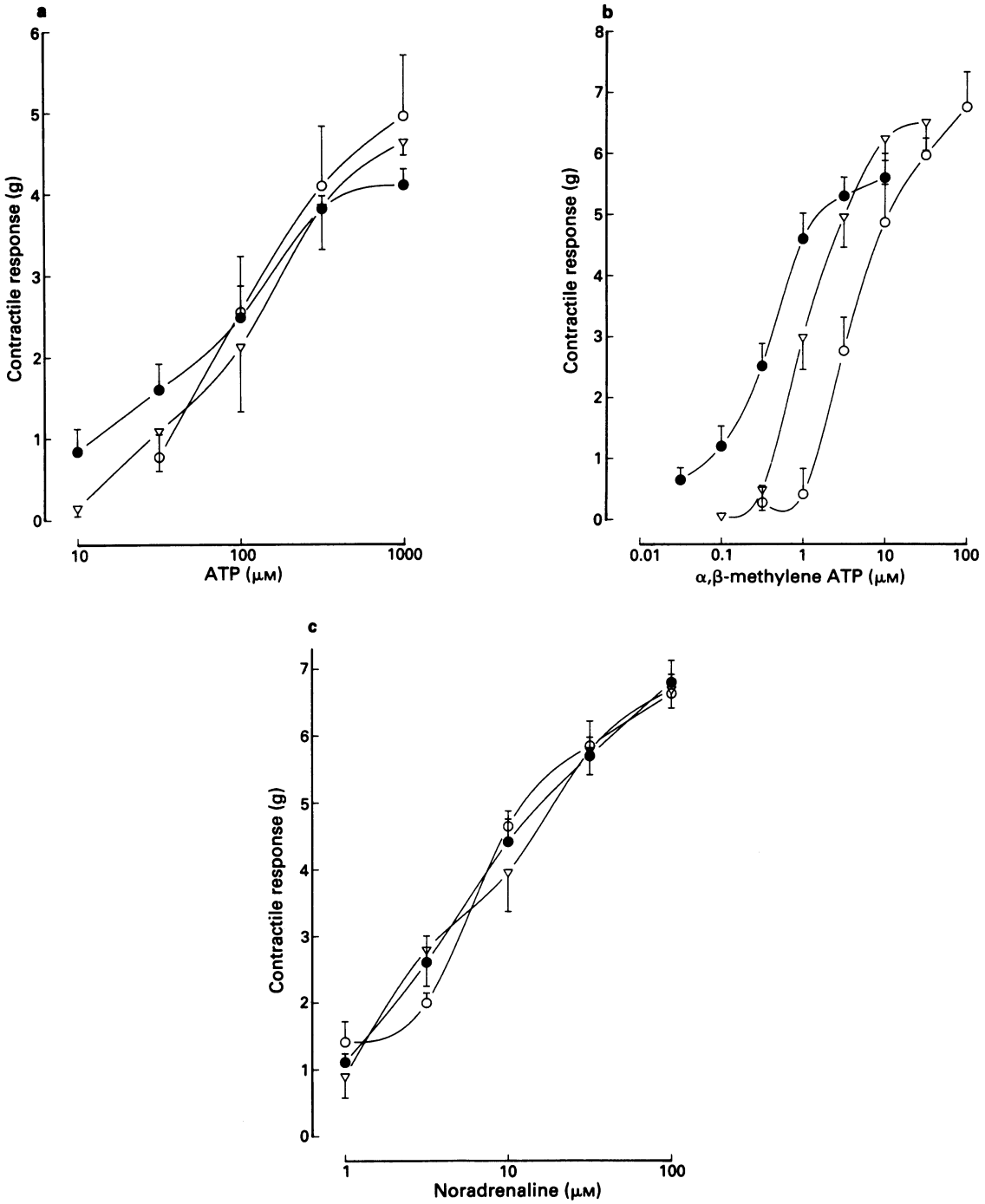

Figure 6 Cat mid-colon circular muscle strip *in vitro*: (a) mean contractile responses to ATP in the absence ($n = 9$) and in the presence of suramin at 100 μM ($n = 5$) and 500 μM ($n = 4$). (b) Mean contractile responses to α,β-methylene ATP in the absence of ($n = 9$) and in the presence of suramin ($n = 5$) at 100 μM ($n = 5$) and 500 μM ($n = 4$). (c) Mean contractile responses to noradrenaline in the absence ($n = 8$) and in the presence of suramin at 100 μM ($n = 4$) and 500 μM ($n = 4$). (a,b,c) Responses in the absence (●) and presence of suramin at 100 μM (▽) and 500 μM (○). Abscissa scale, concentration of agonist. Ordinate scale, tension expressed in g. Values are mean ± s.e.mean.

regression lines were 0.61 ± 0.05 ($n = 6$) and 0.15 ± 0.08 ($n = 6$). The slopes were significantly different, indicating that the rightward shift of the concentration-response curve was not parallel.

$ANAPP_3$ (100 μM) caused a reduction in amplitude of contractile responses to α,β-methylene ATP, and shifted the concentration-response curves to the right (Figure 7b). The EC_{50} values for α,β-methylene ATP in the absence and in the presence of $ANAPP_3$ were 0.516 ± 0.081 μM ($n = 5$) and 5.3 ± 0.86 μM ($n = 4$), respectively and differed significantly ($P < 0.05$) from one another. The slopes of the linear regression lines were 64.5 ± 12.1 ($n = 5$) and 6.7 ± 1.4 ($n = 4$). The slopes were significantly different, indicating that the rightward shift in the concentration-response curve was not parallel. Also, $ANAPP_3$ had no effect on the concentration-response curves to noradrenaline (Figure 7c). This suggests that $ANAPP_3$ irreversibly and non-competitively antagonized postjunctional P_{2X}-purinoceptors on circular smooth muscle.

Effects of α_2-adrenoceptor agonists and antagonists on LCN-evoked contractile responses

Yohimbine (0.1 – 1 μM), an α_2-adrenoceptor antagonist, caused significant ($P < 0.05$) concentration-dependent in-

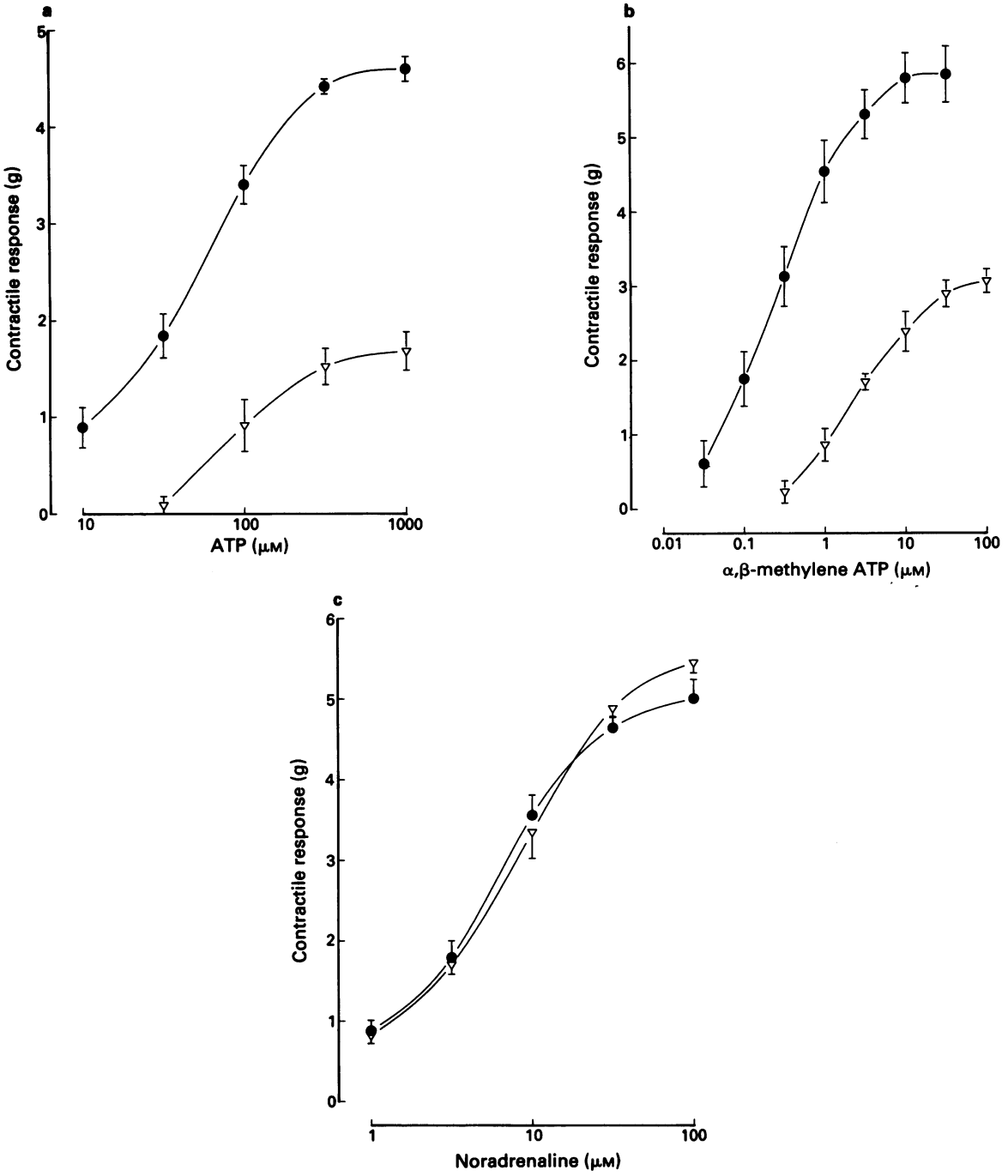

Figure 7 Cat mid-colon circular muscle strip *in vitro*: (a) mean contractile responses to ATP in the absence ($n = 6$) and in the presence of arylazido aminopropyl adenosine triphosphate ($ANAPP_3$) ($n = 6$). (b) Mean contractile responses to α,β-methylene ATP in the absence ($n = 5$) and in the presence of $ANAPP_3$ ($n = 5$). (c) Mean contractile responses to noradrenaline in the absence of ($n = 4$) and in the presence of $ANAPP_3$ ($n = 4$). (a,b,c) Responses in the absence (●) and presence (▽) of $ANAPP_3$. Abscissa scale, concentration of agonist. Ordinate scale, tension expressed in g. Values are mean ± s.e.mean.

creases in amplitude of LCN-evoked contractile responses at 10 Hz whereas clonidine (0.05–1.0 μM), a selective α_2-adrenoceptor agonist, caused significant ($P < 0.05$) concentration-dependent inhibition of them (Figure 8a). Yohimbine (1 μM) and clonidine (1 μM) significantly potentiated and inhibited the amplitude of LCN-evoked contractile responses at all frequencies (Figure 8b). This suggests that prejunctional α_2-receptors regulate the release of neurotransmitter(s) mediating contractile responses. Also, clonidine (0.1–5 μM, $n = 3$) (data not shown) had no effect on the amplitude of spontaneous contractions, nor did it affect resting tension, suggesting that cat colon circular muscle does not contain excitatory postjunctional α_2-adrenoceptors.

Discussion

The results indicate that electrical stimulation of LCN caused a frequency-dependent (2–20 Hz) increase of spontaneous contractions and resting tension of cat colon circular smooth muscle. Contractile responses were abolished by tetrodotoxin and by guanethidine indicating that they were neurogenic and involve the release of neurotransmitter from adrenergic fibres in LCN.

Our data indicate that a component of the LCN-evoked contractile responses was mediated by α_1-adrenoceptors. LCN-evoked contractile responses were reduced approximately 35–45% by the α_1- and α-adrenoceptor antagonists,

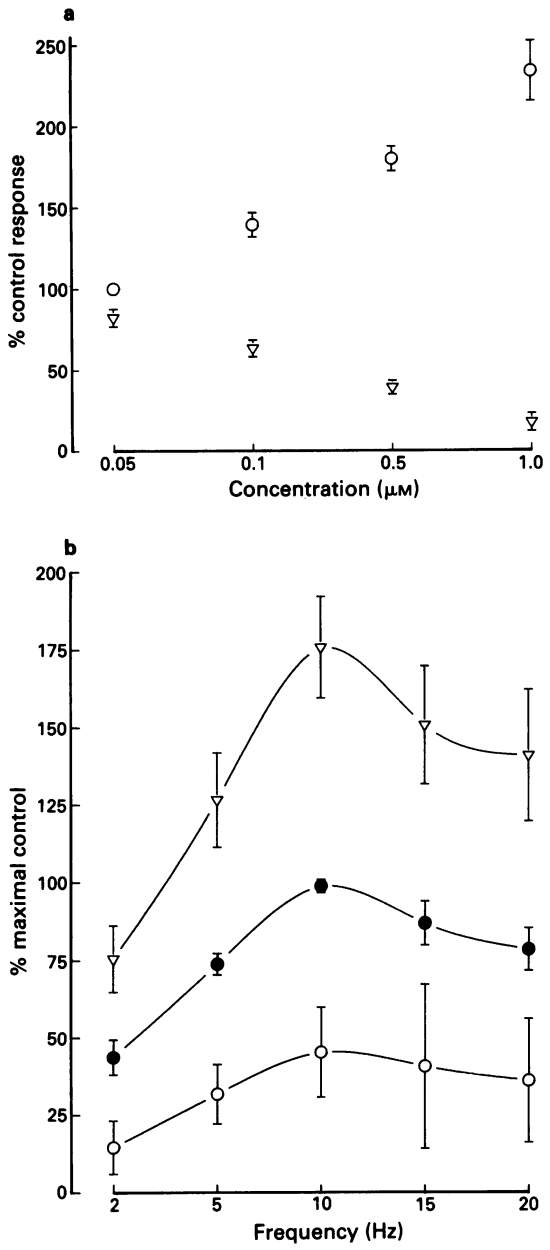

Figure 8 Cat mid-colon circular muscle strip *in vitro*: effects of yohimbine and clonidine on lumbar colonic nerve (LCN)-evoked contractile responses (0.5 ms pulse duration, supramaximal voltage for 45 s at indicated frequencies). Time between stimulus trains was 5 min. (a) Contractile responses at frequency of 10 Hz in presence of clonidine (▽) (0.05–1.0 μM) and yohimbine (○) (0.05–1.0 μM). Ordinate scale, responses expressed as a percentage of control equal to 100%. (b) Contractile responses in the absence of yohimbine or clonidine (●, $n = 10$). Contractile responses in the presence of yohimbine (1 μM, ▽, $n = 5$) and clonidine (1 μM, ○, $n = 5$). Values are mean ± s.e.mean. Effects of yohimbine and clonidine were significant ($P < 0.05$) at all frequencies.

prazosin and phentolamine, but not by yohimbine, an α_2-adrenoceptor antagonist. Furthermore, the α_1-adrenoceptor agonists, phenylephrine and noradrenaline, cause contractions of cat colon circular muscle, that were abolished by prazosin. In contrast, clonidine, an α_2-adrenoceptor agonist, did not alter spontaneous contractions or cause contractile responses. Contractions mediated through α_1-adrenoceptors have been characterized in guinea-pig small intestine (Bauer, 1981; 1982). Postjunctional α_2-adrenoceptors are present in circular muscle of dog small intestine (Ahmad *et al.*, 1991) and mediate contractions of dog proximal colon (Zang *et al.*, 1992) and guinea-pig stomach (Sakyoun *et al.*, 1982). This indicates that the types of adrenoceptors mediating contractions of gastrointestinal smooth muscle are species and/or organ dependent.

Our data support the concept that the prazosin-resistant component of the LCN-evoked contractile response is mediated by the release of ATP or a related purine nucleotide acting on suramin-insensitive and suramin-sensitive P_{2x}-purinoceptors. First, the prazosin-resistant component was not due to the inability of α_1- or α-adrenoceptor antagonists to access the receptors or to noradrenaline activating γ-receptors (Hirst & Edwards, 1989), since contractile responses to phenylephrine and noradrenaline were abolished by α_1-adrenoceptor antagonists. Thus a component of the contractile responses to LCN stimulation is mediated by a nonadrenergic neurotransmitter coreleased with noradrenaline. Second, pre-exposure to the P_{2X}-purinoceptor agonist, α,β-methylene ATP (Kennedy, 1990) desensitizes contractile responses to exogenous ATP and sympathetic nerve stimulation but not to phenylephrine or noradrenaline. In the presence of both prazosin and α,β-methylene ATP contractile responses were nearly abolished. Also, the exogenous administration of ATP and α,β-methylene ATP mimicked sympathetic nerve-evoked contractile responses. These data suggest that ATP or a related nucleotide mediates a component of the contractile response. Third, LCN-evoked contractions and the contractile responses to ATP and α,β-methylene ATP were reduced by $ANAPP_3$, a P_{2X}-purinoceptor antagonist (Burnstock & Kennedy, 1985). The contractile responses to ATP and α,β-methylene ATP were irreversibly and non-competitively inhibited by $ANAPP_3$. In contrast, $ANAPP_3$ did not affect the contractile responses to phenylephrine or noradrenaline. Fourth, suramin, a selective P_{2X}-purinoceptor antagonist (Burnstock, 1991), caused a small reduction in amplitude of LCN evoked contractile responses but caused a large reduction in amplitude and rightward parallel shift in the concentration-response curves to lower concentrations of ATP and to α,β-methylene ATP. The contractile responses to ATP and α,β-methylene ATP were competitively inhibited by suramin while those to phenylephrine or noradrenaline were unaffected. These data suggest that ATP or a related nucleotide released upon nerve stimulation activates predominantly suramin-insensitive P_{2X}-purinoceptors and also, suramin-sensitive P_{2X}-purinoceptors. It is also possible that a related nucleotide released upon nerve stimulation activates a receptor site distinct from the P_2-nucleotide receptor (i.e. pyrimidine receptor, O'Connor *et al.*, 1991). For example, in rabbit ear and basilar artery, UTP activates the pyrimidine receptor (von Kugelgen *et al.*, 1987).

The LCN-evoked contractile responses involve β-adrenoceptors. They mediate an inhibitory component of the contractions to LCN stimulation and to noradrenaline, since the β-adrenoceptor antagonist, propranolol, significantly increased the amplitude of contractions. In contrast, contractile responses to the α_1-adrenoceptor agonist, phenylephrine were not potentiated by propranolol. The subtypes of β-adrenoceptors (β_1- and β_2) involved was not determined in the present study, since propranolol does not distinguish between β_1- and β_2 adrenoceptors. Presumably, LCN-evoked contractions involve both subtypes of β-adrenoceptors since β_1-adrenoceptors are present in the intrinsic neural plexus and β_2-adrenoceptors are present on smooth muscle cells of cat colon (Ek, 1985).

The LCN-evoked contractile responses are regulated by noradrenaline acting on prejunctional α_2-adrenoceptors, since yohimbine increased their amplitude while clonidine decreased them. It is likely that during electrical stimulation, activation of prejunctional α_2-adrenoceptors inhibits the release of both noradrenaline and ATP since contractile responses, comprised of both purinergic and adrenergic components, were nearly abolished by clonidine. These results are consistent with the mechanism of autoinhibition at adrenergic terminals in other tissues, where it has been shown that

prejunctional α_2-adrenoceptors cause negative feedback inhibition of transmitter release (Starke *et al.*, 1989).

In summary, we have identified a lumbar sympathetic pathway that mediates contractions of cat colon circular smooth muscle. The data suggest that the contractions occur through corelease of noradrenaline and ATP or a related nucleotide from adrenergic fibres within lumbar colonic nerves. The physiological role for this excitatory sympathetic pathway is not yet understood. We speculate that it regulates colonic motility *in situ*. For example, when resting tension of the colon is low and spontaneous contractions are generated by myogenic mechanisms (electrical slow waves), reflex activation of lumbar sympathetic nerves causes phasic excitation mediated through postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors. The increase in amplitude of contractions and resting tension occurs by increases in the amplitude and duration of electrical slow waves. In contrast, when resting tension is high, and contractions are generated by excitatory cholinergic and/or peptidergic nerves, lumbar sympathetic nerves suppress colonic contractions by regulating transmitter release at cholinergic and/or peptidergic synapses through prejunctional adrenoceptors (Vizi *et al.*, 1991). These types of antagonistic interactions on colonic motility are yet to be defined.

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Erratum

Br. J. Pharmacol. (1993), **109**, 685–692

Y. Habara & T. Kanno. Dual effects of chlorobutanol on secretory response and intracellular Ca^{2+} dynamics in isolated pancreatic acini of the rat.

In the above article, two errors appeared in Figure 6 as published in the July issue of the journal: the concentrations of carbachol in Figure 6b and Figure 6c were shown as 3 μM and 0.3 μM respectively, but these should have appeared as 0.3 μM and 3 μM respectively. The figure is reproduced here in its correct form.

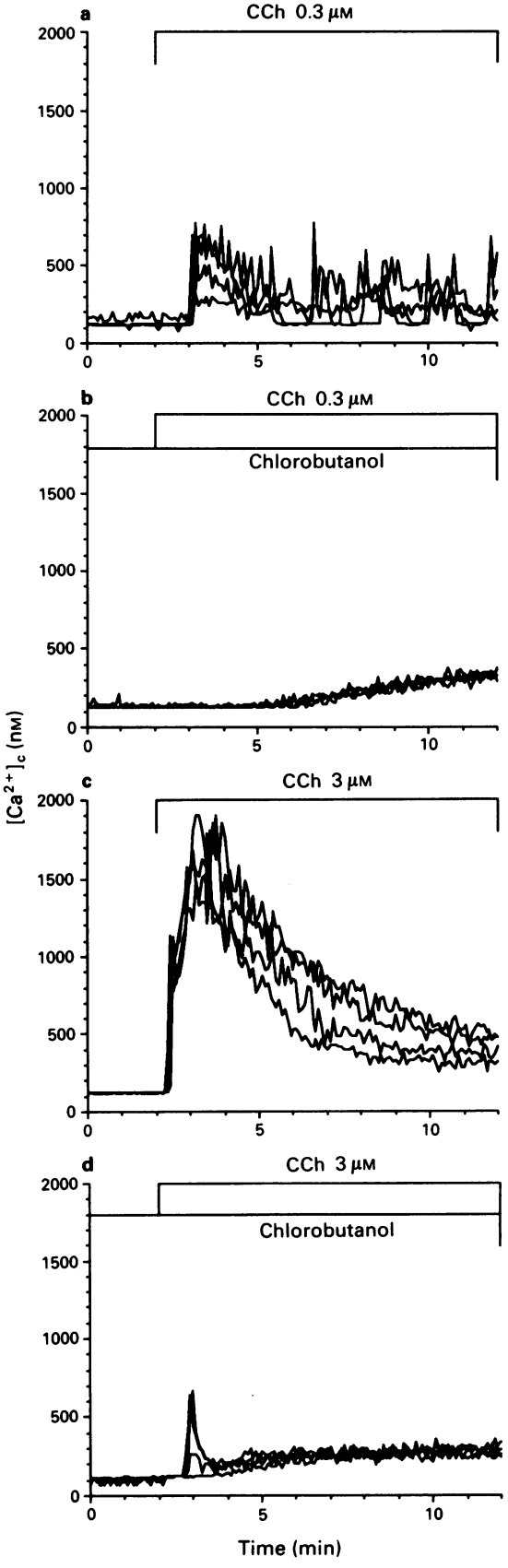

Figure 6 Effect of chlorobutanol on carbachol (CCh)-induced $[Ca^{2+}]_c$ dynamics. Acini loaded with Fura-2 were stimulated with 0.3 μM (a,b) or 3 μM (c,d) carbachol for 10 min in the presence (b,d) or absence (a,c) of 1 mg ml^{-1} chlorobutanol. Fluorescence ratios averaged from 4 μm^2 in four different areas were converted to $[Ca^{2+}]_c$. Each recording represents a typical example obtained from several experiments.

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