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Access of peripherally administered DuP 753 to rat brain angiotensin II receptors

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The *in vivo* access of the nonpeptide angiotensin II (Ang II) antagonist, DuP 753 (10 mg kg^{-1}, i.v.), to Ang II receptors of rat brain was investigated by *in vitro* autoradiography with [^{125}I]-[Sar^1, Ile^8] Ang II as a ligand. DuP 753 markedly inhibited the binding to sites which contain exclusively AT_1 receptors both outside and within the blood brain barrier, such as the circumventricular organs, paraventricular hypothalamic nucleus, median preoptic nucleus and nucleus of the solitary tract. However, binding to other nuclei containing AT_2 receptors was not significantly inhibited. These results demonstrate that DuP 753 and/or its active metabolite readily cross the blood brain barrier *in vivo* and selectively inhibit binding to AT_1 receptors in specific brain nuclei.

Keywords: Rat brain; angiotensin II; receptor subtypes; antagonist

Introduction Angiotensin II (Ang II) plays an important role in maintaining fluid and electrolyte homeostasis and cardiovascular functions by both peripheral and central actions. Ang II receptor binding sites, Ang II-responsive neurones and Ang II containing nuclei have been demonstrated at sites within and outside the blood brain barrier (Mendelsohn *et al.*, 1990). Recently, two distinct subtypes of Ang II receptors, AT_1 and AT_2, have been identified by use of new nonpeptide Ang II antagonists (Chiu *et al.*, 1989). The distribution of these Ang II receptor subtypes in rat brain has been described by *in vitro* autoradiography (Gehlert *et al.*, 1990; Wamsley *et al.*, 1990; Song *et al.*, 1991), and anxiolytic and cognitive-enhancing actions of the AT_1 antagonist, DuP 753, have been reported (Barnes *et al.*, 1990a,b) suggesting that the drug has central actions. However, it is not known whether these compounds have access to Ang II receptor sites within the blood brain barrier *in vivo*. The previously used peptide Ang II receptor antagonists do not penetrate the blood brain barrier, and only have access to the circumventricular organs. Similarly, the angiotensin converting enzyme inhibitors do not readily penetrate the blood brain barrier except after chronic administration of high doses (Sakaguchi *et al.*, 1988). We have therefore examined the effect of peripherally administered DuP 753 on Ang II receptor occupancy in rat brain using *in vitro* autoradiography.

Methods Four adult male Sprague-Dawley rats were injected with DuP 753 (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl) methyl] imidazole) via the tail vein at a dose of 10 mg kg^{-1}, and four other animals received vehicle; 60 min after administration of DuP 753, rats were killed by decapitation. The brains were removed, snap frozen in isopentane–dry ice (−40°C) and stored at −80°C. Sections (20 μm) were cut on cryostat at −18°C, dehydrated overnight under reduced pressure at 4°C, and stored at −80°C in sealed container with silica gel until use.

The methods for *in vitro* autoradiographic localization of Ang II receptors with [^{125}I]-[Sar^1, Ile^8] Ang II have been described previously (Song *et al.*, 1991). Briefly, sections were incubated in 10 mM sodium phosphate (pH 7.4) containing 150 mM NaCl, 5 mM Na_2EDTA, 0.2% bovine serum albumin (BSA) and 0.4 mM bacitracin with 0.2 μCi ml^{-1} (90 pM) of [^{125}I]-[Sar^1, Ile^8] Ang II. Non-specific binding was determined in the presence of 1 μM unlabelled Ang II. After incubation, the sections were washed with ice-cold buffer, loaded into X-ray cassettes and exposed to Agfascopix CR3B X-ray film for 2 weeks. A set of ^{125}I radioactivity standards was included. After exposure, the films were processed in a Kodak RPX-OMAT automatic developer and the optical density was quantitated with an MCID image analysis system (Imaging Research Inc., Ontario, Canada).

Results Ang II receptor binding to the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (10), paratrigeminal nucleus (Pa5), and medial nucleus of the inferior olive (IOM) in a control rat is shown in Figure 1a. *In vivo* administration of DuP 753 resulted in a marked decrease of binding to the nucleus of the solitary tract (29 ± 5%, compared to control), the dorsal motor nucleus of the vagus (31 ± 6%), and the paratrigeminal nucleus (35 ± 9%) (Figure 1b). These are sites where AT_1 receptors occur predominantly (Song *et al.*, 1991). Ang II receptor binding in the circumventricular organs, the subfornical organ, vascular organ of the lamina terminalis, median eminence and area postrema, are also inhibited by more than 50% in rats treated with DuP 753. In addition, binding to many brain structures which contain predominantly AT_1 receptors and are within the blood brain barrier, such as the median preoptic nucleus, amygdala, lateral parabrachial nucleus, spinal trigeminal nucleus, bed nucleus of the stria terminalis, and fusiform nucleus, were also markedly inhibited. By contrast, Ang II receptor binding to sites where AT_2 receptors occur predominantly, such as the medial nucleus of the inferior olive (5 ± 1%, Figure 1b), lateral septal nucleus, many nuclei in the thalamus, subthalamic nucleus (10 ± 2%), superior colliculus (11 ± 3%), locus coeruleus were not influenced significantly by prior administration of DuP 753. These results are consistent with our previous *in vitro* autoradiography studies in which the drug was added to the incubation medium (Song *et al.*, 1991).

Discussion The central actions of Ang II include stimulation of drinking and salt appetite, control of the autonomic nervous system, and regulation of both anterior and posterior pituitary hormone secretion. Using *in vitro* autoradiography, we (Song *et al.*, 1991) and others (Gehlert *et al.*, 1990; Wamsley *et al.*, 1990) have demonstrated that many brain nuclei involved in these functions contain predominantly AT_1 receptors, as determined in the presence of an excess concentration (10 μM) of the AT_2 antagonist, PD 123177. The present study demonstrates that peripherally administered DuP 753 blocks AT_1 receptor binding, not only to the circumventricular organs which are outside the blood brain barrier, but also at sites within the blood brain barrier, such as the median preoptic nucleus, hypothalamic paraventricular nucleus, lateral parabrachial nucleus, spinal trigeminal nucleus, bed

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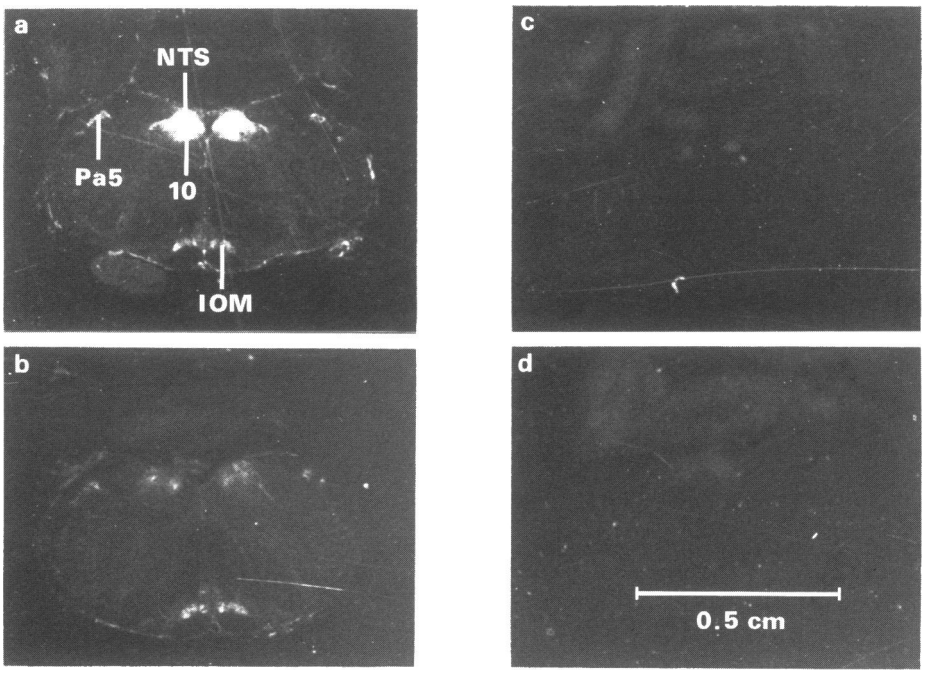


Figure 1 Angiotensin II (Ang II) receptor binding at the level of the nucleus of the solitary tract of rat medulla oblongata: (a) shows binding in a control rat; (b) in a rat previously treated with DuP 753 10 mg kg^{-1}, i.v.; (c) shows non-specific binding in a control rat, and (d) shows non-specific binding in a DuP 753-treated rat. White represents high density of binding while black indicates background levels. Abbreviations are: NTS, nucleus of the solitary tract; 10, dorsal motor nucleus of the vagus; Pa5, paratrigeminal nucleus; IOM, medial nucleus of the inferior olive. Calibration bar: 0.5 cm.

nucleus of the stria terminalis and nucleus of the solitary tract. No difference was observed in inhibition between the sites within and outside the blood brain barrier. By contrast, the effect of DuP 753 administered *in vivo* on AT_2 sites was very weak and related to the ratio of colocalization of AT_1 and AT_2 receptors in each site (Song *et al.*, 1991). For instance, in the subthalamic nucleus, AT_2 receptors account for 80%, and AT_1 receptors for 20% of total binding and here the extent of inhibition by *in vivo* DuP 753 was 10% compared with 50% inhibition on AT_1 receptors in the same sections. The central effect of DuP 753 is therefore highly selective for AT_1 sites.

Although DuP 753 does not cross the blood brain barrier after oral administration of single dose of the drug (Wong *et al.*, 1990a), the inhibition of Ang II receptor binding in rat brain by intravenously administered DuP 753 strongly suggests that, unlike peptide antagonists or converting enzyme inhibitors, DuP 753 and/or its active metabolites can readily cross the blood brain barrier and exert potent and selective inhibition of AT_1 receptors outside or within the blood brain barrier. Whether this effect is mediated by DuP 753 or its active metabolites, such as EXP 3174 (Wong *et al.*, 1990b), remains to be further investigated; however, the current results are relevant to predicting and understanding the central actions of these new classes of Ang II antagonists.

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Differential actions of platelet-activating factor (PAF) receptor antagonists on the vasodilator and vasoconstrictor effects of PAF in the rat perfused heart

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Selectivity for blocking the coronary vasodilator and vasoconstrictor effects of platelet-activating factor (PAF) in the rat perfused heart was observed with different PAF antagonists. CV-6209 showed selectivity for blocking the vasodilator effect of PAF and a higher concentration (10 fold) was required to block the vasoconstrictor effect. The remaining PAF antagonists (FR-900452, WEB 2086 and BN-50739) showed selectivity for blocking the vasoconstrictor effect of PAF (10, 200 and 1000 fold respectively). A combination of low concentrations of CV-6209 (10 nM) with FR-900452 (5 μM) or WEB 2086 (0.5 μM) was effective in blocking both the vasodilator and vasoconstrictor effects of PAF. CV-6209 and WEB 2086 did not affect the vasodilator action of leukotriene B_4 (LTB_4) and the vasoconstrictor action of LTC_4 and LTD_4. Our results support the hypothesis that the functionally opposite effects of PAF in the rat perfused heart may be mediated by different PAF receptor subtypes.

Keywords: PAF receptor subtypes; vasodilatation; vasoconstriction; PAF antagonists

Introduction Platelet-activating factor (PAF) has been shown to possess a variety of biological activities and is a potent activator of platelet aggregation (Braquet *et al.*, 1987). The predominant coronary vascular effect of PAF is vasoconstriction (Piper & Stewart, 1986). However, vasodilatation, vasoconstriction and the combination of both responses could be observed in the rat perfused heart depending on the experimental conditions and the amount of PAF (Man *et al.*, 1990). These results may be explained by the existence of two PAF receptor subtypes (Hu *et al.*, 1991). The activation of the first subtype of PAF receptor produces vasodilatation while the activation of the second subtype of PAF receptor produces vasoconstriction. In the present study, the ability of different PAF antagonists to block the coronary vasodilator and vasoconstrictor effects of PAF selectively in the rat perfused heart were examined.

Methods Following cervical dislocation, hearts from Sprague-Dawley rats (250–350 g) were excised and the aorta was cannulated for coronary perfusion. The Krebs-Henseleit solution had the following composition (mM): NaCl 120, NaH_2PO_4 1.18, $MgSO_4$ 1.18, KCl 4.76, $CaCl_2$ 1.25, $NaHCO_3$ 25.0 and glucose 5.5, and was oxygenated with 95% O_2:5% CO_2. A 10 min equilibration period with normal Krebs-Henseleit solution was followed by 10 min of pretreatment with a solution containing a PAF antagonist. The flow rate was adjusted to maintain a perfusion pressure of 65–70 mmHg before the injection of PAF and the flow rate was kept constant for the rest of the experiment (Man *et al.*, 1990).

CV-6209 (2[N-acetyl-N-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)-aminomethyl]-1-ethylpyridinium chloride) was dissolved in saline (0.9% NaCl) containing 0.25% bovine serum albumin (BSA, Sigma Chemical Co.). FR-900452 (1-methyl-3-(1-(5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidene)-2-piperazinyl)ethyl)-2-indolinone) was dissolved in ethanol then diluted in Krebs-Henseleit solution to a final concentration of 1–50 μM in 0.1% ethanol. 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 dissolved in saline. BN-50739 (tetrahydro-4,7,8,10 methyl-(chloro-2-phenyl)6[(dimethoxy-3,4-phenyl)thio] methythiocarbonyl-9 pyrido[4'3'-4,5]thieno[3,2-f] triazolo-1,2,4[4,3-a] diazepine-1,4) was dissolved in dimethyl sulphoxide (DMSO) and slowly added to warm (50°C) Krebs-Henseleit solution while stirring constantly to a maximum final concentration of 10 μM in 0.4% DMSO. PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (prepared from bovine heart, Sigma Chemical Co.), was prepared fresh daily in saline with 0.25% BSA. Only one injection of PAF was given to each heart. Bolus injections of 100 pmol PAF were given in a volume of 0.1 ml and over a 1 s period, into the perfusion line 5–6 cm proximal to the aortic cannula. This amount of PAF elicited a reproducible vasodilatation followed by a vasoconstriction (Man *et al.*, 1990). LTB_4, LTC_4 and LTD_4 were prepared daily and were administered by continuous infusion (300 pmol min^{-1}).

Results Pretreatment with low concentrations of the PAF antagonist CV-6209 (10 and 50 nM) were effective in blocking the vasodilator effect of 100 pmol PAF and a higher concentration (100 nM) was required to abolish the vasoconstrictor effect (Table 1). Pretreatment with the other PAF antagonists showed a reverse pattern. The vasoconstrictor effect of PAF was abolished by a low concentration of FR-900452 (5 μM), WEB 2086 (0.5 μM) and BN-50739 (0.01 μM) while higher concentrations (50 μM FR-900452, 100 μM WEB 2086 and 10 μM BN-50739) were required to completely abolish the vasodilator effect (Table 1). When the vasodilator effect of PAF was abolished by a low concentration of FR-900452, WEB 2086 or BN-50739, a significantly greater vasodilator effect was observed. Pretreatment with 0.1% ethanol and 0.4% DMSO (the vehicle for FR-900452 and BN-50739 respectively) did not affect the effects of 100 pmol PAF. Bolus injections of 0.1 ml saline did not show any detectable coronary vascular effect. A combination of low concentrations of CV-6209 (10 nM) with FR-900452 (5 μM) or WEB 2086 (0.5 μM) was effective in blocking both the vasodilator and vasoconstrictor effects of 100 pmol PAF in the rat perfused heart (6 and 5 experiments, data not shown). Using the data from Table 1, approximately 10 fold selectivity for blocking the vasodilator effect of PAF than for the vasoconstrictor effect of PAF was observed for CV-6209 while a 10, 200 and 1000 fold selectivity for blocking the vasoconstrictor effect were observed for FR-900452, WEB 2086 and BN-50739. The effects of PAF antagonists on leukotriene responses were evaluated in hearts with and without pretreatment with CV-6209 and WEB 2086. With no pretreatment, infusion of LTB_4 (300 pmol min^{-1}) produced vasodilatation (peak decrease in perfusion pressure,

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Table 1 Effects of the PAF antagonists, CV6209, FR-900452, BN-50739 and WEB 2086, on the vasodilator and vasoconstrictor effects of 100 pmol PAF in the rat perfused heart

	Concentration	*Peak vasodilator effect* (mmHg)[a]	*Peak vasoconstrictor effect* (mmHg)[a]	n
CV-6209	0	−8.4 ± 2.2	29.2 ± 5.8	5
	10 nM	−2.2 ± 1.3**[b]	36.0 ± 9.7	5
	50 nM	−1.2 ± 2.1**	31.0 ± 15.0	5
	100 nM	−2.4 ± 1.9**	1.4 ± 1.9**[b]	5
FR-900452	0	−7.8 ± 1.7	26.3 ± 9.0	6
	1 μM	−13.6 ± 4.7*	23.8 ± 11.8	5
	3 μM	−15.3 ± 2.7*	20.8 ± 5.8	6
	5 μM	−14.5 ± 7.6*	NC[b]	6
	30 μM	−7.5 ± 4.9	NC	6
	50 μM	−1.6 ± 1.5*[b]	0.8 ± 2.0**	5
WEB 2086	0	−9.8 ± 3.9	24.3 ± 4.5	6
	0.1 μM	−15.0 ± 1.4	9.0 ± 3.6**	4
	0.5 μM	−18.4 ± 3.9*	NC[b]	5
	1 μM	−24.8 ± 6.0**	NC	6
	10 μM	−26.6 ± 5.5**	NC	4
	30 μM	−11.0 ± 2.9	0.3 ± 0.5**	5
	50 μM	−12.7 ± 4.0	0.3 ± 0.6**	4
	100 μM	−2.5 ± 2.1*[b]	0.2 ± 0.4**	6
BN-50739	0	−9.8 ± 4.6	23.4 ± 8.1	5
	0.001 μM	−11.7 ± 3.2	31.3 ± 2.1**	5
	0.01 μM	−15.4 ± 4.0*	0.8 ± 1.8**[b]	5
	0.1 μM	−18.0 ± 4.5**	0.3 ± 0.7**	8
	1 μM	−7.0 ± 1.4	NC	5
	5 μM	−3.0 ± 0.4**	NC	5
	10 μM	−0.3 ± 0.6**[b]	0.3 ± 0.5**	5

Values represent mean ± standard deviation, n = number of experiments. NC denotes no detectable change in perfusion pressure. Statistical analyses were performed by analysis of variance followed by Duncan's test. * $P < 0.05$ and ** $P < 0.01$ when compared to the appropriate data in the absence of the PAF antagonist.

[a] With constant flow perfusion, decrease in perfusion pressure represents vasodilatation (negative value), and increase in perfusion pressure represents vasoconstriction.

[b] Since PAF antagonists did not produce a progressive transition of blocking the vasodilator or vasoconstrictor effects of PAF, the lowest concentration that reduced each effect completely was chosen for the calculation of selectivity for the vasodilator and vasoconstrictor effects.

-11.5 ± 2.2 mmHg, $n = 6$) while LTC_4 and LTD_4 produced vasoconstriction (peak increase in perfusion pressure, 27.8 ± 4.5 and 26.3 ± 1.5 mmHg, $n = 6$ and 6 respectively) in the rat perfused heart. CV-6209 (100 nM) and WEB 2086 (100 μM) did not significantly affect the vasodilator effect of LTB_4 (-11.0 ± 1.8 and -10.2 ± 1.1 mmHg, $n = 4$ and 5 respectively) and the vasoconstrictor effect of LTC_4 (20.6 ± 4.4 and 35.6 ± 10.5 mmHg, $n = 5$ and 5 respectively) and LTD_4 (24.0 ± 1.6 and 31.8 ± 2.1 mmHg, $n = 4$ and 5 respectively).

Discussion The present study has demonstrated that functionally opposite vasodilator and vasoconstrictor effects of a single concentration of PAF could be selectively abolished by different PAF antagonists in the perfused heart. These results are compatible with the concept of existence of PAF receptor subtypes. Selectivity for blocking the vasodilator effect of PAF was observed with CV-6209 and selectivity for blocking the vasoconstrictor effect was observed with FR-900452, WEB 2086 and BN-50739. The presence of PAF receptor subtypes can also explain the different time course and the threshold amount of PAF for the PAF-induced vasodilatation and vasoconstriction in the heart (Man *et al.*, 1990).

The existence of PAF receptor subtypes has been postulated in a number of studies using different cell types in the same or different animal species. Based on the lower affinity of kadsurenone for pig peripheral leukocyte aggregation than for PAF-induced chemiluminescence of guinea-pig peritoneal macrophages (91 fold difference in pA_2 values), Lambrecht & Parnham (1986) suggested the presence of PAF receptor subtypes (named PAF_1 and PAF_2 receptors). Differences in potency (6–10 times) of ONO-6240 on PAF receptor binding and PAF-induced aggregation in human leukocytes and platelets also supported the presence of PAF receptor subtypes (Hwang, 1988). A dissociation between PAF-induced superoxide anion generation (high concentration of PAF, μM range), and intracellular degranulation and peroxidase release (low concentration of PAF, nM range) in guinea-pig eosinophils were observed (Kroegel *et al.*, 1989). High and low concentrations of WEB 2086 were also required to block these PAF-induced changes. However, different ionic conditions were present in the test systems because PAF-induced superoxide production was Mg^{2+}-dependent while PAF-induced peroxidase release was Ca^{2+}-dependent. Hence, they concluded that their results could be explained on the basis of two PAF receptor subtypes or a single receptor that can exist in low and high affinity states (Kroegel *et al.*, 1989). Indeed, Hwang *et al.* (1989) demonstrated that the conformation and affinity of the PAF receptor in rabbit platelet membranes could be regulated by the ionic environment, including Ca^{2+} and Mg^{2+} concentrations.

However, PAF receptors conformational change cannot fully explain data that showed selectivity of PAF antagonists on receptor binding in similar ionic environments (Hwang, 1988). Furthermore, the effects of the PAF antagonists, WEB 2086, L-652,731 and BN-52021, on the PAF-induced chemiluminescence and prostacyclin generation by guinea-pig resident peritoneal macrophages and on pig peripheral blood leukocyte and platelet aggregation also showed significant differences in pA_2 values and the rank order of potency of PAF antagonists between macrophages, and platelets and leukocytes (Stewart & Dusting, 1988). In this study, PAF antagonists showed selectivity for blocking the vasodilator and vasoconstrictor effects of PAF in the perfused heart and thereby provided further evidence for the existence of PAF receptor subtypes. Unlike previous studies where receptor subtypes exist in different cell types, our data suggest that receptor subtypes can exist in the same organ and initiate opposite effects although findings with different concentra-

tions of PAF need to be evaluated. However, definitive proof for the presence of PAF receptor subtypes awaits the purification and sequencing of these receptors. Moreover, the role of these putative PAF receptor subtypes in the heart and the functionally opposite vasodilator and vasoconstrictor effects in the regulation of coronary flow remains to be elucidated.

It has been proposed that the coronary vasodilator effect of PAF is mediated by LTB_4 whereas the vasoconstrictor effect of PAF is mediated by LTC_4 and LTD_4 (Hu *et al.*, 1991). It is possible that non-specific actions of the PAF antagonists may influence the leukotrienes-mediated vasodilator and vasoconstrictor effects of PAF. However, our results showed that the coronary vascular effects of leukotrienes were not significantly altered by CV-6209 and WEB 2086. Hence, the selectivity of PAF antagonists on the vasodilator and vasoconstrictor effects of PAF is not likely due to non-specific actions.

Our results showed that if the action of PAF involved multiple receptor subtypes, much higher concentrations of a single PAF antagonist would be required. However, our data suggest that it is possible to combine low concentrations of two selective PAF antagonists to produce the same effect, thus eliminating the need for high concentrations. This may be particularly noteworthy if high concentrations of PAF antagonists are associated with undesirable side effects.

The generous supplies of the PAF antagonists, CV-6209 by Takeda Chemical Industries, Japan, FR-900452 by Fujisawa Pharmaceutical Co., Ltd., Japan, WEB 2086 by Boehringer Ingelheim KG, Federal Republic of Germany and BN-50739 by Institut Henri Beaufour, France are greatly appreciated. We also wish to thank Dr. Ford-Hutchinson of Merck Frosst Canada Inc. for the supply of LTB_4, LTC_4 and LTD_4. This study was supported by the MRC of Canada.

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Neuropeptide Y, peptide YY and C-terminal fragments release histamine from rat peritoneal mast cells

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1 Neuropeptide Y (NPY) and peptide YY (PYY) seem to act on at least two receptor sybtypes, Y_1 and Y_2. The Y_1-receptor requires the whole C-terminally amidated NPY/PYY molecule whereas the Y_2-receptor in addition recognizes C-terminal fragments of the two peptides. The present study was designed to elucidate whether NPY and related peptides were able to release histamine from isolated peritoneal mast cells of the rat.

2 NPY, NPY 15-36, NPY 22-36, NPY 26-36 and desamido-NPY evoked a concentration-dependent release of mast-cell histamine. The pEC_{15} values for NPY 15-36 and NPY 22-36 were higher, while the pEC_{15} value for NPY 26-36 was lower than that for NPY. At the highest concentration tested (0.1 mM), NPY and its C-terminal fragments released between 30 and 40% of the total histamine content. At the same concentration desamido-NPY released about 20%.

3 PYY and PYY 15-36 also evoked a concentration-dependent release of mast-cell histamine. PYY was more effective than PYY 15-36 since, at 0.1 mM, PYY released about 33%, while PYY 15-36 released about 15% of the total histamine content. Pancreatic polypeptide (PP) and the Y_1-receptor-selective agonist [Pro^{34}]NPY were virtually inactive.

4 The effect profile of the NPY/PYY-related peptides suggests that they act on the mast cells by a mechanism that does not involve either of the receptor subtypes hitherto described. The kinetics of the NPY-evoked histamine release may suggest that positively charged amino acid residues of NPY/PYY release mast-cell histamine by a non-receptor mechanism, as has been suggested for substance P and other basic peptides.

Keywords: Neuropeptide Y; peptide YY; C-terminal fragments; histamine release; mast cells of rat

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide with a wide distribution in the peripheral nervous system (Lundberg *et al.*, 1982b). It is found in many sympathetic fibres, notably around blood vessels (Ekblad *et al.*, 1984; Uddman *et al.*, 1985; Sundler *et al.*, 1986). Peptide YY (PYY) is a gut hormone (Lundberg *et al.*, 1982a; Böttcher *et al.*, 1984) that displays 70% homology with NPY. NPY and PYY seem to act on at least two receptor subtypes. The Y_1-receptor is located postjunctionally and is able to evoke vasoconstriction, whereas the Y_2-receptor seems to inhibit transmitter release (for a review see Wahlestedt *et al.*, 1990). We have recently shown that NPY elicits a biphasic blood pressure response (pressor response followed by a depressor response) in the anaesthetized rat, and that the depressor response is abolished by pretreatment with a histamine H_1-antagonist or with the histamine liberator compound 48/80, suggesting that the depressor response reflects release of mast cell histamine (Grundemar *et al.*, 1990). Similarly, recent data suggest that the depressor response evoked by various C-terminal NPY fragments is also abolished by pretreatment with histamine H_1-receptor antagonists (Wahlestedt *et al.*, 1990).

In the present study we examined whether NPY, PYY or C-terminal fragments of NPY and PYY are able to release histamine from rat peritoneal mast cells.

Methods

Adult male Sprague-Dawley rats (freely fed, body weight 300–350 g) were killed by decapitation. Peritoneal cells, consisting of 3–6% mast cells (Padawer, 1963; Kurose & Saeki, 1981), were obtained by peritoneal lavage with 10 ml buffered salt solution (BSS), containing NaCl 145 mM, KCl 2.7 mM and 10% (v/v) Sörensen phosphate buffer ($Na_2HPO_4 + KH_2PO_4$, 67 mM, pH 7.0). After gentle abdominal massage for 5 min, the intra-abdominal mast cell-rich fluid was removed with a pipette. The abdominal fluid was centrifuged at approximately 300 *g* for 5 min at room temperature. The precipitated cells were resuspended in 10 ml BSS to which was added 1 mg ml^{-1} bovine serum albumin (BSA) (Armour, Kankakee, IL, U.S.A.). The cell suspension was washed twice with BSA. Finally, the cells were suspended in 20 ml BSA at room temperature and aliquots of 90 μl were transferred to polypropylene test tubes and preincubated for 5 min at 37°C before adding the peptide in a volume of 10 μl BSS. Each individual experiment included the concentration range of each peptide. The mixture was incubated for 5 min at 37°C and the reaction was interrupted by placing the samples in ice. They were then centrifuged at 600 *g* for 5 min at 4°C. Aliquots (0.1 ml) of the supernatant were taken for direct fluorometric assay of histamine (Håkanson & Rönnberg, 1974). The sediment was suspended in 1 ml boiling redistilled water and centrifuged at 600 *g* for 5 min at 4°C. Aliquots (0.1 ml) of the supernatant were assayed for histamine. All determinations were made in duplicate. The amount of histamine released was expressed as a percentage of the total amount of histamine present in the peritoneal cells at the start of the experiment. The results were corrected for 4–5% spontaneous release.

The pEC_{15} values (the negative logarithm of the concentration that releases 15% of the total histamine content) were estimated by interpolation from the pseudo-rectilinear portions of the concentration-response curves. Results are expressed as means ± s.e.mean; *n*, is the number of independent experiments. Statistical analysis of the difference between pEC_{15} values were performed by means of two-tailed unpaired Student's *t* test and modified by the Bonferroni method for multiple comparisons (Wallenstein *et al.*, 1980). $P < 0.05/4 = 0.012$ was considered significant.

Drugs

Porcine (p) NPY, NPY 22-36, human desamido-NPY (NPY without the C-terminal amide), pPYY and rat pancreatic polypeptide (PP) were purchased from Peninsula, UK. pNPY 15-36 and pPYY 15-36 were synthesized by solid-phase synthesis and purified to at least 96% by high performance liquid

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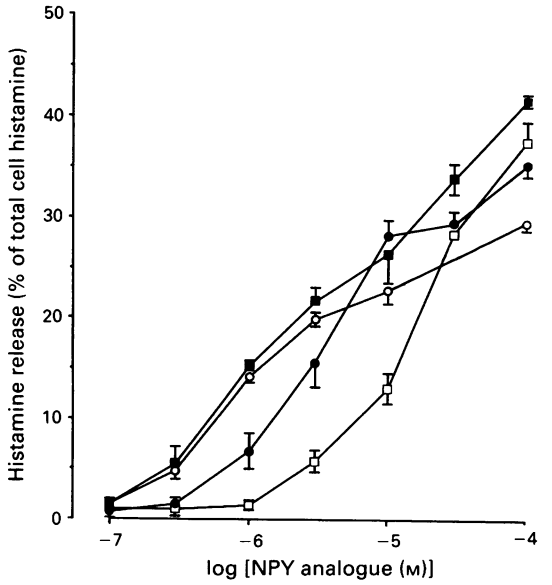


Figure 1 Concentration-response curves for histamine release from rat peritoneal mast cells evoked by neuropeptide (NPY) 1-36 (●), NPY 15-36 (○), NPY 22-36 (■) and NPY 26-36 (□). $n = 6$–9 independent experiments.

chromatography (h.p.l.c.) (W. Krzeminski, Ferring AB, Malmö, Sweden). NPY 26-36 was synthesized by solid phase synthesis and purified to at least 96% by h.p.l.c. (Dr H. Franzén, Dept. of Medical Chemistry, University of Lund, Sweden). p[Pro^{34}]NPY was a kind gift from Prof. T. W. Schwartz, Copenhagen, Denmark. Stock solutions were prepared and diluted with 0.9% saline.

Results

NPY, NPY 15-36, NPY 22-36, NPY 26-36 as well as desamido-NPY released histamine concentration-dependently from rat peritoneal mast cells (Figures 1 and 2). No concentration-response curve reached maximum. The pEC_{15} values for NPY 15-36 and NPY 22-36 were higher, while the pEC_{15} value for NPY 26-36 was lower than that for NPY (Table 1). At the highest concentration tested (0.1 mM) NPY, NPY 15-36, NPY 22-36 and NPY 26-36 released 30–40% of the total histamine content. The histamine release evoked by desamido-NPY was about 20% (Figure 2). In a series of experiments it was shown that the NPY-evoked histamine release reached maximum within 10 s (Figure 3).

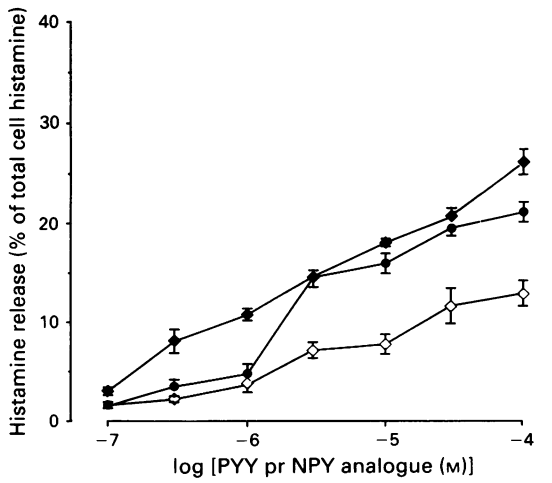


Figure 2 Concentration-response curves for histamine release from rat peritoneal mast cells evoked by peptide YY (PYY) 1-36 (◆), PYY 15-36 (◇) and desamido-NPY (●). $n = 3$–6 independent experiments.

Table 1 Histamine-releasing effect of neuropeptide Y (NPY), peptide YY (PYY), their C-terminal fragments and related peptides

	pEC_{15} values	n
NPY 1-36	5.6 ± 0.1	9
NPY 15-36	5.9 ± 0.0*	6
NPY 22-36	6.0 ± 0.1*	6
NPY 26-36	5.1 ± 0.1*	6
desamido-NPY	(+)	6
PYY	5.5 ± 0.1	5
PYY 15-36	(+)	3
[Pro^{34}]NPY	(+)	3
PP	(+)	3

Values are means ± s.e.mean from 3–6 independent experiments. * $P < 0.012$ for the difference between pEC_{15} values for analogues and fragments versus NPY 1-36. (+), histamine release less than 25% of the total histamine content at 0.1 mM peptide.

PYY and PYY 15-36 also released histamine concentration-dependently (Figure 2). The pEC_{15} values for PYY and NPY were similar (Table 1). PYY 15-36 was much less effective than PYY, since at 0.1 mM PYY released about 27%, while PYY 15-36 released about 13% of the total histamine content (Figure 2). [Pro^{34}]NPY and PP had virtually no effect. At the highest concentration tested (0.1 mM), [Pro^{34}]NPY and PP evoked a histamine release of 6.5 ± 0.3% ($n = 3$) and 7.2 ± 0.2% ($n = 3$), respectively.

Discussion

The depressor component of the biphasic blood pressure response to NPY in the anaesthetized rat can be abolished by pretreatment with histamine H_1-receptor antagonists or with the histamine liberator compound 48/80, implying that NPY is able to release histamine from a mast cell pool (Grundemar *et al.*, 1990). Similarly, several studies have shown that injections of short C-terminal NPY fragments evoke hypotension in the rat (Boublik *et al.*, 1989a, b; Scott *et al.*, 1990) and this response is also blocked by pretreatment with histamine H_1-receptor antagonists (Wahlestedt *et al.*, 1990).

The present study has demonstrated that NPY, NPY 15-36, NPY 22-36, NPY 26-36 as well as desamido-NPY concentration-dependently released histamine from rat peritoneal mast cells. Thus, the present results are in accordance with the suggestion that the hypotensive effect of NPY and its C-terminal fragments can be attributed to mast cell-dependent

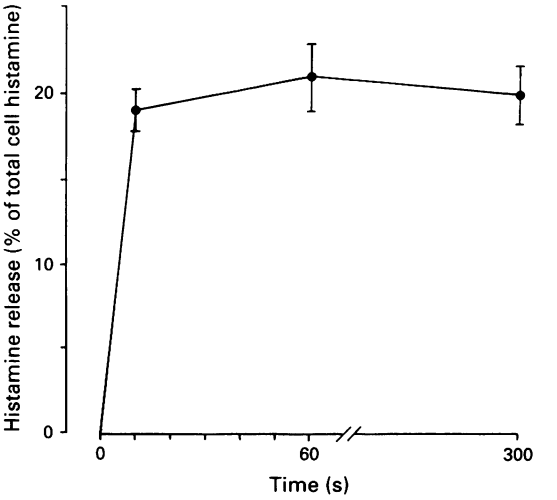


Figure 3 Time course of the neuropeptide Y (NPY)-evoked histamine release. In separate experiments NPY (10 μM, $n = 3$) was incubated for 10 s, 1 and 5 min, respectively.

release of histamine (Grundemar *et al.*, 1990; Wahlestedt *et al.*, 1990). Also PYY and PYY 15-36 evoked a concentration-dependent release of histamine, while PP and [Pro^{34}]NPY were virtually inactive.

NPY and PYY are thought to act on at least two receptor subtypes, referred to as Y_1 and Y_2 (Wahlestedt *et al.*, 1986, 1990). The Y_1-receptor seems to require the whole NPY/PYY molecule to become fully activated; whereas C-terminal fragments of the two peptides are markedly less potent (Rioux *et al.*, 1986; Wahlestedt *et al.*, 1986; Grundemar *et al.*, 1991). Recently, a selective Y_1-receptor agonist was constructed, [Pro^{34}]NPY, which seems to be equipotent with NPY on the Y_1-receptor, while having virtually no affinity to the Y_2-receptor (Krstenansky *et al.*, 1990), The Y_2-receptor recognizes not only the whole NPY/PYY molecule, but also C-terminal fragments of the two peptides. In fact, NPY 13-36/PYY 13-36 are only about one order of magnitude less potent than NPY/PYY (Wahlestedt *et al.*, 1986; Grundemar & Håkanson, 1990). Very short fragments, such as NPY 26-36/PYY 26-36 are inactive (Grundemar & Håkanson, 1990). PYY and NPY are approximately equipotent, while desamido-NPY exerts no activity on either receptor subtype (Wahlestedt *et al.*, 1986; Grundemar & Håkanson, 1990). PP displays 50% homology with NPY but has no activity on either of the Y_1-/Y_2-receptors.

The question arose as to how NPY activates mast cells to release histamine. Since NPY 15-36 and NPY 22-36 were at least as active as NPY and since the selective Y_1-receptor agonist [Pro^{34}]NPY was virtually inactive, the mast cells do not seem to express Y_1 receptors. PYY 15-36 was much less effective than either NPY or PYY, while NPY 26-36 was as effective as NPY but with a lower pEC_{15} value; these results are not in agreement with the properties of the Y_2-receptor. Moreover, desamido-NPY was active, albeit less so than the parent molecule. Taken together, the effect profile of the NPY/PYY-related peptides suggests that they release mast cell histamine by a mechanism distinct from the proposed Y_1-/Y_2-receptor subtypes. Conceivably, the positively charged amino acids of the NPY/PYY molecules activate G proteins in the mast cell membrane by a non-receptor mechanism, as has been suggested for tachykinins and other basic peptides (Mousli *et al.*, 1990). In support for a non-receptor mechanism, the kinetics of the histamine release by substance P and mastoparan are very rapid (less than 10 s) (Mousli *et al.*, 1989; 1990). Similarly, the NPY-evoked histamine release reached maximum within 10 s. Since PP and [Pro^{34}]NPY were inactive, the naturally occurring Pro^{34} in PP and the substitution of Pro^{34} in NPY could have an important conformational effect on the peptides, which may be sufficient to alter the relative disposition of the flanking arginine residues.

Whether the NPY-evoked release of mast cell histamine is physiologically relevant is unclear. Interestingly, mast cells are numerous around blood vessels and nerves (Stead *et al.*, 1989; Nilsson *et al.*, 1990). Possibly, the NPY-evoked release of mast cell histamine contributes to the sympathetic control in certain vascular beds.

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Beneficial effect of beraprost, a prostacyclin-mimetic agent, on post-hypoxic recovery of cardiac function and metabolism in rabbit isolated hearts

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1 The present study was undertaken to determine whether beraprost, a stable prostacyclin-mimetic agent, may exert a beneficial effect on post-hypoxic recovery of cardiac function and metabolism. Isolated rabbit hearts were perfused by the Langendorff method for 20 min under glucose-free hypoxic conditions, followed by 45 min reoxygenation in the presence of glucose, and their functional and metabolic changes with or without beraprost-treatment were examined.

2 Hypoxic insult induced cessation of cardiac contractile force, depletion of myocardial high-energy phosphates, accumulation of tissue calcium, and release of creatine kinase and ATP metabolites. Subsequent reoxygenation resulted in a poor recovery of cardiac contractile force (less than 10% of the pre-hypoxic value), a poor restoration of high-energy phosphates, and increase in calcium content. A further release of creatine kinase and ATP metabolites from the heart was observed during reoxygenation.

3 Treatment with 0.45 μM beraprost during the whole hypoxic period resulted in a significant suppression of the increase in tissue calcium, and the release of creatine kinase and ATP metabolites during hypoxic perfusion. This treatment also elicited a significant post-hypoxic recovery of the cardiac contractile force and the tissue high-energy phosphates. Reoxygenation-induced release of creatine kinase and ATP metabolites was also prevented by treatment with beraprost.

4 When hearts were treated with prostacyclin sodium (0.50 μM) in the same manner for the purpose of comparison, similar improvement of post-hypoxic contractile and metabolic recovery were observed.

5 These results demonstrate that treatment with either beraprost or prostacyclin is beneficial for post-hypoxic recovery of cardiac function and metabolism. Since the observed effects on post-hypoxic contractile recovery were exerted at a concentration of approximately 0.50 μM of these agents (a concentration far from the physiological range) the underlying mechanism appears to be different from the physiological action of prostacyclin.

Keywords: ATP metabolites; beraprost; calcium accumulation; cell membrane permeability; creatine kinase; high-energy phospates; hypoxia, prostacyclin; reoxygenation

Introduction

Beraprost (sodium (+)-(1R*,2R*,3aS*,8bS*)-2,3,3a,8b-tetrahydro-2-hydroxy-1-[(E)-(3S*)-3-hydroxy-4-methyl-1-octen-6-ynyl]-1H-cyclopenta[b]benzofuran-5-butyrate, TRK-100) is a stable and prostacyclin-mimetic agent that can be administered orally (Nishio *et al.*, 1985; Akiba *et al.*, 1986; Umetsu *et al.*, 1986). It induces vasodilatation and inhibition of platelet aggregation in man and experimental animals (Nishio *et al.*, 1988). By virtue of its improvement of peripheral circulation, beraprost has now been developed as a therapeutic agent for peripheral thrombosis of Raynaud's and Beurger's diseases. The agent has also been shown to improve the subjective and neurological symptoms in patients with acute and chronic cerebral infarction (Toda, 1988). However, little information is available concerning its effects on cardiac circulation and metabolism. In particular, there is no information yet concerning the effects of the agent on oxygen deficiency-induced cardiac impairments.

In an early phase of oxygen deficiency, hearts release prostanoids into their coronary circulation. De Deckere *et al.* (1977), Sakai *et al.* (1982), and Karmazyn & Dhalla (1983) have demonstrated that prostacyclin and thromboxane A_2 are the major metabolites of eicosanoids produced in the heart during oxygen-deprived perfusion. Prostacyclin has been shown to be a potent vasodilator and a platelet anti-aggregatory agent (Moncada *et al.*, 1976). In contrast, thromboxane A_2 can oppose such prostacyclin actions (Hamberg *et al.*, 1975; Schrör *et al.*, 1981b). Several investigators have shown that stable prostacyclin-mimetic agents, such as iloprost and nileprost, improved recovery of cardiac energy metabolism after ischaemia and reduced infarct size after ligation of the coronary artery (Schrör *et al.*, 1981a; Thiemermann & Schrör, 1984; De Langen *et al.*, 1985; Darius *et al.*, 1986; Pissarek *et al.*, 1987; Ferrari *et al.*, 1988; Chiariello *et al.*, 1988). Furthermore, it has been shown that thromboxane A_2 antagonists and inhibition of thromboxane A_2 synthesis are capable of reducing myocardial infarct size and inhibiting lactate release (Sakai *et al.*, 1982; Grover *et al.*, 1988). These results suggest that prostacyclin is a possible cardioprotective agent against oxygen deficiency-induced derangements in cardiac function and myocardial metabolism. In contrast, controversial results have been obtained concerning the protective effects of prostacyclin on the ischaemic myocardium. For example, prostacyclin failed to limit ischaemia-induced myocardial injury such as purine release and decline in energy charge value (Edlund *et al.*, 1986). The purpose of the present study was to determine whether beraprost, like iloprost, may improve post-hypoxic recovery of cardiac function and metabolism, and, if so, whether such cardioprotective action may be observed with prostacyclin as well.

Methods

Perfusion of rabbit isolated hearts, determination of high-energy phosphates, tissue calcium, and measurement of ATP metabolites and creatine kinase activity in the perfusate were conducted as described previously (Takeo *et al.*, 1989).

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Perfusion of hearts

Male albino rabbits, weighing 1.8 to 2.2 kg, were anaesthetized i.v. with 35 mg kg^{-1} of sodium pentobarbitone. One thousand units per kg of heparin sodium was also administered simultaneously. After thoracotomy, the rabbit heart was isolated and transferred to a non-working, non-recirculating Langendorff apparatus. The heart was perfused at 37°C with a constant flow rate of 16 ml min^{-1} with Krebs-Henseleit solution of the following composition (mM): NaCl 120, KCl 4.80, KH_2PO_4 1.20, $MgSO_4$ 1.20, $CaCl_2$ 1.25, $NaHCO_3$ 25 and glucose 11. The perfusing solution was equilibrated with a gas mixture of 95% O_2 and 5% CO_2. The heart was preloaded with an initial resting tension of 1.5 g and paced at 180 beats min^{-1}. Cardiac contractile force was estimated by monitoring isometric tension generated via a hook attached to the apex of the heart by means of a force-displacement transducer (TB-612T, Nihonkohden, Tokyo, Japan). Perfusion pressure was monitored through an aortic cannula adjusted just prior to the aortic valve by means of a pressure transducer (TP-101, Nihonkohden, Tokyo, Japan). Changes in contractile force, resting tension and perfusion pressure were displayed on a thermal pen recorded (TB-647G, Nihonkohden, Tokyo, Japan). After 25 min of perfusion with normal Krebs-Henseleit solution (equilibration period), hypoxic perfusion was begun with the Krebs-Henseleit solution without glucose. The glucose was replaced with 11 mM Tris/HCl to reduce rapidly the substrate for anaerobic glycolysis. This perfusing solution was equilibrated previously with a gas mixture of 95% N_2 and 5% CO_2 (hypoxic conditions). Either beraprost or prostacyclin in a concentration of 0.45 or 0.50 μM respectively was infused at an infusion rate of 0.1 ml per min through a needle inserted just prior to the aortic cannula by means of an infusion pump (MP-3B, Tokyorikakikai, Tokyo, Japan), during the whole hypoxic period (20 min). After 20 min of hypoxic perfusion, the heart was perfused for 45 min with Krebs-Henseleit solution saturated with a gas mixture of 95% O_2 and 5% CO_2 in the presence of 11 mM glucose (reoxygenated conditions). The heart was not paced during the initial 10 min of reoxygenation, but thereafter it was stimulated by pacing at the rate of 180 beats min^{-1} as described above. The Po_2 values of the hypoxic and reoxygenated perfusion buffers, measured by means of a blood gas analyzer (DMS-MK2, Denmark Radiometer, Denmark), were 23 to 27 mmHg and 620 to 645 mmHg, respectively. After perfusion under hypoxic or reoxygenated conditions, the hearts were removed from the perfusion apparatus and then examined biochemically.

Sixty rabbit hearts were perfused under the following conditions:

(A) four hearts were perfused for 25 min for equilibration in the presence of glucose (the hearts at 0 min of perfusion); (B) four hearts were perfused for 20 min under glucose-free normoxic conditions; (C) four hearts were perfused under the same conditions as those in (B) but with beraprost-treatment; (D) four hearts were perfused under the same conditions as those in (B) but with prostacyclin-treatment; (E) five hearts were perfused for 20 min under glucose-free hypoxic conditions; (F) four hearts were perfused under the same conditions as those in (E) but with beraprost-treatment; (G) four hearts were perfused under the same conditions as those in (E) but with prostacyclin-treatment; (H) five hearts were perfused for 20 min under glucose-free normoxic conditions, followed by 45 min normoxic reperfusion in the presence of glucose; (I) five hearts were perfused under the same conditions as those in (H) but with beraprost-treatment during 20 min-normoxic perfusion; (J) five hearts were perfused under the same conditions as those in (H) but with prostacyclin-treatment during 20 min-normoxic perfusion; (K) six rabbit hearts were perfused for 20 min under glucose-free hypoxic conditions, followed by 45 min of reoxygenation in the presence of glucose; (L) five hearts were perfused under the same conditions as those in (K) but with beraprost-treatment during 20 min-hypoxic perfusion; (M) five rabbit hearts were perfused under the same conditions as those in (K) but with prostacyclin-treatment during 20 min-hypoxic perfusion.

High-energy phosphate content of the myocardium

After the equilibrating perfusion, hypoxic perfusion or hypoxic/reoxygenated perfusion, small apical portions of left ventricular muscle were quickly cut off with scissors and immersed in liquid nitrogen within 0.5 s. After being left for 30 min in liquid nitrogen, the frozen tissues were weighed and then pulverized in a stainless steel tube with a stainless steel plunger under liquid nitrogen cooling. High-energy phosphate compounds in the frozen tissue were extracted with 0.3 M $HClO_4$ + 0.25 mM disodium ethylenediaminetetraacetate (EDTA). The extract was centrifuged at 1000 *g* for 15 min, and then neutralized with 2.5 M K_2CO_3. The resulting supernatant fluid was used as a sample for determination of adenosine 5'-triphosphate (ATP) and creatine phosphate (CP). Measurement of ATP content was carried out by enzymatic methods (Bücher, 1947). CP content was determined according to the methods of Lowry & Passonneau (1972). To determine the ratio of dry tissue weight to frozen tissue weight, pieces of frozen tissue were weighed and dried at 120°C for 15 h. Then, the dried tissue weights were estimated.

Tissue calcium and water content

After perfusion, 20 ml of the washing solution containing 320 mM sucrose and 20 mM Tris/HCl, pH 7.4 was infused via the aortic cannula to eliminate the perfusing solution from the vascular space. About 100 mg of the left ventricular muscle was sampled for determination of calcium and water content of myocardium. The tissue was cut into four pieces, weighed and dried at 120°C for 15 h. After estimating the dry tissue weight, the myocardium was digested with conc. HNO_3 at 180°C. After digestion, calcium was extracted from the residual tissue with 0.75 N HNO_3 (sample solution). The sample solution for calcium determination was diluted twice with 10 mM $LaCl_3$-0.1 N HCl. The ion content was analyzed by an atomic absorption spectrophotometer (AA-646, Shimazu, Kyoto, Japan). The determination was carried out in triplicate.

Analysis of perfusate eluted from the heart

During the perfusion, the perfusate from the isolated heart was collected and analyzed to determine the net amount of ATP metabolites by means of a high-performance liquid chromatographic analyzer (h.p.l.c.) (L-6000 pump, Hitachi, Tokyo, Japan). ATP metabolites released from the heart were separated through a ODS-cellulose acetate column 4.6 mm in diameter and 15 cm in length (Nakarai Tesque Cosmosil $5C_{18}$, Kyoto, Japan) by elution with 250 mM $NH_4H_2PO_4$ and 4% CH_3CN at a flow rate of 1 ml min^{-1}. Absorbance of the eluate was monitored at 254 nm with an ultraviolet-detector (L-4000, Hitachi, Tokyo, Japan).

Creatine kinase activity of the perfusate

The perfusate was collected during hypoxia and subsequent reoxygenation, respectively. Creatine kinase activity of the perfusate was measured according to the methods of Bergmeyer *et al.* (1970).

Statistics

Results are expressed as the mean ± s.e.mean. Statistical significance between two groups was calculated by either Student's or Welch *t* test, whereas that between three groups, was by analysis of variance, followed by Dunnett's *t* test. Results with a probability of 5% or less were considered to be statistically significant ($P < 0.05$).

Results

Changes in cardiac function of perfused hearts subjected to hypoxia and subsequent reoxygenation

Cardiac contractile force generated with the initial resting tension of 1.5 g was 5.4 ± 0.3 g (n = 60) at 0 min of perfusion (i.e. after the 25 mm equilibration period).

In the first set of experiments, different concentrations of either beraprost or prostacyclin were applied to the perfused heart and their post-hypoxic contractile recovery was monitored to elucidate optimal concentrations for beneficial effects of beraprost and prostacyclin. As shown in Figure 1, the doses which improved post-hypoxic contractile recovery ranged from 0.015 to 1.50 μM for beraprost and 0.018 to 1.80 μM for prostacyclin, respectively (0.1 to 10 μg min^{-1}). We found the maximal or submaximal effective dose with the lowest concentrations to be 0.45 μM of beraprost or 0.50 μM of prostacyclin, respectively. These concentrations were used in the following experiments.

The time course of changes in cardiac contractile force, resting tension and perfusion pressure with and without either beraprost- or prostacyclin-treatment is shown in Figures 2, 3 and 4, respectively.

Contractile function

Contractile force of control hearts decreased to about 75% of the initial value after 20 min of glucose-free normoxic perfusion. However, it recovered almost completely during subsequent 45 min normoxic perfusion in the presence of glucose (more than 95% recovery of the initial value). Cardiac contractile force was not affected by treatment with any concentration of beraprost or prostacyclin during glucose-free, 20 min-normoxic perfusion or during glucose-containing 45 min-normoxic reperfusion (data not shown).

When the hearts were changed to hypoxic perfusion, cardiac contractile force immediately decreased and reached nearly zero after 7 to 10 min of hypoxic perfusion. Reoxygenation of the hearts pre-exposed to hypoxic medium for 20 min resulted in less than 10% recovery of the contractile force (Figure 2).

The hearts which had received either beraprost or prostacyclin during hypoxic perfusion showed a marked recovery of contractile force at 45 min reoxygenation (Figure 2); recovery with beraprost was approximately 60% and that with prostacyclin, 40% of the prehypoxic value.

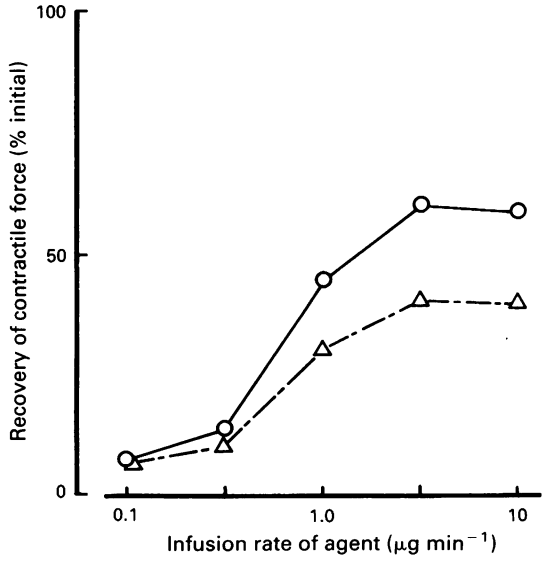


Figure 1 Concentration-dependency of the effects of beraprost (○) and prostacyclin (△) on recovery of cardiac contractile force upon reoxygenation. These agents were administered from 0 to 20 min of hypoxic perfusion. Each value represents the mean value of two experiments.

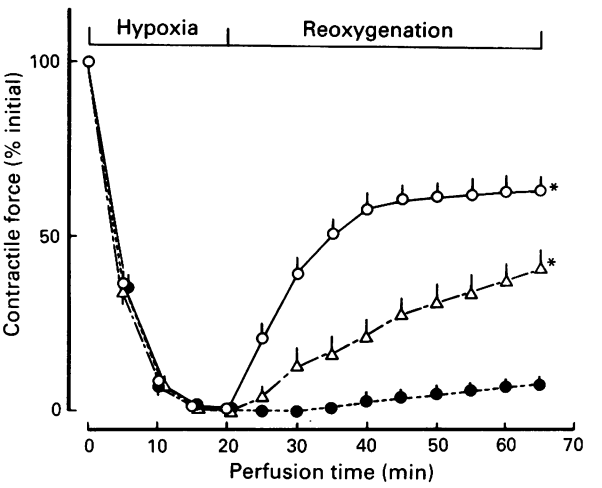


Figure 2 Changes in cardiac contractile force of hearts subjected to 20 min hypoxia and 45 min subsequent reoxygenation with either beraprost (○, n = 9 and 5, respectively) or prostacyclin (△, n = 9 and 5, respectively) treatment and without any treatment during hypoxic insult (●, n = 11 and 6, respectively). Statistical significance between the treated and non-treated groups was evaluated from the values at 20 min-hypoxia and at 45 min-reoxygenation. Significantly different from the value for hearts without treatment *P < 0.05.

In a preliminary study, we also examined periods of beraprost-administration. It was found that recovery of cardiac function and myocardial metabolism of hearts treated with either beraprost or prostacyclin during all perfussion periods, pre-perfusion and hypoxia, or, hypoxia and reoxygenation was substantially similar to that of hearts treated during hypoxia alone. Treatment with the agents either during pre-perfusion alone or during reoxygenation alone recovered cardiac contractile force by less than 10% of the prehypoxic value (data not shown). These results suggest that cardiac impairments, which could be reversed by treatment with prostacyclin and a prostacyclin-mimetic agent, occur during hypoxic perfusion.

Resting tension and perfusion pressure

The prehypoxic value of perfusion pressure was 22.0 ± 0.9 mmHg (n = 60).

There were no significant changes in resting tension and perfusion pressure of hearts during 20 min glucose-free, normoxic perfusion and subsequent 45 min normoxic reperfusion. Treatment of normoxic hearts with either beraprost or prostacyclin did not elicit any change in resting tension and perfusion pressure during 20 min-normoxic perfusion and subsequent 45 min-normoxic reperfusion. Treatment of normoxic hearts with either beraprost or prostacyclin did not elicit significant change in resting tension and perfusion pressure.

When hearts were subjected to hypoxic perfusion, the rise in resting tension occurred about 10 min after the onset of hypoxia, and reached 10.0 ± 0.9 g at 20 min-hypoxia (Figure 3). A further rise in resting tension was observed during the first 10 min of reoxygenation. Although it gradually declined thereafter, the resting tension still remained at a level much higher than the prehypoxic level throughout reoxygenation (6 fold higher than the prehypoxic value).

When the hearts were subjected to hypoxic perfusion, an immediate decline in perfusion pressure was observed. After 5 min of hypoxic perfusion, perfusion pressure maximally declined to 17.2 ± 0.6 mmHg. Thereafter, perfusion pressure was increased. At 20 min of hypoxic perfusion, perfusion pressure increased approximately 2 fold higher than the prehypox-

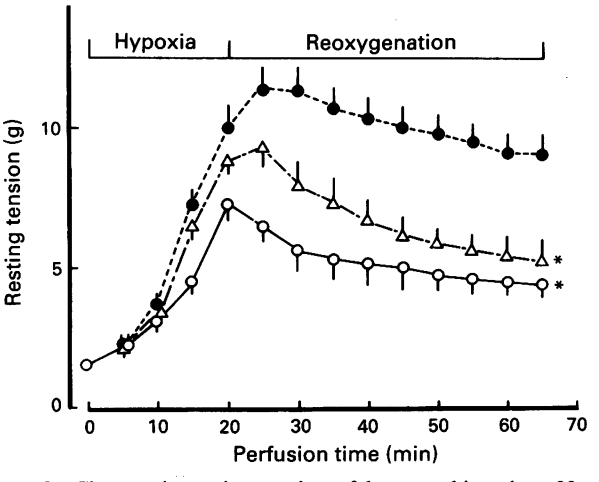


Figure 3 Changes in resting tension of hearts subjected to 20 min hypoxia and 45 min subsequent reoxygenation with either beraprost (○, $n = 9$ and 5, respectively) or prostacyclin (△, $n = 9$ and 5, respectively) treatment and without any treatment during hypoxic insult (●, $n = 11$ and 6, respectively). Statistical significance between the treated and non-treated groups was evaluated from the values at 20 min-hypoxia and at 45 min-reoxygenation. Significantly different from the value for hearts without treatment: $^*P < 0.05$.

ic value. The perfusion pressure remained at a high level throughout reoxygenation (Figure 4).

When the hypoxic hearts were treated with either 0.45 μM beraprost or 0.50 μM prostacyclin, the hypoxia-induced rise in resting tension was significantly suppressed as shown in Figure 3. Similarly, the hypoxia-induced increase in perfusion pressure was significantly suppressed in hearts treated with beraprost, but not with prostacyclin (Figure 4).

Tissue high-energy phosphate content

After 0 min-, 20 min- and 65 min-perfusion, myocardial high-energy phosphate content was determined. The results are shown in Figure 5. The initial values for ATP and CP content (at 0 min-perfusion) were 25.5 ± 0.3 and 39.4 ± 0.4 μmol g^{-1} dry tissue, respectively. When the heart was perfused for 20 min under glucose-free normoxic conditions, myocardial ATP and CP content decreased to 80% and 45% of the initial

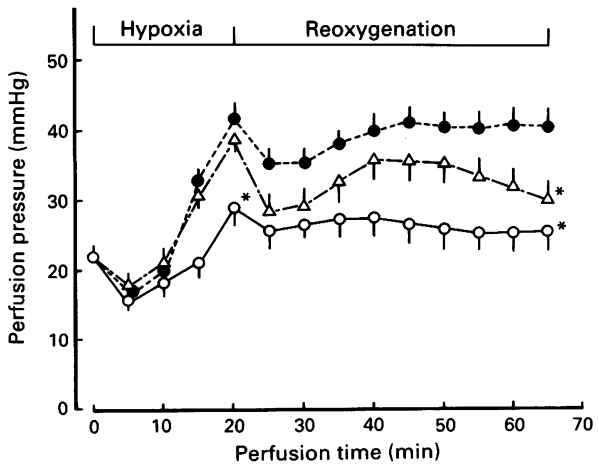


Figure 4 Changes in perfusion pressure of hearts subjected to 20 min hypoxia and 45 min subsequent reoxygenation with either beraprost (○, $n = 9$ and 5, respectively) or prostacyclin (△, $n = 9$ and 5, respectively) treatment and without any treatment during hypoxic insult (●, $n = 11$ and 6, respectively). Statistical significance between the treated and non-treated groups was evaluated from the values at 20 min-hypoxia and at 45 min-reoxygenation. Significantly different from the value for hearts without treatment $^*P < 0.05$.

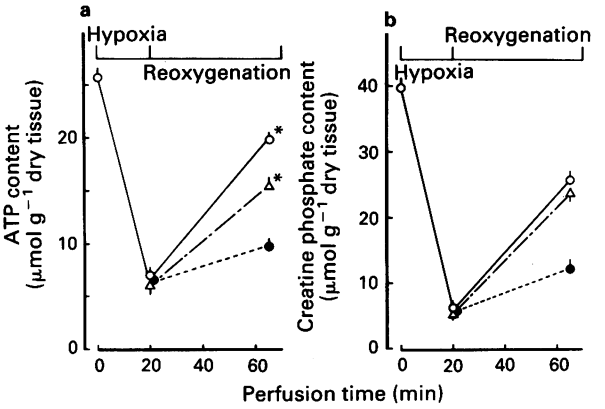


Figure 5 Changes in myocardial ATP (a) and creatine phosphate (b) content of hearts subjected to 20 min hypoxia and 45 min subsequent reoxygenation with either beraprost (○, $n = 4$ and 5, respectively) or prostacyclin (△, $n = 4$ and 5, respectively) treatment and without any treatment during hypoxic insult (●, $n = 5$ and 6, respectively). Significantly different from the value for hearts without treatment $^*P < 0.05$.

value, probably due to a lack of glucose which is the sole exogenous substrate for production of high-energy phospates in the perfused heart. Subsequent 45 min normoxic perfusion in the presence of glucose resulted in an almost complete restoration of the tissue ATP content, whereas the CP content was restored to approximately 90% of the initial value. There were no significant changes in ATP and CP content of hearts at 20 min-normoxia or subsequent 45 min-reperfusion irrespective of treatment with either beraprost or prostacyclin.

Myocardial ATP and CP content at 20 min of hypoxia decreased to approximately 25 and 20% of each initial value, respectively (Figure 6). Subsequent 45 min reoxygenation restored 40% of the initial value for ATP and 35% for CP content. Treatment with either beraprost or prostacyclin during hypoxia did not preserve a higher content of ATP or CP. However, the hearts treated with beraprost significantly restored ATP content upon reoxygenation (80% of the prehypoxic value). The CP content of hearts treated with beraprost, was also restored significantly (65% of the initial value). When the heart had been treated with prostacyclin during hypoxia, restoration of high-energy phosphates was also observed after reoxygenation (65% of the prehypoxic value for both ATP and CP).

Calcium and water content of myocardium

The myocardial calcium content was determined in hearts perfused under normoxic, hypoxic and hypoxic/reoxygenated conditions. The initial value of tissue calcium was 2.50 ± 0.15 μmol g^{-1} of dry tissue. There were no significant differences in calcium content at 20 and 65 min during normoxic perfusion, regardless of treatment with either beraprost or prostacyclin. The tissue calcium content was significantly increased after 20 min hypoxia compared with the pre-hypoxic values (Figure 6). The tissue calcium content was further increased by reoxygenated perfusion. When hearts were treated with either beraprost or prostacyclin, the hypoxia- and reoxygenation-induced increase in calcium content was significantly suppressed.

To express myocardial metabolite content in dry weight, the ratios of dry weight to wet weight were determined. The mean value for this ratio was $21.5 \pm 0.4\%$ ($n = 60$). There were no differences in the ratios between beraprost-, prostacyclin- and non-treated hearts.

ATP metabolites released from the perfused heart

The perfusate eluted from the hypoxic and reoxygenated heart and the glucose-free, normoxic heart was collected and

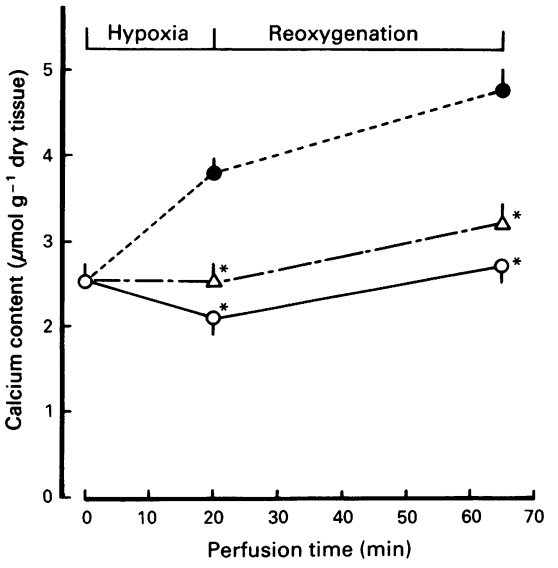


Figure 6 Changes in tissue calcium content of hearts subjected to 20 min hypoxia and 45 min subsequent reoxygenation with either beraprost (○, $n = 4$ and 5, respectively) or prostacyclin (△, $n = 4$ and 5, respectively) treatment and without any treatment during hypoxic insult (●, $n = 5$ and 6, respectively). Significantly different from the value for hearts without treatment $^*P < 0.05$.

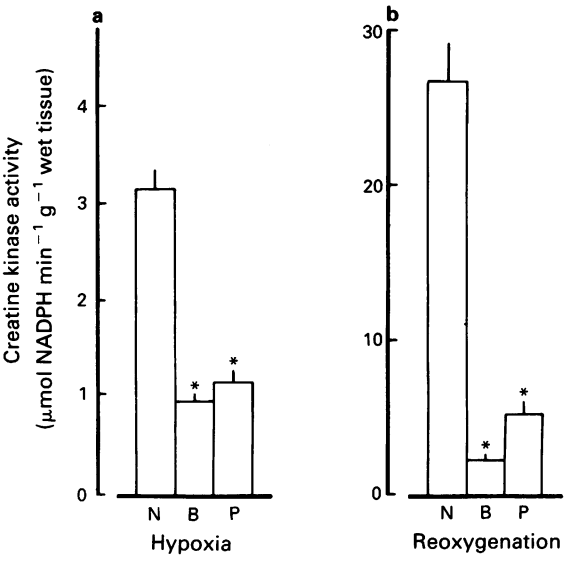


Figure 7 Creatine kinase activity of the perfusate eluted from hypoxic (a) and reoxygenated (b) hearts treated with either beraprost (B) or prostacyclin (P), and without treatment (N). Significantly different from hearts without treatment $^*P < 0.05$.

analyzed by h.p.l.c. The results are shown in Table 1. ATP metabolites released from control hearts throughout normoxic perfusion were less than 100 nmol g^{-1} wet tissue. There were no significant changes in the amount of ATP metabolites released from normoxic hearts regardless of treatment with either beraprost or prostacyclin.

We found previously that the perfusate eluted from hypoxic and subsequent reoxygenated heart contained ATP metabolites such as adenosine, inosine and hypoxanthine (Takeo *et al.*, 1987; 1988a; 1988c). These metabolites are substrates for ATP in the salvage synthesis. Other metabilites such as ADP, AMP, xanthine, adenine and uric acid were negligible or not detectable under the present experimental conditions.

A large amount of ATP metabolites (adenosine + inosine + hypoxanthine) was released during hypoxic perfusion (approximately 2 μmol g^{-1} wet tissue). Subsequently reoxygenation also induced a marked release of ATP metabolites from the heart (approximately 1.6 μmol g^{-1} wet tissue). The loss of ATP metabolites during hypoxia and subsequent reoxygenation was prevented by treatment of hypoxic hearts with beraprost (60 and 75% reduction as compared to the non-treatment group, respectively). Treatment with prostacyclin during hypoxia significantly suppressed the release of ATP metabolites from hypoxic and subsequent reoxygenated heart, but to a lesser degree than that with beraprost-treatment (Table 1).

Creatine kinase activity of perfusate eluted from the heart

Creatine kinase activity of the perfusate eluted from hypoxic and subsequent reoxygenated heart was determined. The results are shown in Figure 7. The control value for total cre-

Table 1 ATP metabolites of the perfusate eluted from hearts subjected to hypoxia/reoxygenation and normoxia/reperfusion

		Hypoxanthine	*Inosine*	*Adenosine*	*Total*
(A) 0–20 min perfusion					
Normoxia					
without treatment	(9)	ND	45 ± 1	ND	45 ± 1
with beraprost	(9)	ND	40 ± 1	ND	40 ± 2
with prostacyclin	(9)	ND	39 ± 3	ND	39 ± 3
Hypoxia					
without treatment	(11)	294 ± 12	662 ± 23	1024 ± 113	1979 ± 127
with beraprost	(9)	61 ± 5*	342 ± 25*	315 ± 46*	739 ± 46*
with prostacyclin	(9)	225 ± 33	502 ± 65	617 ± 99*	1344 ± 162
(B) 20–65 min perfusion					
Normoxic reperfusion					
without treatment	(5)	3 ± 2	41 ± 8	ND	44 ± 8
with beraprost	(5)	ND	42 ± 2	ND	42 ± 2
with prostacyclin	(5)	1 ± 1	41 ± 3	ND	42 ± 3
Reoxygenation					
without treatment	(6)	396 ± 17	1087 ± 57	404 ± 74	1883 ± 121
with beraprost	(5)	ND	384 ± 24*	6 ± 5*	390 ± 26*
with prostacyclin	(5)	243 ± 41	647 ± 93*	291 ± 36*	1191 ± 120*

Values are expressed as nmol g^{-1} of wet tissue. Each value represents the mean ± s.e.mean of four to six experiments. Total values of the groups were compared statistically.

* Significantly different from the non-treatment group ($P < 0.05$). ND means non-detectable. Numbers in parentheses indicate numbers of experiments.

atine kinase activity of the perfusate from the heart perfused for 20 min under glucose-free normoxic conditions and for 45 min under normoxic conditions in the presence of glucose was 0.99 ± 0.08 and 0.80 ± 0.05 μmol NADPH min^{-1} g^{-1} wet tissue, respectively. Under normoxic conditions, there were no significant differences in creatine kinase activity of the perfusate regardless of treatment with either beraprost or prostacyclin.

Hypoxic perfusion significantly increased the enzyme activity of the perfusate (approximately 3 fold higher than glucose-free normoxia). Subsequent reoxygenation induced a further increase in the activity of perfusate (approximately 30 fold higher than glucose-free normoxia). When the hearts were treated with either breaprost or prostacyclin, the increase in creatine kinase activity of the perfusate was significantly suppressed throughout the perfusion (Figure 7).

Discussion

In the present study, we have shown that hypoxia induced cessation of cardiac contractility, a rise in resting tension, an increase in perfusion pressure, a decrease in high-energy phosphate content, accumulation of tissue calcium and release of ATP metabolites and creatine kinase from the perfused heart. Furthermore, subsequent reoxygenation resulted in less than 10% recovery of cardiac contractile force, sustained elevation of resting tension and perfusion pressure, slight improvement of high-energy phosphate levels, further accumulation of tissue calcium and sustained release of ATP metabolites and creatine kinase, as found in our previous studies (Takeo *et al.*, 1987; 1988a, b). When the heart was treated with beraprost during hypoxic perfusion, the hypoxia-induced functional and metabolic changes of the myocardium described above were partially suppressed. Upon subsequent reoxygenation, beraprost-treated hearts showed a significant recovery of cardiac function. Treatment with beraprost also improved recovery of myocardial metabolic parameters. These findings suggest that beraprost-treatment during hypoxic perfusion is beneficial for post-hypoxic contractile and metabolic recovery.

In order to determine whether such beneficial action of beraprost is also exerted by prostacyclin, we treated the hypoxic hearts with prostacyclin in the same manner. It was found that treatment with prostacyclin improved post-hypoxic recovery of cardiac function and myocardial metabolism in a manner similar to that of beraprost. Furthermore, we observed better or more rapid recovery of post-hypoxic contractile force, perfusion pressure, ATP content and suppression of ATP metabolite release in perfused hearts with 0.45 μM beraprost than in those with 0.50 μM prostacyclin. Thus, the potency of cardioprotective effect, although it must be mediated through a similar mechanism, may be greater in hearts treated with beraprost than in those with prostacyclin. One of the possible explanations for the observed difference in the efficacy between beraprost and prostacyclin may be due to their stability in the perfusing buffer since prostacyclin is very unstable in aqueous solution (Lefer *et al.*, 1978).

Das and his co-workers have reported that phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were degraded by phospholipase A_2 in ischaemia/reperfusion (Das *et al.*, 1986; Otani *et al.*, 1986; 1989). Furthermore, it has been demonstrated that arachidonate is produced by phospholipase A_2-activated hydrolysis and is converted to prostacyclin and thromboxane A_2 by cyclooxygenase in the myocardium (De Deckere *et al.*, 1977; Karmazyn & Dhalla, 1983). As described earlier, prostacyclin has physiologically antagonistic effects to thromboxane A_2 on platelet aggregation (Hamberg *et al.*, 1975; Schrör *et al.*, 1981b). Inhibition of thromboxane A_2 production or blocking its receptor during ischaemia has been demonstrated to protect the myocardium against ischaemia/reperfusion injury as evidenced by prevention of an increase in lactate release from the heart, limitation of cardiac risk area and decrease in reperfusion-induced arrhythmias (Sakai *et al.*, 1982; Thiemermann & Schrör, 1984; Grover *et al.*, 1988; 1990; Mehta *et al.*, 1990; Lesenefsky *et al.*, 1990). Furthermore, several investigators have found that treatment with iloprost, a prostacyclin-mimetic agent, improved myocardial metabolism during ischaemia and resulted in an enhanced recovery of cardiac function (Van Glist *et al.*, 1983; Giessen *et al.*, 1986; Pissarek *et al.*, 1987; Chiariello *et al.*, 1988; Ferrari *et al.*, 1988). These results suggest an involvement of prostacyclin-mimetics in the enhancement of post-hypoxic or post-ischaemic functional and metabolic improvement of the myocardium.

Several hypotheses have been proposed for the cardioprotective mechanism of prostacyclin-mimetic agents. As described in the Introduction, beraprost and prostacyclin prevent platelet aggregation and increase coronary flow by their vasodilator action. Lefer *et al.* (1978) suggested that these effects would markedly improve myocardial oxygen supply relative to the myocardial oxygen demand. In the present study, we employed Krebs-Henseleit solution. This perfusing solution does not include platelets. In addition, hearts were perfused at a constant flow-rate. Thus, the protective effect of beraprost is independent of platelet aggregation and, possibly, of increased coronary flow. It is known that propranolol, a β-adrenoceptor blocking agent, and diltiazem, a calcium antagonist, have negative inotropic actions. Since these agents decrease energy demand during hypoxia or ischaemia by suppression of cardiac contractility, they protect the myocardium from depletion of high-energy phosphate levels, that is, they have an energy sparing effect (Nayler *et al.*, 1978). Prostacyclin and iloprost have been shown to preserve high-energy phosphates in the heart following coronary artery ligation (Pissarek *et al.*, 1987). Thus, it is conceivable that prostacyclin and its mimetic agent have an energy-sparing effect on hypoxic or ischaemic myocardium. As described in the Results section, there were no differences in high energy-phosphate contents between treatment with and without the agents at 20 min of hypoxia. In addition, we observed that the negative inotropic action of these agents was not observed when hearts were perfused under glucose-free, normoxic conditions in the presence of the agents (detail not shown). These results suggest a minimal energy-sparing effect of these agents.

Pi & Chen (1989) have shown that iloprost protected against free radical-induced injury in rat isolated working hearts. Ferrari *et al.* (1989) suggested that a mechanism by which iloprost improved recovery of myocardial function may be mediated via a reduction of oxidative stress due to oxygen-derived free radicals. Since superoxide anion is generated by the Fenton reaction including xanthine oxidase, it is possible that oxygen-free radicals produced during the initial phase of reoxygenation, if any, would cause a deleterious effect on the reoxygenated myocardium. However, in the present study, we perfused rabbit isolated hearts which have been reported to reveal undetectable or negligible xanthine oxidase activity (Ketai *et al.*, 1985; Muxfeldt & Schaper, 1987). In addition, we perfused the heart with Krebs-Henseleit solution without leukocytes which is considered to be one of the sites for free-radical production. Therefore, it is unlikely that the protective action of these agents observed in the present study is due to protection of the myocardial cells from free-radical attack.

To elucidate further possible mechanisms underlying the cardioprotective effects of beraprost and prostacyclin, the doses of these agents should be noted. Physiologically effective doses of prostacyclin appear to be 10 nM or 2.7 nmol kg^{-1} min^{-1} infusion (Lefer *et al.*, 1978; Toda, 1984). Ferrari *et al.* (1988; 1989) showed that an optimal dosage of iloprost for cardioprotection against ischaemic derangements was 27 nM. These concentration were much lower than doses employed in the present study. It is conceivable that the hypoxic injury observed in the present study is so severe that prostacyclin at a physiological concentration could not protect the myocardium against hypoxia/reoxygenation-induced derangements. Thus, a higher concentration of pros-

tacyclin or its mimetic would be required to exert its effects. Alternatively, such high doses of these agents might exclude an involvement of their physiological action in the observed cardioprotection.

In the previous studies from our laboratory, we have demonstrated that calcium antagonists, α_1-adrenoceptor blocking agents, and anti-arrhythmic agents exerted a cardioprotective action on hypoxic and reoxygenated myocardium under similar or identical experimental conditions (Takeo *et al.*, 1988c, d; 1989). In these papers, we observed suppression of hypoxia/reoxygenation-induced tissue calcium accumulation, and release of the ATP metabolites and CK from the perfused heart. We postulated that enhancement of post-hypoxic contractile and metabolic recovery was attributed to an inhibition of the transmembrane flux of ions and substrates across the cardiac cell membrane, which presumably is related to suppression of hypoxia/reoxygenation-induced increase in cell membrane permeability. Since we also observed the same effects on hypoxic/reoxygenated myocardium by treatment with either beraprost or prostacyclin in the present study, the beneficial effects of these agents may be related to such inhibitory effects on the transmembrane flux of ions and substrates across the cardiac sarcolemma.

Several reports have shown changes in prostanoid metabolism and/or their possible contribution to cardiac cell membrane damage during or after oxygen-deficiency and subsequent oxygen-replenishment. For example, Das *et al.* (1986) and Otani *et al.* (1986; 1989) showed ischaemia/reperfusion-induced production of non-esterfield arachidonate through phospholipase A_2 activation. Pieper (1990) suggested an involvement of non-esterified arachidonate in cardiac dysfunction during ischaemia/reperfusion. In contrast, Darius *et al.* (1987) showed that iloprost prevented ischaemia-induced breakdown of phosphatidylcholine and phosphatidylethanolamine when administered to rats at an infusion rate of $100\,ng\,kg^{-1}\,min^{-1}$. These findings would support a protective action of prostacyclin-mimetics on the cell membrane against oxygen deficiency-induced deterioration.

In summary, we demonstrated that beraprost and prostacyclin improved post-hypoxic contractile and metabolic recovery associated with a suppression of hypoxic/reoxygenation-induced changes in transmembrane flux of ions, metabolites and enzymes across the cardiac cell membrane. This provides entirely new evidence for the effects of beraprost on the oxygen-deprived and oxygen-replenished myocardium.

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Effects of selective D_1 and D_2 dopamine agonists on cockroach salivary gland acinar cells *in vitro*

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1 The ability of the selective dopamine receptor agonists, fenoldopam and SKF38393 (D_1) and quinpirole and LY163502 (D_2), to mimic the effect of dopamine on cockroach salivary gland acinar cells has been investigated.

2 Intracellular recordings of the membrane potential established that all the agonists mimicked dopamine (i.e. they induced a hyperpolarization which was occasionally followed by a depolarization), whether applied by addition to the superfusate or locally by pressure ejection.

3 The rank order of potency of the agonists to induce a hyperpolarization was (equipotent molar ratio relative to dopamine in parentheses): dopamine (1) ≫ fenoldopam (1000) > SKF38393 (3500) > LY163502 (13750) > quinpirole (35000).

4 The agonists also elicited secretion from the salivary gland when superfused onto the preparation. SKF38393 and quinpirole did not induce the same maximum rate of secretion as dopamine. The rank order of potency of the agonists was (minimum effective concentration in parentheses): dopamine (0.03 μM) ≫ fenoldopam (4.8 μM) > SKF38393 (40.8 μM) > quinpirole (132 μM).

5 Both the hyperpolarizing and secretory responses to all the agonists were antagonized by the selective D_1 antagonist, (+)-SCH23390, but not the selective D_2 antagonist, (±)-sulpiride.

6 These results support the idea that the same receptors mediate both the hyperpolarizing and secretory responses, and that they are similar to the mammalian D_1 receptor.

Keywords: Salivary gland; secretion; hyperpolarization; dopamine; D_1, D_2 receptors; D_1, D_2 receptor agonists

Introduction

Studies of the action of dopamine receptor antagonists on the electrical and secretory responses of the cockroach salivary gland acini (Evans & Green, 1990a, b) suggest that (i) the target receptors for both responses have an identical pharmacology and (ii) they are similar to the mammalian D_1 receptor, but distinct from the mammalian D_2 receptor.

In an effort to confirm these findings we have now investigated the ability of the selective D_1 agonists SKF38393 (Setler *et al.*, 1978; Stoof & Kebabian, 1981) and fenoldopam (Weinstock *et al.*, 1980) and D_2 agonists quinpirole (Tsuruta *et al.*, 1981) and LY163502 (Bymaster *et al.*, 1986) to elicit both secretory and electrical responses, and the ability of D_1 and D_2 dopamine receptor antagonists to inhibit the responses to each agonist tested. The two antagonists used were (+)-SCH23390, which is selective for the D_1 dopamine receptor (Hyttel, 1983) and inhibits the responses of this preparation to dopamine (Evans & Green, 1990a, b), and (±)-sulpiride, which is selective for D_2 dopamine receptors (Jenner & Marsden, 1981) and does not inhibit the responses of this preparation to dopamine (Evans & Green, 1990a, b). The results are consistent with those obtained from the study of the action of dopamine receptor antagonists (Evans & Green, 1990a, b).

Methods

Electrophysiology

Paired salivary glands and associated ducts were dissected from adult cockroaches (*Nauphoeta cinerea* Olivier) as described previously (House, 1973). The preparation was pinned to the base of a shallow perspex chamber lined with silicone resin (Sylgard, Dow Corning). The preparation was kept at room temperature and perfused at a rate of 5 ml min^{-1} with a solution of the following composition (mM): NaCl 160, CaCl 5, KCl 1, glucose 20 and Tris buffer 2.5; pH adjusted to between 7.70 and 7.80 with HCl. Recording microelectrodes were filled with 3 M KCl and had resistances between 20 and 40 MΩ. Membrane potentials were recorded on a pen recorder (Gould, type 565) and on video tape (Sony SL-F 30; with a Sony pulse code modulator PCM-701 ES) via a pre-amplifier (Dagan 8900 patch clamp).

Estimation of agonist potency

Two features of the potency of the agonists were measured; the lowest concentration of the compound required to produce a measurable response (the minimum effective concentration) and the equipotent molar ratio. To obtain an estimate of the minimum effective concentration of an agonist, tissues were exposed to a concentration of the agonist for 60 s. If this did not produce a response, the preparation was washed for 10 min, the concentration doubled and the procedure repeated until a concentration was found that produced a measurable response. If the first concentration of the agonist tested produced a response which was small in amplitude (< 15 mV), or fragmented in appearance (e.g. see Figure 1b, c), this was taken to be the minimum effective concentration, as experience showed that decreasing the concentration by a factor of two produced no measurable response. If the response was greater than 15 mV and sustained, the preparation was washed for 10 min and a two fold lower concentration tested. The equipotent molar ratio for the agonist in each preparation was calculated by dividing the concentration of the test agonist required to produce a submaximal response by the concentration of dopamine producing a response of equivalent amplitude.

Inhibition of hyperpolarizing response by selective antagonists

To assess the ability of the selective antagonists to inhibit the response of the preparation to the agonists, the agonist was applied locally to the acinus by pressure ejection from a

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broken microelectrode positioned close to the acinus (pulse of 35 kPa, duration 0.01–1 s; Picospritzer II, General Valve Corporation). The concentration of the agonist present in the pressure ejection pipette was 1 mM. The antagonists, SCH23390 and (±)-sulpiride, were added to the bathing solution and superfused onto the preparation for 30 min.

Secretion

The method of measuring the secretion rate has been described in detail by House & Smith (1978). Fluid was collected from the secretory duct and transferred to a pool of paraffin oil where it formed a sphere. The volume secreted was determined from the diameter of the sphere.

To enable a direct comparison of the potency of the selective agonists with dopamine, log concentration-response curves for the rate of salivary secretion induced by both dopamine and one of the agonists were obtained from a single preparation. The curves were constructed cumulatively, each concentration of the agonist being in contact with the preparation for 10 min. The responses to dopamine were obtained first, the preparation washed for 30 min and then the responses to the selective agonist measured. The rank order of potency of the agonists was obtained by comparison of the minimum effective concentration, which was estimated to within a factor of three in at least four experiments.

Inhibition of the secretory response by selective antagonists

The actions of (+)-SCH23390 and (±)-sulpiride on the responses to fenoldopam were studied by obtaining a log concentration-response curve to fenoldopam before and 30 min after the addition of each antagonist to the superfusate. The action of these antagonists on responses to SKF38393 and quinpirole was studied similarly, but by 'three point assay' (Edinburgh Staff, 1968).

Drugs

Quinpirole (*trans*-(−)-4aR-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-2H-pyrazolo-(3,4-g)quinoline monohydrochloride) and LY163502 (*trans*-(−)-5,5a,6,7,8,9,9a,10-octahydro-6-propyl-pyrimido[4,5-g]quinolin-2-amine dihydrochloride dihydrate) were donated by Lilly laboratories (Indianapolis, IN, U.S.A.). Fenoldopam (SKF82526-J, 6-chloro-7,8-dihydroxy-1-(*p*-hydroxyphenyl)-2,3,4,5-tetrahydro-(1H)-3-benzazepine) was donated by SK&F Laboratories, Welwyn, Herts. SKF38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine HCl), (+)-SCH23390 hydrochloride (R(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol.HCl) and (±)-sulpiride (5-(aminosulphonyl)-n-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide) were obtained from Semat, St Albans, Herts.

Stock solutions of the agonists (10–50 mM) were made up fresh each day in distilled water. The minimum dilution of the stock solutions in physiological saline was 50 fold. (±)-Sulpiride was dissolved in 5 mg ml^{-1} tartaric acid and after dilution the pH of the solution was returned to between 7.70 and 7.80 with 1 M NaOH. At the concentrations used, tartaric acid had no effect on the preparation.

Results

Electrophysiology

Potency of selective agonists All the agonists induced a hyperpolarization of the acinar cells, which was occasionally followed by a depolarization. Figure 1 shows the result of a typical experiment for each of the agonists. Responses to the minimum effective concentrations of fenoldopam (1 μM), SKF38393 (5 μM), LY163502 (50 μM) and quinpirole (100 μM) are shown in a(i), b(i), c(i) and d(i) respectively. Submaximal responses to each of the agonists and approximately equal responses to dopamine are shown in parts (ii) and (iii) of each section. The equivalent concentrations were a(ii) fenoldopam 50 μM and a(iii) dopamine 100 nM, b(ii) SKF38393 50 μM and b(iii) dopamine 100 nM, c(ii) LY163502 500 μM and c(iii) dopamine 50 nM, d(ii) quinpirole (1 mM) and a(iii) dopamine 50 nM. A more direct comparison of the potencies of three of the agonists is shown in Figure 2 in which the responses of a single cell to a 1 min exposure to (a) quinpirole (1 mM), (b) LY163502 (1 mM) and (c) SKF38393 (100 μM) are shown. The minimum effective concentrations (±s.e.mean) were: 3.3 ± 2.3 μM for fenoldopam ($n = 4$); 5.3 ± 1.8 μM for SKF38393 ($n = 4$); 44 ± 16.6 μM for LY163502 ($n = 4$); and 100 μM for quinpirole ($n = 4$). For submaximal responses the equipotent molar ratios for the agonists relative to dopamine (ratio = 1) were: fenoldopam, 1000 ($n = 4$); SKF38393

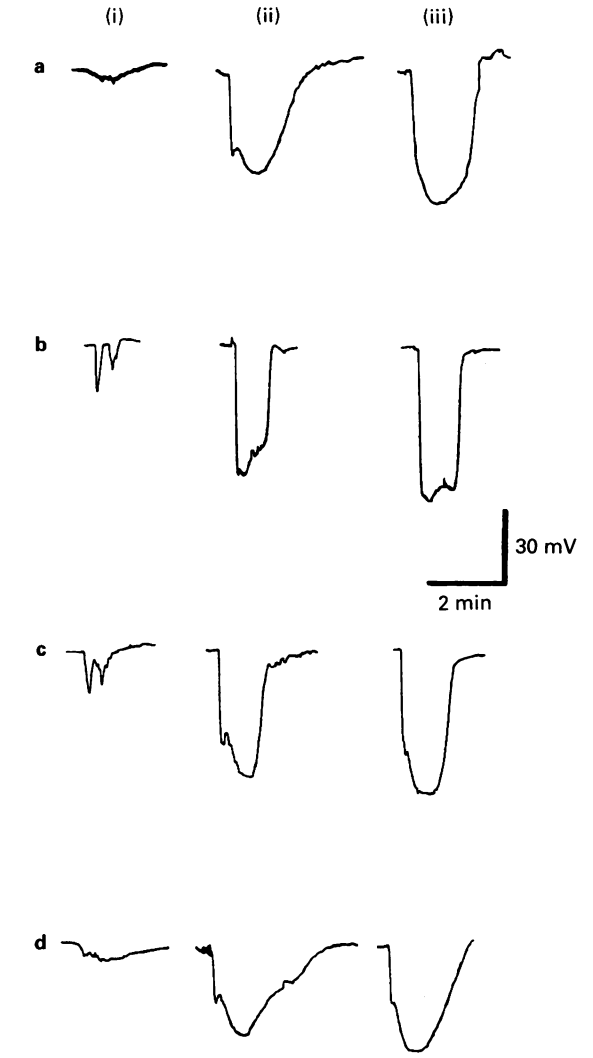


Figure 1 Intracellular recordings of the hyperpolarization of the cockroach salivary gland acinar cells induced by perfusion of dopamine receptor agonists. The hyperpolarization is downwards in this and subsequent records. Agonists were perfused onto the cell for 60 s: (a, i) response to the minimum effective concentration of fenoldopam; (a, ii) submaximal response to fenoldopam; (a, iii) equivalent response to dopamine. In sections (b-d) of this figure the results obtained with SKF38393, LY163502 and quinpirole are illustrated in a corresponding way. Concentrations of test agonists were: (a) fenoldopam, (i) 1 μM, (ii) 50 μM; (b) SKF38393, (i) 5 μM, (ii) 50 μM; (c) LY163502, (i) 50 μM, (ii) 500 μM; (d) quinpirole, (i) 100 μM, (ii) 1 mM. Concentrations of dopamine producing equivalent responses (iii) are 100 nM (a and b) and 50 nM (c and d). Resting membrane potentials were −38 mV, −43 mV, −43 mV, −40 mV, −40 mV in (a, i), (a, ii, iii) (b), (c) and (d) respectively.

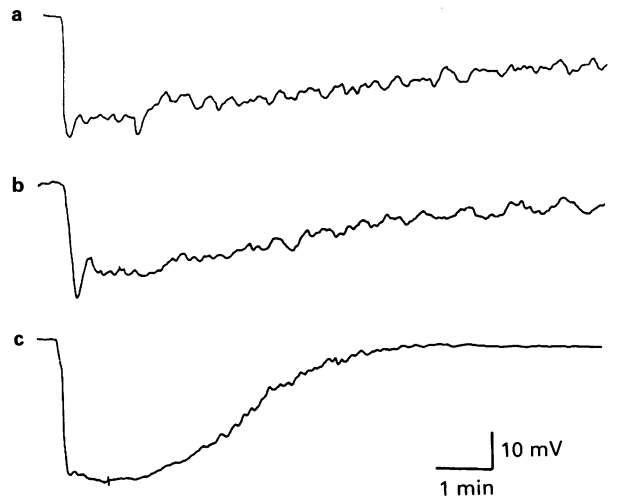


Figure 2 Comparison of the potency of dopamine receptor agonists in a single cell. Perfusion for 60 s of (a) 1 mM quinpirole, (b) 500 μM LY163502 and (c) 100 μM SKF38393. Resting membrane potential was −38 mV throughout. In this cell quinpirole and LY163502 both induced an increase in the baseline noise (which was reversed on washing, not shown). This effect was seen in the presence of each of the agonists tested, but not seen in all preparations. Similarly, the longer duration of the response to the D_2 agonists after the wash was not a consistent effect.

3500 ± 866 (n = 4); LY163502, 13750 ± 3750 (n = 4); quinpirole, 35000 ± 8660 (n = 4). The rank order of potency of the agonists tested was: dopamine ≫ fenoldopam > SKF38393 > LY163502 > quinpirole.

Effect of quinpirole on responses to locally applied dopamine The maximum response produced by quinpirole (1 mM) was considerably smaller than that produced by dopamine. This raised the possibility that quinpirole was acting as a partial agonist. The results of the experiment illustrated in Figure 3 are consistent with this idea. Submaximal hyperpolarizations to locally applied dopamine, pulse duration of 70 and 100 ms, are shown in Figure 3a and b respectively. The preparation was then perfused with 100 μM quinpirole, which produced a hyperpolarization of 20 mV (and an increase in baseline noise); after 10 min the response to the (c) 70 ms pulse of dopamine was scarcely discernible above the baseline noise and the response to the (d) 100 ms pulse was reduced to approximately 40% of control. Figure 3(e) shows the recovery of the response to the 100 ms pulse of dopamine after a 5 min wash. Similar results were obtained in one other experiment.

Antagonism of the hyperpolarization to locally applied agonists by (±)-sulpiride and SCH23390 Although both varieties of selective agonists induced a hyperpolarization, the D_1 compounds were more potent. This raised the possibility that the D_2 compounds were, in fact, acting on D_1 receptors. This idea was tested by the use of selective dopamine antagonists. The

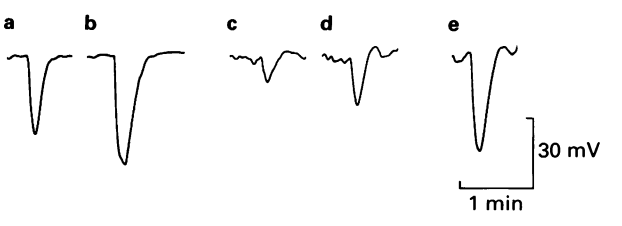


Figure 3 The hyperpolarizations induced by pressure ejected dopamine (0.1 nM), pulse durations of 70 and 100 ms, are shown in (a, b) the absence and (c, d) after 10 min in the presence of 100 μM quinpirole; (e) shows the response to a 100 ms pulse of dopamine 5 min after the removal of quinpirole from the superfusate. The resting potential was −32 mV.

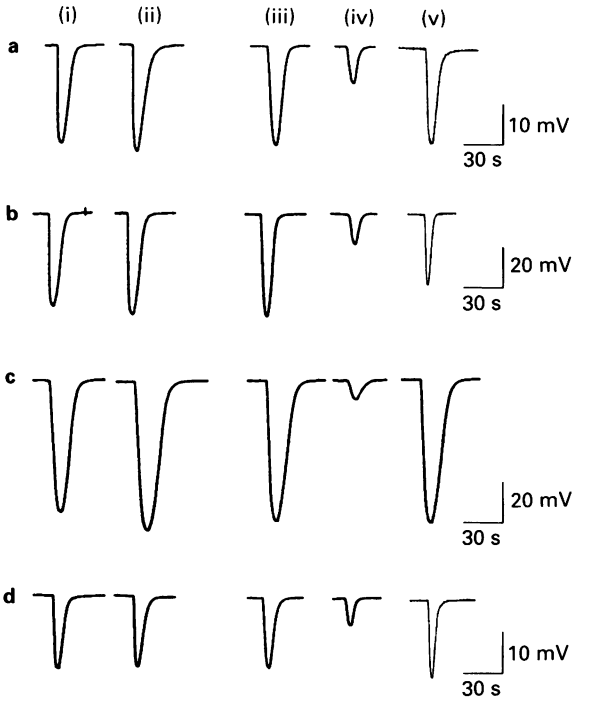


Figure 4 The effects of 100 μM (±)-sulpiride and 20 μM (+)-SCH23390 on the response of acinar cells to local application of dopamine receptor agonists. The agonists tested were (a) fenoldopam (1 mM, 300 ms pulse (i) and (ii), 50 ms pulse (iii), (iv) and (v)), (b) SKF 38393 (1 mM, 150 ms pulse), (c) LY163502 (1 mM, 170 ms pulse) and (d) quinpirole (1 mM, 100 ms pulse). For each of (a), (b), (c) and (d), (i) and (iii) represent the response of the cell to the agonist in antagonist free solution; (ii) is the response after 25 min in the presence of 100 μM (±)-sulpiride, (iv) is the response after 10 min in the presence of 20 μM (+)-SCH23390 and (v) shows the degree of recovery 10 min after the removal of (+)-SCH23390 from the superfusate. The responses in (b), (c) and (d) (i–iv) are from the same cell. In (a) (iii) to (v) the responses from a different cell from those in (i) and (ii). Initial resting membrane potentials: (a) (i and ii) −50 mV, (a) (iii–iv) −40 mV, (b) −44 mV, (c) −40 mV and (d) −52 mV.

results are illustrated in Figure 4: (a) shows the hyperpolarizing response to fenoldopam applied locally by pressure ejection before (i) and 25 min after the addition of 100 μM (±)-sulpiride to the superfusate (ii). The response of a different cell to fenoldopam is shown before (iii) and 10 min after the addition of 20 μM (+)-SCH23390 to the superfusate (iv); (v) shows the response to fenoldopam 10 min after (+)-SCH23390 had been removed from the superfusate. As shown in Figure 4(b–d), similar results were obtained in relation to the agonists SKF38393, LY163502 and quinpirole. In summary, the selective D_1 antagonist (+)-SCH23390 rapidly inhibited the hyperpolarizing response of the acinar cell to all four agonists (n = 4 in each case); the inhibitory action of (+)-SCH23390 could be surmounted and was reversed on washing. In contrast (±)-sulpiride did not inhibit the response to any of the agonists (n = 2 in each case) and in some cells it caused a slight potentiation of the hyperpolarization (Figure 4b, d).

Secretion

Potency of selective agonists Initial experiments with 10 min exposures to single concentrations showed that SKF38393, fenoldopam, quinpirole and LY163502 (not studied further) were all able to increase the rate of fluid secretion from the salivary gland. The rank order of potency for responses to the agonists and to dopamine appeared to be: dopamine > fenoldopam > SKF38393 > LY163502 > quinpirole. In Figure 5, examples of log concentration-response curves for fenoldopam, SKF38393 and quinpirole are compared with those for dopamine. While dopamine produced a substantial

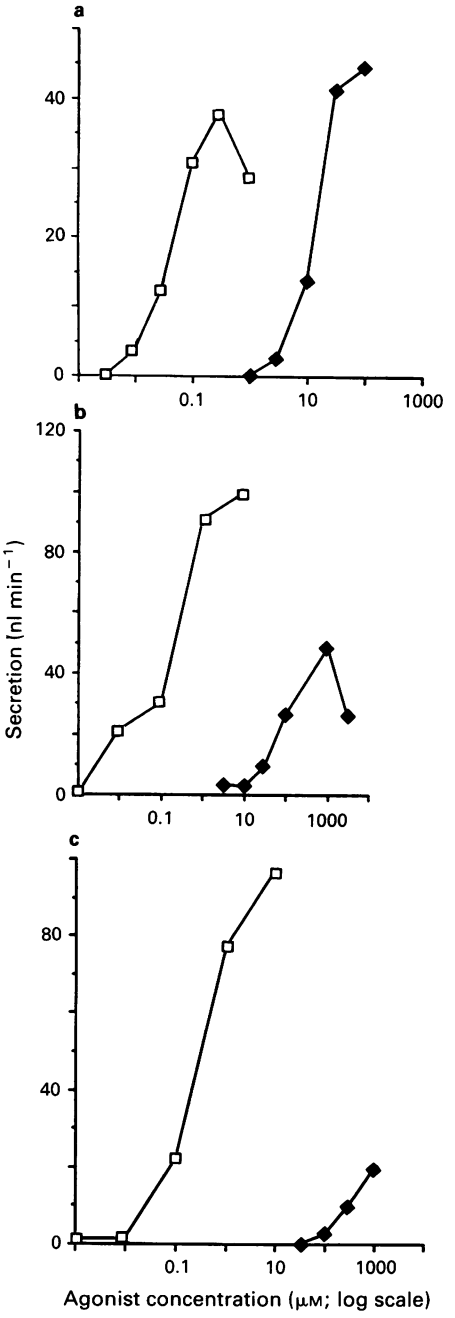


Figure 5 Log-concentration-response curves comparing the increase in secretion from the cockroach salivary gland induced by dopamine (□) with that elicited by (a) fenoldopam, (b) SKF38393 and (c) quinpirole (■). Each section of the figure represents a single experiment.

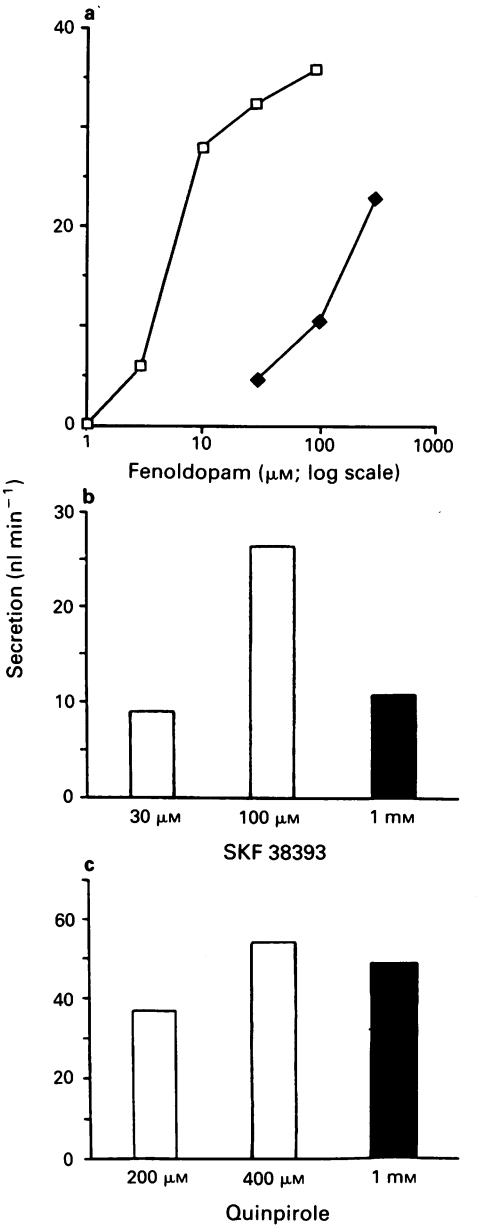


Figure 6 Antagonism by (+)-SCH23390 of secretory responses to dopamine agonists: (a) shows the log concentration-response curve to fenoldopam before (□) and after (■) a 30 min exposure to 10 μM (+)-SCH23390. In (b) and (c), the action of (+)-SCH23390 on the secretory response to SKF38393 and quinpirole, respectively, is illustrated by 'three point assay' with the rate of secretion shown before (open columns) and after (filled columns) a 30 min exposure to 10 μM (+)-SCH23390. (a), (b) and (c) represent the data obtained from single experiments.

increase in the rate of secretion at 0.1 μM, 100 μM SKF38393 and 1 mM quinpirole were required to produce similar responses. The maximum rates of secretion induced by SKF38393 and quinpirole varied considerably between preparations and were never as large as that produced by dopamine. Fenoldopam was more potent than SKF3893 or quinpirole, and in two of the four experiments induced about the same maximal rate of secretion as dopamine. The threshold concentrations of the agonists were 0.03 ± 0.01 μM ($n = 12$) for dopamine, 4.75 ± 1.75 μM ($n = 4$) for fenoldopam, 40.75 ± 20.74 μM ($n = 4$) for SKF38393 and 132 ± 58 μM ($n = 4$) for quinpirole. Thus the rank order of potency appeared to be dopamine > fenoldopam > SKF38393 > quinpirole.

As SKF38393 and quinpirole failed to induce the same maximal rate of secretion as dopamine, it seemed probable that they were partial agonists. In support of this idea, the response to dopamine was reduced by high concentrations of these agonists ($n = 2$ in each case). For example, in an experiment in which the interaction of SKF38393 and dopamine was studied, 100 nM dopamine produced a secretory response of 62 nl min^{-1}, 1 mM SKF38393 produced a response of 40 nl min^{-1} and the two together, a response of 40 nl min^{-1}. The maximum response produced by 10 μM dopamine after a 30 min wash was 126 nl min^{-1}. In a similar experiment, 30 nM dopamine produced a response of 42 nl min^{-1}, 3 mM quinpirole produced a response of 14 nl min^{-1} and the two together, 25 nl min^{-1}. The maximum response produced by 10 μM dopamine after a 30 min wash was 66 nl min^{-1}.

Antagonism of the secretory response to the selective agonists by (+)-SCH23390 and (±)-sulpiride The selective D_1

antagonist, (+)-SCH23390, inhibited the secretory response to fenoldopam, SKF38393 and quinpirole. The inhibitory effect of (+)-SCH23390 on the response to fenoldopam is illustrated in Figure 6a, where the rightward shift of the log concentration-reponse curve in the presence of (+)-SCH23390 (10 μM) can be clearly seen. Similar results were obtained in three other experiments. The inhibitory effect of (+)-SCH23390 (10 μM) on the responses to SKF28393 ($n = 4$) and quinpirole ($n = 4$) was investigated by 'three point assay', as shown in Figure 6b and c. The inhibitory action of (+)-SCH23390 could be surmounted by raising the concentration of agonist and was reversed on removal of SCH23390 from the superfusate. Values for the pA_2 of (+)-SCH23390 (30 min exposure) against each agonist were calculated when possible. The pA_2 values obtained were: 6.16 ± 0.15 ($n = 4$) against fenoldopam; 6.43 ($n = 2$) against SKF38393; 5.77 ($n = 2$) against quinpirole. When one considers the problems associated with the equilibration of SCH23390 and other antagonists and the resultant variability in the data obtained (see Evans & Green, 1990a, b), the pA_2 values against each agonist appear to be in good agreement. This suggests that these three agonists were acting at a common population of receptors.

The secretory response of fenoldopam, SKF38393 and quinpirole remained unaffected after a 30 min incubation of the preparation with 100 μM (±)-sulpiride ($n = 2$ in each case).

Discussion

As a result of studies of the action of dopamine receptor antagonists against dopamine-induced responses of the cockroach salivary gland (Evans & Green, 1990a, b), it was concluded that both the electrical and secretory responses are mediated by the same receptors. This idea receives strong support from the present study in which it has been found that the rank order of potency of the dopamine receptor agonists, dopamine ≫ fenoldopam > SKF38393 > LY163502 > quinpirole, is the same for both responses. Such a conclusion seems valid as these agonists appear to be acting at the same receptor. Thus SCH23390 inhibits the secretory and electrical responses to dopamine (Evans & Green, 1990a, b) and to all four selective agonists, while (±)-sulpiride does not. Moreover SKF38393 and quinpirole were shown to inhibit dopamine while stimulating secretion on their own; an action which also suggests that these two compounds are only partial agonists of the dopamine receptors.

Another suggestion which arose from the previous studies with antagonists was that the cockroach salivary gland dopamine receptor was pharmacologically similar to the mammalian D_1 dopamine receptor. This suggestion is also supported by the results of the present investigation. Thus the rank order of potency, dopamine > fenoldopam > SKF38393, is the same as for most mammalian D_1 receptors (Flaim *et al.*, 1985; Niznik *et al.*, 1988). Although the action of the D_2 agonists, quinpirole and LY163502, appear contradictory, a weak stimulant action of the selective D_2 agonist quinpirole has also been observed on mammalian D_1 receptors subserving dopamine-induced pancreatic secretion in dogs (Horiuchi *et al.*, 1989). As in the cockroach, quinpirole was antagonized by the D_1 antagonist, SCH23390, but not by the D_2 antagonist, (±)-sulpiride. As for LY163502, it is difficult to draw any conclusions as only limited pharmacological studies have so far been carried out on mammalian preparations (Bymaster *et al.*, 1986).

We now know that the dopamine receptors of the cockroach salivary gland are coupled to two second messengers namely inositol (1, 4, 5) triphosphate (IP_3), which appears to be involved in the secretory and electrical response to dopamine (Evans *et al.*, 1991), and adenosine 3′:5′-cyclic monophosphate, which plays a role only in the secretory response (Grewe & Kebabian, 1982; Gray *et al.*, 1984). From the pharmacological data described here and by Evans & Green (1990a, b), it would appear that one class of receptor similar to the mammalian D_1 sub-type mediates both events. However, two D_1 receptors of differing molecular weight have been isolated from the striatum and from mouse NS20Y neuroblastoma cells, the former being coupled to phosopholipase C and the latter being coupled to adenylyl cyclase (Mahan *et al.*, 1990; Monsma *et al.*, 1990). As yet, no pharmacological distinction between these receptors has been shown. Further studies are therefore required to establish beyond doubt whether one or two subclasses of the D_1 receptor are present within the cockroach salivary gland.

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Variations in potassium concentration modify the inhibitory effect of lorcainide on myocardial Na^+-K^+-ATPase activity

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1 The inhibitory action of lorcainide on the myocardial Na^+-K^+-ATPase (EC 3.6.1.3) activity was studied in guinea-pig heart preparations at medium K^+ concentrations of 2.5, 5.0 and 10 mM.

2 Lorcainide exhibited characteristically similar concentration-dependent inhibitory effects at all three K^+ concentrations tested. However, the inhibitory potencies were significantly increased at decreased K^+ concentrations.

3 The IC_{50} values were $10.4 \pm 3.2\,\mu M$ at 2.5 mM, $28.3 \pm 7.9\,\mu M$ at 5.0 mM and $40.7 \pm 9.2\,\mu M$ at 10.0 mM K^+ respectively. Thus, reduction in the K^+ concentration from the 'standard' 5.0 to 2.5 mM enhanced the inhibitory potency of lorcainide, the effective concentrations being shifted towards much lower ranges, while increasing it to 10 mM on the other hand produced opposite but less marked effects.

4 These results show that the inhibition of myocardial Na^+-K^+-ATPase activity by lorcainide depends on the K^+ concentration of the incubation medium. These effects are probably related to the mechanism by which lorcainide interferes with the electrogenic Na^+/K^+ pump activity of the enzyme, and hence may contribute to some of its cardiac actions.

Keywords: Lorcainide; potassium; antiarrhythmic agents; Na^+-K^+-ATPase

Introduction

The ability of antiarrhythmic drugs to initiate or aggravate existing cardiac arrhythmias has been known for some time. It is only recently however, that the clinical significance of this potential risk of antiarrhythmic drug therapy has been appreciated (Bigger & Sahar, 1987; Morganroth, 1987; Rosen & Wit, 1987; Zipes, 1987). This has been further emphasized by the publication of the preliminary report of the Cardiac Arrhythmia Suppression Trial (CAST) Investigators (1989). Lorcainide, like most antiarrhythmic agents in current use, shares this proarrhythmic property (Podrid *et al.*, 1987; Levine *et al.*, 1989). In a recent study (Almotrefi & Dzimiri, 1991a), we have shown that lorcainide is a potent inhibitor of the adenosine 5'-triphosphate (ATP)-hydrolytic action of myocardial Na^+-K^+-ATPase. The implication of this finding with regard to the drug's mode of action is not completely clear. It appears likely, however, that this action could be related to proarrhythmic or arrhythmogenic effects of this compound, in a fashion similar to that ascribed to digitalis-induced arrhythmias (Ferrier, 1977; Kass *et al.*, 1978; Smith *et al.*, 1984). Indeed, by virtue of its role in the active transport of monovalent cations across the cardiac cell membrane, Na^+-K^+-ATPase is a potential target for disturbances in the initiation and propagation of cardiac action potential which lead to cardiac arrhythmias.

The idea that disturbances in K^+ balance probably interfere with antiarrhythmic drug action is quite old (Holland & Klein, 1958; Rosen, 1971; Goldstein *et al.*, 1973; Obayani *et al.*, 1975). There is also compelling evidence pointing to the fact that low K^+ concentrations potentiate the inhibitory effect of cardiac glycosides on Na^+-K^+-ATPase leading to precipitation of cardiac arrhythmias (Klieger *et al.*, 1966; Aaronson & Nordin, 1988). Similarly, disturbance in K^+ balance is also regarded as a risk factor for the development of cardiac arrhythmias in response to class IC antiarrhythmic drugs, such as lorcainide (Morganroth, 1987; Levine *et al.*, 1989). Recently, attempts have been made to elucidate the electrophysiological mechanisms pertaining to the role of low K^+ concentration in the proarrhythmic and arrhythmogenic effects of antiarrhythmic drugs (Bigger & Sahar, 1987; Woosley & Roden, 1987; Morganroth, 1987). On the other hand, however, little has been published concerning the possible biochemical basis of such interactions. In the present study, we therefore address the question whether variations in K^+ concentration have any influence on the inhibition of myocardial Na^+-K^+-ATPase activity by lorcainide and attempt to assess the possible relevance of such interactions to some of its cardiac effects.

Methods

Enzyme preparation

Myocardial Na^+-K^+-ATPase was prepared as described previously (Dzimiri *et al.*, 1987). Guinea-pigs of either sex weighing 500–800 g were killed by a blow on the head and bled. The heart was removed rapidly and placed in ice-cooled isotonic sucrose buffer (180 mM). Four hearts were minced in a bio-homogenizer (Biospec Products Inc., U.S.A.) and homogenized (Stedfast Stirrer, Fisher Scientific) for 10 min in the sucrose buffer to give a 15% suspension. The homogenate was centrifuged for 15 min at 14000 *g* in a Sorvall RC5C Centrifuge (Sorvall Instruments, U.S.A.), the supernatant filtered through a SC 8.0 μm filter (Millipore Corporation, U.S.A.) and then separated on a TSK Toyopearl Gel HW-55F column (Pierce Chemicals, U.S.A.) equilibrated with KCl buffer (KCl 500 mM, imidazole 10 mM, Na_2-EDTA 1 mM, pH 7.4). Ascending elution of the enzyme was carried out at a flow rate of 0.7 ml min^{-1} maintained by means of a peristaltic pump (LKB Bromma). The fractions were collected using LKB-2211 Superrac and dialysed in 20/32 Visking dialysis membrane (Serva Finebiochemica, USA) three times, each time for at least 6 h, twice with 10 mM first, and then once with 100 mM imidazole buffer, pH 7.4. All steps were carried out at 4°C. The enzyme was stored at the same temperature and could be used for up to five days without appreciable loss of activity. The enzyme preparations were free of mitochondrial particles (Fricke & Klaus, 1974).

Determination of enzyme activity

Enzyme assays were run at 37°C in the presence of different drug concentrations. The drug was pre-incubated at this temperature together with 10–12 μg enzyme (protein) for 20 min in

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100 mM imidazole buffer containing (in mM) Mg^{2+} 5, Na^+ 100, K^+ 5 and Na_2-EDTA 1, and the reaction initiated by 2 μM ATP giving a total volume of 1.0 ml. Similar assays were also run in incubation medium containing 2.5 and 10.0 mM K^+. The effects of lorcainide were tested in the range of 1.0 nM–10.0 mM. The liberated inorganic phosphate was determined spectrophotometrically after 20 min at 660 nm by the method of Eibl & Lands (1969), each assay being performed in duplicate. The Na^+-K^+-stimulated ATPase activity was calculated as the difference between the total and the Mg^{2+} plus Na^+-dependent activity. All results were corrected for blank values without the enzyme. The concentration for half maximal inhibition (IC_{50}) of the Na^+-K^+-ATPase were calculated from individual concentration-effect curves as proposed by Hafner *et al.* (1977). Protein concentration of the enzyme preparation was determined by use of Coomassie protein assay reagent (Pierce Chemicals, U.S.A.).

Lorcainide HCl was donated by Janssen Pharmaceutica (Belgium). All the other reagents were of analytical grade. Values are given as mean ± s.d. Statistical significance was calculated with Student's *t* test (paired to compare drug effects at different K^+ concentrations; unpaired for effects at the same K^+ concentrations) and distribution fitting tests (Kolmogorov-Smirnov and Chi tests to compare the geometrical and normal distribution of the mean values) were carried out with the Statgraphics software package version 3.0 (Graphic Software Systems, 1988). Significance criteria refer to $P < 0.05$.

Results

The initial experiments to study the role of K^+ on the ATP-hydrolytic action of myocardial Na^+-K^+-ATPase showed that the enzyme activity increased with increasing K^+ concentration up to approximately 20 mM (Figure 1). Above this concentration, Na^+-K^+-ATPase showed no statistically significant change in activity. At all the tested K^+ concentrations, lorcainide exhibited concentration-dependent inhibitory actions on the Na^+-K^+-ATPase activity, which also showed similar trends in their concentration-effect relationships. However, as depicted in Figure 2, these inhibitory actions were significantly influenced by varying the K^+ concentration. Thus, under 'standard' (5 mM K^+) conditions, lorcainide exerted its inhibitory effects on the enzyme activity (5–95% inhibition) in the range of 0.13–950 μM, with IC_{50} value of 28.3 ± 7.9 μM. Reduction of the K^+ concentration to 2.5 mM on the other hand shifted the inhibitory concentration-range to 0.01–700 μM, thereby reducing the IC_{50} value to 10.4 ± 3.2 μM, while doubling it to 10.0 mM K^+ shifted the range to 0.3–1300 μM, increasing its IC_{50} value to 40.7 ± 9.2 μM. The corresponding IC_{20} values were

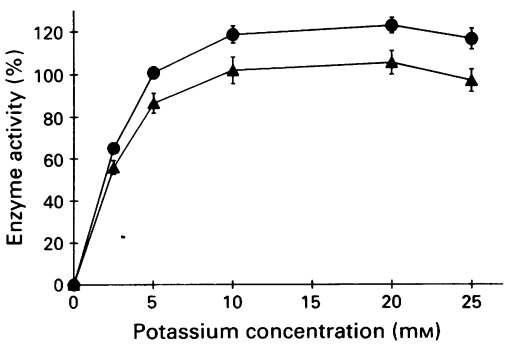


Figure 1 Effect of various K^+ concentrations on myocardial Na^+-K^+-ATPase activity expressed as a percentage of total activity observed in the presence of 5 mM K^+ (●) and as a percentage of maximally attainable enzyme activity observed in presence of 20 mM K^+ (▲). Plotted are the mean values of 8 individual determinations; s.d. shown by vertical bars.

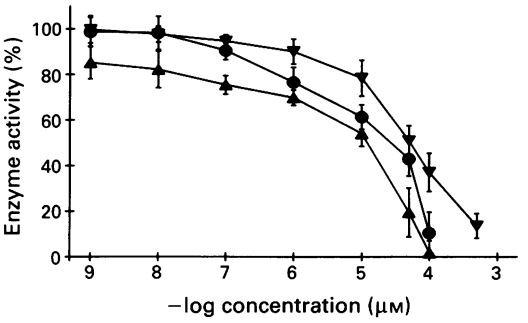


Figure 2 Influence of different concentrations of lorcainide in presence of 2.5 (▲), 5.0 mM (●) and 10.0 mM (▼) K^+ on guinea-pig myocardial Na^+-K^+-ATPase. The mean enzyme baseline activities (100%) were 22.7 ± 6.2, 24.2 ± 5.3 and 26.4 ± 7.4 μM Pi mg^{-1} protein h^{-1} at 2.5, 5.0 and 10.0 mM K^+, respectively. Plotted are the mean values of 7 or 8 individual determinations; s.d. shown by vertical bars.

21.4 ± 3.7 nM at 2.5 mM K^+, 0.9 ± 0.2 μM at 5.0 mM K^+ and 10.1 ± 1.7 μM at 10.0 mM K^+, respectively (Table 1).

Accordingly, reduction of the K^+ concentration by half caused a significant ($P < 0.001$) right-to-left shift in the effective inhibitory concentration range, lorcainide exerting significant actions ($P < 0.05$) at concentrations as low as 10 nM. On the other hand, increase in the K^+ concentration decreased these actions, resulting in a left-to-right shift in effective inhibitory concentration-ranges. These trends are further demonstrated in Figure 3 which compares the changes in enzyme activity at different lorcainide concentrations in relation to the

Table 1 The inhibitory actions of lorcainide on microsomal Na^+-K^+-ATPase activity in guinea-pig heart preparations incubated at different K^+ concentrations

K^+ conc. (mM)	n	*IER (μM)	**IC_{20}	***IC_{50} (μM)
2.5	8	0.01–700	21.4 ± 3.7 nM	10.4 ± 3.2
5.0	7	0.13–950	0.9 ± 0.2 μM	28.3 ± 7.9
10.0	8	0.3–1300	10.1 ± 1.7 μM	40.7 ± 9.2

* The inhibitory effective range, defined as the concentration-range at which the drug inhibits 5–95% of the enzyme activity.
** The concentration required to inhibit the enzyme activity by 20%.
*** The median inhibitory concentration, defined as the concentration required to inhibit the enzyme activity by 50%. IC_{20} and IC_{50} values are expressed as means ± s.d.

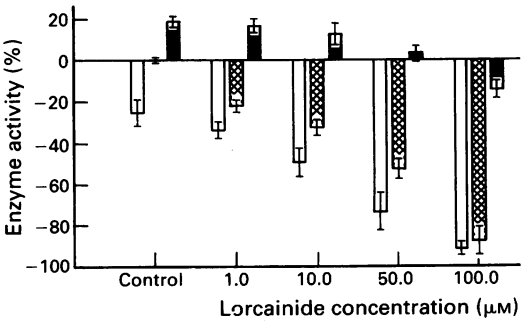


Figure 3 Comparative effects of various incubation medium concentrations of K^+ on the inhibitory actions of lorcainide on guinea-pig myocardial Na^+-K^+-ATPase. The relative effects of lorcainide in presence of 2.5 (open columns) and 10.0 mM (solid columns) K^+ are calculated using as baseline the total enzyme activity at standard 5.0 mM K^+ (cross hatched columns). The mean baseline activity of the enzyme at 5.0 mM K^+ was 26.5 ± 7.8 μM P_i mg^{-1} protein h^{-1}. The first point represents the relative change in enzyme activity in the absence of lorcainide. Plotted are the mean values of 7–10 individual determinations; s.d. shown by vertical bars.

total activity at the standard (5 mM) K^+ level. As depicted in Figure 3, at 10.0 mM K^+ the enzyme activity remained sustained above that of the standard K^+ concentration up to approximately 50 μM. Lorcainide failed to attain 100% inhibition at pharmacologically relevant concentrations. At 2.5 mM K^+ on the other hand, the differences in inhibitory actions became increasingly significant with decreasing lorcainide concentrations.

Discussion

The results demonstrate that in the absence of inhibitor, myocardial Na^+-K^+-ATPase activity increased with K^+ concentration, with maximal activity being attained at approximately 20 mM K^+. A similar influence of K^+ on myocardial Na^+-K^+-ATPase activity has been established previously (see Akera *et al.*, 1979, for review). The present findings confirm the importance of K^+ as a principal determinant of myocardial Na^+-K^+-ATPase activity. The study also shows that in the presence of an inhibitor, such as lorcainide, the inhibitory potency is enhanced significantly by reducing the K^+ concentration level of the incubation medium. Thus, although lorcainide produces similar concentration-dependent inhibitory effects at the tested K^+ concentrations, variation in the 'standard' K^+ concentration exhibits a profound effect on the sensitivity of the enzyme towards inhibition by the drug. It can therefore be concluded that, while K^+ is required for the Na^+-K^+-ATPase activity, its depletion enhances the inhibition of the enzyme activity by lorcainide. Similarly, a reduction in K^+ concentration increases the sensitivity of Na^+-K^+-ATPase inhibition by cardiac glycosides as specific inhibitors of the enzyme (Smith *et al.*, 1984), thereby potentiating their ability to induce cardiac arrhythmias (Aronson & Nordin, 1988). Such interactions of low serum K^+ concentrations with cardiac glycosides have been implicated as important factors in the development of a variety of digitalis-induced arrhythmias (Nordrehaug *et al.*, 1985; Cleland & Dargie, 1988) as well as those induced by antiarrhythmic agents (Levine *et al.*, 1989).

Recently, two plausible suggestions have been made to explain the electrophysiological basis of digitalis-induced arrhythmias (Aronson & Nordin, 1988). Thus, the arrhythmogenic interaction between cardiac glycosides and low K^+ concentration may be a product of the latter enhancing the production of triggered activity by delayed after-depolarizations as a result of its increasing the magnitude of the inward component of the oscillatory current induced by cardiac glycosides. Alternatively, reduced K^+ concentration may decrease the background membrane conductance, thereby increasing the magnitude of the digitalis-induced after-depolarizations, without it affecting the magnitude of the underlying oscillatory current. These after-depolarizations occur as a result of the steroids interfering with the electrogenic pump activity of the enzyme. Thus, by inhibiting the ATP-hydrolytic action of the enzyme, the cardiac glycosides impede the electrogenic pumping of Na^+ out and K^+ into the cell, which interferes with the restoration of resting potential. This may in turn cause after-depolarizations which may trigger arrhythmias (Ferrier, 1977). Class IC antiarrhythmic agents such as lorcainide can trigger early after-depolarizations (Levine *et al.*, 1989). Such secondary depolarizations may also result from low extracellular K^+. It remains to be established whether such electrical disturbances occur as a product of malfunction of the electrogenic pump activity triggered by the inhibition of the ATP-hydrolytic action of myocardial Na^+-K^+-ATPase by lorcainide. Nevertheless, based on the currently available information, it seems reasonable to postulate a relationship between the inhibitory actions of antiarrhythmic drugs on myocardial Na^+-K^+-ATPase and the mechanism of their pro-arrhythmic or arrhythmogenic effects. There were several observations which support such a hypothesis. To begin with, the ability to induce or enhance already existing arrhythmias is a property shown by most of the currently available antiarrhythmic drugs, including lorcainide (Woosley & Roden, 1987; Zipes, 1987). According to recent suggestions, such arrhythmias may be caused, in part at least, by disturbances in ionic balance, particularly that of K^+ and Na^+ (Rosen & Wit, 1987; Bigger & Sahar, 1987; Levine *et al.*, 1989), which may in turn occur as a result of the inhibition of the electrogenic pump activity of Na^+-K^+-ATPase in a fashion similar to that which triggers the digitalis-induced arrhythmias (Ferrier, 1977). It is also well-established that, apart from lorcainide, several other antiarrhythmic agents are also potent inhibitors of Na^+-K^+-ATPase activity (Goldstein *et al.*, 1973; Cook *et al.*, 1983; Prasada Rao *et al.*, 1986; Aomine, 1989; Almotrefi & Dzimiri 1990; 1991b; Dzimiri & Almotrefi, 1990). Likewise, it appears that the reduction in K^+ concentrations also enhances the inhibitory actions of other antiarrhythmic drugs, such as amiodarone, for example (Dzimiri & Almotrefi, 1991).

Therefore, all the above observations seem to point towards a common mechanism for some of the cardiac actions shared by antiarrhythmic drugs and cardiac glycosides, such as their ability to induce cardiac arrhythmias. The present findings also suggest that changes in myocardial K^+ concentration may affect the responsiveness of Na^+-K^+-ATPase towards inhibition by lorcainide. If the hypothesis implicating the inhibition of Na^+-K^+-ATPase activity in the pro-arrhythmic and arrhythmogenic actions of lorcainide is correct, these findings further indicate that hypokalemia may result in aggravation of these arrhythmias during treatment with lorcainide.

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Effects of endothelin-1 on vascular permeability in the conscious rat: interactions with platelet-activating factor

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1 The objectives of the present experiments were to assess the effects of endothelin-1 on the macrovascular permeability in selected vascular beds, to study the involvement of platelet-activating factor (PAF) in vascular responses to endothelin-1 and to examine the vascular effects of combined administration of endothelin-1 and PAF in conscious rats.

2 Intravenous bolus injection of endothelin-1 (0.1–2 nmol kg^{-1}) resulted in a dose-dependent biphasic change in mean arterial blood pressure (MABP) with initial transient hypotension followed by a prolonged pressor action. These changes were accompanied by a dose-dependent increase in haematocrit values.

3 Endothelin-1 (0.1 and 1 nmol kg^{-1}) increased dose-dependently the vascular permeability of the trachea, upper and lower bronchi, stomach, duodenum, spleen and kidney (up to 240%) as measured by the extravasation of Evans blue dye. The permeability of pulmonary parenchyma, liver and pancreas was not affected significantly by endothelin-1 treatment.

4 Pretreatment of animals with the specific PAF receptor antagonist, WEB 2086 (1 mg kg^{-1}, i.v.) or BN 52021 (10 mg kg^{-1}, i.v.) reduced the endothelin-1 (1 nmol kg^{-1})-induced rise in haematocrit by about 50 and 30%, respectively. Both antagonists were highly effective at inhibiting protein extravasation in the stomach, duodenum and kidney. On the other hand, BN 52021, but not WEB 2086, significantly attenuated the effect of endothelin-1 on permeability in the lower bronchi and spleen. Neither WEB 2086 nor BN 52021 modified the changes in MABP evoked by endothelin-1.

5 When low doses of endothelin-1 (0.1 nmol kg^{-1}) and PAF (0.19 nmol kg^{-1}) were administered simultaneously, enhanced protein extravasation was detected in the upper and lower bronchi, whereas neither endothelin-1 nor PAF by themselves affected vascular permeability in these tissues. These changes occurred in the absence of significant changes in MABP.

6 Combined administration of higher doses of endothelin-1 (1 nmol kg^{-1}) and PAF (1.9 nmol kg^{-1}) resulted in marked increases (up to 530%) in protein extravasation in the airways, pancreas, stomach and duodenum. The effect of endothelin-1 on permeability was not affected by PAF in the spleen, whereas it was completely inhibited by PAF in the kidney. Combined injection of endothelin-1 and PAF resulted in a slight, but significant increase in MABP.

7 The present findings show that endothelin-1 is capable of increasing vascular permeability in selected vascular beds including the airways, gastrointestinal tract and kidney, and suggest that PAF may mediate, in part, its action on permeability, but not its hypotensive action. The present data also suggest that endothelin-1 and PAF can act in concert to increase vascular permeability in rat airways and gastrointestinal tract.

Keywords: Endothelin-1; protein extravasation; haemoconcentration; PAF; PAF antagonists; airways; gastrointestinal tract; kidney

Introduction

Endothelial cells are capable of producing a variety of vasoactive substances, including prostacyclin (Moncada *et al.*, 1976), endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980), platelet-activating factor (PAF) (McIntyre *et al.*, 1985) and endothelin-1 (Yanagisawa *et al.*, 1988) that may regulate vascular tone and permeability. Endothelin-1, a 21-amino acid peptide has attracted considerable interest principally because of its highly potent vasoconstrictor and pressor activities. While the contractile action of endothelin-1 on large conduit vessels (Yanagisawa *et al.*, 1988), resistance arterioles (Brain *et al.*, 1988; Armstead *et al.*, 1989), veins and venules (D'Orléans-Juste *et al.*, 1989; Yang *et al.*, 1989; Boric *et al.*, 1990) is well documented in most preparations used, little is known of its effects on vascular permeability. Reports are conflicting since both endothelin-1-induced oedema formation in the forearm in man (Dahlöf *et al.*, 1990) and inhibition of oedema formation by endothelin-1 in rabbit and rat skin (Brain *et al.*, 1989) have been described.

Recent evidence suggests that PAF may mediate certain actions of endothelin-1. Indeed, PAF receptor antagonists have been reported to attenuate the bronchoconstrictor action of endothelin-1 in the guinea-pig both *in vivo* (Lagente *et al.*, 1989) and *in vitro* (Battistini *et al.*, 1990; Filep *et al.*, 1991), to inhibit endothelin-1-induced increase in intracellular free calcium levels in cultured smooth muscle cells (Takayashu *et al.*, 1989) and to protect against endothelin-1-induced sudden death in mice and rats (Terashita *et al.*, 1989). Furthermore, endothelin-1 has recently been shown to stimulate PAF synthesis in glomerular mesangial cells (López-Farré *et al.*, 1990).

Among its numerous actions, PAF is known to enhance vascular permeability (Handley *et al.*, 1984). Elevated plasma concentrations of endothelin-1 and PAF have been reported under the same pathological conditions including ischaemia (Filep *et al.*, 1989; Mózes *et al.*, 1989; Montrucchio *et al.*, 1989; Yasuda *et al.*, 1990; Watanabe *et al.*, 1990) and endotoxin shock (Doebber *et al.*, 1985; Morel *et al.*, 1989). Since enhanced plasma extravasation is a characteristic feature of these conditions, it can be postulated that endothelin-1 and PAF could act together to modulate vascular permeability. The present experiments were designed, firstly, to assess

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whether or not systemically injected endothelin-1 could affect microvascular permeability in selected vascular beds, secondly, to study the possible involvement of PAF in the actions of endothelin-1 by using specific PAF receptor antagonists and, finally, to investigate the vascular responses to combined administration of endothelin-1 and PAF to conscious rats.

Methods

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 250–325 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 glued to the tail. The cuff was connected to a stainless steel spiral and was fed through the top of the metabolism cage. The animals were allowed to recover completely for at least 4 days following the surgical procedures. During the experiments the animals could move freely and had free access to food and water. Mean arterial blood pressure (MABP) was monitored continuously with an electromanometer using a Statham P23 dB strain gauge.

Experimental protocols

In the first series of experiments, after an equilibration period of 2 h, endothelin-1 (0.1, 0.5, 1 and 2 nmol kg^{-1}) was injected i.v. in a volume of 25 μl kg^{-1} body weight followed by 30 μl of sterile 0.9% NaCl solution. Ten min before and after endothelin administration, blood (approximately 20 μl) was obtained through the arterial catheter to determine the haematocrit. On any one day each animal received only one dose of endothelin-1 and at least 3 days were allowed to elapse between two experiments in the same rat.

In subsequent experiments to measure protein extravasation, Evans blue dye was used as a marker of vascular permeability. In this series of experiments, Evans blue (20 mg kg^{-1}, 25 mg ml^{-1} in 0.9% NaCl) was injected intravenously together with vehicle, endothelin-1 (0.1 and 1 nmol kg^{-1}), PAF (0.19 and 1.9 nmol kg^{-1}) or combination of low and high doses of endothelin-1 and PAF (0.1 nmol kg^{-1} endothelin-1 plus 0.19 nmol kg^{-1} PAF and 1 nmol kg^{-1} endothelin-1 plus 1.9 nmol kg^{-1} PAF, respectively). In another set of experiments, the animals were pretreated with the specific PAF receptor antagonists, WEB 2086 (1 mg kg^{-1}, i.v.) (Casals-Stenzel *et al.*, 1987) or BN 52021 (10 mg kg^{-1}, i.v.) (Braquet *et al.*, 1985; Földes-Filep *et al.*, 1987), for 10 min before injection of endothelin-1 (1 nmol kg^{-1}) or PAF (1.9 nmol kg^{-1}). Ten min after the injection of endothelin-1 or PAF, the animals were stunned, then decapitated and exsanguinated. Then the thorax was cut open, the lungs were perfused and trachea, upper bronchi (airways extending from the bifurcation of the trachea to its entry to parenchyma), lower bronchi (defined as major airways surrounded by parenchyma that can be easily dissected without magnification) were prepared as described previously (Sirois *et al.*, 1990). The pulmonary tissues, liver, spleen, pancreas, stomach, duodenum and kidney were weighed and a portion of each tissue was put in formamide (4 ml per g wet weight tissue at 20°C for 24 h) while another part was dried at 60°C for 24 h. The amount of Evans blue extracted in formamide was determined by spectrophotometry (Sirois *et al.*, 1990). Evans blue content of each sample was expressed as μg dye per g dry weight of tissue to avoid underestimation of changes due to oedema formation.

Drugs and chemicals

Endothelin-1 was synthesized at INRS-Santé by solid phase methodology. The purity of the preparation was greater than 97%. Endothelin-1 was dissolved in distilled water and stored under nitrogen at −20°C for up to four weeks. On the day of the experiments an aliquot was removed and diluted further in 0.9% NaCl. BN 52021 (ginkgolide B, 9H-1,7a-(epoxymethanol)-1H,6aH, cyclopenta[c][2-3-b]furo-[3,2′:3,4]cyclopenta-[1,2-d]furan 5,9,12-[4H]trione, 3 *tert*-butylhexahydro 4,7b,11, hydroxy-8-methyl) and 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) were gifts from the Institut Henri Beaufour, Le Plessis Robinson, France and Boehringer-Ingelheim GmbH, Ingelheim, F.R.G., respectively. BN 52021 and WEB 2086 were dissolved in dimethylsulphoxide and 0.9% NaCl, respectively and were further diluted with 0.9% NaCl. Evans blue dye was purchased from Sigma Chemical Co, St. Louis, MO, U.S.A.; PAF (1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine) was purchased from Bachem, Bubendorf, Switzerland.

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical evaluation of the data was performed by one-way analysis of variance using ranks (Friedman's test) followed by a Wilcoxon-Wilcox test (Wilcoxon & Wilcox, 1964), to identify differences between control and repeated measurements on the same animals; by Dunn's multiple contrast hypothesis test (Dunn 1964), when various treatments were compared to the same control; or by 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

Vascular responses to endothelin-1

Bolus i.v. injection of endothelin-1 (0.1–2.0 nmol kg^{-1}) evoked biphasic, dose-dependent effects on MABP with a transient fall followed by a prolonged pressor action. The hypotensive response to endothelin-1 was rapid in onset and MABP returned to base-line within 1 min. Thereafter MABP continued to increase, reaching a maximum within 2–3 min, then slowly declined over the next 10 min. The peak responses in MABP to endothelin-1 are summarized in Table 1. These changes were accompanied by a dose-dependent increase in haematocrit values (Table 1). The lowest tested dose of endothelin-1 required to cause significant increase in haematocrit was 0.5 nmol kg^{-1} (Table 1). Following administration of endothelin-1, 1 and 2 nmol kg^{-1}, the haematocrit increased on average by 11 and 15%, respectively. Higher doses of endothelin-1 were not tested because severe bleeding from the orbital sinus was observed in all animals receiving 2 nmol kg^{-1} endothelin-1.

In another set of experiments the effects of 1 nmol kg^{-1} endothelin-1 on extravasation of Evans blue dye were studied in selected vascular beds. Injection of this dose of endothelin-1 increased tissue Evans blue content in the upper and lower bronchi on average by 83 and 140%, respectively (Figure 1). Although Evans blue content tended to be higher in the trachea and pulmonary parenchyma, these changes did not reach statistical significance (Figure 1). Endothelin-1 injection evoked increases of 109, 132, 34 and 72% in Evans blue content in the stomach, duodenum, spleen and kidney, respectively (Figure 2). Endothelin-1 did not affect the vascular permeability of the liver (Figure 2).

Effects of PAF receptor antagonists on endothelin-1-induced vascular permeability

In order to study the possible involvement of PAF in the vascular responses to endothelin-1, the animals were pretreated with one or other of the specific PAF receptor antagonists WEB 2086 (1 mg kg^{-1}, i.v.) or BN 52021 (10 mg kg^{-1}, i.v.) for 10 min before injection of endothelin-1, 1 nmol kg^{-1} and Evans blue. Neither WEB 2086 nor BN 52021 affected the

Table 1 Effects of endothelin-1 on mean arterial blood pressure (MABP) and haematocrit values in conscious rats

		Peak Δ MABP (mmHg)		*Haematocrit* (vol %)		
		Decrease	*Increase*	*Before endothelin-1*	*10 min after injection*	P
Vehicle	$n = 7$	—	—	39.7 ± 0.9	40.3 ± 0.7	NS
Endothelin-1, 0.1 nmol kg^{-1}	$n = 5$	6.6 ± 1.2	7.0 ± 1.4	38.3 ± 0.9	40.3 ± 0.7	NS
Endothelin-1, 0.5 nmol kg^{-1}	$n = 5$	10.6 ± 2.9	34.2 ± 6.5	40.1 ± 1.2	43.4 ± 1.1	<0.05
Endothelin-1, 1.0 nmol kg^{-1}	$n = 7$	17.6 ± 2.3	37.3 ± 3.1	39.9 ± 0.6	44.4 ± 0.5	<0.01
Endothelin-1 2.0 nmol kg^{-1}	$n = 4$	17.8 ± 2.2	57.3 ± 1.3	40.3 ± 0.9	46.7 ± 1.1	<0.05

Values are means ± s.e.mean.
P values were obtained by the Wilcoxon's signed rank test.

changes in MABP in response to endothelin-1 (Figure 3). Although both PAF receptor antagonists appeared to be effective in attenuating the endothelin-1-induced rise in haematocrit, the haematocrit was still significantly higher following administration of endothelin-1 in rats treated with either WEB 2086 or BN 52021 than in control animals. Haematocrit rose from 40.2 ± 0.5 to 42.3 ± 0.7% ($n = 7$, $P < 0.05$) and from 39.3 ± 0.7 to 42.2 ± 1.0% ($n = 6$, $P < 0.05$) in response to endothelin-1 in WEB 2086-, and BN 52021-treated animals, respectively.

Neither of the PAF receptor antagonists affected the level of permeability in the trachea, upper bronchi and pulmonary parenchyma (Figure 1). However, in the lower bronchi, BN 52021 reduced protein extravasation by 67%, while WEB 2086 caused only a slight, statistically non-significant decrease in tissue Evans blue content (Figure 1). Similarly, BN 52021, but not WEB 2086, nearly completely abolished endothelin-1-induced extravasation in the spleen (Figure 2). On the other hand, both PAF receptor antagonists were effective in inhibiting endothelin-1-induced increases in vascular permeability in the stomach, duodenum and kidney (Figure 3). However, in these tissues, WEB 2086 appeared to be slightly more potent than BN 52021 (100, 83 and 82% inhibition by WEB 2086 versus 81, 60 and 36% inhibition by BN 52021 in the three tissues, respectively). Although Evans blue content in the pancreas of WEB 2086-treated rats were statistically significantly higher than in control animals, these values did not differ significantly from endothelin-1 or BN 52021 plus endothelin-1-treated animals (Figure 2).

Since the inhibitory effects of WEB 2086 and BN 52021 on endothelin-1-induced protein extravasation were different in some tissues, the effects of these antagonists on the vascular permeability changes elicited by exogenous PAF were also compared. Intravenous injection of 1.9 nmol kg^{-1} PAF

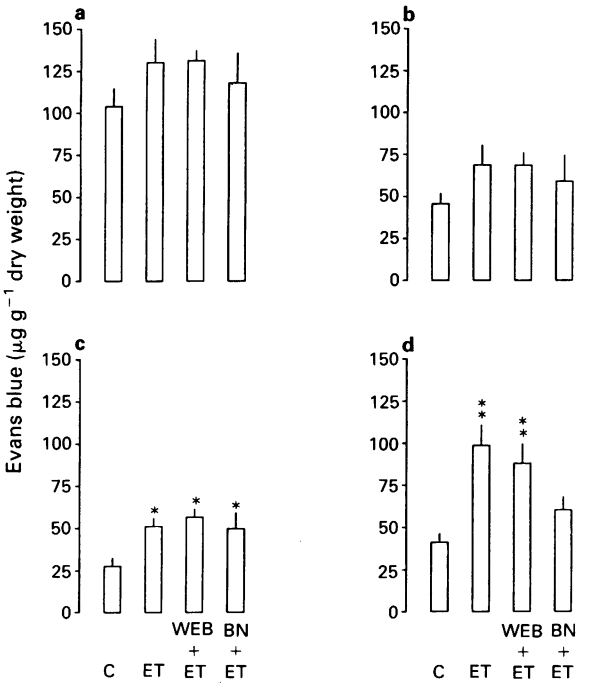


Figure 1 Effects of the PAF receptor antagonists, WEB 2086 and BN 52021 on endothelin-1-induced protein extravasation in rat airways: (a) trachea, (b) parenchyma, (c) upper bronchi and (d) lower bronchi. The animals were pretreated with WEB 2086 (WEB, 1 mg kg^{-1}), BN 52021 (BN, 10 mg kg^{-1}) or 0.9% NaCl (control, C) for 10 min before i.v. bolus injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after the injection of endothelin-1. Values are means with s.e.mean shown by vertical lines. $n = 9$ for C, $n = 7$ for endothelin-1 and WEB 2086 plus endothelin-1 and $n = 6$ for BN 52021 plus endothelin-1. * $P < 0.05$; ** $P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

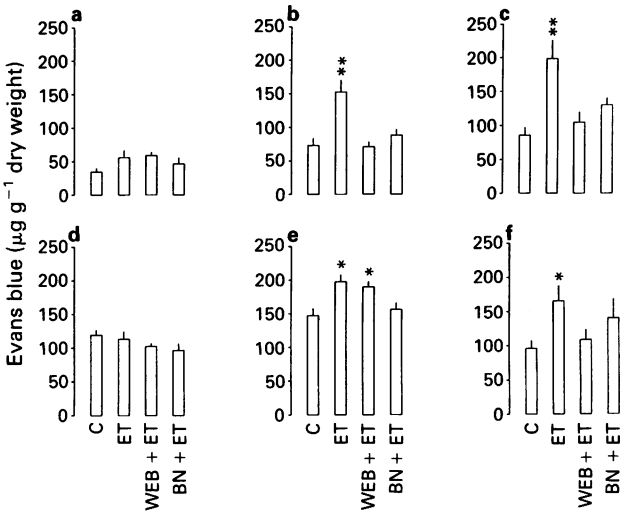


Figure 2 Effects of the PAF receptor antagonists, WEB 2086 and BN 52021 on endothelin-1-induced protein extravasation in the rat pancreas (a), stomach (b), duodenum (c), liver (d), spleen (e) and kidney (f). The animals were pretreated with WEB 2086 (WEB, 1 mg kg^{-1}), BN 52021 (BN, 10 mg kg^{-1}) or 0.9% NaCl (control, C) for 10 min before i.v. bolus injection of endothelin-1 (endothelin, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after the injection of endothelin-1. Values are means with s.e.mean shown by vertical lines. $n = 9$ for C, $n = 7$ for endothelin-1 and WEB 2086 plus endothelin-1 and $n = 6$ for BN 52021 plus endothelin-1. * $P < 0.05$; ** $P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

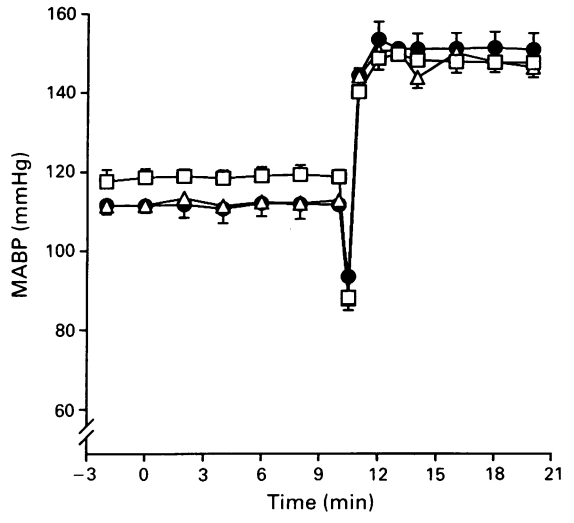


Figure 3 Endothelin-1-induced changes in mean arterial blood pressure (MABP) in conscious rats pretreated with WEB 2086 (△), BN 52021 (□) or 0.9% NaCl (●). The animals were given WEB 2086 (1 mg kg^{-1}, i.v.), BN 52021 (10 mg kg^{-1}, i.v.) or saline at 0 min, endothelin-1 (1 nmol kg^{-1}) was injected at 10 min. Values are means with s.e.mean shown by vertical lines for 7 experiments.

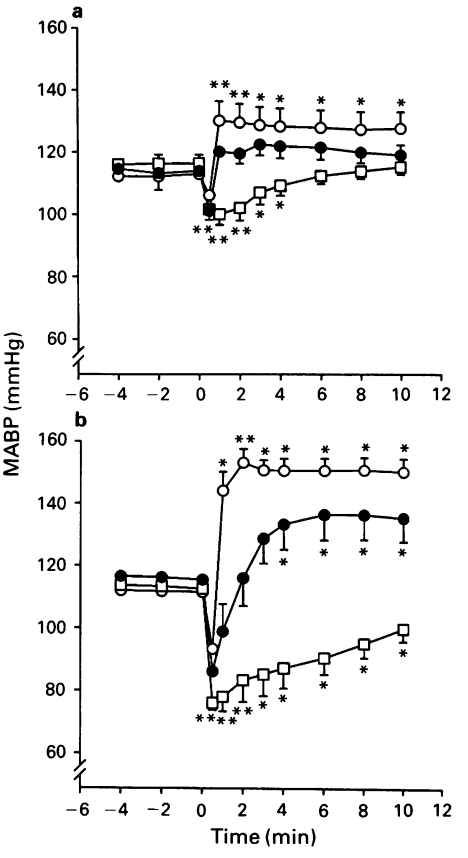


Figure 4 Blood pressure responses to combined administration of endothelin-1 and PAF in conscious rats. Endothelin-1 (○), PAF (□) or endothelin-1 plus PAF (●) were injected intravenously at 0 min as follows: (a) endothelin-1, 0.1 nmol kg^{-1} ($n = 5$), PAF, 0.19 nmol kg^{-1} ($n = 7$), endothelin-1, 0.1 nmol kg^{-1} plus PAF, 0.19 nmol kg^{-1} ($n = 7$); (b) endothelin-1, 1 nmol kg^{-1} ($n = 7$), PAF, 1.9 nmol kg^{-1} ($n = 7$) and endothelin-1, 1 nmol kg^{-1} plus PAF, 1.9 nmol kg^{-1} ($n = 5$). Values are means with s.e.mean shown by vertical lines. * $P < 0.05$; ** $P < 0.01$ (compared to the average of MABP between −4 and 0 min in the same group by Wilcoxon-Wilcox test).

resulted in a significant elevation of tissue Evans blue content in the trachea, upper and lower bronchi, pancreas, stomach and duodenum (Table 2). These changes were inhibited significantly by both BN 52021 and WEB 2086. The antagonists were equiactive in the trachea and duodenum. Compound WEB 2086 appeared to be more potent in the stomach than BN 52021, whereas BN 52021 was more potent than WEB 2086 in the upper and lower bronchi and pancreas (Table 2).

When given in the absence of either PAF or endothelin-1, the PAF receptor antagonists did not modify MABP, haematocrit or protein extravasation in any of the tissues studied.

The effectiveness of BN 52021 and WEB 2086 treatments were tested in separate experiments. Intravenous injection of 0.19 or 1.9 nmol kg^{-1} of PAF reduced MABP by 17.3 ± 2.6 and 43.0 ± 5.4 mmHg in the absence, and by 0.5 ± 1.2 ($n = 4$, $P < 0.05$) and 7.0 ± 1.1 mmHg ($n = 4$, $P < 0.05$) in the presence, of BN 52021, 10 mg kg^{-1}, respectively. Similar inhibition was observed following treatment with 1 mg kg^{-1} WEB 2086; MABP decreased by 18.3 ± 2.5 and 0.8 ± 1.1 mmHg ($n = 4$, $P < 0.05$), in the absence and presence of WEB 2086, and by 38.6 ± 1.4 and 7.5 ± 1.3 mmHg ($n = 4$, $P < 0.05$), in the absence and presence of the antagonist, in response to 0.19 and 1.9 nmol kg^{-1} PAF, respectively.

Table 2 Effects of the PAF receptor antagonists, WEB 2086 and BN 52021 on PAF-induced protein extravasation in conscious rats

Evans blue (μg g^{-1} dry weight)	*Control* ($n = 6$)	*PAF* (1.9 nmol kg^{-1}) ($n = 6$)	*WEB 2086* (1 mg kg^{-1}) + *PAF* (1.9 nmol kg^{-1}) ($n = 4$)	*BN 52021* (10 mg kg^{-1}) + *PAF* (1.9 nmol kg^{-1}) ($n = 4$)
Trachea	99.8 ± 8.4	185.5 ± 17.1*	143.8 ± 25.8	143.6 ± 27.7
Upper bronchi	39.6 ± 7.5	95.2 ± 5.7*	55.0 ± 13.8	35.6 ± 8.5§
Lower bronchi	40.6 ± 6.2	127.7 ± 17.3*	58.9 ± 13.5§	35.9 ± 2.2§
Pulmonary parenchyma	39.4 ± 8.3	46.1 ± 6.2	48.6 ± 6.9	21.7 ± 3.4
Pancreas	38.6 ± 3.0	210.5 ± 19.7*	60.5 ± 4.1*§	50.1 ± 11.1§
Stomach	72.9 ± 8.5	137.9 ± 4.8*	75.9 ± 6.0§	98.6 ± 14.0§
Duodenum	100.3 ± 10.8	220.1 ± 8.4*	92.8 ± 9.6§	95.6 ± 10.6§
Liver	119.0 ± 5.9	91.6 ± 5.8	90.8 ± 13.4	96.0 ± 5.2
Spleen	139.6 ± 14.3	149.7 ± 10.7	130.0 ± 14.1	162.4 ± 6.8
Kidney	96.7 ± 9.5	119.6 ± 9.4	98.5 ± 14.5	90.8 ± 3.2

Values are means ± s.e.mean. The animals were pretreated with WEB 2086 (1 mg kg^{-1}), BN 52021 (10 mg kg^{-1}) or their vehicle (control) 10 min before i.v. injection of PAF (1.9 nmol kg^{-1}) together with Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after administration of PAF. * $P < 0.05$ compared to controls. § $P < 0.05$ compared to PAF-treated animals by Dunn's multiple contrast hypothesis test.

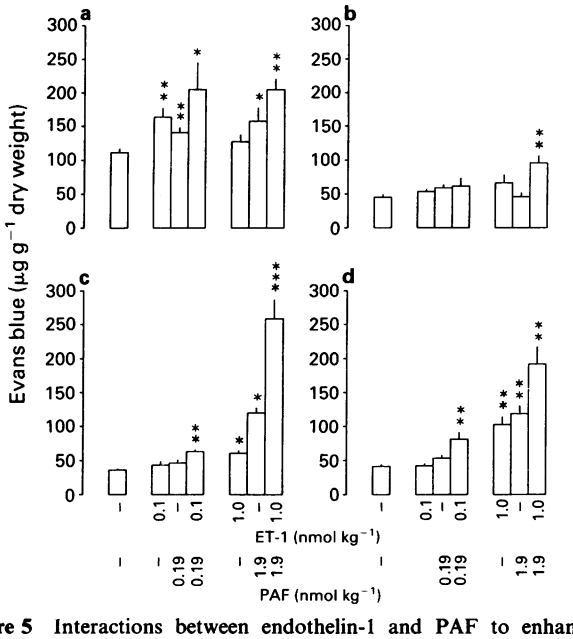


Figure 5 Interactions between endothelin-1 and PAF to enhance vascular permeability in rat airways: (a) trachea, (b) parenchyma, (c) upper bronchi and (d) lower bronchi: Endothelin-1 (0.1 or 1 nmol kg^{-1}), PAF (0.19 or 1.9 nmol kg^{-1}), endothelin-1, 0.1 nmol kg^{-1} plus PAF, 0.19 nmol kg^{-1}, endothelin-1, 1 nmol kg^{-1} plus PAF, 1.9 nmol kg^{-1} or saline were injected intravenously together with Evans blue dye, as indicated. The animals were killed 10 min after the injection. $n = 16$ for control (saline injection), $n = 5$ for endothelin-1, 0.1 nmol kg^{-1} and for combined administration of endothelin-1 plus PAF, $n = 7$ for endothelin-1, 1 nmol kg^{-1}, for PAF, 0.19 nmol kg^{-1} and for PAF, 1.9 nmol kg^{-1}. Values are means with s.e.mean shown by vertical lines. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to control by Dunn's multiple contrast hypothesis test).

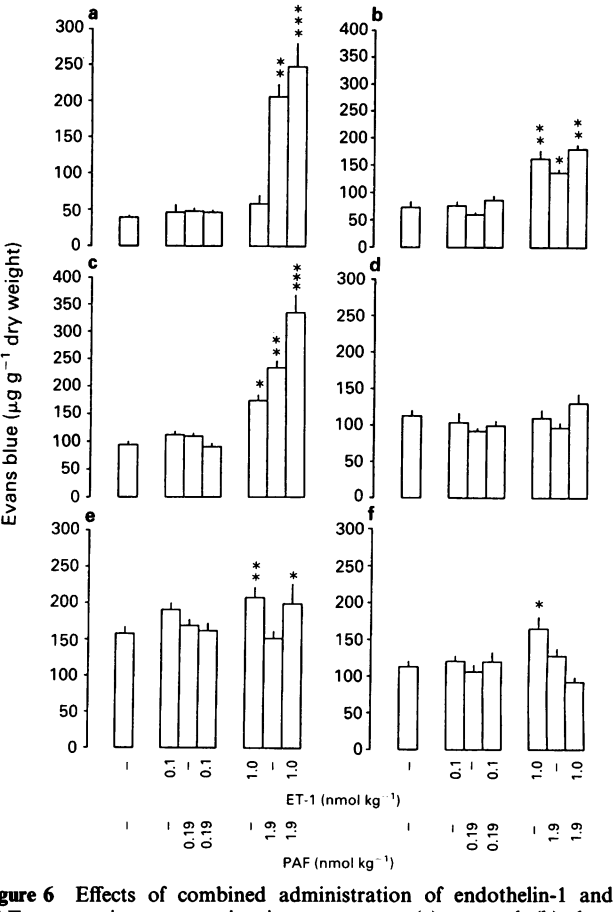


Figure 6 Effects of combined administration of endothelin-1 and PAF on protein extravasation in rat pancreas (a), stomach (b), duodenum (c), liver (d), spleen (e) and kidney (f). Endothelin-1 (0.1 or 1 nmol kg^{-1}), PAF (0.19 or 1.9 nmol kg^{-1}), endothelin-1, 0.1 nmol kg^{-1} plus PAF, 0.19 nmol kg^{-1}, endothelin-1, 1 nmol kg^{-1} plus PAF, 1.9 nmol kg^{-1} or saline were injected intravenously together with Evans blue dye, as indicated. The animals were killed 10 min after the injection. $n = 16$ for control (saline injection), $n = 5$ for endothelin-1, 0.1 nmol kg^{-1} and for combined administration of endothelin-1 plus PAF, $n = 7$ for endothelin-1, 1 nmol kg^{-1}, for PAF, 0.19 nmol kg^{-1} and for PAF, 1.9 nmol kg^{-1}. Values are means with s.e.mean shown by vertical lines. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (compared to control by Dunn's multiple contrast hypothesis test).

Effects of combined administration of endothelin-1 and PAF on vascular permeability

In view of the above results that suggested the involvement of PAF in endothelin-1-induced increase in vascular permeability, the vascular responses to combined administration of PAF and endothelin-1 were also studied. In the first set of experiments, endothelin-1 and PAF were injected at doses of 0.1 nmol kg^{-1} and 0.19 nmol kg^{-1}, respectively. At these doses, neither endothelin-1 (Table 1) nor PAF affected significantly haematocrit values which were 40.3 ± 0.5 and 41.2 ± 0.7% 20 min before, and 10 min after, administration of 0.19 nmol kg^{-1} PAF, respectively, $n = 7$, $P > 0.05$. As expected, injection of 0.19 nmol kg^{-1} PAF resulted in an immediate, transient hypotension, whereas 0.1 nmol kg^{-1} endothelin-1 evoked a biphasic response in MABP (Figure 4a). When endothelin-1 and PAF injections were combined, a slight increase in MABP was detected following an initial, transient hypotension (Figure 4a). The hypotensive response to injection of endothelin-1 plus PAF was similar to those observed after endothelin-1 injection (Figure 4a). Haematocrit rose from 39.9 ± 0.6 to 41.6 ± 0.5% ($n = 5$, $P > 0.05$) following injection of endothelin-1 plus PAF. Neither endothelin-1, nor PAF by itself, affected significantly Evans blue content in the tissues studied except in the trachea (Figure 5), where both endothelin-1 and PAF increased vascular permeability by 47 and 26%, respectively. However, when endothelin-1 and PAF were administered simultaneously, a significant increase in protein extravasation was observed in the upper and lower bronchi (Figure 5). In the trachea, the effects of endothelin-1 and PAF were additive (Figure 5).

Intravenous injection of 1.9 nmol kg^{-1} PAF resulted in similar changes in haematocrit values (39.6 ± 0.4 versus 45.5 ± 0.3%, $n = 7$, $P < 0.01$) to those observed following administration of 1 nmol kg^{-1} endothelin-1 (Table 1). Combined administration of these doses of endothelin-1 and PAF increased haematocrit from 39.4 ± 0.8 to 44.0 ± 0.9% ($n = 5$, $P < 0.05$). PAF, 1.9 nmol kg^{-1}, induced severe hypotension; MABP decreased by 36 mmHg within the first min after PAF injection, and 10 min after PAF injection, it was still 14 mmHg lower than control values (Figure 4b). Endothelin-1, 1 nmol kg^{-1} evoked biphasic changes (Table 1). Combined administration of endothelin-1 and PAF significantly reduced the magnitude and the duration of hypotensive response to PAF, and MABP returned to control levels 2 min after the injection of endothelin-1 plus PAF (Figure 4b). MABP then increased further and stabilized at around 130 mmHg for the entire observation period (Figure 4b). However, this level of MABP was 14 mmHg lower than that observed when 1 nmol kg^{-1} endothelin-1 was injected by itself.

Administration of 1.9 nmol kg^{-1} PAF significantly increased tissue Evans blue content in the trachea, and the upper and lower bronchi (Figure 5). When PAF and endothelin-1 injections were combined, protein extravasation was markedly enhanced in all three tissues (Figure 5). A significant increase in Evans blue content was observed even in

the pulmonary parenchyma following injection of endothelin-1 plus PAF, despite the fact that neither PAF nor endothelin-1 by itself modified vascular permeability in this tissue (Figure 5). PAF also increased protein extravasation in the pancreas by 430% (Figure 6). While 1.0 nmol kg^{-1} endothelin-1 caused only a slight increase in Evans blue content in this tissue, it potentiated the effect of PAF resulting in a 537% increase in Evans blue content (Figure 6). In the stomach and duodenum, the permeability effects of endothelin-1 and PAF appeared to be additive (Figure 6). Administration of 1.9 nmol kg^{-1} PAF affected neither protein extravasation nor the response to 1.0 nmol kg^{-1} endothelin-1 in the spleen (Figure 6). Interestingly, while PAF by itself had no effect on vascular permeability and endothelin-1 enhanced protein extravasation in the kidney, tissue Evans blue content was similar to that of control animals when the two mediators were injected simultaneously (Figure 6). No significant changes were detected in protein extravasation in the liver following administration of endothelin-1, PAF or endothelin-1 plus PAF (Figure 6).

Discussion

The present results show that i.v. administration of endothelin-1 evoked a dose-dependent haemoconcentration and increased protein extravasation in rat large airways, gastrointestinal tract and kidney. Some of these effects of endothelin-1 were significantly attenuated by specific PAF receptor antagonists. Furthermore, combined administration of PAF with endothelin-1 markedly enhanced protein extravasation in the airways and gastrointestinal tract.

In confirmation of earlier observations (Yanagisawa *et al.*, 1988; De Nucci *et al.*, 1988; Goetz *et al.*, 1988), the present study also shows a dose-dependent biphasic change of MABP in response to i.v. bolus injection of endothelin-1. Since pretreatment of the animals with the PAF receptor antagonists, WEB 2086 or BN 52021 failed to affect the time-course of changes in MABP, one may conclude that PAF neither mediates the initial hypotensive response nor counteracts the prolonged pressor action of endothelin-1. Endothelin-1-induced changes in MABP were accompanied by an acute haemoconcentration, as evidenced by the increase in haematocrit. The effect of endothelin-1 on haematocrit was dose-dependent, but there was only a narrow range between no response (0.1 nmol kg^{-1} endothelin-1) and an apparently maximal response (1–2 nmol kg^{-1}) to i.v. injection of endothelin-1. Since, at a dose of 2 nmol kg^{-1}, endothelin-1 caused severe bleeding from the orbital sinus, higher doses of the peptide were not tested and consequently the maximum effect was not precisely determined. Since endothelin-1 produces only a slight increase in mean circulatory filling pressure (Waite & Pang, 1990), an index of body venous tone (Yamamoto *et al.*, 1980), it seems unlikely that endothelin-1 affected the venous system as a whole in conscious rats. On the other hand, isolated veins have been reported to be more sensitive to endothelin-1 than arteries. For example, endothelin-1 was found to be a considerably more potent constrictor of the human mammary vein than of mammary arteries (Yang *et al.*, 1989) and it has a longer duration of action in rat mesenteric veins than mesenteric arteries (Warner, 1990). An increase in both pre-, and post-capillary resistance by endothelin-1 in certain vascular beds could lead to elevation of capillary hydrostatic pressure and consequently to enhanced transcapillary fluid transfer. The findings of Dahlöf *et al.* (1990) using a rat hindquarter preparation give experimental support to this notion. Although in this latter study no sign of increased vascular permeability was observed, it does not exclude the possibility that endothelin-1 is capable of modifying vascular permeability.

In order to examine this latter possibility, we studied the effects of endothelin-1 on extravasation of Evans blue dye, a marker of protein extravasation. Intravenous injection of 1 nmol kg^{-1} endothelin-1 resulted in a marked increase in protein extravasation in the upper and lower bronchi, stomach, duodenum and kidney, but not in the pulmonary parenchyma, pancreas and liver. Paradoxically, endothelin-1 at a dose of 0.1 nmol kg^{-1}, which affected neither haematocrit values nor protein extravasation in nine vascular beds studied, significantly enhanced tissue Evans blue content in the trachea, whereas at a higher dose it failed to affect protein extravasation in the same tissue. A likely explanation might be that the vasoconstriction evoked by the higher dose of endothelin-1 in the tracheal vascular bed could have masked its action on vascular permeability. In contrast to the present findings, topical application of endothelin-1 significantly reduced oedema formation elicited by chemotactic agents in rat and rabbit skin (Brain *et al.*, 1989; Chander *et al.*, 1989). This inhibitory action of endothelin-1 can be attributed to the markedly reduced local blood flow secondary to selective contraction of arterioles (Brain *et al.*, 1988). Interestingly, intradermal injection of endothelin-1 into human skin evoked a similar response to that observed after intradermal injection of histamine except for the formation of the wheal (Brain *et al.*, 1989). These observations suggest that the constrictor effect of endothelin-1 could mask any oedema-inducing effect of the peptide in the skin. The susceptibility of different vascular beds to endothelin-1 thus may reflect differential sensitivity of the vasculature to this peptide, differences in endothelin-1 receptor distribution and/or differences in local production of secondary mediators. Recent evidence suggests that PAF may be one of these mediators. Indeed, certain effects of endothelin-1 can be attenuated by PAF receptor antagonists (Lagente *et al.*, 1989; Takayashu *et al.*, 1989; Terashita *et al.*, 1989; Battistini *et al.*, 1990; Filep *et al.*, 1991), and endothelin-1-induced PAF synthesis has also been reported (López-Farré *et al.*, 1990).

Pretreatment of the animals with either of the structurally unrelated PAF receptor antagonists, WEB 2086 or BN 52021, attenuated the endothelin-1-induced rise in haematocrit by about 50 and 30%, respectively. However, haematocrit values were still significantly higher in WEB 2086- or PAF-treated rats than control values. Although WEB 2086 and BN 52021 at the doses employed were equally potent in inhibiting the hypotensive response to exogenous PAF, they exerted somewhat different inhibitory effects on endothelin-1-induced protein extravasation. Both PAF receptor antagonists were highly effective in inhibiting protein extravasation in the stomach, duodenum and kidney. However, WEB 2086 appeared to be a somewhat more potent inhibitor than BN 52021 in these vascular beds. On the other hand, BN 52021, but not WEB 2086, significantly attenuated the permeability effect of endothelin-1 in the lower bronchi and spleen. Similar differences in the inhibitory actions of WEB 2086 and BN 52021 were detected when comparison was made of their effects on the protein extravasation elicited by exogenous PAF. The different responses to WEB 2086 and BN 52021 suggest that one of the antagonists might act on a different PAF receptor population (Voelkel *et al.*, 1986). It is also possible that the bioavailability of these antagonists is different (Kubes *et al.*, 1990). Taken together, these findings suggest that PAF may be an important mediator of the action of endothelin-1 on vascular permeability. Mediator-stimulated increase in protein extravasation is primarily attributable to an increase in the hydraulic conductivity of the microvascular membrane (Grega *et al.*, 1986). All inflammatory mediators evaluated to date, including autacoids, anaphylatoxins and free radicals, induce interendothelial cell gap formation exclusively in the venules (Bjork & Smedegard, 1983; Grega *et al.*, 1986). This leads to opening of the variable large-pore system, the dominant macromolecular transport pathway operant in inflammation (Grega *et al.*, 1986). It is not known at present if endothelin-1 could induce the formation of venular large junctional gaps directly. However, endothelin-1 could possibly induce gap formation through release of PAF. Furthermore, it is reasonable to assume that mediators other than PAF could

also mediate or modulate the permeability action of endothelin-1, or both, since PAF receptor antagonism did not result in a complete inhibition of endothelin-1-induced increase in vascular permeability and haematocrit.

Enhanced plasma levels of endothelin-1 have recently been reported in pathological conditions, including ischaemia (Yasuda *et al.*, 1990; Watanabe *et al.*, 1990) and endotoxin shock (Morel *et al.*, 1989) when enhanced blood PAF levels can also be detected (Doebber *et al.*, 1985; Filep *et al.*, 1989; Montrucchio *et al.*, 1989; Mózes *et al.*, 1989). Since PAF and endothelin-1 exert opposite effects on the vascular tone in most vascular beds (PAF is usually a vasodilator while endothelin-1 is a vasoconstrictor), one might assume that they could modify each other's effects on vascular permeability. Indeed, combined injection of PAF and endothelin-1 significantly enhanced protein extravasation in most of the tissues studied. Thus, significantly enhanced protein extravasation was detected following the combined administration of low doses of endothelin-1 ($0.1\,nmol\,kg^{-1}$) and PAF ($0.19\,nmol\,kg^{-1}$) in the trachea, upper and lower bronchi despite the fact that neither endothelin-1 nor PAF by themselves affected vascular permeability in the two latter vascular beds. On the other hand, there were no changes in haematocrit and vascular permeability in other tissues following low doses of endothelin-1 plus PAF. Furthermore, when higher doses of endothelin-1 ($1\,nmol\,kg^{-1}$) and PAF ($1.9\,nmol\,kg^{-1}$) were injected simultaneously, markedly increased protein extravasation was detected in the airways, pancreas, duodenum and stomach. Interestingly, combined administration of endothelin-1 plus PAF evoked a significant increase in protein extravasation in the pulmonary parenchyma, where the classical inflammatory mediators fail to produce changes in macromolecular permeability (Maron, 1982; Grega *et al.*, 1986; Sirois *et al.*, 1990). The permeability effect of endothelin-1 was not affected by PAF in the spleen, whereas it was completely inhibited by PAF in the kidney. The rise in haematocrit in endothelin-1 plus PAF-treated animals were similar to those observed following PAF or endothelin-1 injections, suggesting that combined administration of endothelin-1 and PAF might have reduced extravasation in organs other than the kidney, which were not examined in the present study. A likely explanation for the markedly increased vascular permeability following combined injection of endothelin-1 and PAF might be that these mediators act in concert, but separately to open the variable large-pore system. The additive actions of endothelin-1 and PAF in the stomach, duodenum and pancreas lend support to this hypothesis. Another possibility might be that interendothelial gap formation could be attributed to PAF injected i.v. or released locally by endothelin-1, or both, and protein extravasation is further enhanced by the increased microvascular pressure due to endothelin-1-induced contraction of both arterioles and veins (see above). The permeability effects of endothelin-1 and PAF appear to be independent of changes in MABP. Indeed, low doses of endothelin-1 plus PAF increased protein extravasation in the trachea, upper and lower bronchi at a stable MABP (i.e. the vasopressor action of endothelin-1 counteracted the hypotensive effect of PAF). Administration of the higher doses of endothelin-1 ($1\,nmol\,kg^{-1}$) and PAF ($1.9\,nmol\,kg^{-1}$) significantly enhanced protein extravasation whereas only a slight elevation in MABP was observed. These observations are consistent with a view that changes in perfusion pressure or vasodilatation are not primary determinants of macromolecular permeability (Amelang *et al.*, 1981; Grega *et al.*, 1986). The inhibitory action of PAF on endothelin-1-induced protein extravasation in the kidney might be attributed to its potent contractile action in the renal vascular bed, and consequently to marked reduction in the renal blood flow (Scherf *et al.*, 1986).

The present observations may have relevance in pathological conditions where plasma or local tissue levels of endothelin-1 are elevated. In addition to vascular spasm, ischaemia and contraction of airway smooth muscle, endothelin-1-induced plasma extravasation with local oedema would induce or predispose to tissue damage. For example, it has been postulated that local oedema formation might directly contribute to bronchial hyperresponsiveness by causing airway narrowing and increasing mucosal thickness (Moreno *et al.*, 1986), might increase the susceptibility of gastric mucosa to damage through causing endothelial injury (Szabo *et al.*, 1985) and might contribute to the development of glomerular injury (Simonson & Dunn, 1991).

In conclusion, the present data demonstrate the capacity of endothelin-1 to induce haemoconcentration and to increase vascular permeability in the rat airways, gastrointestinal tract and kidney. These effects of endothelin-1 are mediated, in part, by the release of PAF. The present findings also suggest that endothelin-1 and PAF can act in concert to enhance vascular permeability in the airways and gastrointestinal tract independently of changes in mean arterial blood pressure.

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The cardioprotective effect of γ-glutamylcysteine ethyl ester during coronary reperfusion in canine hearts

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1 The cardioprotective effect of γ-glutamylcysteine ethyl ester was investigated on ischaemia-reperfusion-induced myocardial damage in anaesthetized dogs.

2 Open chest anaesthetized dogs were divided into four groups: 2 h occlusion of the left anterior descending coronary artery (LAD); 2 h LAD occlusion followed by 1 h reperfusion; 2 h LAD occlusion followed by 1 h reperfusion with administration of γ-glutamylcysteine ethyl ester (10 mg kg^{-1} just before reperfusion); 2 h LAD occlusion followed by 1 h reperfusion with administration of GSH (the reduced form of glutathione, 10 mg kg^{-1} just before reperfusion).

3 After occlusion or reperfusion, heart mitochondria were prepared from the normal area and the occluded or the reperfused area, and mitochondrial function (rate of oxygen consumption in State III, and respiratory control index) was measured polarographically.

4 Mitochondrial GSH and GSSG (the oxidized form of glutathione) concentrations, and activities of glutathione peroxidase and glutathione reductase were measured.

5 Two h of LAD occlusion induced mitochondrial dysfunction with depletion of mitochondrial GSH concentration. One h of reperfusion after 2 h LAD occlusion induced significant mitochondrial dysfunction associated with a marked depletion of mitochondrial GSH concentration.

6 γ-Glutamylcysteine ethyl ester reduced mitochondrial dysfunction and depletion of mitochondrial GSH concentration after 2 h LAD occlusion and 1 h reperfusion. In contrast, GSH did not prevent depletion of mitochondrial GSH concentration and mitochondrial dysfunction after 2 h LAD occlusion followed by 1 h reperfusion.

7 The activities of glutathione peroxidase and glutathione reductase did not change significantly in each group.

8 One h of reperfusion after 2 h occlusion of LAD induced ventricular arrhythmias. γ-Glutamylcysteine ethyl ester markedly reduced the development of reperfusion arrhythmias, whilst GSH showed no protective effect.

9 γ-Glutamylcysteine ethyl ester maintained mitochondrial GSH concentration, prevented reperfusion myocardial damage, and reduced reperfusion arrhythmias.

Keywords: γ-Glutamylcysteine ethyl ester; glutathione; mitochondria; ischaemia; reperfusion; arrhythmia

Introduction

Management of acute myocardial infarction is a major concern of many physicians, because myocardial infarction is a life threatening disease and might often be a cause of chronic heart failure. To reduce the amount of necrotic myocardium might lead to prevention of heart failure (Braunwald, 1976). Several invasive procedures such as intracoronary thrombolysis, percutaneous coronary angioplasty, and coronary bypass surgery for patients with acute myocardial infarction are performed to salvage jeopardized myocardium and beneficial effects of these procedures have been confirmed (DeWood *et al.*, 1983; Kloner *et al.*, 1983). Nevertheless, restoration of coronary blood flow is not always beneficial to ischaemic myocardium and exacerbation of ischaemia-induced myocardial dysfunction, i.e., reperfusion injury, is also recognized (Hearse, 1977).

Various pharmacological interventions have been proposed to prevent reperfusion injury. Since the generation of oxygen free radicals has been implicated as a pathophysiological mechanism involved in the transition from reversible to irreversible myocardial damage (Gauduel & Duvelleroy, 1984; Peterson *et al.*, 1985; Romaschin *et al.*, 1987; Ferrari *et al.*, 1989) treatment using radical scavengers has attracted much interest.

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It is widely accepted that the reduced form of glutathione (GSH) is one of the most important physiological antioxidants, working alone and in concert with glutathione peroxidase and glutathione reductase. Curello *et al.* (1985) reported that ischaemia-reperfusion decreased tissue GSH concentration and that this decrease might be linked with cellular dysfunction. Accordingly, it is expected that administration of GSH would alter favourably reperfusion injury. However, GSH itself as a medicament is not suitable because GSH is not taken up by cells in its intact form, and is degraded into its constituent amino acids before entering cells (Jensen & Meister, 1983). Recently, γ-glutamylcysteine ethyl ester, a precursor of glutathione biosynthesis, has been developed (Kamimura *et al.*, 1990). γ-Glutamylcysteine ethyl ester may penetrate into cells in its intact form and then be converted by glutathione synthetase resulting in an increase in intracellular GSH concentration (Ohtsu *et al.*, 1991). Accordingly, in this paper, we examined effects of γ-glutamylcysteine ethyl ester on mitochondrial function during coronary reperfusion and on reperfusion arrhythmias in relation to changes in mitochondrial GSH and GSSG concentrations.

Methods

Animal preparations

This study was performed in accordance with the guidelines for animal experimentation set by the University of Nagoya.

Fifty-two adult mongrel dogs of either sex weighing from 8 to 13 kg were anaesthetized with intraperitoneal sodium pentobarbitone (50 mg kg^{-1}). After endotracheal intubation, artificial ventilation was instituted with a Harvard ventilator (Igarashi Ika Kogyo, Tokyo, Japan). Lead II of the electrocardiogram was monitored continuously throughout the experiment with a VC-640G oscillographic recorder (Nihon Kohden Co., Ltd., Tokyo, Japan). The right femoral vein was dissected and cannulated for the administration of test drug and adequate hydrating fluid. The right femoral artery was dissected and cannulated for the recording of arterial blood pressure (AD-600; Nihon Kohden). The chest of each dog was opened with a left fourth or fifth intercostal incision, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free distal to the first major diagonal branch. A 3-0 silk suture was placed under the vessel, and polyethylene tubing was threaded over the suture. Occlusion was produced by ligating the vessel and the tubing together, and reperfusion was induced by cutting the suture and tubing at the same time. Reperfusion was shown by the immediate appearance of hyperaemia in the reperfused area.

Experimental protocol

The dogs were divided into four groups. In group 1 ($n = 9$), the LAD was occluded for 2 h. In group 2 ($n = 17$), the LAD was occluded for 2 h followed by 1 h of reperfusion. In group 3 ($n = 10$), the LAD was occluded for 2 h and γ-glutamylcysteine ethyl ester (10 mg kg^{-1}) was administered intravenously just before 1 h of reperfusion. In group 4 ($n = 16$), GSH (10 mg kg^{-1}) was administered intravenously just before 1 h of reperfusion following 2 h occlusion of the LAD.

Estimation of mitochondrial function

Heart mitochondria were prepared from the normal area and the occluded or the reperfused area by differential centrifugation according to the method of Hatefi *et al.* (1961), and the rate of oxygen consumption in State III (St. III O_2) and the respiratory control index (RCI) were measured. St. III O_2 was calculated from mitochondrial oxygen consumption in ng atoms (1 mole O_2 = 2 gram atoms O_2) of oxygen consumed mg^{-1} mitochondrial protein min^{-1} during State III respiration. RCI was taken as the ratio between the rate of oxygen consumption before and after the addition of adenosine diphosphate (ADP). Oxygen consumption in mitochondria was measured polarographically with an oxygen electrode and a closed cell (UC-12; Central Kagaku, Tokyo, Japan) as described previously (Sano *et al.*, 1990).

Estimation of mitochondrial GSH and GSSG concentrations

Mitochondrial total glutathione (GSH + GSSG) concentration was determined by the DTNB-glutathione reductase recycling method described by Tietze (1969) and mitochondrial GSSG concentration was determined according to the method of Griffith (1980). Heart mitochondria were prepared from the normal area and the occluded or the reperfused area as described above, and 0.5 ml of the prepared heart mitochondria was added 0.05 ml of sulphosalicylic acid (27.5% w/v) and agitated with a mechanical device. The sulphosalicylic acid supernatant solution was obtained after centrifuging at 10,000 *g* for 10 min and used for the glutathione assay. Mitochondrial GSH concentration was deduced by subtracting the GSSG concentration from the total glutathione concentration.

Estimation of mitochondrial glutathione peroxidase and glutathione reductase activities

Glutathione peroxidase was determined according to the method of continuous monitoring of GSSG formation described by Floré & Günzler (1984) with minor modifications (Sugiyama *et al.*, 1989). Prepared mitochondria diluted with 0.05 M potassium phosphate buffer was used as the enzyme source. The activity of glutathione peroxidase was expressed in units mg^{-1} mitochondrial protein. The amount of glutathione peroxidase inducing a net GSH decrease to 10% of the initial concentration in 1 min at 37°C at pH 7.0 is defined as 1 unit. Glutathione reductase was determined at 30°C according to the method of Carlberg & Mannervik (1985) with minor modifications. Prepared mitochondria diluted with 0.05 M potassium phosphate buffer was used as the enzyme source. The activity of glutathione reductase was expressed in units mg^{-1} mitochondrial protein, where 1 unit was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH min^{-1}.

Estimation of haemodynamic data

Arterial blood pressure was measured immediately before (0 min) and every 30 min during LAD occlusion and reperfusion. The number of ventricular premature beats and the total duration of ventricular tachycardia, which was defined as more than three successive ventricular premature beats, was also measured during LAD occlusion and reperfusion. In order to avoid artifacts such as LAD ligation and ventricular premature beats, heart rate was calculated only by sinus beats immediately before (0 min) and every 30 min after LAD ligation.

Statistics

The significance of all results was determined by Dunnett's test of analysis of variance except for mortality, for which the chi-squared test was used. Probability (P) values of less than 0.05 were considered statistically significant. All data in this paper are expressed as mean ± s.d.

Results

Mortality

Of the 52 dogs that underwent coronary artery occlusion, 5 died (1/9 in group 1; 1/17 in group 2; 2/10 in group 3; 1/16 in group 4). These were excluded from the subsequent analysis. During reperfusion, 15 dogs died from ventricular fibrillation (8/16 in group 2; 7/15 in group 4). Mortality in groups 2 and 4 was thus significantly higher ($P < 0.05$) than that in group 3. Data for the remaining 32 dogs form the basis of this paper.

Comparison of mitochondrial function

Figures 1 and 2 show St. III O_2 and RCI prepared from both the normal area and the occluded or the reperfused area in each group, respectively. In group 1 (2 h occlusion) mitochondrial function from the occluded area decreased significantly compared with the normal area. In group 2 (2 h occlusion followed by 1 h reperfusion) more severe mitochondrial dysfunction was observed. Administration of γ-glutamylcysteine ethyl ester significantly lessened mitochondrial dysfunction induced by 1 h reperfusion following 2 h occlusion, and indices of mitochondrial function from the reperfused area were improved significantly compared with the occluded area in group 1. Administration of GSH did not maintain mitochondrial function, and a similar decrease in mitochondrial function was observed to that in group 2.

Comparison of mitochondrial GSH and GSSG concentrations and activities of glutathione peroxidase and glutathione reductase

Table 1 shows mitochondrial total glutathione (GSH + GSSG), GSH, and GSSG concentrations prepared from both the normal area and the occluded or the reperfused area in each group, respectively. Two hour occlusion (group 1)

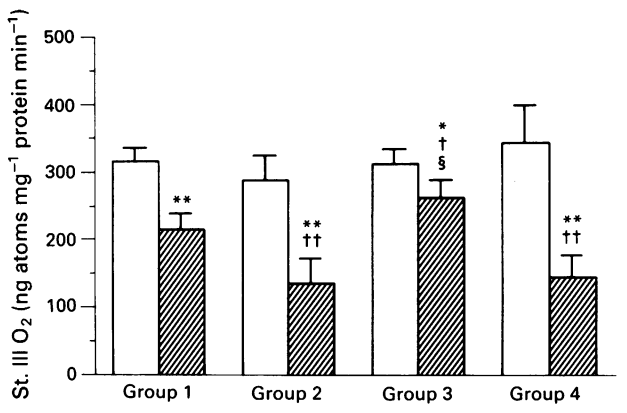


Figure 1 The rate of oxygen consumption in State III (St. III O_2) in heart mitochondria from the normal area (open columns) and the occluded or the reperfused area (hatched columns) in each group. Group 1 = 2 h occlusion; Group 2 = 2 h occlusion/1 h reperfusion; Group 3 = 2 h occlusion/1 h reperfusion with γ-glutamylcysteine ethyl ester administration; Group 4 = 2 h occlusion/1 h reperfusion with GSH administration.
* $P < 0.05$; ** $P < 0.01$ versus normal area of the corresponding group.
† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.
§ $P < 0.01$ versus reperfused area of Group 2.
In each group, St. III O_2 of the occluded or the reperfused area decreased significantly compared with the normal area of the corresponding group. However, in group 3, St. III O_2 of the reperfused area was maintained significantly compared with the occluded or the reperfused area in groups 1 and 2. Each column shows the mean and vertical bars indicate s.d.

Table 1 Mitochondrial total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG) concentrations in each experimental group (nmol mg^{-1} protein)

	Normal area	*Occluded or reperfused area*
Total glutathione		
Group 1	4.03 ± 0.57	3.26 ± 0.51*
Group 2	4.12 ± 0.40	2.53 ± 0.66**†
Group 3	4.06 ± 0.52	3.57 ± 0.46§
Group 4	3.94 ± 0.33	2.44 ± 0.57**††
GSH		
Group 1	3.59 ± 0.46	2.87 ± 0.40*
Group 2	3.67 ± 0.45	2.16 ± 0.64**†
Group 3	3.68 ± 0.54	3.12 ± 0.40§
Group 4	3.67 ± 0.37	2.17 ± 0.52**†
GSSG		
Group 1	0.44 ± 0.28	0.40 ± 0.17
Group 2	0.45 ± 0.14	0.37 ± 0.15
Group 3	0.38 ± 0.09	0.45 ± 0.15
Group 4	0.27 ± 0.08	0.27 ± 0.09

Values are mean ± s.d.
* $P < 0.05$; ** $P < 0.01$ versus normal area of the corresponding group.
† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.
§ $P < 0.01$ versus reperfused area of Group 2.
In group 2, mitochondrial total glutathione and GSH concentrations of the reperfused area decreased significantly compared with the occluded area in group 1. However, in group 3, they were maintained significantly compared with that in group 2.
No significant changes were observed in mitochondrial GSSG concentration among the normal areas and the occluded or the reperfused areas in all groups.

decreased mitochondrial total glutathione and GSH concentrations in the occluded area. This decrease was accelerated by 1 h reperfusion (group 2). Administration of γ-glutamylcysteine ethyl ester lessened the depletion of mitochondrial total glutathione and GSH concentrations induced by 1 h reperfusion following 2 h occlusion, and no significant changes were observed in mitochondrial total glutathione and GSH concentrations between the normal area and the reperfused area. On the contrary, administration of GSH itself did not maintain mitochondrial total glutathione and GSH concentrations, and significant decreases were observed similar to those in group 2. Mitochondrial GSSG concentration did not change significantly among the normal areas and the occluded or the reperfused areas in all groups. Table 2 shows activities of mitochondrial glutathione peroxidase and glutathione reductase. No significant changes were observed in activities of mitochondrial glutathione peroxidase and glutathione reductase among the normal areas and the occluded or the reperfused areas in all groups.

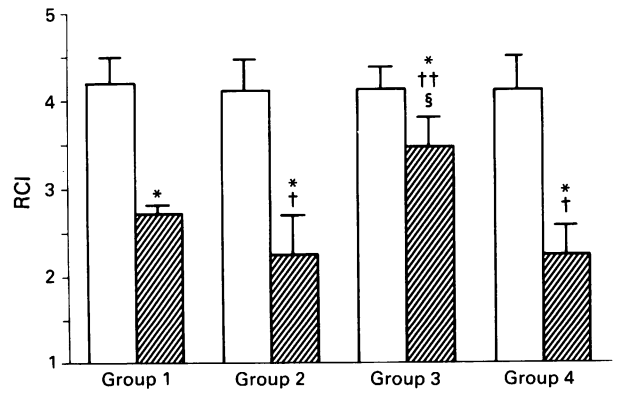


Figure 2 The respiratory control index (RCI) in heart mitochondria from the normal area (open columns) and the occluded or the reperfused area (hatched columns) in each group.
* $P < 0.01$ versus normal area of the corresponding group.
† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.
§ $P < 0.01$ versus reperfused area of Group 2.
In each group, RCI of the occluded or the reperfused area decreased significantly compared with the normal area of the corresponding group. However, in group 3, RCI of the reperfused area was maintained significantly compared with the occluded or the reperfused area in groups 1 and 2. Each column shows the mean and vertical bars indicate s.d.

Haemodynamic data

Figure 3 shows the time course of arterial blood pressure in each group. No significant difference in arterial blood pressure was seen among the groups. Tables 3 and 4 show the time

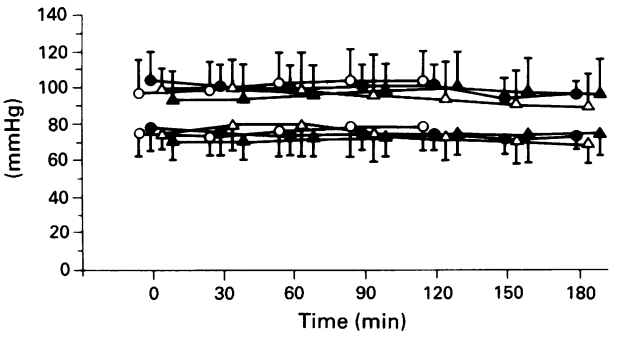


Figure 3 The time course of arterial blood pressure (systolic and diastolic) in each group. The time immediately before left anterior descending coronary artery (LAD) ligation is expressed as 0 min. (○) Group 1 (2 h occlusion); (●) group 2 (2 h occlusion/1 h reperfusion); (△) group 3 (2 h occlusion/1 h reperfusion with γ-glutamylcysteine ethyl ester administration); (▲) group 4 (2 h occlusion/1 h reperfusion with GSH administration). No significant difference in arterial blood pressure was observed among the groups. Each symbol represents the mean and vertical bars indicate s.d.

Table 2 Mitochondrial glutathione peroxidase and glutathione reductase activities in each experimental group (m units mg^{-1} protein)

	Glutathione peroxidase		*Glutathione reductase*	
	Normal area	*Occluded or reperfused area*	*Normal area*	*Occluded or reperfused area*
Group 1	17.03 ± 3.18	15.40 ± 3.57	7.99 ± 1.83	7.00 ± 1.85
Group 2	15.40 ± 5.32	12.73 ± 3.18	7.46 ± 1.34	5.83 ± 1.24
Group 3	16.95 ± 3.87	15.93 ± 3.51	7.44 ± 1.52	6.28 ± 1.67
Group 4	14.82 ± 3.17	11.54 ± 2.78	6.60 ± 1.27	5.35 ± 1.20

Values are mean ± s.d.
No significant changes were observed in activities of mitochondrial glutathione peroxidase and glutathione reductase among the normal areas and the occluded or the reperfused areas in all groups.

course of heart rate, the number of ventricular premature beats and the total duration of ventricular tachycardia in each group. No significant difference was observed during occlusion among the groups. However, administration of γ-glutamylcysteine ethyl ester reduced the number of ventricular premature beats and the total duration of ventricular tachycardia during reperfusion compared with those in groups 2 and 4 without inducing significant difference in heart rate among the groups.

Discussion

Mechanisms of reperfusion injury are considered to be complex biological phenomena and a number of factors have been suggested to contribute to the myocardial damage associated with ischaemia and reperfusion. These include myocardial high energy phosphate depletion (Jennings *et al.*, 1981; Jennings & Reimer, 1983), intracellular calcium overload (Fleckenstein *et al.*, 1974; Schanne *et al.*, 1979; Clusin *et al.*, 1983), inflammatory response (Fantone & Ward, 1982), loss of ionic homeostasis (Corr & Witkowski, 1983), labilization of lysosomes (Ogawa *et al.*, 1989), catecholamine (Singal *et al.*, 1981; 1982), and phospholipase-mediated membrane injury (Nagai *et al.*, 1985).

It is well known that adenine nucleotide breakdown to hypoxanthine and xanthine proceeds in ischaemic conditions and that superoxide anions are generated by xanthine oxidase after reperfusion (McCord, 1985). Neutrophils are also a major source of oxygen free radicals, and neutrophil migration is observed in necrotic myocardium (Romson *et al.*, 1982). Hence, generation of oxygen free radicals is expected to be enhanced in the ischaemia-reperfusion process. On the other hand, a living cell is provided with various radical scavenging systems such as superoxide dismutase, catalase, and glutathione. Glutathione is ubiquitously distributed, and has a wide range of radical scavenging functions. That is, its antioxidant function includes the removal of hydroperoxides, lipid peroxides, myeloperoxidase products, and also includes the removal of superoxide anions. Accordingly, glutathione is one of the most powerful intracellular antioxidant defences. Mitochondria contain most of the intracellular glutathione. Oxygen free radicals are produced endogenously by the mitochondrial electron transport system, and mitochondrial glutathione together with glutathione peroxidase might play an important role in scavenging these free radicals. Kajiyama *et al.* (1987) found that ischaemia-reperfusion decreased mitochondrial GSH concentration. Since oxygen free radical scavenging action by the glutathione redox system depends on GSH concentration itself within the range of cellular concentration, deterioration of the radical scavenging system in mitochondria might result from reperfusion. Since ischaemia-reperfusion might accelerate oxygen free radical formation, mitochondria during reperfusion phase are highly vulnerable to oxygen free radicals. Because mitochondria occupy a crucial role in cellular energy metabolism, mitochondrial dysfunction is directly linked with cellular dysfunction. Accordingly, a decrease in mitochondrial GSH concentration might play an important role in ischaemia-reperfusion-induced myocardial damage.

Table 3 Time course of heart rate (beats min^{-1}) in each group

	0	30	60	90	120	150	180 (min)
Group 1	119 ± 16	112 ± 13	104 ± 11	97 ± 14	94 ± 10		
Group 2	125 ± 10	114 ± 14	109 ± 15	102 ± 15	96 ± 12	95 ± 14	93 ± 12
Group 3	121 ± 12	117 ± 9	109 ± 9	99 ± 8	95 ± 4	92 ± 6	90 ± 5
Group 4	124 ± 18	117 ± 18	106 ± 16	95 ± 20	91 ± 17	91 ± 10	88 ± 8

Values are mean ± s.d.
The time immediately before left anterior descending coronary artery (LAD) ligation is expressed as 0 min.

Table 4 Number of ventricular premature beats and total duration of ventricular tachycardia (s) during occlusion and reperfusion

	Ventricular premature beats		*Ventricular tachycardia*	
	Occlusion	*Reperfusion*	*Occlusion*	*Reperfusion*
Group 1	30 ± 31		3 ± 6	
Group 2	11 ± 15	1376 ± 956	0 ± 1	1029 ± 671
Group 3	40 ± 51	32 ± 30*	1 ± 2	3 ± 5*
Group 4	42 ± 43	1731 ± 1574	1 ± 2	983 ± 959

Values are mean ± s.d.
* $P < 0.01$ versus reperfusion period of Group 2.

In this paper, we confirmed that ischaemia-induced mitochondrial dysfunction was accelerated by reperfusion, and that mitochondrial GSH concentration decreased significantly during occlusion, this decrease being exacerbated by reperfusion. Administration of γ-glutamylcysteine ethyl ester maintained mitochondrial GSH concentration and prevented reperfusion-accelerated mitochondrial dysfunction. GSH, a hydrophilic compound, cannot penetrate through cell membranes, and GSH administered exogenously might not be effective as an intracellular antioxidant. In these experiments it was confirmed that administration of GSH itself did not maintain mitochondrial GSH concentration and did not mitigate mitochondrial dysfunction associated with reperfusion.

The mechanism by which the concentrations of mitochondrial GSH decrease has not been fully elucidated. Kurosawa *et al.* (1990) found that proton gradient is necessary to maintain GSH in the mitochondrial matrix. Mitochondria deteriorated by ischaemia could not maintain proton gradient, and GSH might be washed out by reperfusion. In spite of administration of γ-glutamylcysteine ethyl ester, mitochondrial GSH concentration did not increase significantly in the normal area. GSH regulates its own biosynthesis via feedback inhibition (Gander *et al.*, 1983). However, it is reported that mitochondria do not contain enzymes for glutathione synthesis (Griffith & Meister, 1985). Accordingly, GSH concentration in mitochondria might regulate its own transport to mitochondria.

Reperfusion arrhythmias are another concern associated with reperfusion, because reperfusion arrhythmias might be a cause of sudden death. Since oxygen free radicals cause peroxidation of lipids in cell membrane resulting in alteration in the ionic permeability, oxygen free radicals are likely to be involved in reperfusion arrhythmias (Manning & Hearse, 1984). In the present paper, administration of γ-glutamylcysteine ethyl ester also mitigated markedly the development of reperfusion arrhythmias. No protective effects of GSH itself were observed in reperfusion arrhythmias. In previous studies (Sugiyama *et al.*, 1980; Hanaki *et al.*, 1989), a relationship between disturbances in energy metabolism and the development of ventricular arrhythmias was proposed. We would like to point out here that both ventricular arrhythmias and mitochondrial dysfunction were reduced by γ-glutamylcysteine ethyl ester.

Drugs such as propranolol (Shatney *et al.*, 1976) and thromboxane synthetase inhibitor (Toki *et al.*, 1988) reduce infarct size, and these drugs are also reported to reduce ischaemia-induced mitochondrial dysfunction (Nagai *et al.*, 1984; Ogawa *et al.*, 1988). Accordingly, γ-glutamylcysteine ethyl ester might contribute to the reduction of infarct size.

In conclusion γ-glutamylcysteine ethyl ester maintained mitochondrial GSH concentration, and reduced reperfusion-induced mitochondrial dysfunction. γ-Glutamylcysteine ethyl ester also reduced the development of reperfusion arrhythmias.

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Effect of carbenoxolone on the biological activity of nitric oxide: relation to gastroprotection

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1 The interactions between carbenoxolone and nitric oxide (NO) were examined by investigating their effects on human platelet aggregation, on rat aortic strips precontracted by phenylephrine and on protection of rat gastric mucosa against ethanol-induced injury.

2 Carbenoxolone (100–300 μM) caused a significant and concentration-dependent potentiation of rat peritoneal neutrophil (RPN)-, 3-morpholino-sydnonimine (SIN-1)- or iloprost-induced inhibition of platelet aggregation. Higher concentrations (500 μM) of carbenoxolone alone markedly inhibited platelet aggregation. Pretreatment with carbenoxolone (100–300 μM) antagonized the reversal of the RPN- or SIN-1-induced antiaggregatory effect by oxyhaemoglobin (10 μM).

3 Rat aortic strips with intact endothelium precontracted by phenylephrine (0.1–0.3 μM) were relaxed by carbenoxolone (100–300 μM) in a concentration-dependent manner. Relaxations were abolished by mechanical removal of the endothelium or by incubation with methylene blue (10 μM) or N^G-nitro-L-arginine (L-NNA, 100 μM). Sodium nitroprusside (10 nM)-induced relaxations of endothelium-denuded rat aortic strips were potentiated by carbenoxolone (100 μM).

4 The carbenoxolone (200 mg kg^{-1}, p.o.)-induced gastroprotection against ethanol was antagonized by L-NNA (5–40 mg kg^{-1}) in a dose-dependent manner. Pretreatment of rats with indomethacin (10 mg kg^{-1}, s.c.) increased the effect of L-NNA.

5 The results suggest that the activity of carbenoxolone in the experimental systems tested is due to phosphodiesterase inhibition, although radical scavenging properties of the drug could contribute to some of the effects observed. In the rat gastric mucosa both increased prostaglandin levels and effects on the NO system could contribute to the protective action of carbenoxolone.

Keywords: Carbenoxolone; nitric oxide; gastroprotection; platelet aggregation; rat aortic strips; platelet leukocyte interaction; N^G-nitro-L-arginine (L-NNA); 3-morpholino-sydnonimine (SIN-1)

Introduction

Carbenoxolone is the synthetic hemisuccinate of the triterpenoid 18β-glycyrrhetinic acid, which in turn is derived from glycyrrhizic acid, a biologically active ingredient of the liquorice root. Carbenoxolone protects the gastric mucosa against a variety of noxious agents in experimental animals (Derelanko & Long, 1981; Martin *et al.*, 1984) and accelerates peptic ulcer-healing in man (Doll *et al.*, 1962) without inhibiting acid secretion (Baron, 1977). The gastroprotective effect is diminished, but not abolished, by the cyclo-oxygenase inhibitor, indomethacin (Wan & Gottfried, 1985) suggesting that endogenous prostanoids mediate part of the gastroprotection. Other mediators could contribute to the carbenoxolone effect. We have, therefore, investigated the effect of carbenoxolone on the biological activity of endothelium-derived relaxing factor/nitric oxide (NO), another gastroprotective mediator system (Peskar *et al.*, 1991). Carbenoxolone was tested in two NO-producing experimental models, rat peritoneal neutrophils (RPN) and rat aorta. In addition, we have studied whether the protective effect of carbenoxolone against ethanol-induced gastric mucosal damage in rats is affected by pretreatment with an inhibitor of endogenous NO biosynthesis.

Methods

Preparation of rat peritoneal neutrophils

RPN were harvested from male Wistar rats (200–250 g) by peritoneal lavage 6 h after i.p. injection of 15 ml of a 0.6% solution of oyster glycogen similar to the procedure described by Palmer *et al.* (1980). After hypoosmotic lysis of contaminating erythrocytes in 0.2% saline, RPN were resuspended in oxygenated calcium-free Tyrode solution at a final concentration of 2×10^7 cells ml^{-1}. The RPN preparation contained at least 95% polymorphonuclear neutrophils and the viability was more than 97% as assessed by the trypan blue exclusion test.

Preparation of washed human platelets and aggregation bioassay

Blood was obtained from healthy volunteers who had not taken any medication for at least 2 weeks. Washed platelets were prepared as described by Radomski & Moncada (1983). The cells were suspended in oxygenated calcium-free Tyrode solution. After addition of calcium and magnesium ions (1 mM each), 1 ml aliquots of the platelet suspensions were incubated at 37°C for 3 min in an Eppendorf aggregometer. Aggregation was induced by addition of a submaximal (30–60 mu ml^{-1}) concentration of bovine thrombin. The decrease in optical density was monitored for 4 min after thrombin addition. In experiments with oxyhaemoglobin the aggregation was monitored for up to 15 min after addition of thrombin. Inhibition of platelet aggregation was calculated as the percentage decrease of optical density determined after 4 min induced by thrombin alone.

Inhibition of aggregation by rat peritoneal neutrophils, 3-morpholino-sydnonimine (SIN-1) or iloprost

RPN ($0.5–8.0 \times 10^5$ cells in a volume of 10–40 μl) or SIN-1 (final concentration 0.35–7 μM) or iloprost (final concentration 0.05–1 nM) were added to the platelet suspensions 1 min before thrombin. In experiments with carbenoxolone the drug was added to the platelets 30 s before RPN, SIN-1 or iloprost,

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respectively. Oxyhaemoglobin (10 μM) was added either 30 s or 10 min after thrombin.

SIN-1 was dissolved immediately before use in distilled water acidified to pH 5.2 with 1 N HCl. Carbenoxolone disodium salt and oxyhaemoglobin were dissolved in saline. A stock solution of iloprost (100 $\mu g\,ml^{-1}$) was diluted in saline to the concentrations desired. In control experiments the solvents were added to the platelet suspensions instead of the drugs.

Drug effects on endothelium-dependent relaxations of rat aortic strips

Spirally cut strips of rat thoracic aorta with intact endothelium were mounted in 5 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.

The solution was continuously gassed with 95% O_2/5% CO_2 at 37°C. The preload applied to the strips was 0.7 g. Contractions and relaxations were recorded with an isotonic lever transducer (Hugo Sachs, Hugstetten, F.R.G.) and displayed on a Watanabe multipen recorder. The tissues were allowed to equilibrate for 60 min with buffer changes every 10–15 min. Then the strips were precontracted by phenylephrine (0.1–0.3 μM) and the functional preservation of endothelium was tested by the induction of relaxations with carbachol (0.1–1 μM). Carbenoxolone was used in concentrations between 100 and 300 μM. In all experiments sodium nitroprusside (NaNP, 1–20 nM), which elicits endothelium-independent relaxations (Rapoport & Murad, 1983), was used as a reference compound. Results are expressed as NaNP equivalents.

In further experiments, the effects of a 1 h preincubation with methylene blue (10 μM) or N^G-nitro-L-arginine (L-NNA, 100 μM) on drug-induced relaxations of the rat aortic strips were investigated.

In an additional series of experiments, the relaxing effects of carbachol and carbenoxolone were tested before and after mechanical removal of the endothelium from the aortic strips.

Studies on gastric mucosal protection

Male Wistar rats (180–220 g) were fasted overnight with free access to water. Carbenoxolone disodium salt (200 $mg\,kg^{-1}$, suspended in 0.25% carboxymethylcellulose) was administered by oral intubation. Controls received the vehicle (2 $ml\,kg^{-1}$). Groups of 5–7 rats received i.v. injections of graded doses of L-NNA (5–40 $mg\,kg^{-1}$, dissolved in saline) or solvent 20 min before carbenoxolone. Thirty min after carbenoxolone administration, 1.5 ml of absolute ethanol was instilled into the stomach. Rats were killed 5 min later, the stomachs were removed, opened and gross mucosal damage was assessed by calculating a lesion index based on the number and length of haemorrhagic mucosal necroses as described previously (Peskar *et al.*, 1986). In additional experiments, rats were treated with L-arginine HCl or D-arginine HCl (100 $mg\,kg^{-1}$ each) immediately prior to injection of 40 $mg\,kg^{-1}$ L-NNA. L-Arginine HCl and D-arginine HCl were freshly dissolved in isotonic saline and administered i.v. in a volume of 2 $ml\,kg^{-1}$. Further groups of rats received indomethacin (10 $mg\,kg^{-1}$, s.c.) 60 min before i.v. injection of L-NNA (30 $mg\,kg^{-1}$) or saline. Ten min after administration of L-NNA, rats were treated with carbenoxolone (200 $mg\,kg^{-1}$, p.o.) followed by intragastric instillation of 1.5 ml of absolute ethanol after a further 30 min period.

Reagents

3-Morpholino-sydnonimine (SIN-1) was a gift from Cassella-Riedel-Pharma, Frankfurt, F.R.G. Carbenoxolone disodium salt was a gift from Biorex Laboratories, Enfield, Middlesex, and iloprost was kindly provided by Schering AG, Berlin, F.R.G. Methylene blue, phenylephrine, carbachol, sodium nitroprusside, N^G-nitro-L-arginine (L-NNA), L-arginine HCl, D-arginine HCl, indomethacin and oyster glycogen (type II) were purchased from Sigma Chemicals, St. Louis, MO, U.S.A. Haemoglobin was purified from bovine haemoglobin type I (Sigma) as described by Martin *et al.* (1985). Bovine thrombin was obtained from Behringwerke, Marburg, F.R.G.

Statistics

Results are expressed as means ± s.e.mean. Statistical analysis of parametric data was performed by use of Student's t test for unpaired data. In the case of multigroup comparisons t-statistics were corrected by the Bonferroni method. The Wilcoxon rank test was used for statistical comparison of non-parametric data. A probability of $P < 0.05$ was considered as statistically significant.

Results

Effects of carbenoxolone and oxyhaemoglobin on the inhibition of platelet aggregation by rat peritoneal neutrophils, SIN-1 and iloprost

RPN (0.5–6.0 × 10^5 cells) added to 1 ml of platelet suspension, SIN-1 (0.5–3 μM) and iloprost (0.1–1.0 nM) inhibited thrombin (30–60 mu)-induced platelet aggregation. The sensitivity of platelets varied between donors and a concentration of the inhibitors was selected that moderately inhibited aggregation (Figure 1a–c). The effects of RPN, SIN-1 and iloprost were significantly and concentration-dependently enhanced by carbenoxolone (Figure 1a–c). Carbenoxolone itself inhibited thrombin-induced platelet aggregation to a variable extent. Thus, at concentrations of 100 to 300 μM, inhibition by 10–100% was observed (data not shown). Sensitivity of platelets to carbenoxolone was constant within one experiment, but varied between donors. For the experiments shown in Figure 1, only platelets were used that exhibited maximally 15% inhibition of aggregation on exposure to 300 μM carbenoxolone. Complete inhibition of aggregation by RPN (1.5–8 × 10^5 cells ml^{-1}) or SIN-1 (3–10 μM) was abolished by oxyhaemoglobin (10 μM). This compound was effective, when added 30 s, but not 10 min, after thrombin. Pretreatment with a concentration of carbenoxolone (100–300 μM) that potentiated the antiaggregatory effects of RPN, but inhibited platelet aggregation maximally by 15% when given alone, antagonized the action of oxyhaemoglobin (Figure 2). Similarly, carbenoxolone antagonized the oxyhaemoglobin effect on the inhibition of platelet aggregation by SIN-1 (data not shown). On the other hand, oxyhaemoglobin did not influence the antiaggregatory effect of high concentrations of carbenoxolone alone (300–500 μM).

Drug effects on the phenylephrine-induced tone of rat aortic strips

Rat aortic strips with intact endothelium precontracted with phenylephrine (0.1–0.3 μM) were relaxed by carbachol (0.1–1 μM, data not shown) and carbenoxolone (Figure 3) in a concentration-dependent manner. On a molar basis carbenoxolone was about 300–500 times less potent than carbachol.

Mechanical removal of the endothelium or incubation of the aortic strips with methylene blue (10 μM) or L-NNA (100 μM) inhibited the carbachol- (data not shown) and carbenoxolone (300 μM)-induced (Figure 4) relaxations. Carbenoxolone (100 μM) potentiated the NaNP-induced relaxations of endothelium-denuded rat aortic strips. When NaNP (10 nM)-induced relaxations were taken as 100%, relaxations in the presence of carbenoxolone (100 μM) were 153 ± 17% ($P < 0.02$, $n = 6$).

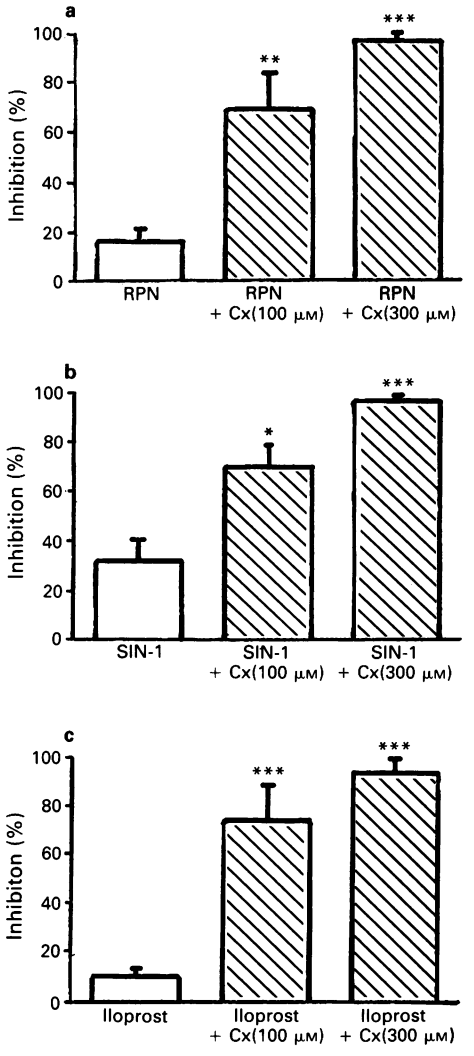


Figure 1 Effect of carbenoxolone (Cx, 100 and 300 μM) on the inhibition of thrombin (30–60 mu ml^{-1})-induced aggregation of human platelets by rat peritoneal neutrophils (RPN, 0.5–6.0 $\times$ 10^5 cells, a), 3-morpholino-sydnonimine (SIN-1, 0.5–3 μM, b) and iloprost (0.1–1.0 nM, c). In these experiments 300 μM carbenoxolone alone caused maximally 15% inhibition of aggregation. Results, shown as % inhibition of thrombin-induced platelet aggregation, are expressed as mean of 4–9 experiments per group; s.e.mean shown by vertical bars. Significant difference from RPN, SIN-1 and iloprost, alone is shown as $*P < 0.02$, $**P < 0.01$ and $***P < 0.002$, respectively.

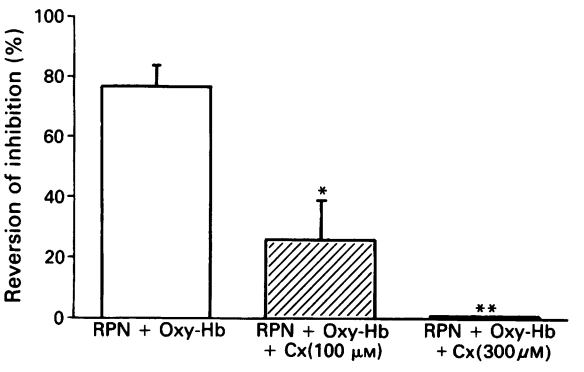


Figure 2 Effect of carbenoxolone (Cx, 100 and 300 μM) on the reversal of the antiaggregatory effect of rat peritoneal neutrophils (RPN, 1.5–8.0 $\times$ 10^5 cells ml^{-1}) by oxyhaemoglobin (Oxy-Hb, 10 μM) added 30 s after thrombin. In these experiments a concentration of RPN was selected that induced 100% inhibition of platelet aggregation, while exposure to 300 μM carbenoxolone alone caused maximally 15% inhibition of aggregation. Results show % reversion of inhibition of the antiaggregatory effect of RPN and are expressed as mean of 4–5 experiments per group; vertical bars show s.e.mean. $*P < 0.02$, $**P < 0.002$ as compared to the effect of oxyhaemoglobin alone.

Drug effects on ethanol-induced gastric mucosal damage

Gastric mucosal damage caused by ethanol was dose-dependently aggravated by i.v. administration of L-NNA (5–40 mg kg^{-1}) (Figure 5). Treatment with 200 mg kg^{-1}

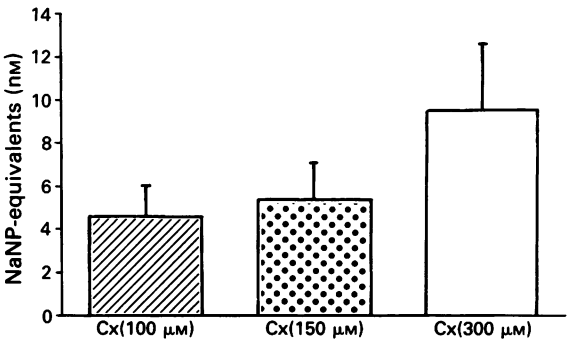


Figure 3 Relaxation of rat aortic strips with endothelium by carbenoxolone (100–300 μM). Results are given in sodium nitroprusside (NaNP)-equivalents (nM) and represent the mean of 8 experiments per group; vertical bars show s.e.mean.

Table 1 Effects of administration of N^G-nitro-L-arginine (L-NNA), L-arginine, D-arginine and indomethacin on the gastroprotective action of carbenoxolone

Treatment	*Dose* (mg kg^{-1})		*Dose* (mg kg^{-1})	*Lesion index*	(n)
Controls					
+saline				41 ± 3	(6)
+L-NNA	40			58 ± 3[a]	(7)
+indomethacin	10			44 ± 3	(6)
Carbenoxolone	200				
+saline				2 ± 1[b]	(6)
+L-NNA	40			42 ± 4**	(7)
+L-NNA	40	+L-arginine	100	10 ± 3[c]	(6)
+L-NNA	40	+D-arginine	100	35 ± 5	(6)
+L-NNA	30			28 ± 5**	(8)
+indomethacin	10			17 ± 3*	(10)
+indomethacin	10	+L-NNA	30	44 ± 2**	(7)

Results, shown as lesion index of gross mucosal damage, are mean ± s.e.mean of n animals. Statistical difference is shown as [a]$P < 0.01$ and [b]$P < 0.001$ vs. saline controls; [c]$P < 0.001$ vs. carbenoxolone + L-NNA (40 mg kg^{-1}); $*P < 0.01$; $**P < 0.001$ vs. carbenoxolone + saline. For sake of clarity, statistical differences of combined treatments vs. the corresponding controls are not included.

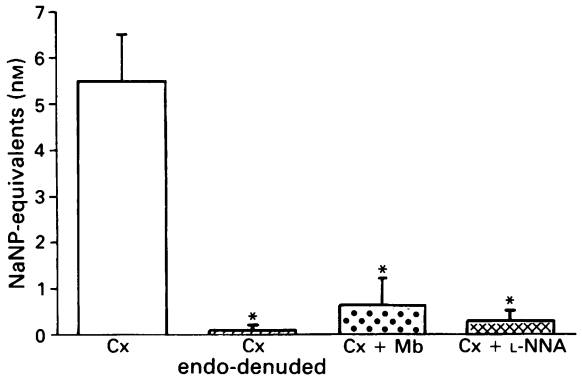


Figure 4 Effect of removal of endothelium (endo-denuded, $n = 6$), methylene blue (Mb, 10 μM, $n = 6$) and N^G-nitro-L-arginine (L-NNA, 100 μM, $n = 6$) on carbenoxolone (Cx, 300 μM)-induced relaxations of rat aortic strips. Results are given in sodium nitroprusside (NaNP)-equivalents (nM) and represent the mean with s.e.mean shown by vertical bars. Significant difference from carbenoxolone ($n = 18$)-induced relaxation is shown as $^*P < 0.05$.

carbenoxolone conferred significant protection against damage produced by ethanol. This protection was counteracted in a dose-dependent manner by pretreatment with L-NNA (Figure 5). Thus, while 200 mg kg^{-1} carbenoxolone reduced ethanol-induced gastric mucosal damage by 95 ± 2% in rats injected i.v. with saline, inhibition of gastric injury by carbenoxolone was only 26 ± 7% after administration of 40 mg kg^{-1} L-NNA. As shown in Table 1, the inhibitory effect of L-NNA (40 mg kg^{-1}) on the protective activity of carbenoxolone was reversed by concurrent administration of L-arginine HCl, but not D-arginine HCl (100 mg kg^{-1} each). Pretreatment with indomethacin (10 mg kg^{-1}, s.c.), a dose resulting in

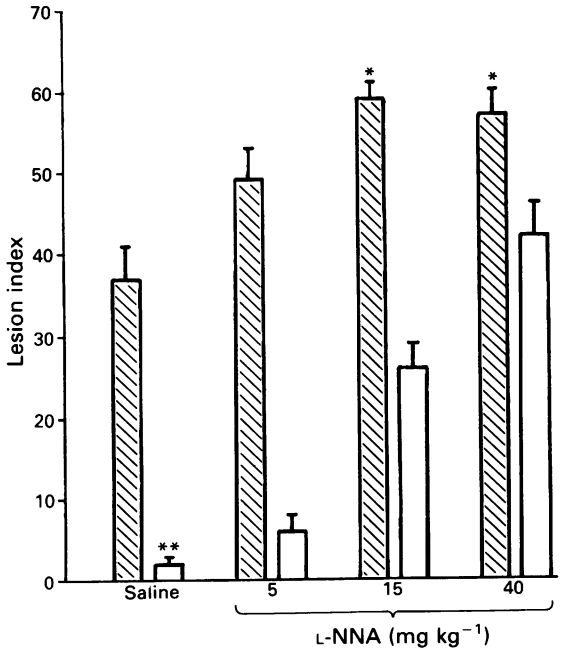


Figure 5 Inhibition of the carbenoxolone-induced gastroprotection by i.v. injections of N^G-nitro-L-arginine (L-NNA, 5–40 mg kg^{-1}). L-NNA augmented gross mucosal damage caused by i.g. instillation of ethanol (1.5 ml) and simultaneously counteracted the protection conferred by carbenoxolone (200 mg kg^{-1}, p.o.) Results give the lesion index and represent the mean of 6–7 rats per group; s.e.mean shown by vertical bars. Hatched columns: rats treated with saline or L-NNA before ethanol challenge; open columns: rats treated with saline or L-NNA followed by administration of carbenoxolone before ethanol challenge. $^*P < 0.01$; $^{**}P < 0.001$ as compared to saline-treated controls.

near-maximal inhibition of gastric mucosal prostaglandin formation, partially antagonized the protection conferred by carbenoxolone (Table 1). The inhibitory action of indomethacin on carbenoxolone-induced protection was additive to the effect of L-NNA (Table 1).

Discussion

The present results demonstrate increased biological activity of NO in the presence of carbenoxolone in two test systems, rat aortic strips and the antiaggregatory activity of RPN. The carbenoxolone-induced relaxations of rat aortic strips probably involve NO, since they are inhibited by mechanical removal of the endothelium (Furchgott & Zawadzki, 1980), treatment with the inhibitor of guanylate cyclase methylene blue (Martin *et al.*, 1985) or treatment with the inhibitor of NO biosynthesis L-NNA (Moore et al., 1990; Mülsch & Busse, 1990; Ishii *et al.*, 1990). The increased biological activity of NO observed could result from increased biosynthesis and/or decreased inactivation of NO or direct effects on the second messenger systems in the target tissues.

It has been demonstrated that NO is destroyed by superoxide anions (Gryglewski *et al.*, 1986) and that inactivation of superoxide anions protects and prolongs the action of NO (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986). Thus, superoxide dismutase induces relaxation of rat aortic strips with intact endothelium (Rubanyi & Vanhoutte, 1986; Rimele *et al.*, 1988) and increases the antiaggregatory activity of neutrophils (Radomski *et al.*, 1987). Compounds with steroidal structures were recently shown to be potent O_2^--scavengers and to be active in protecting neurones against peroxide injury (Hall *et al.*, 1987). Carbenoxolone in micromolar concentrations has been shown to suppress the generation of superoxide anions and hydrogen peroxide in macrophages (Suzuki *et al.*, 1983). Thus, the scavenging property of carbenoxolone could contribute to the potentiation of the antiaggregatory activity of RPN and SIN-1 as well as to the carbenoxolone-induced endothelium-dependent relaxation of the rat aorta.

In addition, glycyrrhetinic acid and carbenoxolone have been demonstrated to be potent inhibitors of phosphodiesterases that inactivate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Amer *et al.*, 1974; Vapaatalo *et al.*, 1978). Other phosphodiesterase inhibitors such as the specific inhibitor of cyclic GMP inactivation, M&B 22948, and the non-specific inhibitors, isobutylmethylxanthine (IBMX) and papaverine, were shown to relax rat aortic strips (Martin *et al.*, 1986). In rat aorta the K_i values of glycyrrhetinic acid for cyclic GMP and cyclic AMP phosphodiesterases were found to be 2.5 and 6.0×10^{-5} M, respectively (Amer *et al.*, 1974). These concentrations are in the same order of magnitude as those effective in our experimental systems. As to rat aorta, the lower K_i value for inhibition of cyclic GMP degradation indicates some selectivity for cyclic GMP-mediated effects such as NO-induced smooth muscle relaxation.

The potentiation of the relaxing effect of NaNP on endothelium-denuded aortic strips further supports the suggestion that phosphodiesterase inhibition is a major mechanism of action of carbenoxolone. Similar results have been obtained after combined administration of glyceryl trinitrate, which stimulates guanylate cyclase in blood vessels (Schultz *et al.*, 1977) and the selective cyclic GMP-phosphodiesterase inhibitor, M&B 22948 (Kukovetz *et al.*, 1979; Martin *et al.*, 1986). Furthermore, the antagonistic effect of carbenoxolone on the action of oxyhaemoglobin on RPN- and SIN-1-induced inhibition of platelet aggregation resembles that of IBMX and is compatible with phosphodiesterase inhibition (Salvemini *et al.*, 1990). Both IBMX (Salvemini *et al.*, 1990) and carbenoxolone, counteract the effect of oxyhaemoglobin, added 30 s after thrombin, on RPN- and SIN-1-induced inhibition of platelet aggregation. Finally, the carbenoxolone-

induced potentiation of the inhibition of platelet aggregation by iloprost can be attributed to phosphodiesterase inhibition, but not radical scavenging, since the iloprost effect on platelets is mediated by stimulation of adenylate cyclase (Stürzebecher & Losert, 1987) and is insensitive to superoxide anions.

We have previously shown that carbenoxolone inhibits the prostaglandin inactivating enzymes, 15-hydroxy-prostaglandin-dehydrogenase and Δ^{13}-prostaglandin-reductase (Peskar *et al.*, 1976) suggesting that prolongation of the biological activity of locally generated prostaglandins contributes to the protective and ulcer-healing activity of the drug. Furthermore, in patients with peptic ulcer disease administration of carbenoxolone was reported to increase the secretion of prostaglandin E_2 into the gastric lumen (Rask-Madsen *et al.*, 1983). However, as shown by our results, pretreatment with indomethacin in a dose that causes near-maximal suppression of gastric mucosal prostaglandin formation only partially antagonized the protective activity of carbenoxolone against ethanol-induced rat gastric mucosal damage confirming results published by Wan & Gottfried (1985). The present findings demonstrate that the indomethacin-resistant part of the gastroprotective activity of carbenoxolone is sensitive to inhibition of NO biosynthesis. We have shown recently that protection against ethanol-induced mucosal damage elicited by stimulation of afferent sensory neurones by intragastric capsaicin depends on endogenous NO formation (Peskar et al., 1991). The capsaicin-induced gastroprotection is not affected by indomethacin (Holzer *et al.*, 1990) and is substantially antagonized by pretreatment with L-NNA in a dose as low as $1.5\,mg\,kg^{-1}$ (Peskar *et al.*, 1991). In contrast, higher doses of L-NNA are necessary to counteract the protection conferred by carbenoxolone. Our results with L-NNA and indomethacin suggest that both the NO and prostaglandin systems contribute to the protective effect of carbenoxolone. Due to its characteristic pharmacokinetics with significant absorption in the stomach and strong binding to gastric mucosal proteins high tissue levels of carbenoxolone can be expected to occur in the gastric mucosa (Johnston *et al.*, 1975). Concentrations of carbenoxolone in human gastric mucosa reached after oral administration of the drug are in the order of magnitude shown to be active under our experimental conditions (Peskar, 1980). This correlation further supports the concept that local pharmacological effects of carbenoxolone, e.g. the increase in mucosal blood flow (Wan & Gottfried, 1985), might be related to increased activity of the endogenous NO system due to the inhibitory action on phosphodiesterases. It remains to be investigated, to what extent the gastroprotection observed with other phosphodiesterase inhibitors such as papaverine (Hagel et al., 1982) is related to increased biological activity of locally formed NO.

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Evidence for inhibition of sympathetic neurotransmission by endogenously released acetylcholine in the guinea-pig trachea

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1 Interactions between pulmonary cholinergic and noradrenergic nerves were studied in the innervated tracheal tube preparation isolated from guinea-pigs anaesthetized with urethane. Relaxations of the trachealis smooth muscle in response to postganglionic stimulation of the sympathetic nerve were recorded as decreases in the intraluminal pressure of the tracheal tube after the pressure had been raised with the stable thromboxane-mimetic, U46619. In contrast, contractions following preganglionic stimulation of the vagal nerve trunk were recorded as increases in intraluminal pressure.

2 In approximately half of the preparations studied, concurrent stimulation of the vagal nerve trunk inhibited relaxation responses elicited by stimulation of the sympathetic nerves. The vagi were stimulated at parameters which caused no change in intraluminal pressure, excluding the involvement of postjunctional mechanisms.

3 The effect of simultaneous stimulation of the sympathetic nerve trunk was studied on contractile responses evoked by preganglionic stimulation of the vagus nerve. In 80% of the preparations tested the vagal responses were inhibited. This inhibitory effect of sympathetic nerve stimulation was antagonized by propranolol.

4 The potassium channel agonist, cromakalim, endothelins 1 and 3 and the neuropeptides, vasoactive intestinal peptide, neurokinin A and substance P, did not significantly modulate sympathetic nerve-induced relaxations.

5 The anticholinesterase drug, physostigmine, induced a concentration-dependent increase in the intraluminal pressure of the tracheal tube and potentiated the postjunctional action of exogenously applied acetylcholine to contract the guinea-pig trachealis muscle. In the presence of higher concentrations of physostigmine both vagally-induced contractions and sympathetic nerve-induced relaxations were reduced. Atropine blocked both the inhibitory effect of physostigmine on sympathetic relaxations and its postjunctional contractile action on the trachealis smooth muscle.

6 It is concluded that, in the guinea-pig trachea, acetylcholine released endogenously from pulmonary parasympathetic nerves, either by anticholinesterase drugs or in response to nerve stimulation, can inhibit transmission in the adjacent sympathetic nerves via activation of prejunctional muscarinic heteroreceptors, probably of the M_3 subtype.

Keywords: Inhibitory muscarinic heteroreceptors; endogenous acetylcholine; sympathetic neurotransmission; guinea-pig trachea

Introduction

The airways receive a dual innervation from both the parasympathetic and sympathetic nervous systems. In all species the dominant control of airway smooth muscle tone is exerted via the parasympathetic nerves, whereas the sympathetic innervation to airway smooth muscle is very species-dependent and may be sparse or absent (Mann, 1971). However, even in species which lack a direct sympathetic supply to the airway smooth muscle, cholinergic and noradrenergic varicosities have been shown to lie in close apposition (Jones *et al.*, 1980; Daniel *et al.*, 1986). The close proximity of the two branches of the autonomic nervous systems suggests that they may interact at the prejunctional level and that either nerve may be able to modulate neurotransmission in the other type of nerve and thus modulate airway smooth muscle tone. This suggestion is supported by the observation that in the guinea-pig, stimulation of the sympathetic nerve trunk inhibits vagally-induced contractions of the innervated tracheal tube preparation via activation of β-adrenoceptors on the cholinergic nerve endings (McCaig, 1987). Similarly, in the dog (Cabezas *et al.*, 1971) and cat (Baker & Don, 1987) sympathetic nerve stimulation inhibited the bronchoconstrictor effect of vagal stimulation *in vivo*.

The reverse relationship, that is, modulation of sympathetic neurotransmission by acetylcholine released from the adjacent parasympathetic nerves, has not yet been fully investigated in the airways. However, in other tissues which receive a dual innervation, such as the gut (Manber & Gershon, 1979) and heart (Löffelholz & Muscholl, 1970; Levy & Blattberg, 1976; Boyle & Pollock, 1988), there is evidence that endogenously released acetylcholine can inhibit noradrenaline release via activation of prejunctional muscarinic heteroreceptors on the sympathetic nerve terminals.

We have previously demonstrated, using the guinea-pig isolated innervated tracheal tube preparation, that exogenously applied muscarinic agonists can inhibit noradrenaline release via activation of muscarinic M_3 cholinoceptors on pulmonary sympathetic nerve terminals (Pendry & Maclagan, 1989; 1991). The aim of the present study was to determine whether these inhibitory muscarinic heteroreceptors could be activated by endogenous acetylcholine released from pulmonary parasympathetic nerves and whether stimulation of the parasympathetic nerves might inhibit the release of noradrenaline from the adjacent sympathetic nerves via this mechanism. A preliminary account of these findings has previously been reported to the British Pharmacological Society (Pendry & Maclagan, 1990).

Methods

Male guinea-pigs (200–400 g) of the Dunkin-Hartley strain were killed with an anaesthetic overdose (urethane 1.5 g kg^{-1}, i.p.). The trachea with the sympathetic nerve trunk on the right hand side and both vagal nerves and recurrent laryngeal

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branches attached was removed. During the dissection, the pulmonary nerves were kept moist with Krebs-Henseleit solution (composition mM: $NaCl_2$ 118.4, KCl 4.7, $NaHCO_3$ 25.0, glucose 11.1, KH_2PO_4 1.16, $MgSO_47H_2O$ 1.19 and $CaCl_2$ 2.6) gassed with 95% O_2 and 5% CO_2. The trachea was cannulated at both ends and mounted horizontally, at its *in vivo* length, in an organ bath (25 ml) containing gassed Krebs-Henseleit solution maintained at 37°C. The lumen of the trachea was filled with Krebs-Henseleit solution; one end was closed and the other end was attached to a Statham (P23AC) transducer to measure changes in the intraluminal pressure (ILP). Increases or decreases in ILP reflected smooth muscle contraction or relaxation, respectively.

All ILPs were expressed as values in mmH_2O above the minimum ILP in the presence of excess isoprenaline (10^{-3} M) which was established at the end of each experiment. At the beginning of each experiment, tissues were left to equilibrate for 1 h in the presence of indomethacin (5×10^{-6} M) to remove any prostaglandin-induced tone. Indomethacin was present in the Krebs-Henseleit solution throughout all experiments.

Effect of concurrent vagal and sympathetic stimulation

The vagal nerve trunks and sympathetic stellate ganglion were stimulated through two separate bipolar platinum electrodes with trains of rectangular pulses delivered from two Grass S44 stimulators, via stimulator isolation units to avoid any 'leakage' of current.

In the first part of this study, the tone of the guinea-pig trachealis muscle was raised with U46619 in order to observe relaxation responses following supramaximal postganglionic stimulation of the sympathetic nerve trunk (40 Hz max voltage, 0.2 ms for 5 s at 90 s intervals) (Pendry & Maclagan, 1991). When the tone had reached a plateau, sympathetic nerve-induced relaxations were measured in the absence and presence of concurrent submaximal vagal stimulation (20 Hz, 0.2 ms for 180 or 270 s). The stimulation voltage was chosen such that stimulation of the vagus alone caused no significant change in ILP in order to exclude involvement of physiological antagonism and of tone changes.

In the second part of this study contractile responses following supramaximal preganglionic stimulation of the vagal bundles (30 Hz, max voltage, 0.2 ms for 5 s at 45 s intervals) were measured. When a stable response had been obtained, the effect of concurrent submaximal sympathetic stimulation (40 Hz, 0.2 ms, 90 s) on these vagally-induced contractions was investigated. Sympathetic parameters were chosen so that stimulation of the sympathetic nerve trunk alone did not alter the ILP, to exclude physiological antagonism.

Effect of an anticholinesterase drug

The effect of the anticholinesterase, physostigmine, was studied (1) on the ILP, (2) on contractile responses of the guinea-pig trachea following addition of acetylcholine, and (3) on contractions evoked by stimulation of the preganglionic cholinergic nerve fibres (30 Hz, max voltage, 0.2 ms, 5 s). Sympathetic nerve-induced relaxations of the guinea-pig trachea (40 Hz, max voltage, 0.2 ms, 5 s) were also measured following at least 15 min preincubation with physostigmine (10^{-7} or 10^{-6} M); in these experiments the intraluminal pressure was raised with U46619.

Drugs

U46619 (11α, 9α epoxymethano-prostaglandin H_2; Sigma/Semat), physostigmine sulphate, urethane, indomethacin (Sigma), acetylcholine bromide, atropine sulphate (BDH), propranolol hydrochloride (ICI) and hexamethonium bromide (Koch Light), cromakalim (Beecham), neurokinin A, substance P, vasoactive intestinal polypeptide (VIP), endothelin 1 and 3 (Peninsula) and isoprenaline sulphate (Wellcome) were used.

Indomethacin (1 mg ml^{-1}) was dissolved daily in buffer, (KH_2PO_4 19.76 mM and Na_2HPO_4 118.34 mM; pH adjusted to 7.8 with NaOH by warming and sonication before addition to the Krebs-Henseleit solution) final concentration 5×10^{-6} M. Stock solutions of U46619 were prepared by dissolving 1 mg in 1 ml ethanol and adding 0.9% saline to give a 1 mM solution. Cromakalim was dissolved in saline and sonicated for at least 10 min. Stock solutions of neurokinin A, substance P and VIP were prepared in distilled water. Endothelin 1 and 3 were dissolved in 0.001 M acetic acid. Aliquots of these stock solutions were stored at −70°C and diluted daily in 0.9% saline. All resultant solutions were stored on ice throughout the experiment. All other drugs were dissolved and diluted in 0.9% saline. Stock solutions of isoprenaline were never kept for longer than 5 min and therefore no antioxidant was used.

Statistical analysis of data

Sympathetic nerve-induced relaxations in the absence and presence of physostigmine, cromakalim and the peptides were compared by unpaired Student's *t* tests and were deemed significantly different when $P < 0.05$.

Results

Effect of concurrent vagal and sympathetic stimulation

Figure 1 shows an experiment in which the tone of the guinea-pig trachea was raised with U46619 (10^{-7} M). As previously described, sympathetic nerve-induced relaxations increased as the ILP was increased (Pendry & Maclagan, 1991). When the tone had reached a plateau, repetitive stimulation at supramaximal voltage of the postganglionic sympathetic nerve trunk resulted in reproducible relaxations. When the vagal nerves were stimulated simultaneously with the sympathetic nerve trunk at submaximal voltages which caused no increase in ILP, inhibition of succeeding sympathetic relaxation responses was seen.

This inhibitory effect of the parasympathetic nervous system on responses to sympathetic nerve stimulation was observed in approximately 50% of the preparations and was not dependent upon the reverse relationship (that is, inhibition of vagal responses by the sympathetic nervous system); nor was it related to stimulation of either the right or the left vagal nerve trunk. The inhibitory effect of the parasympathetic nervous system on the sympathetic neurotransmission was: (i) very variable between tissues; (ii) not reproducible in the same tissue; and (iii) declined with successive periods of stimulation. It was therefore not possible to investigate the effect of muscarinic antagonists on this inhibitory effect or to determine whether attenuation was greater

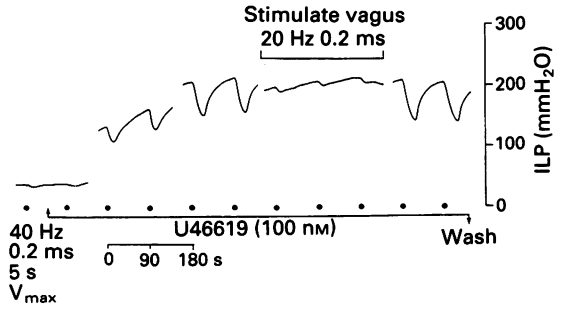


Figure 1 Effect of concurrent stimulation of the vagal nerve trunk (shown by the brackets; preganglionic 20 Hz, 0.2 ms) on relaxation responses of the guinea-pig trachea induced by stimulation of the preganglionic sympathetic nerve (indicated by the dots; 40 Hz, max voltage, 0.2 ms, 5 s). Concurrent stimulation of the vagal nerve trunk did not alter the intraluminal pressure (ILP) but inhibited sympathetic nerve-induced relaxations.

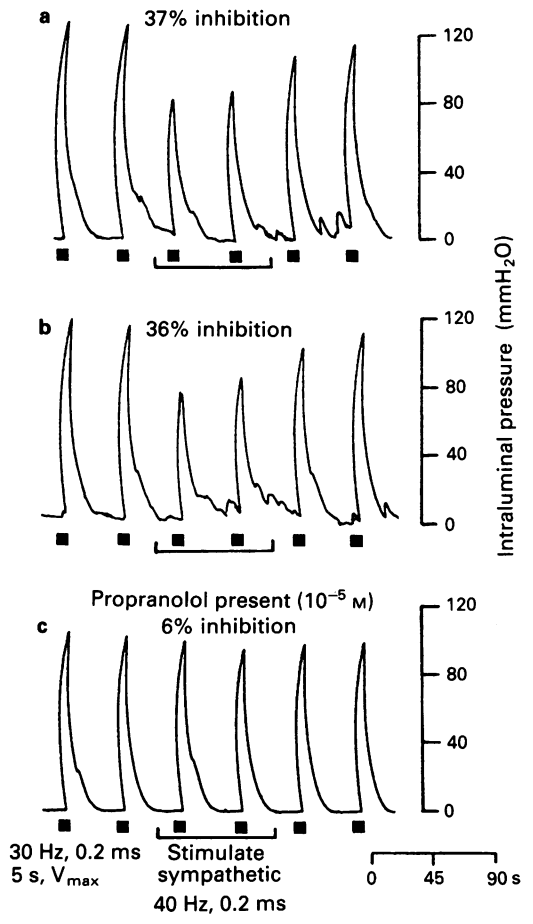


Figure 2 Effect of concurrent stimulation of the sympathetic nerve trunk (shown by the brackets; 40 Hz, 0.2 ms), on contractile responses of the guinea-pig trachealis muscle induced by preganglionic stimulation of the vagal nerve trunk (shown by the dots; 30 Hz, max voltage, 0.2 ms, 5 s). (a) and (b) show two separate periods of concurrent sympathetic stimulation in the same tissue; (c) shows a period of concurrent sympathetic stimulation after the tissue had been preincubated with propranolol (10^{-5} M). Propranolol (10^{-5} M) antagonized the inhibitory effect of the sympathetic nervous system on contractions induced by vagal stimulation.

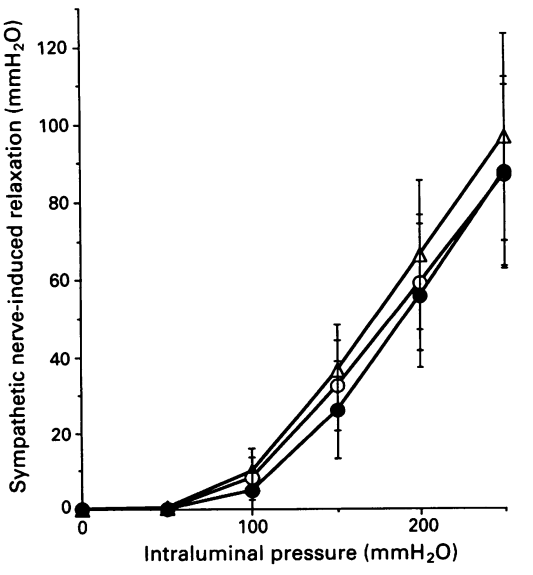


Figure 3 Relaxations of the guinea-pig trachea induced by stimulation of the sympathetic nerve trunk (40 Hz, max voltage, 0.2 ms, 5 s) in the absence (△) and presence of cromakalim 10^{-6} M (○) and 10^{-5} M (●). The intraluminal pressure was raised with U46619.

Table 1 Effect of physostigmine on the intraluminal pressure (ILP) and postjunctional contractile action of acetylcholine to increase the tone of the guinea-pig trachealis smooth muscle

Physostigmine concentration ($-\log_{10}$ M)	*ILP increase* (mmH_2O)	*Increase (%) in contractile response to acetylcholine* (10^{-5} M)
8.0	2.9 ± 1.4	—
7.5	2.1 ± 1.8	—
7.0	11.3 ± 4.2	69.8 ± 32
65	24.8 ± 5.9	—
6.0	86.6 ± 9.6	122.9 ± 40

All values are means ± s.e.mean of at least five experiments.

when both vagi were stimulated simultaneously compared to the effect of stimulation of a single nerve. When vagal stimulation ceased, sympathetic nerve-induced relaxations returned immediately to control values (Figure 1).

The reverse relationship is shown in Figure 2, where sympathetic nerve stimulation was superimposed on the effect of vagal nerve stimulation. Stimulation of the preganglionic vagal nerve fibres with supramaximal voltages resulted in reproducible contractile responses, which could be abolished with atropine. These contractile responses increased with increasing frequency, a maximum being observed at 30 Hz and were rapid in onset (0.5 s). In almost 80% of the preparations concurrent stimulations of the sympathetic nerve trunk resulted in a reduction in the size of the vagal contractile response. The degree of inhibition was reproducible in any one tissue. (Figure 2a, b). This inhibitory effect of the sympathetic nervous system on the parasympathetic nervous system was reduced by propranolol (10^{-5} M; Figure 2c).

The neuropeptides, VIP (10^{-9}–10^{-8} M; $n = 4$), neurokinin A (10^{-9}–10^{-6} M; $n = 5$) and substance P (10^{-8}–10^{-6} M; $n = 3$) and endothelins 1 and 3 (10^{-9}–10^{-8} M; $n = 4$) did not inhibit sympathetic nerve-induced relaxations; nor were such relaxations significantly different in the absence and presence of the potassium channel opener, cromakalim (10^{-6}, 10^{-5} M; Figure 3).

Effect of an anticholinesterase drug

In the absence of nerve stimulation, the anticholinesterase drug, physostigmine (in the range 10^{-8} to 10^{-6} M) caused a concentration-dependent increase in the ILP of the fluid-filled trachea which was slow to develop and reached a maximum after about 15 min exposure, after which time the contraction was maintained or slowly declined. Physostigmine was also found to potentiate the postjunctional contractile action of exogenously applied acetylcholine in a concentration-dependent manner (Table 1).

Figure 4 shows that physostigmine at the lowest concentrations used (10^{-8}, 3×10^{-8} M) had very little effect on vagally-induced contractions; at intermediate concentrations (10^{-7}, 3×10^{-7} M) it caused an overall potentiation of vagally-induced contractions, whereas in the presence of the highest concentration of physostigmine (10^{-6} M), vagally-induced contractions were reduced. Physostigmine, particularly at the higher concentrations, increased the duration of vagally-induced contractions. The large error bars on Figure 5 are, however, an indication of the variability between tissues.

The effect of physostigmine (10^{-7} M and 10^{-6} M) on sympathetic nerve-induced relaxations is shown in Figure 5. Sympathetic relaxations were inhibited in the presence of physostigmine (10^{-6} M).

In five experiments, atropine (10^{-7} M) completely blocked both the inhibitory effect of physostigmine (10^{-6} M) on sympathetic nerve-induced relaxations and its postjunctional action to contract the guinea-pig trachealis smooth muscle indicating the involvement of muscarinic receptors in both of these responses.

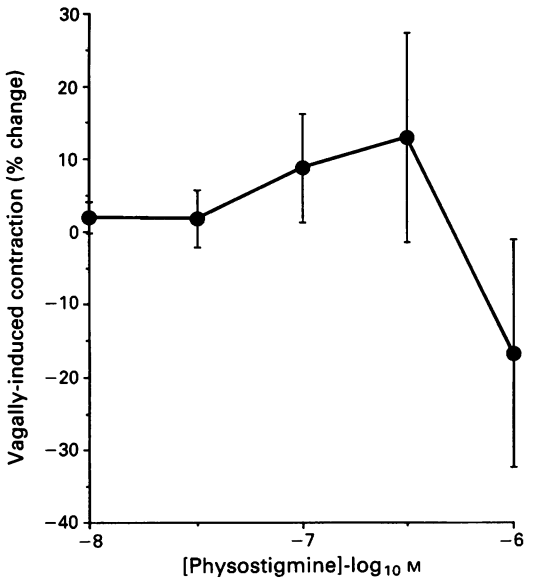


Figure 4 Effect of physostigmine on contractions of the guinea-pig trachea induced by stimulation of the vagal nerve trunk (30 Hz, max voltage, 0.2 ms, 5 s). Points are mean ($n > 5$) with s.e.mean shown by vertical bars.

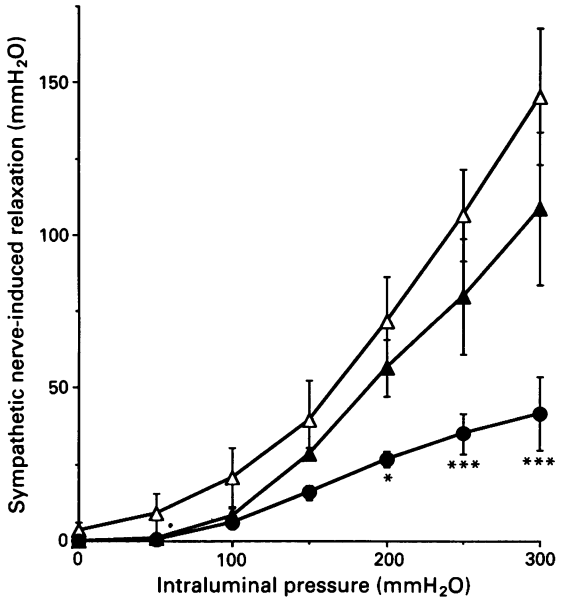


Figure 5 Sympathetic nerve-induced relaxation responses of the guinea-pig trachea (40 Hz, max voltage, 0.2 ms, 5 s), in the absence (△) and presence of physostigmine 10^{-7} M (▲) and 10^{-6} M (●). The intraluminal pressure was raised with U46619. Sympathetic nerve-induced relaxations were inhibited in the presence of the higher concentration of physostigmine (10^{-6} M) (*$P < 0.05$; ***$P < 0.005$; mean of $n > 5$; s.e.mean shown by vertical bars).

Discussion

Interrelationships have been described between the two branches of the autonomic nervous system for tissues, such as the heart (Löffelholz & Muscholl, 1970; Boyle & Pollock, 1988) and ileum (Manber & Gershon, 1979), which receive a dual innervation from both branches of the autonomic nervous system. Although in the airways a prejunctional inhibitory effect of the sympathetic nervous system on the parasympathetic nervous system has been reported, very little is known about the reverse relationship. It has, however, been demonstrated in our laboratory that, in the guinea-pig trachea, exogenously applied muscarinic agonists can inhibit noradrenaline release via activation of muscarinic M_3 cholinoceptors present on the sympathetic nerve terminals (Pendry & Maclagan, 1991). It was important to establish whether the inhibitory muscarinic receptors on pulmonary sympathetic nerve endings could be activated by acetylcholine released under physiological conditions from the adjacent cholinergic nerves.

Using discrete stimulation of the pulmonary parasympathetic and sympathetic nerve trunks, we were able to confirm this interaction. Concurrent stimulation of the vagal nerve trunk with submaximal voltages inhibited sympathetic nerve-induced relaxations in 50% of the preparations. The involvement of postjunctional functional antagonism was excluded by stimulating the vagal nerve trunk at parameters which did not contract the trachealis smooth muscle. It proved to be very difficult to judge these parameters, and the lack of inhibition observed in half of the preparations may be due to variability between tissues in the metabolism of the transmitter by cholinesterase enzymes before reaching the muscarinic receptors on the airway smooth muscle and the sympathetic nerve endings. Stimulation of the preganglionic vagal nerve trunk not only stimulates the cholinergic fibres but also non-adrenergic, non-cholinergic (NANC) fibres which arise in the vagal bundle. The possibility that the inhibitory effect of concurrent vagal stimulation was due to release of neuropeptides from NANC nerves is, however, unlikely because when VIP, neurokinin A or substance P were administered exogenously, they did not inhibit sympathetic nerve-induced relaxations. In addition McCaig (1986) was unable to elicit vagally mediated NANC responses in the guinea-pig trachea *in vitro* during cholinergic blockade using similar stimulus parameters to those used in the present study. In McCaig's experiments, sustained stimulation with pulses of 2–4 ms duration and frequencies as great as 160 Hz were required to observe NANC responses. In contrast, in the experiments described in this paper the lower parameters of 0.2 ms and 20 Hz were sufficient to elicit an inhibitory effect of concurrent vagal stimulation upon sympathetic nerve-induced relaxations. Sympathetic nerve-induced relaxations were also unaffected by the presence of either endothelin 1 or endothelin 3, which are known to be formed by airway epithelial cells (Black *et al.*, 1989).

Thus it appears from the above results that, in the airways, acetylcholine released following preganglionic stimulation of the cholinergic nerves can activate inhibitory muscarinic receptors present on the adjacent sympathetic nerve endings.

It is known that certain subtypes of muscarinic receptors which inhibit cell function are linked to the opening of potassium channels such as those studied in the heart (Sakmann *et al.*, 1983). However, in the present experiments sympathetic nerve-induced relaxation responses were not altered in the presence of concentrations of the potassium channel opening drug, cromakalim which had previously been shown to inhibit vagally induced contraction by at least 50% (Hall & Maclagan, 1988). This suggests that the inhibitory muscarinic receptors on the pulmonary sympathetic nerves are not linked to potassium channels of the type opened by cromakalim, and a different second messenger system may be involved.

Other workers have reported a prejunctional inhibitory effect of noradrenaline from sympathetic nerves on cholinergic transmission in the airways of various species (Vermiere & Vanhoutte, 1979; Jones *et al.*, 1980; McCaig, 1987; Rhoden *et al.*, 1988). We also recorded inhibition of vagally-induced contractile responses by noradrenaline released endogenously following concurrent stimulation of the sympathetic nerve trunk. The adrenoceptors involved, which are present on the cholinergic nerve endings, appear to be predominantly of the β subtype.

These findings are consistent with the observations that, in the airways, the two branches of the autonomic nervous system lie in close proximity and may interconnect (Jones *et al.*, 1980; Daniel *et al.*, 1986; Daniel, 1988). The present results confirm that functional interactions between these two nervous systems may also occur.

As we were able to show that endogenous acetylcholine can inhibit sympathetic neurotransmission, anticholinesterase drugs would be expected to potentiate this interaction. It is well known that anticholinesterase drugs, such as physostigmine and neostigmine, cause contraction of the trachealis muscle. It has been suggested that this contractile effect may not depend totally on their ability to prevent breakdown of acetylcholine but may also be due to potentiation of acetylcholine release from cholinergic nerve endings (Douglas, 1951; Carlyle, 1963; Kirkpatrick & Rooney, 1979).

Although potentiation of vagally-induced contractions of the guinea-pig trachea by the anticholinesterases, neostigmine and physostigmine, has been reported (McCaig, 1986; Widmark & Waldeck, 1986), other workers have described inhibition of potassium-evoked release of [^{3}H]-acetylcholine from the rat bronchi by the anticholinesterase, soman (Åas *et al.*, 1986; 1987).

In our experiments, the anticholinesterase, physostigmine, potentiated contractions at high concentrations and caused inhibition at low concentrations. Previous conflicting reports in the literature may be due to the concentration of the anticholinesterase used and to the contact time with the tissue.

Åas (1988) found that exposure for 40 h to the anticholinesterase, soman, reduces the acetylcholine-induced contraction of the rat bronchi, probably via a reduction in the number of muscarinic receptors. However, the inhibition of vagally-induced contractions following 15 min preincubation with 10^{-6} M physostigmine, in our experiments, does not appear to be due to desensitization of postjunctional muscarinic receptors, as 10^{-6} M physostigmine caused a greater increase in the postjunctional contractile action of acetylcholine than was obtained with the lower concentration of 10^{-7} M physostigmine. The inhibitory effect is therefore probably a result of acetylcholine accumulating in the synapse and exerting a negative feedback via muscarinic autoreceptors on the cholinergic nerve endings so inhibiting its own release. This complication has previously been observed in gut experiments involving measurement of acetylcholine release in the presence of anticholinesterases to prevent its breakdown (Kilbinger, 1984).

The importance of our finding is that the concentration of physostigmine which inhibits sympathetic nerve-induced relaxations is in the same concentration range as that which inhibits vagally-induced contractile responses. This inhibitory effect of physostigmine was blocked by atropine (10^{-7} M). Similarly, McCaig (1986) reported an inhibitory effect of the anticholinesterase, neostigmine, on sympathetic relaxations, which was shown to be reduced by atropine. The inhibitory effect of the anticholinesterase drugs on sympathetic responses is likely to be due to accumulation of acetylcholine which activates inhibitory muscarinic heteroreceptors on the nearby sympathetic nerve terminals. This accumulation of transmitter is probably due to a combination of the anticholinesterase effects of physostigmine on spontaneously released ACh and a direct stimulatory effect of the drug on the cholinergic nerve terminals. This sympathoinhibitory effect may contribute to the bronchospasm observed with anticholinesterase drugs.

The results described in this paper reinforce the hypothesis that in the airways, parasympathetic and sympathetic nerves can interact at the prejunctional level. Thus, either branch of the autonomic nervous system can modulate neurotransmission in the other branch, increasing the complexity of the neural control of airway smooth muscle tone.

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The *a*- and *β*-adrenoceptor blocking activities of labetalol and its **RR-SR** (50 : 50) stereoisomers

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1 We compared the α_1-, α_2- and β_1-adrenoceptor blocking potencies of labetalol with those of its two stereoisomers (**RR** and **SR**) in pithed rats and in homogenized rat cerebral cortex and heart.

2 In pithed rats, labetalol and the **RR-SR** combination were given orally either at doses of 25 and 50 mg kg^{-1} body wt. or intravenously at doses of 1 and 5 mg kg^{-1} body wt. Prazosin 4 and 20 μg kg^{-1} body wt. and propranolol 1 and 5 mg kg^{-1} body wt., were given intravenously for comparison studies of potency at α_1- and β_1-adrenoceptors, respectively. Effects were studied before and after i.v. administration of either phenylephrine (at doses which increased the mean arterial pressure by approximately 80 mmHg) or isoprenaline (at doses that increased heart rate by approximately 100 beats min^{-1}).

3 In pithed rats, labetalol and the **RR-SR** combination antagonized, in a dose-dependent manner, the pressor effect of phenylephrine ($P < 0.05$) and the chronotropic effect of isoprenaline ($P < 0.05$). Following both oral and intravenous dosing, the **RR-SR** combination was twice potent as labetalol in terms of α_1- and β_1-adrenoceptor antagonism at equivalent doses.

4 Labetalol and the enantiomers lacked affinity at α_2-adrenoceptors while at α_1-adrenoceptors the order of potency was prazosin $\gg$ **RR-SR** > labetalol. At β_1-adrenoceptors, the affinity of the compound **RR-SR** was about 3 times that of labetalol.

5 As labetalol is a mixture of active (**RR** and **SR**) and inactive (**SS** and **SR**) enantiomers (in terms of α- and β-receptor actions), the combination of **RR** and **SR** may be a valuable substitute for labetalol in the treatment of systemic hypertension. Although the potential for non-specific side effects (common to all four enantiomers) could be expected to be diminished, recent reports by postmarketing surveillance indicate that the **RR** isomer (dilevalol) can induce liver toxicity. Interestingly, labetalol is devoid of this effect; whether the combination of **RR** and **SR** enantiomers could be of clinical importance warrants further investigation.

Keywords: Labetalol; labetalol **RR-SR** isomers; adrenoceptors; arterial pressure; heart rate; radioligand binding

Introduction

Labetalol, an antihypertensive agent that possesses competitive α_1- and β_1-adrenoceptor antagonist activity, contains two asymmetric centres, and thus four stereoisomers exist. The formulation used clinically and experimentally is a mixture of equal proportions of the four stereoisomers **RR**, **SR**, **SS**, and **RS**. The α- and β-adrenoceptor antagonist potencies of the individual stereoisomers differ considerably such that most of the α_1-blocking activity of labetalol is attributable to the **SR** isomer, whereas most of the β_1-adrenoceptor blocking activity is attributable to the **RR** isomer (dilevalol), while the **SS** and **RS** isomers are devoid of α_1- and β_1-antagonist activity (Baum *et al.*, 1981; Brittain *et al.*, 1982; Chrisp & Goa, 1990; Sybertz *et al.*, 1981).

Since non-specific drug toxicity is commonly independent of chirality (Materson *et al.*, 1989; Shoenberger *et al.*, 1989; Chrisp & Goa, 1990), it can be expected that each enantiomer contributes equally to labetalol's potential for side effects (such as dizziness, dyspepsia, diarrhoea and nausea, the latter two being dose-related). However, this is not always true and the **RR** isomer (dilevalol) has recently been withheld from the market due to liver toxicity. We do not know whether the combination of the **RR** and **SR** isomers may represent a valuable substitute for both labetalol and dilevalol. Therefore, we have characterized the α_1- and β_1-blocking activity of these enantiomers in combination.

In this study, we attempted to establish whether the **RR-SR** combination possesses greater potency for blockade at α_1- and β_1-receptors than labetalol. We took a 50:50 combination of the two active enantiomers (**RR-SR**) and compared the α_1-blocking activity with that of labetalol and prazosin (measured as the ability to inhibit the increase in arterial pressure following phenylephrine) and the β_1-blocking activity to that of labetalol and propranolol (measured as the ability to inhibit the increase in heart rate following isoprenaline). We also studied the *in vitro* binding of labetalol and the **RR-SR** (50:50) on α_1- and α_2-adrenoceptors in the rat cerebral cortex and with β_1-adrenoceptors in the rat heart by radioligand binding techniques.

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Methods

Animals

Male CD-COBS rats (300–400 g body wt., Charles River, Italy) were used for all experiments. Rats were fasted overnight and allowed to drink water freely. All animals received humane care in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 80-23, revised 1978).

In vivo *studies*

Rats were anaesthetized with ether and pithed according to the technique described by Gillespie & Muir (1967). Pancuronium bromide and atropine were injected intravenously at a dose of 0.2 mg kg^{-1} body wt. and 1 mg kg^{-1} body wt., respectively. The animals were ventilated with room air, at a stroke volume of approximately 8 ml kg^{-1} body wt. and at a rate of 55 strokes min^{-1}, after vagotomy and tracheostomy. Ventilation was adjusted to maintain arterial blood gases at physiological levels. Body temperature was maintained with a heating lamp. The carotid artery was cannulated for arterial

blood pressure monitoring (Gould P23 ID, U.S.A.) and blood sampling for gas analysis. The femoral vein was cannulated for drug dosing. The electrocardiogram was recorded via standard limb leads (Battaglia Rangoni Polygraph, Bologna, Italy).

The drug solutions were made fresh daily. For oral dosing, labetol (25 and 50 mg kg^{-1} body wt.) and the **RR-SR** combination (25 and 50 mg body wt.), were dissolved in 0.4% carboxymethylcellulose. For the intravenous dosing, atropine sulphate (1 mg kg^{-1}), labetalol hydrochloride (25 and 50 mg kg^{-1} body wt.), **RR-SR** (50:50) hydrochloride (25 and 50 mg kg^{-1} body wt.), propranolol hydrochloride (1 and 5 mg kg^{-1} body wt.) and prazosin hydrochloride (4 and 20 μg kg^{-1} body wt., with 5% w/v sucrose) were dissolved in distilled water; phenylephrine hydrochloride (0.1–10 μg kg^{-1} body wt.), isoprenaline hydrochloride (0.005–5 μg kg^{-1} body wt., with 0.1% w/v ascorbic acid) and pancuronium bromide (200 μg kg^{-1} body wt., brought to pH = 4 with acetic acid) were dissolved in saline. The concentration of each drug is expressed as the free base.

Five groups of animals (5–6 rats/group) were used for oral administration studies. Rats were given 25 and 50 mg kg^{-1} body wt. of either labetalol or **RR-SR**, by gavage, 1 h before surgical preparation, with the control group receiving the same volume of the vehicle (0.4% carboxymethylcellulose). Following surgical preparation, the animals were allowed to stabilize for 20–30 min. Following this time, phenylephrine, at increasing doses (from 0.1 to 10 μg kg^{-1} body wt.) was given at intervals of 10 min until an increase in mean arterial blood pressure of approximately 80 mmHg was achieved. Isoprenaline, at increasing doses (from 0.005 to 5 μg kg^{-1} body wt.), was then given at intervals of 10 min until an increase in heart rate of 100 beats min^{-1} was reached.

Nine groups of animals (3–5 rats/group) were used for intravenous administration studies. Following the surgical preparation, the animals were allowed to stabilise for 20–30 min and then rats were given 1 or 5 mg kg^{-1} body wt., of labetalol, 1 or 5 mg kg^{-1} body wt. of the **RR-SR**, or 4 or 20 μg kg^{-1} body wt. of prazosin intravenously; with two control groups receiving 1 ml kg^{-1} body wt. of distilled water or saline. After a 15–30 min interval, phenylephrine (5 μg kg^{-1} body wt.) or isoprenaline (0.5 μg kg^{-1} body wt. given over 60 s) was administered.

Exclusion criteria

Rats with cardiac arrhythmias caused by pithing (atrio-ventricular block) or isoprenaline (ventricular tachycardia) were excluded. Any animal that was excluded, was immediately replaced by another.

In vitro *studies*

For the receptor binding studies, drugs (hydrochloride salts) were dissolved fresh daily in distilled water giving concentrations ranging from 10^{-5} M to 10^{-10} M and added to incubation mixture in a volume of 10 μl.

α-Adrenoceptor binding was studied with crude membrane preparations from the rat cerebral cortex. Tissue was excised and homogenized in 50 volumes of 50 mM Tris HCl buffer (pH = 7.4) and centrifuged at 50 000 *g* for 10 min. Pellets were washed with Tris buffer, incubated for 15 min at 37°C and washed again with Tris buffer. Finally, pellets were resuspended in 100 volumes of Tris HCl 50 mM containing 0.1% ascorbic acid (pH = 7.7) and 10 μM pargyline. To evaluate α_1-binding, 0.35 nM [^{3}H]-prazosin (specific activity of 76.6 Ci mmol^{-1}, NEN) plus 1 ml of tissue preparation were incubated for 30 min at 25°C, in the presence or absence of an excess (3 μM) of phentolamine to delineate non-specific binding (Greengrass & Bremner, 1979). To evaluate α_2-binding, 0.4 nM [^{3}H]-clonidine (specific activity of 41.5 Ci mmol^{-1}, NEN) plus 1 ml of tissue preparation were incubated for 20 min at 25°C in the presence or absence of an excess (100 μM) of noradrenaline to delineate non specific binding (Greenberg *et al.*, 1976).

β_1-Adrenoceptor binding was studied in heart homogenate by the technique of Baker & Potter (1980), with minor modifications (Gobbi *et al.*, 1988). The heart was homogenized in 20 volumes of 10 mM Tris HCl buffer (pH = 7.7). An equal volume of 1 M KCl was then added to the tissue preparation. After 10 min at 0°C, the sample was centrifuged for 10 min at 50 000 *g* three times, with an intermediate incubation for 10 min at 37°C. Prior to the last centrifugation the homegenate was passed through a 4 gauze layer to remove connective tissue elements. Finally, the pellet was resuspended in 80 volumes of 50 mM Tris HCl buffer (pH = 7.7) containing 0.1% ascorbic acid and 10 μM pargyline. One nM [^{3}H]-dihydroalprenolol (specific activity of 102 Ci mmol^{-1}, NEN) was added to 1 ml of tissue preparation and incubated for 20 min at 25°C in the absence or presence of an excess (10 μM) of propranolol to delineate non-specific binding.

Incubations were terminated by vacuum filtration on Whatman GF/B (α-receptors) or GF/C glass fibre filters, followed by washing with 5 ml Tris buffer three times. The filter was counted in 10 ml Filter Count Solution using a liquid scintillator spectrometer (Beckman, LS-7500), at approximately 50% counting efficiency.

Inhibition curves were obtained by using 9–12 drug concentrations in triplicate. Data from the *in vitro* binding study were analysed by non-linear fitting programmes (Sacchi Landriani *et al.*, 1983) according to the 'logistic function' (De Lean *et al.*, 1978) calculated as follows:

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

y = percent of ^{3}H-ligand bound; a = minimum effect (maximum percentage of ^{3}H-ligand binding); d = maximum effect (minimum percentage of ^{3}H-ligand binding, non-specific binding); x = concentration of the displacing drug; c = molar concentration of the displacing drug giving 50% of the effect between a and d or IC_{50}; b = slope factor.

Binding affinities (K_i) for inhibitors were calculated from IC_{50} according to the equation of Cheng & Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

IC_{50} = concentration of the drug giving 50% of radioligand binding inhibition at the site, obtained by the non-linear fitting of the experimental points according to the logistic function; [L] = concentration of the radioligand; K_d = affinity constant of the radioligand.

Values are expressed as mean ± variation coefficient (s.d. of the parameter). Student's *t* test between parameters was used in the *in vitro* study for comparison of affinity of **RR-SR** vs labetalol.

Measurement of haemodynamic variables

Arterial blood pressure and heart rate (derived from the ECG) were recorded throughout the experiment.

In the dose-response studies, 10 min intervals were imposed between each dose of phenylephrine or isoprenaline to allow haemodynamic variables to stabilize: plateau blood pressure and heart rate values were recorded and expressed as the pre-drug control value.

In rats given labetalol and **RR-SR** 50 mg kg^{-1} orally, phenylephrine and isoprenaline at doses higher than 10 μg kg^{-1} and 5 μg kg^{-1} respectively, were required in order to obtain the maximal response on arterial blood pressure and heart rate (as defined earlier). However, at those doses toxic effects occurred (e.g., isoprenaline higher than 5 μg kg^{-1} induced ventricular arrhythmias in 100% of the animals), precluding

maximum values being reached. Thus, results from dose-response studies of phenylephrine on blood pressure and isoprenaline on heart rate, have been evaluated in terms of the concentration of agonist ($\mu g\,kg^{-1}$ body wt.) that increased mean arterial blood pressure by 15 mmHg and heart rate by 35 beats min^{-1}.

Partial pressures of oxygen (Po_2) and carbon dioxide (Pco_2) were measured in the arterial blood (1 ml) from pithed rats randomly selected ($n = 4$) and were found to be in the physiological range ($Po_2 = 73 \pm 3$ mmHg, $Pco_2 = 43 \pm 2$ mmHg).

Arterial blood pressure and heart rate are expressed as mean ± s.e.mean. Analysis of variance was followed by Duncan's test for multiple comparison (Rocchetti & Recchia, 1982). Differences were considered statistically significant at $P < 0.05$.

Consideration of pharmacokinetics

Intravenous administration was chosen in order to limit the influence of absorption and first-pass metabolism. First-pass extraction by both the liver and the gut may result in differences in systemic availability for enantiomeric pairs of high extraction ratio drugs (Williams & Lee, 1985). Significant stereoselective hepatic extraction has been demonstrated for metoprolol (Lennard *et al.*, 1983) and propranolol (Walle & Walle, 1979).

Comparison between the in vivo *and the* in vitro *studies*

The concentration of antagonists in equilibrium with receptors after oral administration was assumed to be equivalent to the plasma concentration reported previously with identical doses (Martin *et al.*, 1976). To compare the results obtained *in vivo* with the binding affinities from the *in vitro* studies, we calculated the apparent dissociation constant (K_B) of the agonist-receptor complex from the oral dose-response study (Furchgott, 1972) as follows:

$$K_B = \frac{[B]}{\frac{[A']}{[A]} - 1}$$

B = plasma concentration of antagonist; [A'] and [A] = concentrations of agonist giving an equal response in the presence and absence of the antagonist respectively, referred to as the dose-ratio.

The plasma concentration of the antagonist was calculated from the plasma elimination curve of labetalol described elsewhere (Martin *et al.*, 1976). In absence of published data, we assumed that the enantiomers are metabolized at the same rate.

Results

In vivo *studies*

Control values for arterial blood pressure and heart rate were similar in all the groups. Table 1 shows values of mean arterial blood pressure and heart rate in pithed rats pretreated with adrenoceptor blocking drugs, prior to phenylephrine and isoprenaline dosing. A significant reduction in arterial blood pressure was caused by i.v. **RR-SR** (50:50) compared with other treatments. On theoretical grounds, both labetalol and the **RR-SR** combination can be expected to decrease and increase blood pressure, respectively owing to their relative α- and β-selectivity.

The dose-response effects of i.v. phenylephrine on mean arterial blood pressure and isoprenaline on heart rate in pithed rats pretreated orally with adrenoceptor blocking drugs, are shown in Figures 1 and 2, respectively. Labetalol and the **RR-SR** combination significantly inhibited ($P < 0.05$), in a dose-dependent manner, the effects of phenylephrine on the arterial blood pressure and of isoprenaline on heart rate compared with control. The doses of phenylephrine and isoprenaline that increased the mean arterial blood pressure by 15 mmHg and heart rate by 35 beats min^{-1} are shown in Table 2. The **RR-SR** (50:50) combination was more potent than labetalol, at equivalent doses, both at α_1- and β_1-adrenoceptors.

Table 1 Mean arterial blood pressure (MAP) and heart rate (HR) in pithed rats pretreated with adrenoceptor blocking drugs prior to i.v. phenylephrine and isoprenaline

Treatment	*MAP* (mmHg)	*HR* (beats min^{-1})
Oral study ($n = 5$–6)		
Control (vehicle 1 ml kg^{-1})	41 ± 1	333 ± 19
Labetalol (25 mg kg^{-1})	40 ± 1	326 ± 17
Labetalol (50 mg kg^{-1})	42 ± 2	344 ± 10
RR-SR (25 mg kg^{-1})	40 ± 3	357 ± 11
RR-SR (50 mg kg^{-1})	41 ± 4	359 ± 9
Intravenous study ($n = 3$–5)		
Control (water 1 ml kg^{-1})	46 ± 1	322 ± 10
Control (saline 1 ml kg^{-1})	48 ± 1	322 ± 18
Labetalol (1 mg kg^{-1})	46 ± 3	313 ± 23
Labetalol (5 mg kg^{-1})	47 ± 3	356 ± 14
RR-SR (1 mg kg^{-1})	33 ± 1*	337 ± 5
RR-SR (5 mg kg^{-1})	37 ± 4*	338 ± 7
Propranolol (1 mg kg^{-1})	52 ± 1	334 ± 8
Propranolol (5 mg kg^{-1})	48 ± 1	358 ± 5
Prazosin (4 $\mu g\,kg^{-1}$)	46 ± 2	334 ± 16
Prazosin (20 $\mu g\,kg^{-1}$)	47 ± 4	315 ± 7

Values are mean ± s.e.mean.
*$P < 0.05$ versus control.

Changes in mean arterial blood pressure (ΔMAP) and heart rate (ΔHR) in pithed rats pretreated intravenously with adrenoceptor blocking drugs following single doses of phenylephrine (5 $\mu g\,kg^{-1}$) and isoprenaline (0.5 $\mu g\,kg^{-1}$) are shown in Table 3. Labetalol, **RR-SR** (50:50) combination, and prazosin significantly inhibited ($P < 0.05$), in a dose-dependent manner, the effects of phenylephrine on arterial blood pressure compared to control rats (Table 3). Labetalol, the **RR-SR** combination and propranolol, significantly inhibited ($P < 0.05$), in

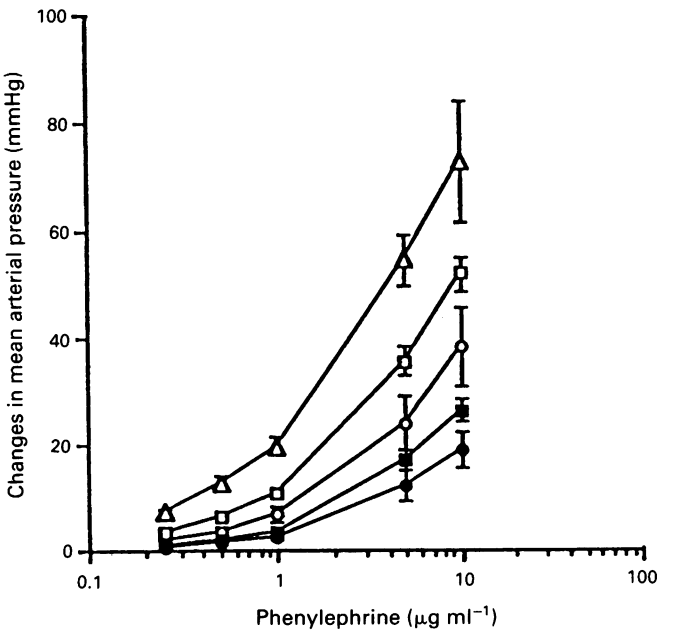


Figure 1 Dose-response effects of intravenous phenylephrine on mean arterial blood pressure changes in rats given vehicle (△) ($n = 6$), labetalol 25 mg kg^{-1} (□) ($n = 5$), labetalol 50 mg kg^{-1} (○) ($n = 5$), the **RR-SR** combination 25 mg kg^{-1} (■) ($n = 5$) and **RR-SR** combination 50 mg kg^{-1} (●) ($n = 5$) orally, 1 h prior to dose-response study.

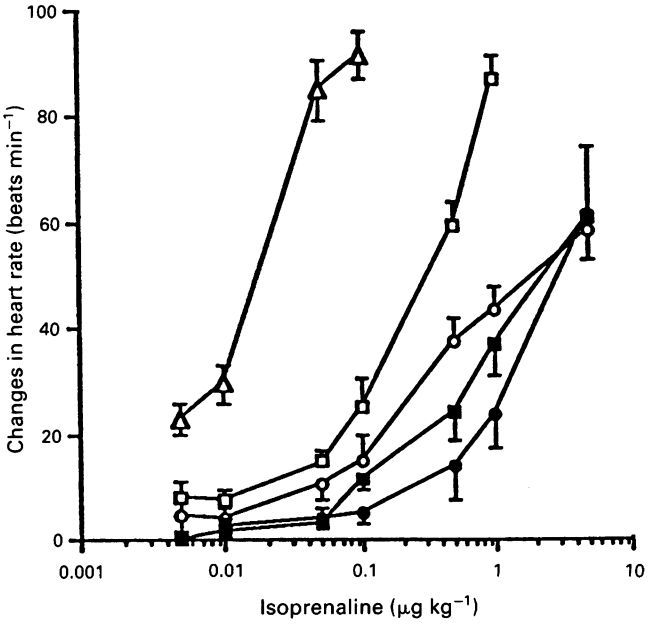


Figure 2 Dose-response effects of intravenous isoprenaline on heart rate changes in rats given vehicle (△) ($n = 5$), labetalol 25 mg kg^{-1} (□) ($n = 3$), labetalol 50 mg kg^{-1} (○) ($n = 3$), the **RR-SR** combination 25 mg kg^{-1} (■) ($n = 3$) and **RR-SR** (50:50) 50 mg kg^{-1} (●) ($n = 3$) orally, 1 h prior to dose-response study.

a dose-dependent manner, the effects of isoprenaline on heart rate, compared with control (Table 3).

In vitro *studies*

The affinities of labetalol and the **RR-SR** combination at α_1-, α_2- and β_1-adrenoceptors are shown in Table 4. Both labetalol and the **RR-SR** combination had low affinity for α_2-adrenoceptors and high affinity for α_1-adrenoceptors (Table 4). The order of potency at the α_1-adrenoceptor was prazosin > **RR-SR** (50:50) > labetalol. Affinity constants (K_i) at α_1-adrenoceptors were different for labetalol and the **RR-SR** combination, the latter being about twice as potent as labetalol.

In Table 4 are also shown the results obtained from displacement of [^{3}H]-dihydroalprenolol at β_1-adrenoceptors from the rat heart. Labetalol and the **RR-SR** combination displaced [^{3}H]-dihydroalprenolol with slope values approaching unity (as did propranolol). The **RR-SR** (50:50) isomers were significantly more active (approximately 2–3 times) than labetalol at cardiac β_1-adrenoceptors.

Table 2 Doses of i.v. phenylephrine and isoprenaline required to increase the mean arterial blood pressure by 15 mmHg or heart rate by 35 beats min^{-1} in pithed rats pretreated with adrenoceptor blocking drugs

Treatment	*Phenylephrine* (μg kg^{-1} body wt.)	*Isoprenaline* (μg kg^{-1} body wt.)
Oral study ($n = 5$–6)		
Control (vehicle 1 ml kg^{-1})	0.68 ± 0.10	0.01 ± 0.002
Labetalol (25 mg kg^{-1})	1.72 ± 0.14*	0.21 ± 0.05
Labetalol (50 mg kg^{-1})	4.75 ± 0.59*	1.15 ± 0.36*
RR-SR (25 mg kg^{-1})	3.95 ± 1.41*	1.26 ± 0.87*
RR-SR (50 mg kg^{-1})	8.28 ± 1.34*	2.90 ± 0.87*

Values are mean ± s.e.mean.
*$P < 0.05$ vs control.

Table 3 Changes in mean arterial blood pressure (ΔMAP) and heart rate (ΔHR) following phenylephrine (5 μg kg^{-1}) and isoprenaline (0.5 μg kg^{-1}) in pithed rats pretreated with adrenoceptor blocking agents

Treatment	*ΔMAP* (mmHg)	*ΔHR* (beats min^{-1})
Intravenous study ($n = 3$–5)		
Control (water 1 ml kg^{-1})	+58 ± 5	+139 ± 7
Labetalol (1 mg kg^{-1})	+26 ± 1*	+43 ± 7*
Labetalol (5 mg kg^{-1})	+15 ± 2*	+16 ± 3*
RR-SR (1 mg kg^{-1})	+10 ± 1*	+21 ± 2*
RR-SR (5 mg kg^{-1})	+6 ± 2*	+5 ± 1*
Control (saline 1 ml kg^{-1})	+58 ± 1	+122 ± 13
Propranolol (1 mg kg^{-1})	—	+38 ± 4*
Propranolol (5 mg kg^{-1})	—	+9 ± 1*
Prazosin (4 μg kg^{-1})	+19 ± 3*	—
Prazosin (20 μg kg^{-1})	+7 ± 4*	—

Values are mean ± s.e.mean.
*$P < 0.05$ vs control.

Comparison between the in vivo *and* in vitro *studies*

We have compared the *in vitro* and *in vivo* affinities of labetalol and the **RR-SR** combination of α_1- and β_1-adrenoceptors on the basis of their K_i (Table 4) and K_B values. In Table 5 are shown the dissociation constants (K_B) at α- and β-adrenoceptors for labetalol and the **RR-SR** (50:50) isomers calculated from the *in vivo* studies and their affinity constants (K_i) obtained from the *in vitro* studies. The dissociation constants for labetalol and the **RR-SR** combination at α_1-adrenoceptors were similar *in vivo* ($K_B = 1.9 \times 10^{-7}$ and 7.6×10^{-8} respectively) and *in vitro* ($K_i = 1.2 \times 10^{-7}$ and 5.9×10^{-8} respectively), thus, giving estimates of the relative α_1/β_1 potency ratio of approximately 15 *in vivo* and 10 *in vitro*.

Table 4 Inhibition of radioligand binding (K_i) at adrenoceptors in the rat cerebral cortex (α_1- and α_2-adrenoceptors) and heart (β_1-adrenoceptor)

	Cerebral cortex		*Heart*
Andrenoceptors	α_1-	α_2-	β_1-
Antagonists	[^{3}*H*]-*prazosin*§	[^{3}*H*]-*clonidine*§	[^{3}*H*]-*dihydroalprenolol*§
Prazosin	$0.29 \pm 0.02 \times 10^{-9}$	$>5 \times 10^{-5}$	—
Propranolol	—	—	$1.3 \pm 0.4 \times 10^{-9}$
Labetalol	$1.20 \pm 0.01 \times 10^{-7}$	$2.6 \pm 0.7 \times 10^{-5}$	$1.3 \pm 0.2 \times 10^{-8}$
RR-SR	$5.90 \pm 0.03 \times 10^{-8}$*	$1.7 \pm 0.2 \times 10^{-5}$	$0.5 \pm 0.1 \times 10^{-8}$*

Values are mean ± s.d. of parameter.
* $P < 0.05$ from labetalol with Student's t test on considered parameter.
§ Concentration and affinity constant for [^{3}H]-prazosin were 0.35 nM and 0.5 nM respectively, for [^{3}H]-clonidine were 0.4 nM and 2.5 nM respectively, and for [^{3}H]-dihydroalprenolol were 1.0 nM and 0.5 nM respectively. Inhibition curves were obtained by using 9–12 drug concentrations in triplicate.

Table 5 α- and β-Adrenoceptor blocking activities of labetalol and **RR-SR** expressed as dissociation constant (K_B) of the complex receptor-antagonist *in vivo* and as affinity constant (K_i) *in vitro*

	α_1-	β_1-	α_1/β_1 ratio
In vivo *dissociation constant* (K_B)			
Labetalol	1.9×10^{-7}	1.29×10^{-8}	14.7
RR-SR	7.6×10^{-8}	0.53×10^{-8}	14.3
Labetalol/**RR-SR** Ratio	2.5	2.4	
In vitro *affinity constant* (K_i)			
Labetalol	1.2×10^{-7}	1.3×10^{-8}	9.2
RR-SR	5.9×10^{-8}	0.5×10^{-8}	11.8
Labetalol/**RR-SR** Ratio	2.0	2.6	

Calculations based on data from Brittain & Levy ($n = 3$ rats for i.v. and $n = 5$ rats for p.o. kinetics).

The higher potency of the **RR-SR** (50:50) isomers compared with labetalol (approximately double) is also similar both *in vivo* and *in vitro*.

Discussion

Labetalol is a mixture of four optical isomers (**RR, SS, SR, RS**) the relative potency of which on adrenoceptors differs considerably. The **RR** stereoisomer was found to be a potent, non-selective antagonist at β-adrenoceptors and the **SR** stereoisomer a potent antagonist at α_1-adrenoceptors as shown previously (Brittain *et al.*, 1982). Negligible α- and β-blocking activity was possessed by the **SS** and **SR** isomers, as shown previously (Brittain *et al.*, 1982).

In this study we compared the adrenoceptor blocking activities of labetalol with those of a 50:50 combination of the two active enantiomers, **RR** and **SR** both *in vivo* and *in vitro*.

In the pithed rat, labetalol and the **RR-SR** combination possess α- and β-blocking properties at adrenoceptors and the **RR-SR** combination was 2–3 times more potent than labetalol both at α- and β-adrenoceptors. The results obtained from i.v. and p.o. dosing studies were similar, this indicates that the kinetics of labetalol and the **RR** stereoisomer after the two routes of administration are not markedly different.

Prazosin, a selective α_1-adrenoceptor antagonist, possesses a higher affinity at the α-adrenoceptor than either labetalol or the **RR-SR** combination, both in the pithed rat and in the rat cerebral cortex homogenate.

Propranolol possessed a potency intermediate between labetalol and the **RR-SR** combination at β_1-adrenoceptors. In the guinea-pig isolated atrium, previous studies have shown that propranolol was 4–5 times more potent than labetalol at β-adrenoceptors (Brittain & Levy, 1976). In the rat, very little is known about labetalol kinetics and differences between labetalol and propranolol in terms of absorption, protein binding, tissue/plasma partition or other pharmacokinetic factors, and their contributions to differences in potency between isolated atrium and *in vivo* studies. It was of interest therefore, to compare the activities at α_1- and β_1-adrenoceptors of labetalol and **RR-SR** (50:50) in the pithed rat with affinities for α_1- and β-adrenoceptors obtained *in vitro* from the cerebral cortex and rat heart. There was a good agreement between labetalol/**RR-SR** isomer potency ratio and affinities for α_1- and β_1-adrenoceptors between *in vivo* and *in vitro* studies. The relative α_1/β_1 potency ratio for labetalol and the **RR-SR** isomer *in vivo* and *in vitro* studies was 15 and 10, respectively.

The **RR-SR** isomer/labetalol potency ratio at α- and β-adrenoceptors was approximately 2. This is expected since labetalol contains four optical stereoisomers, two of which are devoid of adrenoceptor activity.

It must be acknowledged that the possibility exists that the pharmacodynamics and/or pharmacokinetics of labetalol differ from that of its stereoisomers, as previously reported for several β-adrenoceptors blocking drugs (Walle & Walle, 1979; Lennard *et al.*, 1983; Williams & Lee, 1985). Also, we are aware that some of our assumptions are necessarily limited by their having being based on the only published paper on labetalol pharmacokinetics in the rat (Martin *et al.*, 1976).

Rather than defining the potential therapeutic implications of the tested compounds, the aim of our study was to compare the pharmacological characteristics of labetalol and the **RR-SR** combination *in vivo* and *in vitro*.

In conclusion, the active isomers of labetalol, **RR** and **SR**, can be expected to be a good substitute for labetalol in the clinical setting in the treatment of systemic arterial hypertension, since 50% of labetalol comprises **SS** and **RS** isomers which although devoid of α- and β-adrenoceptor blocking properties might contribute to the overall toxicity of the drug.

However, the recent withdrawal of the **RR** isomer preparation (dilevalol) from the market, due to liver toxicity, suggests that further work to explore the potential therapeutic advantages of the **RR** and **SR** combination is required.

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An analysis of the nitrate-like and K channel opening actions of KRN2391 in canine coronary arterial smooth muscle

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1 To clarify the mechanism of action of KRN2391, a new vasodilator containing a nitroxy group, its effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and force of contraction in canine coronary artery were compared with those of two derivatives lacking the nitro group.

2 KRN2391, its hydroxy and acetoxy derivative (Compound 2 and Compound 3, respectively) partially reduced $[Ca^{2+}]_i$ in 5 or 30 mM KCl physiological salt solution (PSS), effects which were antagonized by glibenclamide. No KRN2391-induced change in $[Ca^{2+}]_i$ was observed in 90 mM KCl-PSS.

3 The order of potency in reducing $[Ca^{2+}]_i$ and inhibiting the contracture in 30 mM KCl-PSS was: KRN2391 > Compound 3 > Compound 2.

4 In 30 mM KCl-PSS, KRN2391 shifted the $[Ca^{2+}]_i$-force relationship so that a greater increase in $[Ca^{2+}]_i$ was needed to produce force. Compounds 2 and 3 were ineffective. The $[Ca^{2+}]_i$-force curve obtained in 90 mM KCl-PSS was shifted to the right by KRN2391 (10^{-4} M).

5 The ability of KRN2391 to reduce the force of contraction in both 30 mM and 90 mM KCl-PSS was inhibited by 10^{-5} M methylene blue.

6 KRN2391 inhibited U46619-induced contractions; this effect was associated with a reduction of $[Ca^{2+}]_i$, which decreased below the basal level.

7 Thus, KRN2391 is a potent vasodilator in canine coronary artery. It possesses nitrate-like and potassium channel opening actions and can be designated as a nitrate-potassium channel opener (N-K) hybrid. The denitrate derivatives of KRN2391 are specific K channel openers. The nitroxy moiety in KRN2391 is important for not only its action as a nitrate but also its potency as a K channel opener.

Keywords: N-K hybrid; K channel opener; nitrate; KRN2391; glibenclamide; fura-2; intracellular calcium concentration; relaxation; dog coronary artery

Introduction

A hybrid nitrate and potassium (K) channel activating (N-K hybrid) mechanism was proposed by Taira (1989), as the mechanism of action of nicorandil which has the dual vasodilator mechanism of a nitrate (Holzmann, 1983; Endoh & Taira, 1983) and a K channel opener (Karashima *et al.*, 1982; Weir & Weston, 1986; see reviews, Taira, 1987; Edwards & Weston, 1990). Nicorandil is the only N-K hybrid that is clinically used for the treatment of patients with angina pectoris at present. Recently, it has been reported that KRN2391, N-cyano-N'-(2-nitroxyethyl)-3-pyridinecarboximidamide methansulphonate (Figure 1), has vasorelaxant and antihypertensive effects (Kashiwabara *et al.*, 1990). The vasodilator effect of KRN2391 was partly inhibited by K channel blockers, e.g., glibenclamide, tetraethylammonium and 4-aminopyridine, and also by methylene blue and oxyhaemoglobin (Kashiwabara *et al.*, 1991). KRN2391 also increased ^{86}Rb efflux from rat aorta. Nicorandil possesses both K channel opening and nitrate-like actions in canine coronary arteries (Yanagisawa *et al.*, 1990) which suggests that relaxations produced by KRN2391 could also be associated with a similar dual mechanism (Kashiwabara *et al.*, 1991).

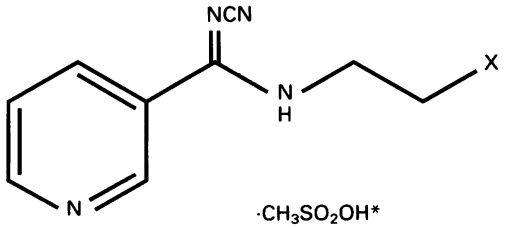


Figure 1 Chemical structures of KRN, Compound 2 (Ki3315) and Compound 3 (Ki4032). * The free bases of KRN2391 and Comp. 2 were used throughout these experiments.

Yanagisawa *et al.* (1989, 1990) found that the effects of K channel openers and nitrates could be clearly characterized by simultaneous measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) measured by fura-2 (Grynkiewicz *et al.*, 1985) and force of contraction in canine coronary arteries. K channel openers, like cromakalim, reduce the $[Ca^{2+}]_i$ due to the closure of voltage-dependent Ca^{2+} channels by hyperpolarization (Weston, 1989; Nelson *et al.*, 1990) without affecting the Ca^{2+} sensitivity of contractile proteins (Yanagisawa *et al.*, 1990). In contrast, nitrates, like nitroglycerin, relax the contracture produced by KCl by reducing the sensitivity of contractile proteins to Ca^{2+} but without affecting $[Ca^{2+}]_i$ (Yanagisawa *et al.*, 1989).

The present study was performed to clarify the mechanisms of vasodilatation of KRN2391 by examining its effects on both $[Ca^{2+}]_i$ and force in canine coronary arteries. Since KRN2391 has a nitroxy moiety in its structure (Figure 1), we determined whether this substituent plays an important role in its pharmacological activity by comparing its effects with those of two derivatives lacking the nitro group (denitrate derivatives), Compound 2 (Comp. 2, Ki3315) and Compound 3 (Comp. 3, Ki4032).

Methods

Preparation of arterial rings

Hearts were obtained from mongrel dogs of either sex, weighing 5 to 15 kg, anaesthetized with pentobarbitone sodium

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(30 mg kg^{-1}, i.v.). Coronary arterial rings (0.7–1.2 mm in diameter, about 1 mm in width) were dissected and connective tissues were carefully removed in a dissecting chamber under a binocular microscope (SZ, Olympus, Tokyo, Japan). The endothelium was removed by gentle rubbing with forceps and the effectiveness of removal was checked by the abolition of acetylcholine-induced relaxation. The luminal side of the arterial ring was turned to face outwards.

Fluorescence measurements

We performed fluorescence measurements as previously described (Yanagisawa *et al.*, 1989). In brief, coronary arterial rings were exposed to 10 μM fura-2 acetoxymethyl ester (fura-2 AM) for about 2 h at 37°C. The non-cytotoxic detergent, pluronic F-127 (0.1% w/v), was premixed in the loading physiological salt solution (PSS) to help dissolve the fura-2 AM in PSS. After the fura-2 loading, muscles were rinsed with normal PSS for more than 1 h and then used for experiments. Fluorescence was measured in a fluorimeter equipped with a dual wavelength excitation device (CAM-200 or CAM-220, Japan Spectroscopic, Tokyo, Japan) connected to an inverted microscope (TMD, Nikon, Tokyo, Japan). The fluorescence image was obtained by focusing on the smooth muscle cells in the medial layer with a Nikon CF u.v. (Fluor) 10× objective lens. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml min^{-1}. The muscle ring was stretched to a resting tension of about 5 mN between two tungsten needles, one of which was glued to a transducer element (AE801, AME, Horten, Norway or U-10230, Shinko, Tokyo, Japan). The photosignals and the mechanical activity were measured simultaneously and both were recorded on a chart recorder (Recti-horiz-8k, NEC-San-ei, Tokyo, Japan). They were also digitized by A/D converters and fed into a microcomputer (PC-9801, NEC, Tokyo, Japan) for further calculation and graphical analysis (Himpens & Somlyo, 1988). Every experiment started with perfusion of 90 mM KCl-PSS for 10 min. At the end of each experiment, the cell membrane was lysed with a detergent, Triton X-100 (1%), and autofluorescence signals were determined by quenching fura-2 signals with $MnCl_2$. We subtracted the F_{340} and F_{380} values due to autofluorescence from the corresponding values of F_{340} and F_{380} obtained under conditions of fura-2 loading to derive a recalculated $ratio_{rc}$. Since it is difficult to calculate the absolute concentration of $[Ca^{2+}]_i$ (Konishi *et al.*, 1988; Karaki, 1989), we used the relative F_{340}/F_{380} ratio as an indicator of $[Ca^{2+}]_i$ (Sato *et al.*, 1988; Yanagisawa *et al.*, 1989). Changes in the $ratio_{rc}$ (changes in $[Ca^{2+}]_i$) and those in force were expressed as percentages of the differences between basal values and those obtained with a 10 min perfusion with 90 mM KCl-PSS. None of the drugs and chemicals used in the present study, with the exception of fura-2, $MnCl_2$ and methylene blue affected fluorescence signals at the concentrations used.

Drugs and solutions

The composition (mM) of normal or 5 mM KCl physiological salt solution (PSS) was as follows: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2.5, glucose 11.1, HEPES 3 (pH 7.4). The solution was equilibrated with 100% O_2 at 37°C. High KCl-PSS was made by substituting NaCl with equimolar KCl. Ca^{2+}-free PSS was made by removing $CaCl_2$ from normal PSS and sometimes by adding 1 mM EGTA to the solution.

Drugs and chemicals were obtained from the following sources: KRN2391, Comp. 2 and Comp. 3 (Kirin Brewery, Tokyo, Japan), glibenclamide (Yamanouchi, Tokyo, Japan), methylene blue trihydrate (Kanto Chemical, Tokyo, Japan), U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; Sigma, St. Louis, MO, U.S.A.), EGTA (glycol-etherdiamine-tetraacetic acid), HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid), fura-2 AM and pluronic F-127 (Dojin, Kumamoto, Japan), DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). The free bases of KRN2391 and Comp. 2 were used throughout the experiments. KRN2391, Comp. 2 and Comp. 3 were diluted with PSS immediately before use and the pH of the solution was adjusted to 7.4. Fura-2 AM was dissolved in DMSO at a concentration of 1 mM. Pluronic F-127 was dissolved in 25% w/v in DMSO. Glibenclamide was dissolved in DMSO to give a concentration of 10 mM. U46619 was dissolved in ethanol to give a concentration of 1 mM. Other drugs or chemicals were dissolved in distilled water and diluted to the desired concentrations by PSS.

Analysis of concentration-effect curves

The concentration-effect curves for drugs in reducing the force of contraction, and the increased $[Ca^{2+}]_i$ produced by high KCl or U46619, were expressed as a % reduction from the pre-drug values, and computer fitted to a logistic equation:

$$E = 100 - M \times A^p/(A^p + K^p) \quad (1)$$

where E is the normalized effect, M is the maximum effect, A is the drug concentration, K is the EC_{50} value of each drug and p is the slope parameter. When the maximum effect of each drug was not obtainable because of its limited solubility, M was fixed at 100. The concentration-effect curves for KRN2391 in reducing $[Ca^{2+}]_i$ below the basal level in 5 mM KCl-PSS were also computer fitted to equation (1). The maximum effect, M, however, was the maximum reduction of $[Ca^{2+}]_i$ produced on perfusion with Ca^{2+}-free PSS containing 1 mM EGTA which was taken as 100%. The concentration-effect curves for extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) were also computer fitted to a logistic equation:

$$E = M \times A^p/(A^p + K^p) \quad (2)$$

where E is the effect normalized by the differences between basal values and those of 90 mM KCl at 10 min, A is $[Ca^{2+}]_o$, K is EC_{50} value of $[Ca^{2+}]_o$ and p is the slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$). Experimental values are given as mean ± s.e.mean. Statistical significance of results was evaluated by a one-way analysis of variance followed by Dunnett multiple-comparison test or Kruskal-Wallis test followed by Student's multiple-comparison test after Bartlett's test for pre-analysis of variance. A P value smaller than 0.05 was considered to be significant.

Results

Effect of KRN2391 on $[Ca^{2+}]_i$ and KCl-induced contractures

In canine coronary arteries loaded with fura-2, KRN2391 (10^{-8}–10^{-4} M) reduced the $[Ca^{2+}]_i$ below the based level in 5 mM KCl-PSS, without affecting the resting force (Figure 2a). The pD_2 value for this effect was 6.79 ± 0.10 (n = 6) (Table 1). Perfusion with 30 mM KCl-PSS increased $[Ca^{2+}]_i$ and elicited a contracture. KRN2391 (3×10^{-8}–10^{-4} M) reduced both $[Ca^{2+}]_i$ and contracture in a concentration-dependent manner (Figure 2b). The contracture was nearly abolished by 10^{-4} M KRN2391 but the increased $[Ca^{2+}]_i$ was only partially inhibited. The effects of KRN2391 could be reversed by washing. The summarized data from five muscles are shown in Figure 2c, and the results of the analysis of the concentration-effect curves for KRN2391 to reduce the increased $[Ca^{2+}]_i$ and contracture produced by 30 mM KCl-PSS are summarized in Table 1. Thus, although the pD_2 value for inhibition of contracture (6.06) was slightly but not significantly smaller than that associated with a reduction of the increase in $[Ca^{2+}]_i$ (6.25), the maximum inhibitory effect on force (91%) was much larger than on $[Ca^{2+}]_i$ (39%) (Table 1). The ability

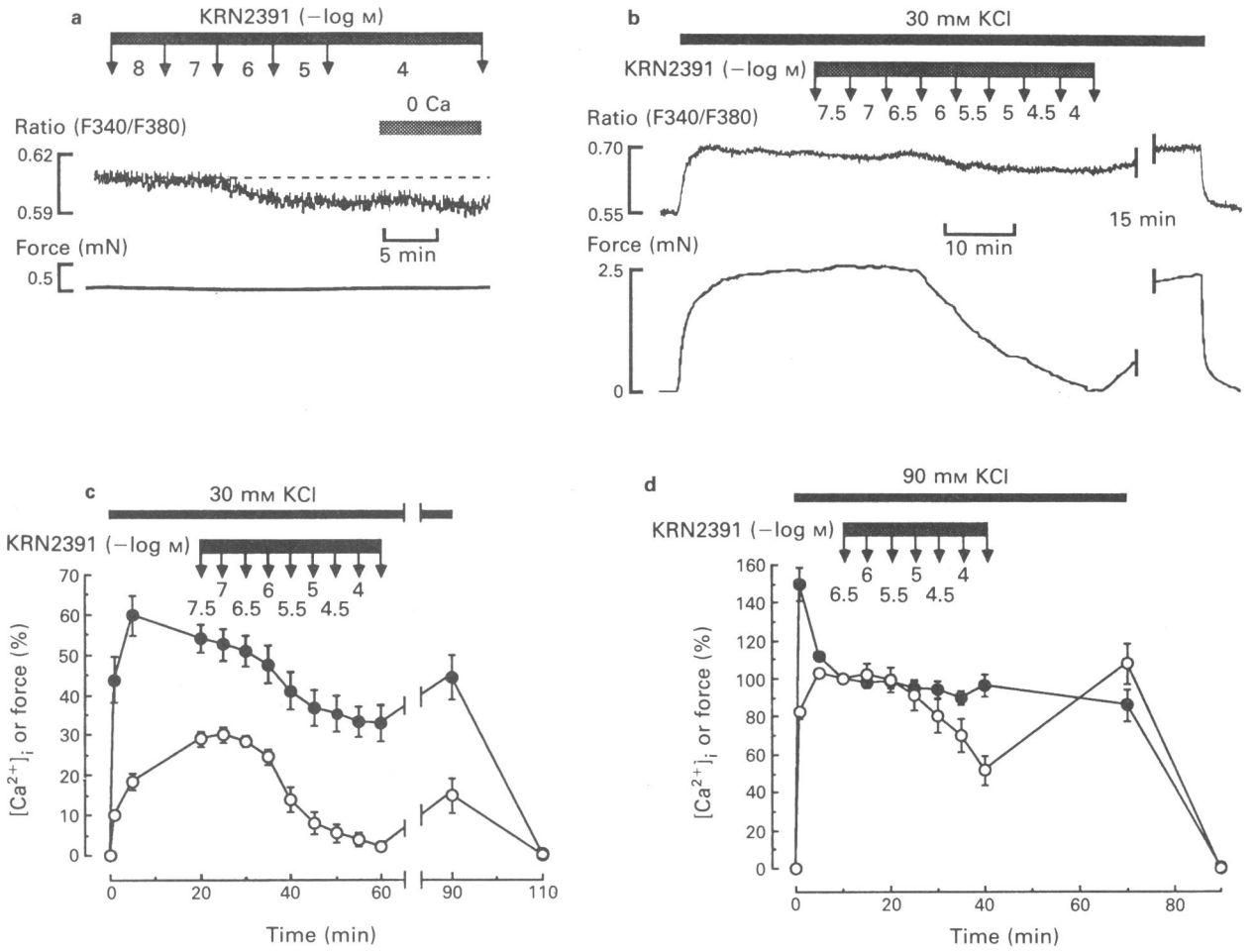


Figure 2 Effects of KRN2391 on the increase in $[Ca^{2+}]_i$ and force of contraction produced by high KCl-PSS in canine coronary arteries. (a) Typical traces of the force and $[Ca^{2+}]_i$ (indicated as fluorescence ratio) under resting conditions (5 mM KCl-PSS). The concentrations of KRN2391 (10^{-8}–10^{-4} M) were increased cumulatively. (b) Typical traces of the force and $[Ca^{2+}]_i$ in 30 mM KCl-PSS. (c) The summarized data obtained from six preparations in 30 mM KCl-PSS. The force of contraction (○) and the increase in $[Ca^{2+}]_i$ (●) are expressed as a percentage of each effect to 90 mM KCl-PSS at 10 min. The force of contraction and the difference in the $ratio_{rc}$ in the presence of 90 mM KCl-PSS at 10 min were 9.9 ± 0.7 mN and 0.21 ± 0.05, respectively. Vertical bars show s.e.mean. (d) Effects of KRN2391 (3×10^{-7}–10^{-4} M) on the force of contraction (○) and increase in $[Ca^{2+}]_i$ (●) in 90 mM KCl-PSS. Data are expressed as mean ($n = 6$); s.e.mean shown by vertical bars.

Table 1 Effects of KRN2391 and its denitrate derivatives on the $[Ca^{2+}]_i$ and force of contracture

	n	*Maximum effect* (%)	*Reduction of* $[Ca^{2+}]_i$ pD_2	p	*Maximum effect* (%)	*Reduction of force* pD_2	p
KRN2391							
5 mM KCl	6	66.1 ± 5.0	6.79 ± 0.10	1.11 ± 0.16	No effect (<4)		
30 mM KCl	5	39.1 ± 5.6	6.25 ± 0.11	1.23 ± 0.11	91.4 ± 3.2	6.06 ± 0.13	1.37 ± 0.24
30 mM KCl + Glib	6	25.8 ± 6.0	4.61 ± 0.17**	1.31 ± 0.26	88.9 ± 6.0	4.92 ± 0.28**	0.83 ± 0.06*
30 mM KCl + MB	6	37.5 ± 4.6	6.16 ± 0.14	1.46 ± 0.24	86.1 ± 5.4	5.54 ± 0.14	0.92 ± 0.05*
90 mM KCl	6		no effect (<4)		100[a]	4.25 ± 0.16	0.64 ± 0.06
90 mM KCl + MB	5		no effect (<4)		—	<4	—
U46619 (3×10^{-7} M)	5	145.7 ± 8.0	6.58 ± 0.08	1.72 ± 0.18	91.7 ± 1.2	6.45 ± 0.04	1.52 ± 0.19
Compound 3							
30 mM KCl	5	55.3 ± 9.2	5.22 ± 0.77**	1.21 ± 0.11	70.4 ± 4.5**	4.89 ± 0.07**	1.49 ± 0.14
30 mM KCl + Glib	6	—	<3.52	—	—	<3.52	—
90 mM KCl	6		No effect (<3.52)		No effect (<3.52)		
Compound 2							
30 mM KCl	5	—	<3	—	—	<3	—

The concentration-effect curves for KRN2391, Compound 3 and Compound 2 were analysed by computer fitting to the logistic equation (1) described in the methods. $pD_2 = -\log EC_{50}$ and p is the slope parameter. [a] Maximum effect was fixed at 100. Glib and MB are glibenclamide (10^{-5} M) and methylene blue (10^{-5} M), respectively. —: Not determined. Data show mean ± s.e.mean. Significantly different from 30 mM KCl values: *$P < 0.05$; **$P < 0.01$.

of KRN2391 to reduce $[Ca^{2+}]_i$ was dependent on the concentration of KCl. In 90 mM KCl-PSS, although the increased $[Ca^{2+}]_i$ was not changed by KRN2391, it inhibited the force of contraction in a concentration-dependent manner (Figure 2d). The relaxant potency of KRN2391 was also dependent on KCl concentration, i.e., the greater the depolarization of muscle, the lower the pD_2 values (Table 1). These results indicate that KRN2391 has a dual vasodilator mechanism of action, i.e., reduction of $[Ca^{2+}]_i$ and an inhibition of contracture independent of a reduction of the increased $[Ca^{2+}]_i$.

Effects of the denitrate derivatives of KRN2391 on KCl-induced increase in $[Ca^{2+}]_i$ and contractures

In the presence of 30 mM KCl-PSS, Comp. 3 (10^{-7}–3×10^{-4} M) reduced both $[Ca^{2+}]_i$ and contracture in a concentration-dependent manner (Figure 3), with pD_2 values of 5.22 ± 0.07 and 4.89 ± 0.07, respectively. Since the concentration-response curves did not reach maximal effects, the calculated values in Table 1 are the approximations of true values for each compound. Although the maximum inhibitory effect of Comp. 3 on $[Ca^{2+}]_i$ was slightly larger than that of KRN2391, the maximum mechano-inhibitory effect of Comp. 3 was less than that of KRN2391 (Table 1). Comp. 2 (3×10^{-5}–10^{-3} M) slightly reduced both the increase in $[Ca^{2+}]_i$ and contracture induced by 30 mM KCl-PSS (Figure 3). When arterial rings were perfused with 90 mM KCl-PSS, Comp. 3 had no effect on either the force of contraction or on the increased $[Ca^{2+}]_i$ (data not shown).

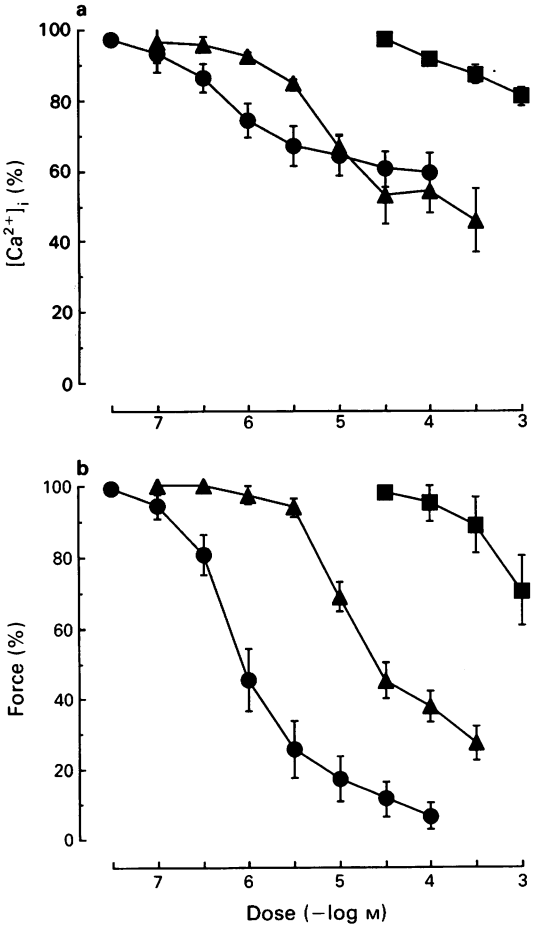


Figure 3 Effects of KRN2391 (●), Compound 3 (▲) and Compound 2 (■) on the increase in $[Ca^{2+}]_i$ (a) and force of contraction (b) produced by 30 mM KCl. The $[Ca^{2+}]_i$ and force are expressed as a percentage of each pre-drug value. The pre-drug value of $[Ca^{2+}]_i$ and force expressed as a percentage of the effect to 90 mM KCl-PSS at 10 min was 54.1 ± 3.4 and 29.0 ± 1.9% (KRN2391), 51.1 ± 4.2 and 28.1 ± 5.1% (Compound 3) and 48.9 ± 2.5 and 19.3 ± 2.4% (Compound 2), respectively. Data are expressed as mean ($n = 5$); s.e.mean shown by vertical bars.

Modification by glibenclamide of the effects of KRN2391 and its denitrate derivatives

Figure 4 shows the influence of 10^{-5} M glibenclamide on the ability of KRN2391 and Comp. 3 to reduce increased $[Ca^{2+}]_i$ and force of contraction produced by 30 mM KCl-PSS. Although glibenclamide (10^{-5} M) did not significantly change $[Ca^{2+}]_i$ or the contracture induced by 30 mM KCl, it antagonized the effect of KRN2391 (10^{-7}–10^{-4} M) on these parameters (Figure 4a). The ability of KRN2391 to reduce $[Ca^{2+}]_i$ was more effectively inhibited by glibenclamide than the inhibition of force of contraction (Figure 4a; Table 1). The influence of glibenclamide (10^{-5} M) on the effects of Comp. 3 is shown in Figure 4b. The rightward shift by glibenclamide was almost the same for both the $[Ca^{2+}]_i$ and the force of contraction. Although glibenclamide similarly antagonized the reduction of $[Ca^{2+}]_i$ produced by both KRN2391 and Comp. 3, it more effectively antagonized the reduction of contracture caused by Comp. 3 than that by KRN2391 (Figure 4, Table 1). The ability of Comp. 2 to reduce $[Ca^{2+}]_i$ and contracture in 30 mM KCl-PSS was also inhibited by glibenclamide; $[Ca^{2+}]_i$ and force (%) were 81.0 ± 2.7 and 70.4 ± 9.8% in the presence of Comp. 2 (10^{-3} M) alone, but 96.8 ± 2.5 and 100.5 ± 4.1% in the presence of both Comp. 2 and glibenclamide (10^{-5} M), respectively ($P < 0.01$ for the changes in both $[Ca^{2+}]_i$ and force; $n = 5$).

Effect of KRN2391 or its denitrate derivatives on the relationship between $[Ca^{2+}]_i$ and force in 30 mM KCl-depolarized artery

Figure 5 shows the relationship between $[Ca^{2+}]_i$ and force in the absence (control) and the presence of KRN2391 (3×10^{-8}–10^{-4} M), KRN2391 plus glibenclamide, Comp. 3 (10^{-7}–3×10^{-4} M) and Comp. 2 (3×10^{-5}–10^{-3} M) in 30 mM KCl-depolarized canine coronary arteries. The relationship was almost linear in the presence or absence of KRN2391 in 30 mM KCl-PSS. The line for KRN2391 was steeper than that of the control, and in the presence of glibenclamide it was much steeper than in both the control and KRN2391 alone groups (Figure 5a). In contrast, the slopes in the presence of Comp. 2 and 3 were almost the same as that of the control. These results show that, in addition to its ability (glibenclamide-sensitive) to reduce $[Ca^{2+}]_i$, KRN2391 also reduces the sensitivity of contractile proteins to Ca^{2+} in 90 mM KCl-PSS (Figure 3) and even in 30 mM KCl-PSS. In contrast, the relaxant effects of Comp. 2 and 3 are due to a decrease in $[Ca^{2+}]_i$ and not to a change in Ca^{2+} sensitivity of the contractile proteins.

Effect of KRN2391 on extracellular Ca^{2+}-induced changes in $[Ca^{2+}]_i$ and mechanical activity in 90 mM KCl-depolarized artery

As previously described, the relaxant potency of KRN2391 was dependent on the KCl concentration (Table 1) and furthermore KRN2391 was able to exert vasorelaxant effects without a detectable reduction in $[Ca^{2+}]_i$. To gain an insight into this mechanism, the effects of KRN2391 were examined under conditions in which $[Ca^{2+}]_i$ was changed without alteration of the membrane potential. A segment of artery was at first perfused with Ca^{2+}-free PSS for 10 min and then with Ca^{2+}-free 90 mM KCl-PSS. Removal of extracellular Ca^{2+} reduced $[Ca^{2+}]_i$ below the basal level (-20.7 ± 1.6%; expressed as a percentage of the increase following 10 min exposure to 90 mM KCl-PSS), whereas the basal force was not changed. Subsequent exposure of the muscle to 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ (-20.4 ± 3.8%) or any change in force. During continued exposure to 90 mM

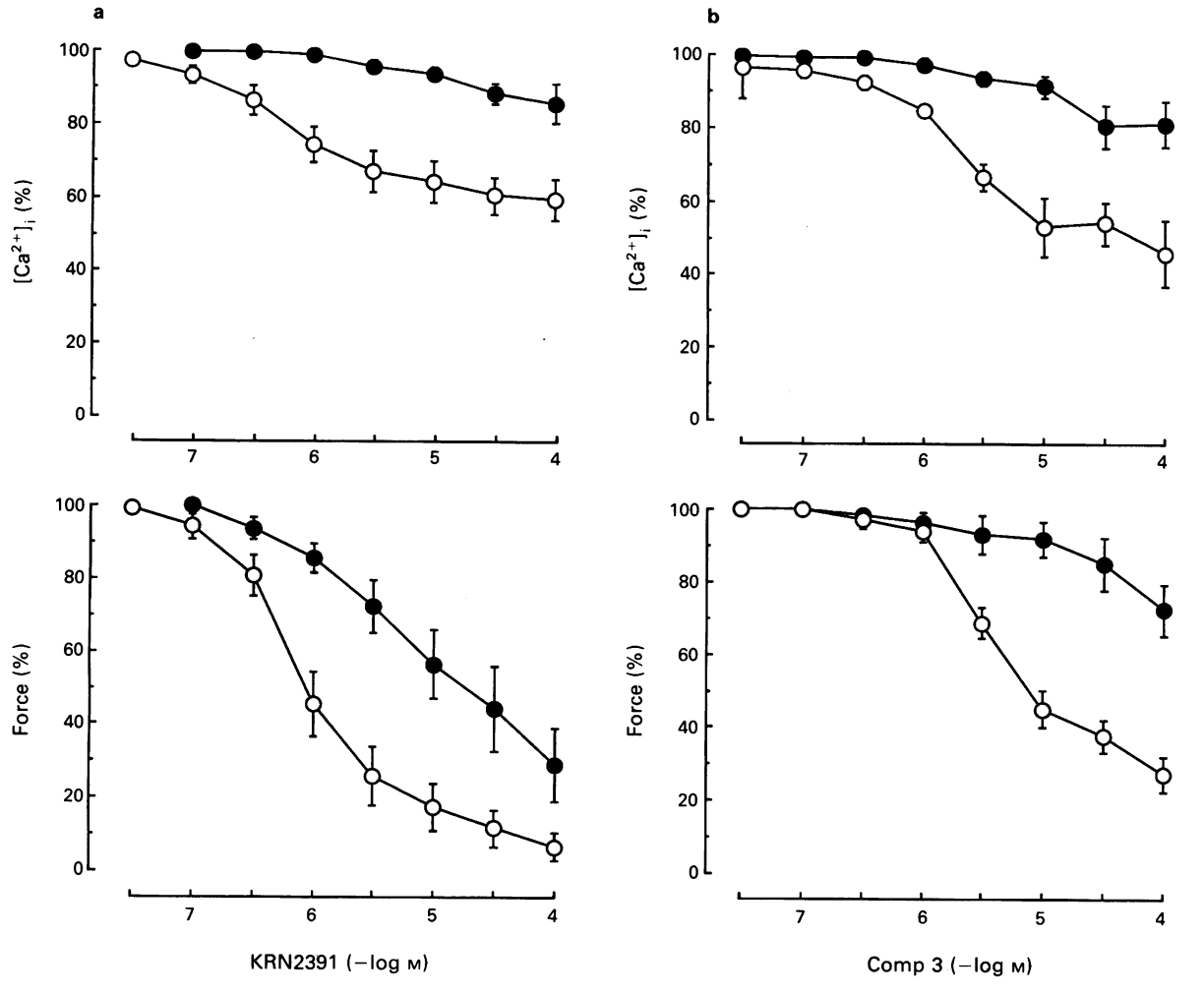


Figure 4 Influence of glibenclamide on the KRN2391- and Compound 3-induced reduction of $[Ca^{2+}]_i$ and contraction produced by 30 mM KCl-PSS. Effects of KRN2391 (a) and Compound 3 (b) in the absence (○) and the presence (●) of 10^{-5} M glibenclamide. Glibenclamide was added 10 min after treatment with 30 mM KCl-PSS and KRN2391 was added 20 min after the addition of glibenclamide. The $[Ca^{2+}]_i$ and force are expressed as a percentage of each pre-drug value. $[Ca^{2+}]_i$ and force (%) were 61.9 ± 2.4 and 16.5 ± 3.3% before the addition of glibenclamide, and 54.7 ± 2.7 and 12.1 ± 1.6% after the addition of glibenclamide, respectively. Data are expressed as mean (n = 5); s.e.mean shown by vertical bars.

KCl-PSS, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 0.03 to 15 mM with a consequent increase in both $[Ca^{2+}]_i$ and force (Figure 6a). Summarized data obtained from five muscles are shown in Figure 6b. The results of computer fitting the curve to the logistic equation (2) are summarized in Table 2. The maximum effect shown in Table 2 is the calculated value. The pD_2 value for $[Ca^{2+}]_o$ to increase $[Ca^{2+}]_i$ (3.28 ± 0.13) is the apparent K_d value of $[Ca^{2+}]_o$ for binding at voltage-dependent Ca^{2+} channels opened by depolarization. The difference between the pD_2 value of $[Ca^{2+}]_o$ to increase $[Ca^{2+}]_i$ and that to produce contracture are a consequence of the nonlinear $[Ca^{2+}]_i$-force relationship (Figure 7). When KRN2391 (10^{-4} M) was applied to muscle perfused with Ca^{2+}-free 90 mM KCl-PSS, $[Ca^{2+}]_i$ was not changed (Figure 6, Table 2). Although the increases in $[Ca^{2+}]_o$ produced the same increments in $[Ca^{2+}]_i$ in the absence (control) and the presence of KRN2391, the force produced by the increased $[Ca^{2+}]_o$ was smaller in the presence of KRN2391. Furthermore, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly decreased in the presence of KRN2391 ($P < 0.05$). These inhibitory effects of KRN2391 were reversible (Table 2). Figure 7 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and the presence of 10^{-4} M KRN2391 in 90 mM KCl-depolarized canine coronary artery. The curve in the presence of KRN2391 was different from that of the control and was shifted to the right. These results obtained from the analysis of $[Ca^{2+}]_i$-force relationship in the presence of KRN2391 suggest that KRN2391 reduces the Ca^{2+}-sensitivity of the contractile proteins.

Modification by methylene blue of the effect of KRN2391

Although methylene blue (10^{-5} M) reduced F_{340} and F_{380} slightly, the values of F_{340}/F_{380} and $ratio_{rc}$ were not changed. Thus, we have assumed that it is possible to estimate changes in $[Ca^{2+}]_i$ from the $ratio_{rc}$ even in the presence of methylene blue. Methylene blue (10^{-5} M) antagonized the inhibitory effect of KRN2391 on contracture produced by exposure to 30 mM KCl-PSS, but had no effect on KRN2391-induced reduction of $[Ca^{2+}]_i$ (Figure 8a). The concentration-effect curve for force was shifted to the right by methylene blue and the results of analysis of the concentration-effect curves are shown in Table 1. Although the pD_2 value and the maximum effect of KRN2391 in reducing $[Ca^{2+}]_i$ in the presence of methylene blue were the same as in its absence (control), the pD_2 value of KRN2391 for inhibition of contracture was slightly smaller in the presence of methylene blue. The slope parameter of the concentration-effect curve of KRN2391 for reduction of force of contraction was reduced by methylene blue. In 90 mM KCl-PSS, methylene blue antagonized the ability of KRN2391 to inhibit the force of contraction (Figure 8b). The rightward shift of the curve by methylene blue was greater in 90 mM KCl-PSS than in 30 mM KCl-PSS (Figure 8a,

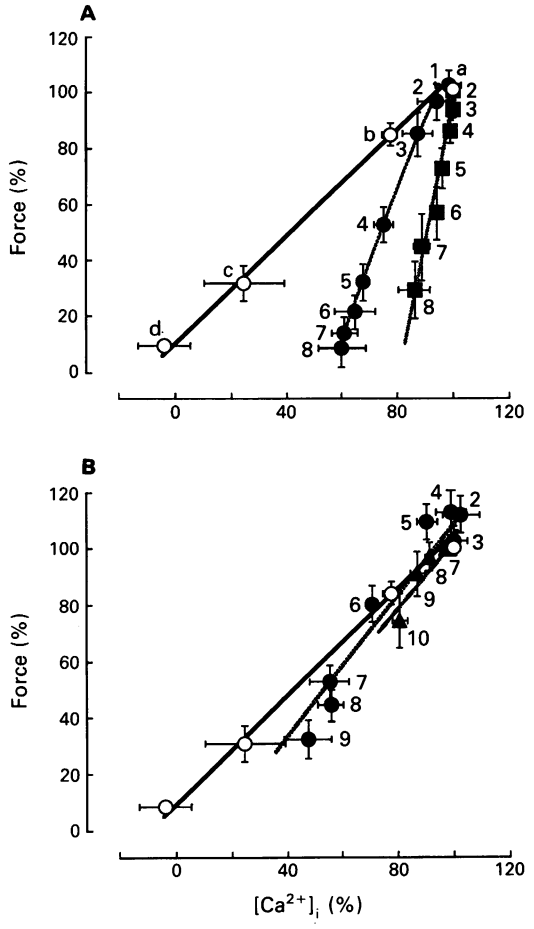


Figure 5 (A) Relationship between $[Ca^{2+}]_i$ and force in the absence (control; ○) and presence of KRN2391 (●) or KRN2391 + glibenclamide 10^{-5} M (■) in 30 mM KCl-PSS. (B) Relationship between $[Ca^{2+}]_i$ and force in the absence (control; ○) and the presence of Compound 3 (●) or Compound 2 (▲) in 30 mM KCl-PSS. Control curves were obtained by decreasing $[Ca^{2+}]_o$ (a: 2.5, b: 1.0, c: 0.3, d: 0.1 mM $[Ca^{2+}]_o$) in 30 mM KCl. Each point of KRN2391, Compound 3 or Compound 2 (1: 3×10^{-8}, 2: 10^{-7}, 3: 3×10^{-7}, 4: 10^{-6}, 5: 3×10^{-6}, 6: 10^{-5}, 7: 3×10^{-5}, 8: 10^{-4}, 9: 3×10^{-4}, 10: 10^{-3} M) was adapted from Figures 3 and 4, respectively. Data are expressed as mean of 5–6 experiments. Vertical and horizontal bars are s.e.mean.

b). The increased $[Ca^{2+}]_i$ was not changed by KRN2391 in the presence of methylene blue (Figure 8b).

Effect of KRN2391 on U46619-induced contractures and increase in $[Ca^{2+}]_i$

When coronary arterial rings were perfused with U46619 (3×10^{-7} M), $[Ca^{2+}]_i$ increased transiently and then remained at an elevated level. The force of contraction was increased in a similar manner (Figure 9a). KRN2391 (10^{-8}–10^{-4} M) reduced the increased $[Ca^{2+}]_i$, and the $[Ca^{2+}]_i$ was decreased below the basal level by KRN2391 (10^{-6}–10^{-4} M). Thus, the maximum effect of KRN2391 was 145.7 ± 8.0% (Table 1). KRN2391 almost completely inhibited the force of contraction produced by U46619. The pD_2 values and the slope parameter for reduction of $[Ca^{2+}]_i$ and force of contraction were almost the same (Table 1).

Discussion

In this study, KRN2391 partially reduced $[Ca^{2+}]_i$ in a concentration-dependent manner in both 5 and 30 mM KCl-PSS, but failed to modify $[Ca^{2+}]_i$ in 90 mM KCl-PSS. The reduction of $[Ca^{2+}]_i$ caused by KRN2391 in 30 mM KCl-PSS was antagonized by 10^{-5} M glibenclamide. The other K channel openers, cromakalim, pinacidil and nicorandil, each reduced the increase in $[Ca^{2+}]_i$ induced by 30 mM KCl, but that induced by 90 mM KCl was unaffected (Yanagisawa *et al.*, 1990). The KRN2391 reduction of $[Ca^{2+}]_i$ was thus influenced by the KCl concentration; a major characteristic of K channel openers, but not Ca^{2+} channel blockers (Yanagisawa *et al.*, 1990). In normal (5 mM KCl) PSS, KRN2391 reduced $[Ca^{2+}]_i$ below the basal level; a similar effect had also been observed with cromakalim, pinacidil and nicorandil (Yanagisawa *et al.*, 1990). Yanagisawa *et al.* (1990) suggested that certain Ca^{2+} channels are open in normal PSS, and that a hyperpolarization of the plasma membrane by a K channel opener is capable of closing this Ca^{2+}-entry pathway. Nelson *et al.* (1990) have shown that voltage-dependent Ca channels in arterial smooth muscle in the steady state can be open and very sensitive to membrane potential changes in a range that occurs in resistance arteries with tone, and that many vasodilators work, in part, through membrane hyperpolarization caused by ATP-sensitive K (K_{ATP}) channel activation. Although there have not been any reports about the effects of KRN2391 on the membrane potentials or membrane currents of vascular smooth muscle cells, KRN2391 increased ^{86}Rb efflux from rat aorta, indicating that it opened K channels (Kashiwabara *et al.*, 1991). This has also been observed with various other K channel openers (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast & Cook, 1989). Recently, sulphonylureas like glibenclamide have been shown to block cromakalim-induced electrical and mechanical changes in cardiac (Escande *et al.*, 1988; Fosset *et al.*, 1988; Sanguinetti *et al.*, 1988; Satoh *et al.*, 1990) and in vascular smooth muscle (Buckingham *et al.*, 1989; Eltze, 1989; Quast & Cook, 1989; Winquist *et al.*, 1989), suggesting a role for K_{ATP} channels (Standen *et al.*, 1989). The antagonism of KRN2391-induced reduction of $[Ca^{2+}]_i$ by glibenclamide indicates that, like other K channel openers, KRN2391 reduces $[Ca^{2+}]_i$ by hyperpolarizing the membrane of coronary arterial smooth muscle cells and thus closes Ca channels (Yanagisawa *et al.*, 1990). Therefore, it is conceivable that KRN2391 is one of the K_{ATP} channel openers, although the selectivity of glibenclamide for the K_{ATP} channel has not been established in smooth muscles.

Although in 30 mM KCl-PSS, KRN2391 reduced the force of contraction with a reduction of $[Ca^{2+}]_i$, the reduction of $[Ca^{2+}]_i$ is not the sole mechanism of its vascular relaxation. In 30 mM KCl-PSS, KRN2391 reduced $[Ca^{2+}]_i$ by about 40% but it almost abolished the force of contraction at high concentrations. In the presence of glibenclamide, the reduction of $[Ca^{2+}]_i$ was effectively inhibited, but the reduction of force of contraction remained. KRN2391 relaxed the artery without reducing the increased $[Ca^{2+}]_i$ in 90 mM KCl-PSS, and changed the relationship between $[Ca^{2+}]_i$ and force in 30 and 90 mM KCl-PSS. Therefore, in addition to its K channel opening action, KRN2391 reduces the sensitivity of contractile proteins to Ca^{2+}. This effect of KRN2391 was inhibited by methylene blue (Figure 8), a supposed guanylate cyclase inhibitor (Ignarro *et al.*, 1981). KRN2391 contains a nitroxy moiety within its structure and increased intracellular levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in canine coronary artery (in our laboratory, data not shown). Furthermore, the inhibitory effect of KRN2391 on contracture induced by noradrenoline was reduced by methylene blue or oxyhaemoglobin in the rat aorta (Kashiwabara *et al.*, 1991). Comp. 3, a denitrate derivative of KRN2391, did not reduce the sensitivity of contractile proteins to Ca^{2+} (Figure 5). These findings indicate that KRN2391 can also act as a nitrate in that it reduces the sensitivity of contractile proteins to Ca^{2+}. Nitroglycerin, a classical nitrate, also relaxes canine coronary arteries by this mechanism of action (Yanagisawa *et al.*, 1989), and sodium nitroprusside relaxes KCl-induced contractures accompanied by a small decrease in both $[Ca^{2+}]_i$ and ^{45}Ca uptake in the rat aorta (Karaki *et al.*, 1988). The amount of myosin light chain phosphorylation was reduced by sodium

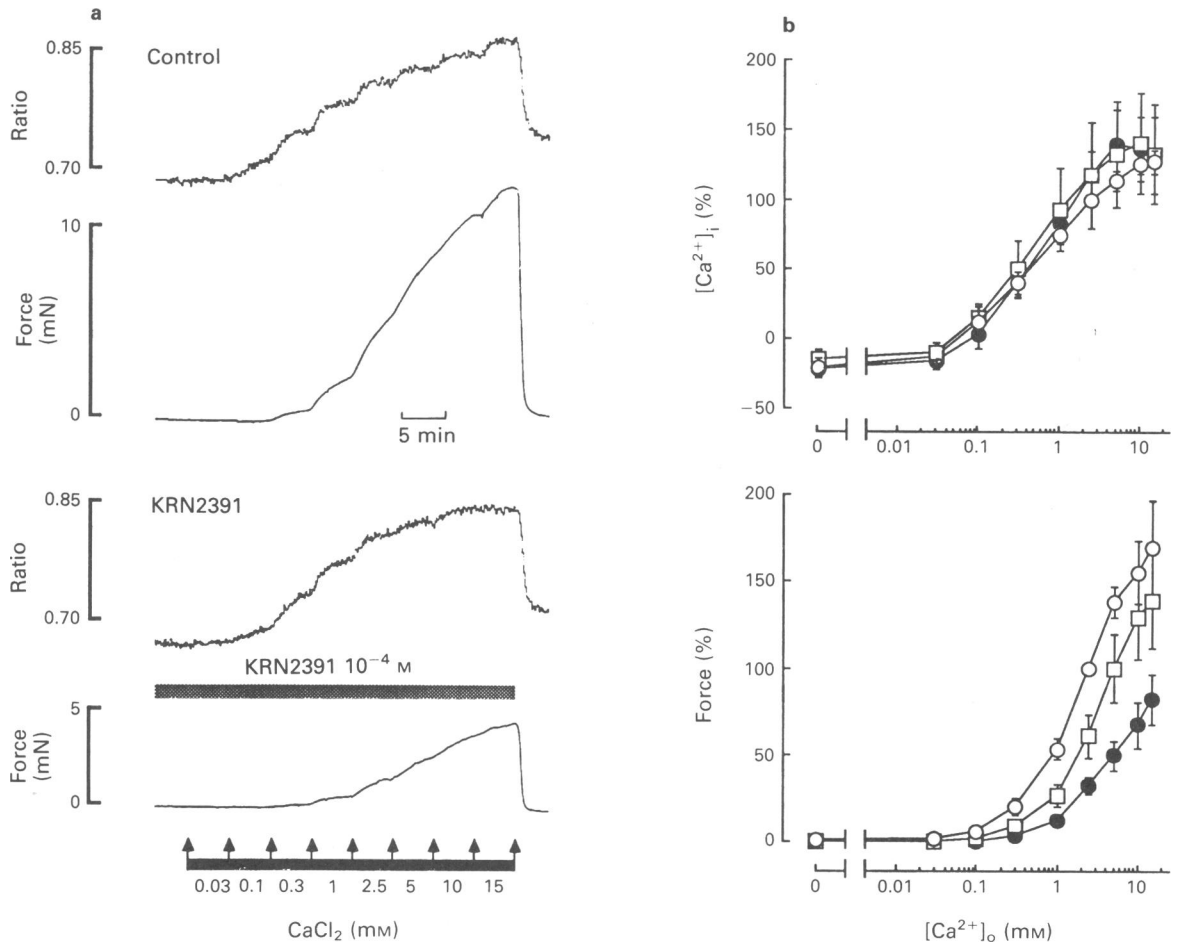


Figure 6 (a) Changes in $[Ca^{2+}]_i$ and force produced by increasing $[Ca^{2+}]_o$ in a canine coronary arterial muscle depolarized with 90 mM KCl-PSS and the effects of KRN2391 on these changes. (b) Summarized data of control (○), 10^{-4} M KRN2391 (●) and washout (30 min, □) obtained from five experiments. Data are expressed as mean with s.e.mean shown by vertical bars. The force of contraction and the difference in the $ratio_{rc}$ in the presence of 15 mM $CaCl_2$ and 90 mM KCl-PSS at 5 min were 9.7 ± 1.6 mN and 0.28 ± 0.06, respectively, in the control group.

nitroprusside and 8-bromo cyclic GMP (Rapoport *et al.*, 1982; Draznin *et al.*, 1986). Thus, it is likely that nitrates, including KRN2391, relax canine coronary arteries by reducing the amount of myosin light chain phosphorylation normally associated with an increased $[Ca^{2+}]_i$ following Ca^{2+} influx on depolarization.

KRN2391 thus possesses a dual action, both as a K channel opener and a nitrate, and in this respect its action is similar to that of nicorandil in canine coronary arteries (Yanagisawa *et al.*, 1990). Since the effects of Comp. 2 and Comp. 3 (Figure 1) were blocked by 10^{-5} M glibenclamide and the relationships between $[Ca^{2+}]_i$ and force in the presence of Comp. 2 and Comp. 3 were the same as those in their absence, the mechanism of action of these two denitrate derivatives could be solely attributed to an opening of K channels. The order of potency of this ability to reduce $[Ca^{2+}]_i$, which reflects a K channel opening action, was KRN2391 > Comp. 3 > Comp. 2. Comp. 2, in which a nitroxy moiety of KRN2391 is replaced by a hydroxy group, is much less potent than KRN2391. Comp. 3, with an acetoxy group, was about 10 times less potent than KRN2391 (Figure 3 and Table 1), but its maximum effect was slightly greater than that of KRN2391. Thus, the structure-activity relationships of pyridine derivatives as K channel openers indicates that the nitroxy substituent is very important for this activity. Such a structure-activity relationship has already been shown for nicorandil (Maruyama *et al.*, 1982; for review Edwards & Weston, 1990).

Finally, we also examined the effect of KRN2391 on the increased $[Ca^{2+}]_i$ and contracture induced by U46619, a

Table 2 Effects of KRN2391 on the $[Ca^{2+}]_i$-$[Ca^{2+}]_o$ and force-$[Ca^{2+}]_o$ relationships in 90 mM KCl-depolarized canine coronary artery

	Maximum effect (%)	*Increase in* $[Ca^{2+}]_i$ pD_2	p	*Maximum effect* (%)	*Increase in force* pD_2	p
Control	158.7 ± 15.15	3.28 ± 0.13	0.90 ± 0.09	209.0 ± 35.98	2.65 ± 0.16	1.22 ± 0.14
KRN2391 (10^{-4} M)	170.8 ± 28.87	3.28 ± 0.10	1.05 ± 0.07	114.5 ± 22.79*	2.23 ± 0.07*	1.10 ± 0.07
Washout	170.0 ± 28.57	3.16 ± 0.21	0.94 ± 0.16	161.7 ± 31.46	2.45 ± 0.05	1.38 ± 0.11

Increase in $[Ca^{2+}]_i$ was calculated from the difference between the value obtained in Ca^{2+}-free 90 mM KCl and the value in the presence of $[Ca^{2+}]_o$. The maximum value of $[Ca^{2+}]_i$ in each experiment was usually obtained at 15 mM $[Ca^{2+}]_o$. Maximum effect (%) in $[Ca^{2+}]_i$ and force is the calculated values of each experiment by computer fitting to the logistic equation (2). p is the slope parameter. Significant difference from the control values: $^*P < 0.05$ ($n = 5$).

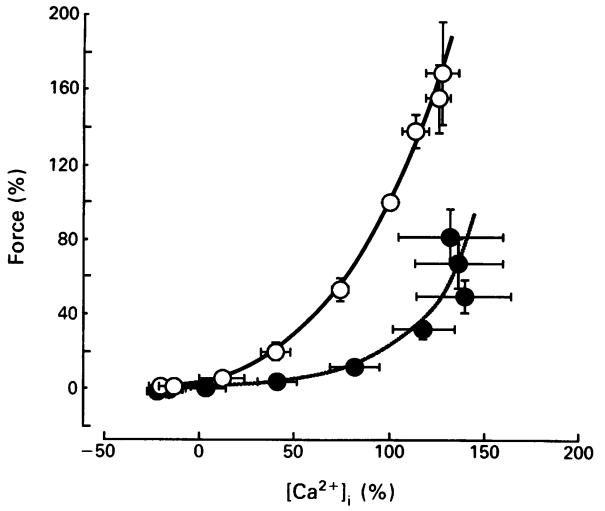


Figure 7 Relationship between $[Ca^{2+}]_i$ and force in the absence (control; ○) and the presence of 10^{-4} M KRN2391 (●). Each point was obtained from the data shown in Figure 6. Vertical and horizontal bars are s.e.mean ($n = 5$). The curves were fitted by eye.

stable thromboxane A_2 analogue. The contracture induced by U46619 was inhibited by KRN2391 in parallel with the reduction of $[Ca^{2+}]_i$ (Figure 8, Table 1). The $[Ca^{2+}]_i$ in the presence of 10^{-6}–10^{-4} M KRN2391 was decreased below the basal level as in the absence of U46619. Since the pD_2 value of KRN2391 for reduction of $[Ca^{2+}]_i$ in the presence of U46619 was almost the same as that in 5 mM KCl-PSS, the effect of KRN2391 seems to be mainly due to K channel opening action. Thus, the tonic increase in $[Ca^{2+}]_i$ produced by U46619 is mainly due to Ca^{2+} influx through voltage-dependent Ca channels (Nelson *et al.*, 1990) as is the case with histamine in the coronary artery of the pig (Mori *et al.*, 1990).

In conclusion, KRN2391 acts as an N-K hybrid compound and the nitroxy moiety in KRN2391 is important not only for its action as a nitrate but also for its potency as a K channel opener.

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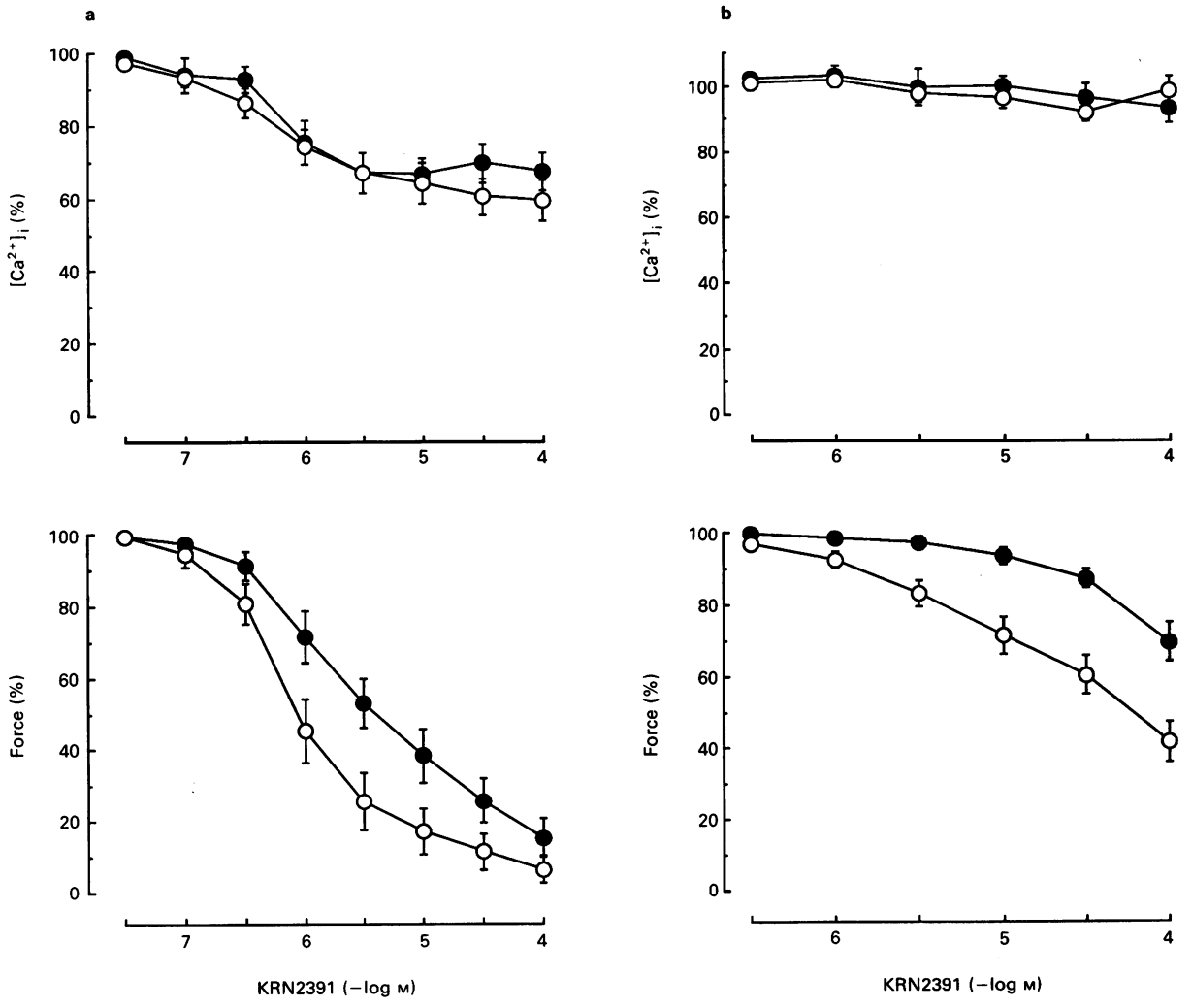


Figure 8 Influence of methylene blue on the ability of KRN2391 to reduce the increased $[Ca^{2+}]_i$ and force of contraction in high KCl-PSS. The effects of KRN2391 on $[Ca^{2+}]_i$ or the force produced by 30 mM KCl-PSS (a) and on the force produced by 90 mM KCl-PSS (b) in the absence (○) and the presence of 10^{-5} M methylene blue (●). Methylene blue was added 20 min before exposure to high KCl-PSS and then KRN2391 was administered 30 min later. The concentrations of KRN2391 were increased cumulatively. The $[Ca^{2+}]_i$ and force are each expressed as a percentage of each value before the addition of KRN2391. Data are expressed as mean ($n = 5$–6); s.e.mean shown by vertical bars.

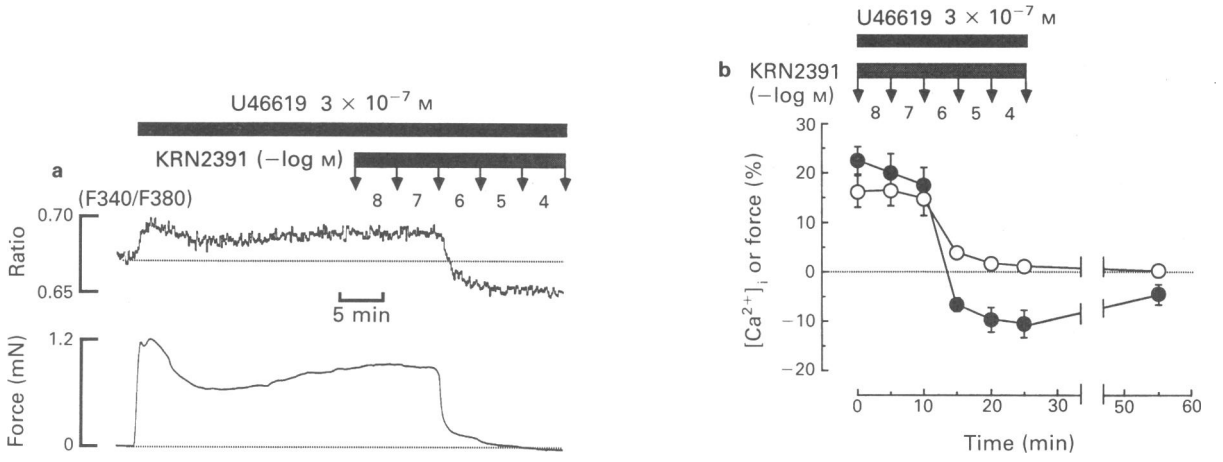


Figure 9 Effects of KRN2391 (10^{-8}–10^{-4} M) on the increase in $[Ca^{2+}]_i$ and the force of contraction produced by 3×10^{-7} M U46619. (a) Typical traces of the force and $[Ca^{2+}]_i$ (indicated as fluorescence ratio). KRN2391 (10^{-8}–10^{-4} M) was applied cumulatively. (b) The summarized data were obtained from five preparations. The increase in $[Ca^{2+}]_i$ (●) and the force of contraction (○) are expressed as a percentage of each effect to 90 mM KCl-PSS at 10 min. Data are expressed as mean with s.e.mean shown by vertical bars. Dotted lines indicate basal values.

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Histamine-induced increases in cyclic AMP levels in bovine adrenal medullary cells

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1 The effect of histamine on cellular cyclic AMP levels in cultured bovine adrenal medullary cells has been studied.

2 Histamine (0.3–30 μM) increased cyclic AMP levels transiently, with a maximal response after 5 min, a smaller response after 20 min, and no increase seen after 80 or 180 min. The EC_{50} at 5 min was approximately 2 μM. Histamine had no effect on cyclic AMP release from the cells over 5 min, but increased it after 90 min.

3 The cyclic AMP response to 5 μM histamine was reduced by 45% by 1 μM mepyramine and by almost 30% by 1 μM cimetidine, and was abolished by the combination of both antagonists. Cimetidine at 100 μM did not inhibit the response to histamine more than 1 μM cimetidine. The H_3-receptor antagonist, thioperamide (1 μM), had no effect on the response to histamine.

4 The H_1-receptor agonist, 2-thiazolylethylamine (5–100 μM) and the H_2-receptor agonist, dimaprit (5–100 μM), each induced a cyclic AMP response, and gave more-than-additive responses when combined. The H_3 agonist (**R**)α-methylhistamine (100 μM) had no effect either on its own or in combination with either the H_1 or the H_2 agonist. The response to 100 μM 2-thiazolylethylamine was unaffected by cimetidine (100 μM).

5 The cyclic AMP responses to 5 μM histamine, 100 μM thiazolylethylamine and 100 μM dimaprit were each weakly enhanced in the presence of 1 mM 3-isobutyl-1-methylxanthine. The response to dimaprit was enhanced more than 10 fold in the presence of 0.3 μM forskolin, while the responses to histamine and thiazolylethylamine were weakly enhanced.

6 The cyclic AMP response to 5 μM histamine was partially reduced in the absence of extracellular Ca^{2+}, and the residual response was fully antagonized by 1 μM cimetidine and was unaffected by 1 μM mepyramine. In the absence of Ca^{2+}, the cyclic AMP response to 100 μM thiazolylethylamine was abolished, while that to 100 μM dimaprit was unaffected.

7 Reincubation of 5 μM histamine solutions with a second set of chromaffin cells, following prior incubation with another set of cells, induced a cyclic AMP response in the fresh cells. This response was reduced by a combination of mepyramine and cimetidine to the same degree as the response to fresh 5 μM histamine solutions.

8 The results indicate that histamine increases cellular cyclic AMP levels in bovine chromaffin cells by three mechanisms: by acting on H_1 receptors, by acting on H_2 receptors, and by an interaction between H_1 and H_2 receptors. The H_1 response does not require concomitant activation of H_2 receptors, is fully dependent on extracellular Ca^{2+}, does not depend on secreted chromaffin cell products, and is not due to reduced cyclic AMP degradation or export. The H_2 cyclic AMP response is the first functional response reported for H_2 receptors on chromaffin cells, is independent of Ca^{2+}, is not due to reduced cyclic AMP export or degradation, and is likely to be mediated via a direct action through G_s. The role of these different mechanisms in the regulation of cyclic AMP-dependent processes in chromaffin cells by histamine is under investigation.

Keywords: Adrenal medulla; adenosine-3′:5′-cyclic monophosphate; histamine receptors; chromaffin cells; calcium; second messengers

Introduction

The second messenger adenosine-3′:5′-cyclic monophosphate (cyclic AMP) has been implicated in a number of regulatory processes in adrenal medullary chromaffin cells. Conflicting results have been reported from studies on the effects of cyclic AMP on catecholamine secretion. In some studies, low concentrations of the diterpine forskolin, which increases cellular cyclic AMP levels by direct activation of adenylate cyclase, inhibited acetylcholine- or nicotine-induced catecholamine secretion from cultured bovine chromaffin cells (Cheek & Burgoyne, 1987; Marriott *et al.*, 1988; Negishi *et al.*, 1989). In others, it enhanced the nicotinic secretory response or had no effect (Adams & Boarder, 1987; Higgins & Berg, 1988; Morita *et al.*, 1987a,b). Similar contradictory data have been obtained with other drugs that affect cellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels, such as phosphodiesterase inhibitors, or that mimic the actions of cyclic AMP, such as the permeant analogues 8-bromo- and dibutyryl-cyclic AMP (Adams & Boarder, 1987; Marriott *et al.*, 1988; Morita *et al.*, 1985; Tsujimoto *et al.*, 1986; Serck-Hanssen, 1974). The cause of the discrepancies between these studies remains unclear but may include different concentrations or time-courses of cyclic AMP generated in the cells by the procedures used and the different types and concentrations of secretagogues used to evoke catecholamine secretion (see for example Morita *et al.*, 1987a,b; Marriott *et al.*, 1988).

In contrast, several studies have consistently found that tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis, is rapidly phosphorylated on multiple sites in chromaffin cells by a cyclic AMP-dependent protein kinase (Masserano & Weiner, 1979; Meligeni *et al.*, 1982; Niggli *et al.*, 1984; Waymire *et al.*, 1988). This results in a reduction in the K_m of the enzyme for its tetrahydropterine cofactor, which activates the enzyme (Meligeni *et al.*, 1982). The rate of transcription of the gene encoding tyrosine

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hydroxylase is also enhanced by cyclic AMP (Kumakura et al., 1979; Lewis et al., 1983; 1987). The expression of several other chromaffin granule components is increased by cyclic AMP, including proenkephalin A (the precursor of numerous opioid peptides found in chromaffin granules and secreted together with catecholamines) and vasoactive intestinal polypeptide (Eiden & Hotchkiss, 1983; Eiden et al., 1984; Pruss et al., 1985; Wan et al., 1991). These observations suggest that cyclic AMP may play a role in regulating the biosynthesis of catecholamines and of other secretory products in adrenal chromaffin cells.

Levels of cyclic AMP in adrenal chromaffin cells are increased by nicotinic cholinergic stimulation, which also causes catecholamine secretion (Boonjaviroy & Gutman, 1977; Eiden et al., 1984; Guidotti & Costa, 1974; Marriott et al., 1988; Morita et al., 1987a,b; Tsujimoto et al., 1980; Wilson, 1988). Since cyclic AMP activates catecholamine biosynthesis, these findings have suggested that cyclic AMP (together with Ca^{2+}) may be intimately involved in stimulation-secretion-synthesis coupling, by which the synthesis of catecholamines is activated concomitantly with their secretion. It is not known, however, whether other receptor-mediated secretagogues also increase cyclic AMP levels in these cells or regulate catecholamine biosynthesis concurrently with secretion in a cyclic AMP-dependent manner. In the present study we have investigated the regulation of chromaffin cell cyclic AMP formation by another well-characterized secretagogue for these cells, histamine.

Methods

Incubation of cultured bovine adrenal chromaffin cells

Bovine adrenal glands from adult animals were obtained from a local abattoir. Adrenal medullary cells were isolated by collagenase digestion and purified by Percoll density gradient centrifugation, as described in detail previously (Livett et al., 1987). Cells were cultured on 6-well tissue culture plates, precoated with rat tail collagen, at a density of 1.25×10^6/well (see Livett et al., 1987, for conditions).

Cells were cultured for three days before use, to allow secure attachment of the cells. Cells were then given three consecutive incubations at 37°C in 1 ml of prewarmed buffer comprising (mM): NaCl 154, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, $CaCl_2$ 2.2 and D-glucose 10, supplemented with 0.5% bovine serum albumin and adjusted to pH 7.4. The incubations were made with the culture plates floating on a 37°C water bath. The first incubation was a 5 min wash and the second a 5 or 15 min wash in the presence of receptor antagonists if used. The third incubation was for the times indicated in the presence of receptor agonists together with antagonists, forskolin or phosphodiesterase inhibitors where used.

Following the third incubation period, the incubation medium was removed and the cellular cyclic AMP extracted by addition of 1 ml of acid ethanol (0.01 M HCl in 100% ethanol). When the incubation medium was also saved for cyclic AMP determinations, the medium was mixed with two volumes of acid ethanol. Extracts were stored at −20°C before being processed for cyclic AMP determinations.

In experiments where Ca^{2+}-free buffer was used, the 2.2 mM $CaCl_2$ in the incubation buffer was replaced by 2.2 mM $MgCl_2$, and all three incubations were performed in Ca^{2+}-free buffer.

Determination of cyclic AMP in cell and medium extracts

Extracts were evaporated to dryness in a vacuum centrifuge (Speed Vac Concentrator, Savant). The cell and medium extracts were reconstituted in 600 µl and 1200 µl of assay buffer (see below) respectively, and 600 µl of each were acetylated by the rapid sequential addition of 12 µl triethylamine and 6 µl acetic anhydride. Duplicate 100 µl aliquots were then assayed for cyclic AMP by radioimmunoassay.

Radioimmunoassay of cyclic AMP

The radioimmunoassay for cyclic AMP followed the methods of Steiner et al. (1972) and Brooker et al. (1979). Polyclonal antisera to cyclic AMP were raised in rabbits immunised with 2'-O-monosuccinyladenosine-3':5'-cyclic monophosphate conjugated to keyhole limpet haemocyanin with 1-ethyl-(3-diethylaminopropyl)- carbodiimide. Antiserum Ab5102 was used at a final dilution of 1 in 15,000. The tracer was prepared by radioiodination of 2'-O-monosuccinyladenosine-3':5'-cyclic monophosphate tyrosylmethylester by the chloramine T method, and purified by elution with a methanol gradient in 1% trifluoroacetic acid from a C18 reverse-phase Sep-Pak cartridge (Millipore). Acetylated standards were prepared as for the cell and medium extracts, using synthetic adenosine-3':5'-cyclic monophosphate. The standard was calibrated by its u.v. absorption at 258 nm with a molar extinction coefficient of 14,100.

Assays were performed with a sample volume of 100 µl in a final volume of 300 µl of a 50 mM sodium acetate buffer containing 1 mM theophylline and 0.1% bovine serum albumin, pH 5.0. After incubation for 48 h at 4°C, unbound tracer was separated from antibody-bound tracer by the addition of bovine serum albumin-coated charcoal (Activated Carbon Norit A, Aldrich Chemical Co.) in 50 mM sodium phosphate buffer, pH 7.4, followed by centrifugation.

The cyclic AMP radioimmunoassay had an IC_{50} for cyclic AMP of 160 (±6.4) fmol/100 µl sample (mean (±s.e.mean) of 10 assays) and could detect 5 fmol of cyclic AMP. The assay had less than 0.05% cross-reactivity with cyclic GMP, and exhibited no detectable cross-reactivity (<0.0005%) with ATP, ADP, AMP, adenosine, GTP, GDP, GMP or guanosine. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine caused no interference in the assay when used in the experiments at a concentration of 1 mM.

Data presentation and statistical analysis

All results are presented as means (± s.e.mean) for the stated number of observations. Basal cyclic AMP levels varied greatly between cell preparations, in the range 65–950 fmol/10^6 cells (mean ± s.e.mean = 308 ± 51.7 fmol/10^6 cells for 22 cell preparations). Consequently, cyclic AMP levels have been normalised to the basal levels for each cell preparation. Results were analysed by Student's t test, where indicated in the figure legends. Interactions between combinations of agonists were assessed by determining whether the observed effect of the combination of drugs lay outside the 95% confidence limits for the expected effect if the two agents were simply additive (see Moeller et al., 1989).

Materials

Cimetidine, histamine dihydrochloride, 3-isobutyl-1-methylxanthine (IBMX), mepyramine (pyrilamine maleate), and all adenosine and guanosine compounds were from Sigma Chemical Co. Forskolin was from Calbiochem Co. Dimaprit dihydrochloride and 2-thiazolylethylamine dihydrochloride were generous gifts from Dr V.G. Balmer (Smith, Kline and French), and (R)α-methylhistamine dihydrochloride and thioperamide were generous gifts from Dr J.-M. Arrang (INSERM, Paris). Forskolin was dissolved in ethanol, and cimetidine, IBMX and thioperamide in dimethylsulphoxide, before dilution in buffer to their working concentrations. Appropriate solvent controls were performed.

Results

Pharmacology of histamine-induced cyclic AMP responses in bovine chromaffin cells

Histamine increased cellular cyclic AMP levels in cultured bovine adrenal medullary cells in a concentration- and time-dependent manner (Figure 1). The threshold concentration for an effect was approximately 300 nM, the EC_{50} was about 2 μM and the maximal response was obtained at about 10 μM. The cellular cyclic AMP response to histamine was transient: the increase was maximal over a 5 min stimulation, but reduced after 20 min, and was no longer visible after 80 or 180 min.

The histamine-induced increase in cellular cyclic AMP levels was only weakly inhibited by the H_2 receptor antagonist, cimetidine (1 μM, Figure 2a). Higher concentrations of cimetidine (100 μM) had no greater effect than 1 μM (Figure 2c). The response to histamine was also partially reduced by the H_1-antagonist, mepyramine, at 1 μM (Figure 2a), a concentration that fully antagonized the H_1-receptor effects on catecholamine release and inositol phosphate production in these cells (Livett & Marley, 1986; Wan *et al.*, 1989a). A combination of both mepyramine and cimetidine each at 1 μM, however, completely blocked the cyclic AMP response to histamine. The potent and selective H_3-antagonist, thioperamide, had no effect on the histamine response (Figure 2b). None of the antagonists had any effect on cellular cyclic AMP levels on their own (Figure 2).

The selective but weak H_1-receptor agonist, 2-thiazolylethylamine, increased cellular cyclic AMP levels in bovine adrenal medullary cells at concentrations of 5–100 μM (Figure 3a,c). The response to 100 μM 2-thiazolylethylamine was unaffected by 100 μM cimetidine (Figure 2c), indicating that, at 100 μM, 2-thiazolylethylamine retains its H_1-selectivity in this preparation. The H_2-receptor agonist, dimaprit, also increased cellular cyclic AMP levels over the concentration-range 5–100 μM (Figure 3a,b). Combinations of increasing concentrations of dimaprit with a single high concentration of 2-thiazolylethylamine gave more-than-additive cyclic AMP responses (Figure 3a), indicating H_1- and H_2-receptors can interact in increasing cellular cyclic AMP levels.

In contrast, the selective H_3-receptor agonist, (**R**)-α-methylhistamine, had no effect on cyclic AMP levels on its own (Figure 3b,c). Increasing concentrations of either dimaprit (Figure 3b) or 2-thiazolylethylamine (Figure 3c) in combination with a single high concentration of (**R**)-α-methylhistamine produced cyclic AMP responses that were no different from those in the absence of (**R**)-α-methylhistamine.

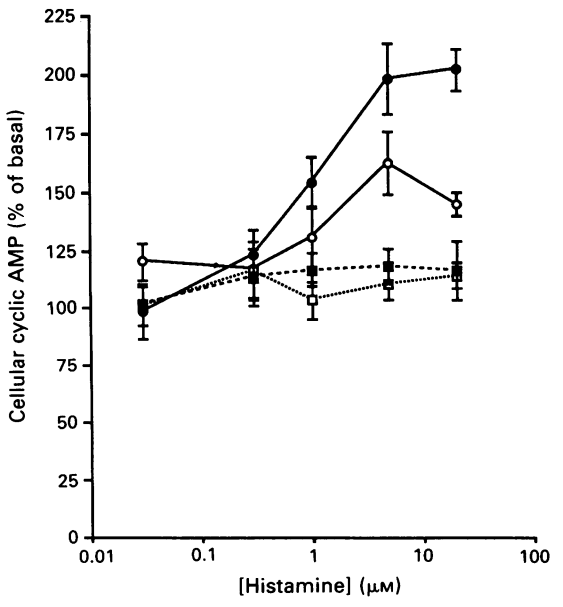


Figure 1 Time-course and concentration-dependance of histamine-induced cellular cyclic AMP responses in cultured bovine adrenal medullary cells: (●) 5 min, (○) 20 min, (□) 80 min, (■) 180 min. Data are mean for $n = 6$–9 (s.e.mean shown by vertical bars), and are pooled from three different cell preparations.

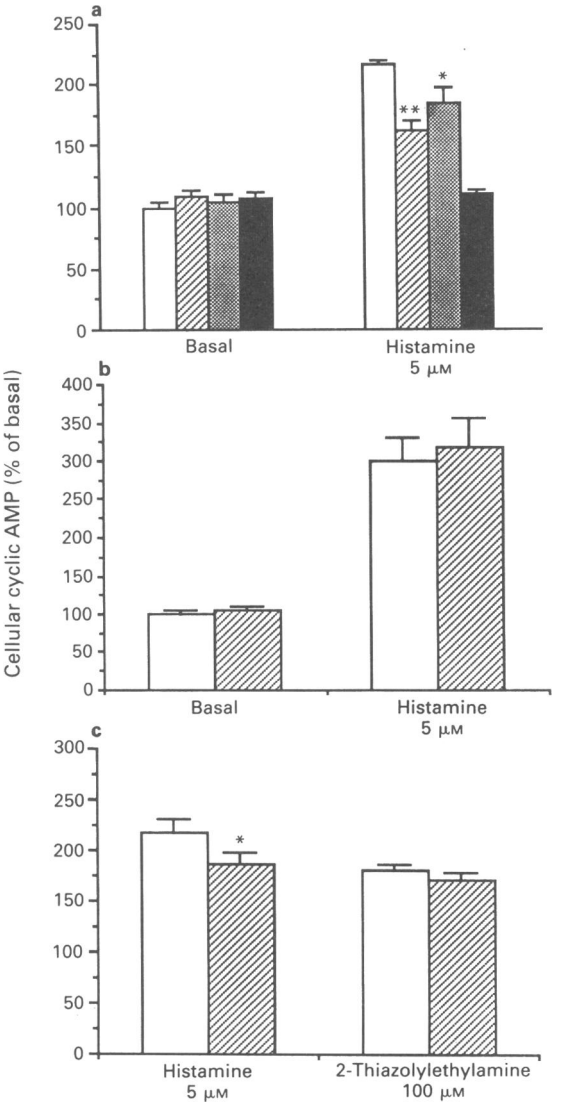


Figure 2 Effect of selective histamine receptor antagonists on basal and histamine-induced cellular cyclic AMP responses in cultured bovine adrenal medullary cells. (a) Effect of 1 μM mepyramine (hatched columns), 1 μM cimetidine (stippled columns) or a combination of both (solid columns) and (b) effect of 1 μM thioperamide (hatched columns) on basal and 5 μM histamine-induced cellular cyclic AMP levels over 5 min. (c) Effect of 100 μM cimetidine (hatched columns) on 5 μM histamine- and 100 μM 2-thiazolylethylamine-induced cyclic AMP responses. Results are means (± s.e.mean) for $n = 8$ or 9 (a), and $n = 10$ or 11 (b), data pooled from two cell preparations; vertical bars show s.e.mean. Results in (c) are $n = 5$ from a single cell preparation and representative of similar results from two cell preparations. * $P < 0.05$; ** $P < 0.01$ compared with 5 μM histamine alone, Student's t test.

Mechanism of histamine-induced cyclic AMP responses in bovine adrenal medullary cells

Use of selective agonists and antagonists for H_1, H_2 and H_3 receptors, as described above, indicated that the cyclic AMP response to histamine in bovine adrenal medullary cells was due to both H_1 and H_2 receptors. It was of interest to determine whether the mechanisms for the cyclic AMP responses to these two receptor types were similar or different.

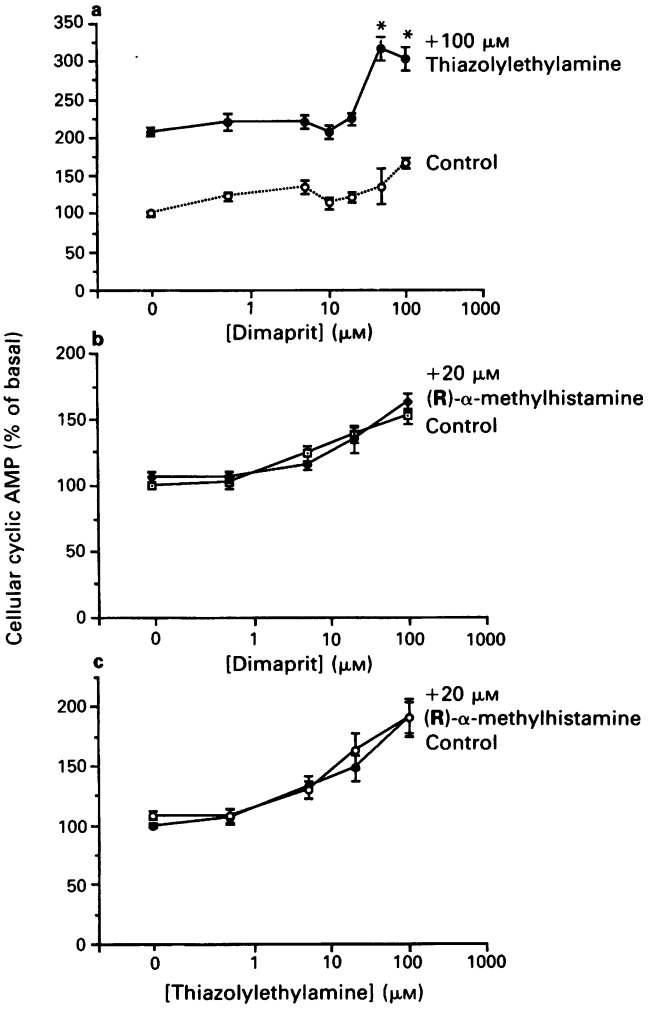


Figure 3 Effect of selective H_1-, H_2- and H_3-agonists, alone and in combination, on cellular cyclic AMP levels over 5 min in cultured bovine adrenal medullary cells. (a) Concentration-response curve for dimaprit in the absence (open symbols) and presence (filled symbols) of 100 μM 2-thiazolylethylamine. (b) Concentration-response curve for dimaprit in the absence (open symbols) and presence (filled symbols) of 20 μM (R)-α-methylhistamine. (c) Concentration-response curve for 2-thiazolylethylamine in the absence (filled symbols) and presence (open symbols) of 20 μM (R)-α-methylhistamine. Results are means for $n = 8$–12, data pooled from two different cell preparations for each panel; vertical bars show s.e.mean. The difference in the shape of the dimaprit concentration-response curves in (a) and (b) reflects results on different cell preparations. * $P < 0.01$ for interaction (see Methods and Moeller *et al.*, 1989).

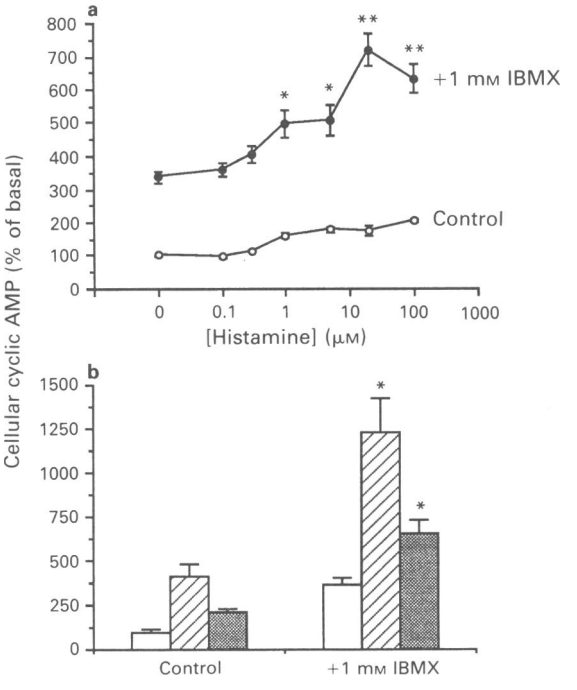


Figure 4 Effect of 1 mM 3-isobutyl-1-methylxanthine (IBMX) on the cellular cyclic AMP response to (a) histamine and (b) H_1- and H_2-agonists, over 5 min in cultured bovine adrenal medullary cells. (a) Results are means for $n = 10$–15, data pooled from three different cell preparations; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ for interaction between histamine and IBMX (see Methods). (b) Effect of IBMX on responses to 100 μM 2-thiazolylethylamine (H_1-agonist, hatched columns) and 100 μM dimaprit (H_2-agonist, stippled columns). Basal responses are indicated by the open columns. Results are means (± s.e.mean) for $n = 4$, data from a single cell preparation and representative of similar results on three cell preparations. * $P < 0.01$ for interaction (see Methods).

Histamine was still able to increase cellular cyclic AMP levels in bovine adrenal medullary cells after inhibition of all phosphodiesterase isozymes with the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM) (Figure 4a). Indeed the response to histamine was enhanced by blocking cyclic AMP degradation with IBMX. Furthermore, the separate responses to both H_1- and to H_2-agonists were also enhanced by IBMX (Figure 4b). These results indicate that neither H_1- nor H_2-receptors increased cyclic AMP levels in these cells by reducing cyclic AMP degradation by inhibiting phosphodiesterases.

Histamine had no effect on (Figure 5a) or slightly increased the export ('egress') of cyclic AMP from bovine adrenal medullary cells into the incubation medium over a 5 min incubation period, indicating neither H_1- nor H_2-receptors increased cellular cyclic AMP by inhibiting cyclic AMP export. Over long time periods (e.g.: 90 min incubation), histamine increased extracellular cyclic AMP levels, even though cellular cyclic AMP had returned to basal levels (Figure 5b). In the presence of IBMX, the cellular cyclic AMP response to histamine over 5 min was increased while the extracellular levels were unaffected, and both cellular and extracellular cyclic AMP responses were increased after 90 min (Figure 5a,b).

By extracting together both cellular and extracellular cyclic AMP following incubation with histamine in the presence of IBMX (to block degradation by phosphodiesterases), it was found that histamine stimulated cyclic AMP formation only for 20 min (data not shown). This result suggests that in the absence of IBMX, the transient increased formation of cyclic AMP induced by histamine is counteracted by subsequent increased cyclic AMP degradation and removal from the cell, such that the cellular cyclic AMP levels return to resting levels after 20 min or so, even in the maintained presence of histamine.

Receptors that are coupled to activation of adenylate cyclase via the stimulatory G protein G_S have been reported to interact synergistically with forskolin in increasing cyclic AMP formation (Seamon & Daly, 1983). Histamine gave significantly more-than-additive cyclic AMP responses in the presence of a low concentration of forskolin (Figure 6a), indicating that at least part of its response was likely to be mediated through a receptor coupled to G_S. The effect of the H_2-agonist, dimaprit, was very substantially enhanced in the presence of forskolin (Figure 6b): the response to dimaprit in the presence of 0.3 μM forskolin was 10.8 times the response in the absence of forskolin (mean of 3 experiments). In contrast, the effect of the H_1-agonist, 2-thiazolyethylamine, only increased weakly (2.6 fold, mean of 3 experiments) in the presence of forskolin (Figure 6b).

In the absence of extracellular Ca^{2+} (equimolar substitution with Mg^{2+}), histamine was still able to increase cellular cyclic

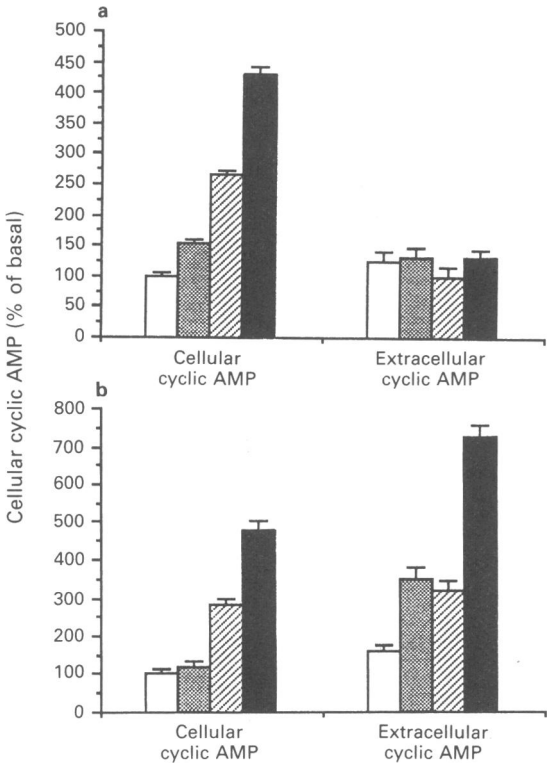


Figure 5 Effect of 5 μM histamine in the absence and presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) on cellular and extracellular cyclic AMP levels in cultured bovine adrenal medullary cells over (a) 5 min, and (b) 90 min. Open columns, controls; stippled columns, 5 μM histamine; hatched columns, 1 mM IBMX; solid columns, 5 μM histamine with 1 mM IBMX. Results are means for $n = 4$ or 5; vertical bars show s.e.mean; data from a single cell preparation and representative of similar results from three cell preparations.

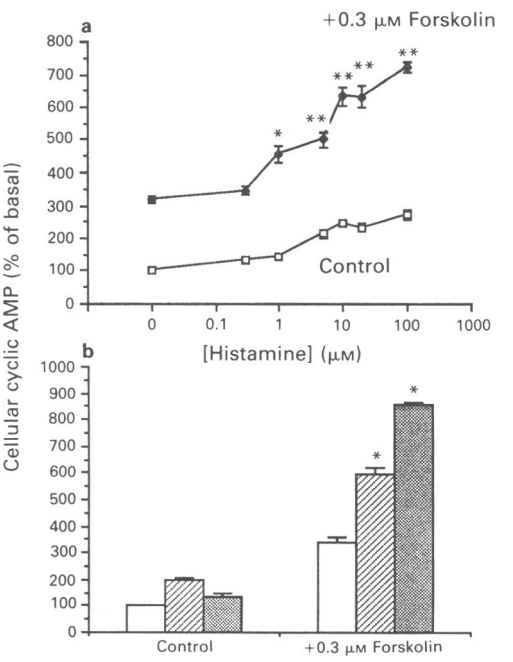


Figure 6 Effect of 0.3 μM forskolin on the cellular cyclic AMP response to (a) histamine and (b) H_1- and H_2-agonists, over 5 min in cultured bovine adrenal medullary cells. (a) (□) Control; (●) +0.3 μM forskolin; results are means for $n = 5$–15, data pooled from three different cell preparations; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ for interaction between histamine and forskolin (see Methods). (b) Effect of forskolin on responses to 100 μM 2-thiazolylethylamine (H_1-agonist, hatched columns) and 100 μM dimaprit (H_2-agonist, stippled columns). Results are means for $n = 4$, s.e.mean shown by vertical bars. Data from a single cell preparation and representative of similar results on three preparations. * $P < 0.01$ for interaction (see Methods).

AMP levels; however the response was greatly reduced (Figure 7a). The ability of dimaprit to increase cellular cyclic AMP levels was unaffected by removing extracellular Ca^{2+} while the response to 2-thiazolylethyalmine was abolished (Figure 7b). Furthermore, the response to histamine in the absence of extracellular Ca^{2+} was antagonized by 1 μM cimetidine in 5 out of 7 experiments, and was unaffected by 1 μM mepyramine in all 7 experiments (Figure 8). These results support those with forskolin in indicating distinct mechanisms for the regulation of cyclic AMP by H_1- and H_2-receptors in bovine adrenal medullary cells.

Histamine H_1-receptors induce secretion of granule contents from chromaffin cells in a Ca^{2+}-dependent manner. The H_1-receptors might therefore increase cyclic AMP levels by releasing components from chromaffin granules which then act on their own receptors on chromaffin cells in an autocrine manner to produce a cyclic AMP response. Reincubation of cultured chromaffin cells with buffer containing histamine which had previously been incubated with another batch of chromaffin cells still induced a cyclic AMP response, and this response was still antagonized by a combination of H_1- and H_2-receptor antagonists (Figure 9). This suggests that constituents of the chromaffin granules that are released during histamine stimulation play a minor (if any) role in the stimulation of cyclic AMP formation by histamine.

Discussion

Histamine receptor subtypes and the regulation of chromaffin cell cyclic AMP levels

The present study indicates that histamine can stimulate cyclic AMP formation in cultured bovine adrenal chromaffin cells. This effect appears to be the result of three distinct mechanisms: the action of histamine on H_1-receptors, the action of histamine on H_2-receptors, and an interaction between these two actions to give more-than-additive cyclic AMP responses. The contribution of the direct H_2 component was small, given the weak and variable antagonism by cimetidine (Figure 2a,c) and the low efficacy of dimaprit (Figures 3, 4, 6). It was very significant however in facilitating the H_1-response, as seen in the ability of mepyramine with low concentrations of cimetidine to block the histamine response.

These results with bovine adrenal medullary cells are very similar to previous findings on the effects of histamine on cellular cyclic AMP levels in brain slices. In such intact slice preparations, histamine increased cyclic AMP levels by a combined action on H_1 and H_2 receptors, and the responses to these two receptor subtypes interacted (Hill, 1987; Palacios *et al.*, 1978).

Substantial information has previously been published about H_1-receptors on adrenal chromaffin cells. Ligand binding studies on adrenal medullary membranes with [^{3}H]-mepyramine have found only a single class of high affinity binding sites, with K_d of 1.1–8.5 nM depending on species (Chang *et al.*, 1979). In contrast, several classes of H_1 binding site have been found in other tissues, including adrenal cortex (Chang *et al.*, 1979). H_1-receptors increase cytosolic Ca^{2+} in isolated chromaffin cells (Staudermann & Pruss, 1990) and they have been known for over 70 years to induce catecholamine secretion (Elliott, 1912; Szczygielski, 1932; see Livett & Marley, 1986). This secretory action is fully dependent on extracellular Ca^{2+} (Poisner & Douglas, 1966; Livett & Marley, 1986; Noble *et al.*, 1988). In addition, the H_1-receptors are coupled to increased formation of inositol phosphates (Noble *et al.*, 1986; Plevin & Boarder, 1988; Wan *et al.*, 1989a; Staudermann & Pruss, 1990; Bunn *et al.*, 1990), a

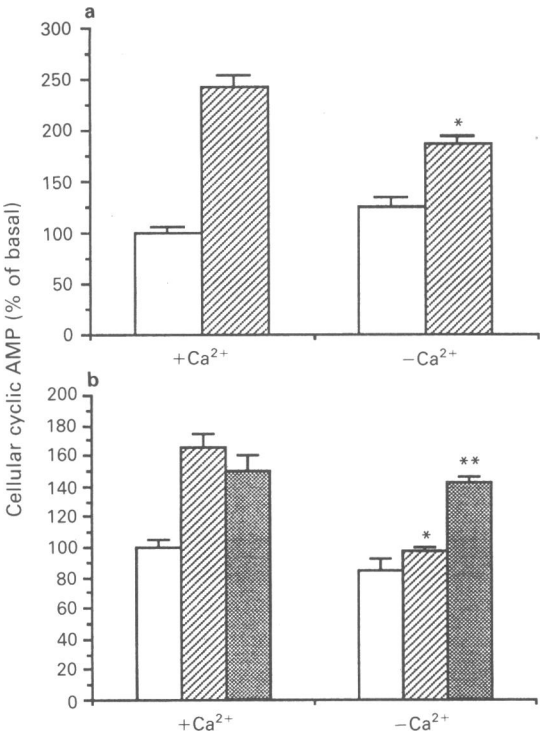


Figure 7 Effect of (a) 5 μM histamine and (b) H_1- and H_2-agonists on cellular cyclic AMP levels over 5 min in cultured bovine adrenal medullary cells in the presence ($+Ca^{2+}$) and absence ($-Ca^{2+}$) of extracellular Ca^{2+}. For experiments in the absence of extracellular Ca^{2+}, cells received two 5 min washes in buffer in which the 2.2 mM $CaCl_2$ was replaced with $MgCl_2$, followed by a 5 min stimulation with the indicated agonists in the same buffer. (a) Hatched columns, 5 μM histamine. Results are means for $n = 12$, data pooled from two different cell preparations; s.e.mean shown by vertical bars; * $P < 0.005$ compared with either 5 μM histamine in the presence of Ca^{2+} or the control response in the absence of Ca^{2+}, Student's t test. (b) Effect of 100 μM 2-thiazolylethylamine (H_1-agonist, hatched columns) and 100 μM dimaprit (H_2-agonist, stippled columns) in the presence and absence of extracellular Ca^{2+}. Results are means for $n = 4$, data from a single preparation and representative of similar results from three cell preparations; vertical bars show s.e.mean. * $P < 0.005$ compared with the thiazolylethylamine response in the presence of Ca^{2+} and $P > 0.15$ compared with the control response in the absence of Ca^{2+}. ** $P < 0.005$ compared with the control response in the absence of Ca^{2+} and $P > 0.25$ compared with the H_2-agonist response in the presence of Ca^{2+}, Student's t test.

response that over a time course of several minutes is also fully dependent on extracellular Ca^{2+} (Wan *et al.*, 1989a). Furthermore, histamine induces expression of proenkephalin A mRNA in chromaffin cells via H_1-receptors (Wan *et al.*, 1989b).

In contrast, there have been no previous reports of the presence of functional H_2-receptors in the adrenal medulla. The present study provides evidence that both H_1- and H_2-receptors can increase cyclic AMP levels in bovine adrenal medullary cells, and that at least some of the cells possess both types of receptor. Although it has been demonstrated by several laboratories that H_2-receptors do not play a role in histamine-induced catecholamine secretion, inositol phosphate responses or Ca^{2+} influx in these cells, the present data shows that they do mediate a cyclic AMP response, and that this response interacts with the H_1-receptor's cyclic AMP response.

Mechanisms of the H_1- and H_2-induced cyclic AMP responses in bovine adrenal medullary cells

Histamine is well known to increase cyclic AMP levels in a wide variety of cell types. In most of these, histamine and

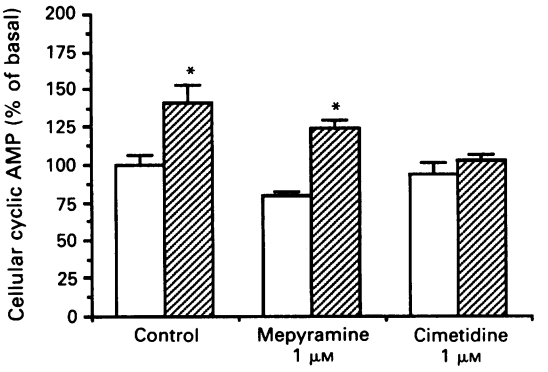


Figure 8 Effects of 1 μM mepyramine and 1 μM cimetidine on basal (open columns) and 5 μM histamine-induced (hatched columns) cellular cyclic AMP levels over 5 min in cultured bovine adrenal medullary cells, in the absence of extracellular Ca^{2+}. Results are means for $n = 3$ or 4, data from a single cell preparation and representative of similar results from three different cell preparations; s.e.mean shown by vertical bars. * $P < 0.005$ compared with the respective responses in the absence of 5 μM histamine, Student's t test.

H_2-agonists can increase cyclic AMP in cell-free membrane preparations, suggesting that the H_2-receptors are directly coupled to adenylate cyclase by G_s. In contrast, in intact slices of brain (e.g. guinea-pig hippocampus or cortex slices), histamine increased cyclic AMP levels by a combined effect on H_1- and H_2-receptors (Rogers *et al.*, 1975; Palacios *et al.*, 1978; Hill *et al.*, 1981), while the cyclic AMP response to histamine in homogenates of these tissues was mediated exclusively by H_2-receptors (Hegstrand *et al.*, 1976; Green *et al.*, 1977). Subsequent studies showed that the H_1 contribution was dependent on extracellular Ca^{2+} (Schwabe *et al.*, 1978) and was mediated indirectly, possibly by potentiating the H_2-receptor stimulation (Palacios *et al.*, 1978; Hollingsworth & Daly, 1985).

In the present study, the effects of histamine on cyclic AMP levels clearly involve two distinct mechanisms: an H_1-response which is completely dependent on the presence of extracellular Ca^{2+}, and an H_2 response which is unaffected by removing extracellular Ca^{2+}. These two receptor subtypes are

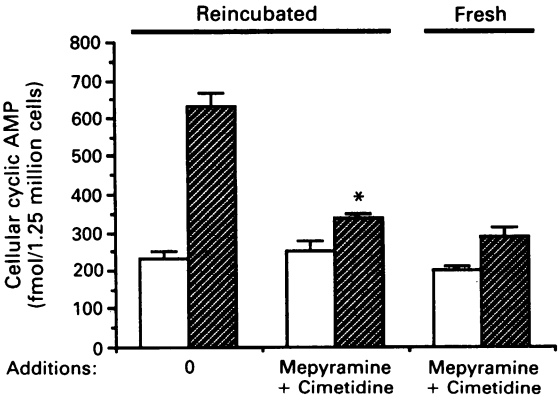


Figure 9 Effect of incubating two sets of bovine adrenal medullary cells sequentially with the same histamine solutions. One set of cells were incubated with control buffer (open columns) or 5 μM histamine (hatched columns) for 5 min. These solutions were saved, and reincubated with a second set of chromaffin cells in the absence or presence of a combination of 1 μM each mepyramine and cimetidine (added in 1% of the volume at 100 μM). Fresh control and 5 μM histamine solutions were also incubated with the second set of cells in the presence of the antagonists. Results are means for $n = 4$, data from a single cell preparation and representative of similar results from two different cell preparations; s.e.mean shown by vertical bars. * $P < 0.005$ compared to reincubated 5 μM histamine response, and $P > 0.05$ compared to fresh 5 μM histamine in the presence of antagonists, Student's t test.

independently capable of increasing cellular cyclic AMP in these cells. Neither receptor achieves its effect by inhibiting cyclic AMP release from the cells, since cyclic AMP release is unaffected by short incubations with histamine and is increased by histamine over longer periods or in the presence of phosphodiesterase inhibition. Furthermore, neither receptor increases cyclic AMP by inhibiting specific phosphodiesterase isozymes in these cells, since their responses are enhanced, not blocked, by IBMX. Taken together, these observations indicate that both H_1- and H_2-receptors increase cellular cyclic AMP levels in chromaffin cells by increasing adenylate cyclase activity and not by affecting cyclic AMP degradation or release from the cell.

The more-than-additive effects of histamine and of the H_2-agonist, dimparit, when combined with low concentrations of forskolin suggest that the H_2-receptors on chromaffin cells may be coupled directly through the G_S regulatory G protein to adenylate cyclase. In contrast the H_1-receptors interacted only weakly with forskolin-stimulated cyclic AMP production. The mechanism for this interaction, which presumably, like all the responses to H_1-receptors on chromaffin cells, is dependent on extracellular Ca^{2+}, is not known.

Ca^{2+}-dependence of the H_1 cyclic AMP response in chromaffin cells is similar to the Ca^{2+} -dependence of the H_1 cyclic AMP response in brain slices and is of considerable interest. The mechanism by which Ca^{2+} activates cyclic AMP formation in these cells is not known. Several possible mechanisms have been suggested from other work. One isozyme of adenylate cyclase is known to possess an allostearic binding site for and to be activated by Ca^{2+}/calmodulin, and a Ca^{2+}/calmodulin-sensitive form of adenylate cyclase is present in bovine adrenal medulla (LeDonne & Coffee, 1980). The Ca^{2+} influx induced by H_1-receptor activation may hence act via calmodulin to activate adenylate cyclase.

Alternatively, adenylate cyclase activity has been shown in a number of cell types to be increased by protein kinase C (Yoshimasa *et al.*, 1987; Choi & Toscano, 1988). Protein kinase C is activated by increases in cytosolic Ca^{2+} in three ways, by a direct action of ionic Ca^{2+} on the kinase, by Ca^{2+} inducing translocation of the kinase to the plasma membrane, and by Ca^{2+} activating phospholipase C, which increases the production of diacylglycerol, which is a known activator for protein kinase C.

Any of these mechanisms might mediate the H_1-induced cyclic AMP response in chromaffin cells. Our recent work using K^+ depolarization of these cells has found that Ca^{2+} entry is a sufficient requirement for increasing cyclic AMP levels (Keogh & Marley, unpublished observations). In addition, nicotine-induced cyclic AMP responses (which are completely dependent on extracellular Ca^{2+}) are blocked by low concentrations of the calmodulin antagonist, W7, and are not blocked by down-regulation of protein kinase C by chronic phorbol ester treatment (Anderson, Robinson and Marley, unpublished observations). These observations suggest that Ca^{2+} may increase cyclic AMP formation by activating adenylate cyclase through calmodulin.

It is not known whether histamine increases catecholamine biosynthesis in adrenal chromaffin cells, and, if it does, whether this effect is mediated by cyclic AMP. In the case of nicotinic activation of catecholamine synthesis, both cyclic AMP and Ca^{2+} may be involved. Both of these second messengers mediate a phosphorylation of tyrosine hydroxylase and increase its activity (Niggli *et al.*, 1984). Several common sites on tyrosine hydroxylase are phosphorylated both by a Ca^{2+}-dependent mechanism and by a cyclic AMP-dependent mechanism (Niggli *et al.*, 1984). The increase in phosphorylation of tyrosine hydroxylase produced by nicotinic stimulation is completely dependent on extracellular Ca^{2+} (Waymire *et al.*, 1988), and we have found that the nicotine-induced cyclic AMP response is also completely Ca^{2+}-dependent (Anderson, Robinson and Marley, unpublished observation). Since histamine, like nicotine, mediates a concomitant rise in cytosolic Ca^{2+} and of cyclic AMP, it is tempting to speculate that histamine also can regulate tyrosine hydroxylase activity and expression by mechanisms involving both Ca^{2+} and cyclic AMP. This possibility is under investigation.

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Contraction of rat thoracic aorta strips by endothelin-1 in the absence of extracellular Ca^{2+}

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1 Endothelin-1 (ET-1) caused a concentration-dependent contraction of helical strips from rat thoracic aorta in the absence of extracellular Ca^{2+}. The Ca^{2+}-depleted muscle strips, prepared by three repeated applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+}-free buffer, were contracted by 10^{-8} M ET-1 in the same manner as non-treated strips.

2 In the absence of extracellular Ca^{2+}, 10^{-7} M phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, induced a small but sustained contraction of the rat thoracic aorta strips within 60 min. Preincubation of the strips with 10^{-7} M PMA for 60 min in Ca^{2+}-free buffer, did not affect the 10^{-8} M ET-1-induced contraction, but decreased the 5×10^{-8} M phorbol 12,13-dibutyrate (PDB)-, or the 10^{-7} M PMA-induced contraction, and potentiated the contraction induced by 10^{-8} M urotensin II. Preincubation with 10^{-8} M ET-1 (which induced maximum contraction) for 25 min in Ca^{2+}-free buffer did not change the subsequent contraction induced by PMA (10^{-7} M) or urotensin II (10^{-8} M) but gave a somewhat lower maximum tension than in non-treated strips.

3 Calyculin-A, a potent inhibitor of phosphatase, also induced a contraction of the Ca^{2+}-depleted muscle strips in Ca^{2+}-free buffer. Preincubation of the strips with ET-1 (10^{-8} M) or PMA (10^{-7} M) decreased the calyculin-A (3×10^{-8} M)-induced contraction.

4 These results suggest that ET-1 may induce phosphorylation of an unknown protein either without an increase in myoplasmic Ca^{2+} concentration or, alternatively, with mobilization of intracellular Ca^{2+} from noradrenaline- and caffeine-insensitive Ca^{2+} sources, through a mechanism different from that of phorbol ester.

Keywords: Endothelin; phorbol 12-myristate 13-acetate; phorbol 12,13-dibutyrate; urotensin II; calyculin-A; intracellular Ca^{2+}

Introduction

Endothelin (ET), a peptide originally isolated from the culture medium of porcine endothelial cells, is one of the most potent vasoconstrictor substances known (Yanagisawa *et al.*, 1988). Several mechanisms have been proposed to explain the potent vasoconstrictor properties of ET. Early studies postulated that ET mediated its vasoconstrictor effect solely through activation of voltage-dependent Ca^{2+} channels (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). Subsequent studies indicated that the contractile response to ET consists of two components, one independent of Ca^{2+} influx, the other dependent on Ca^{2+}-influx and insensitive to voltage-dependent Ca^{2+} channel blockers (Auguet *et al.*, 1988; Blackburn & Highsmith, 1990; Bolger *et al.*, 1990). In some instances, ET initiates the metabolism of inositol phosphates and releases intracellularly stored Ca^{2+} (Van Renterghem *et al.*, 1988; Marsden *et al.*, 1989; Sugiura *et al.*, 1989; Takuwa *et al.*, 1990). In aortic smooth muscle cells derived from Wistar rats, ET appears to have no effect on phospho-inositide turnover (Hirata *et al.*, 1988). It has also been suggested that ET causes a stimulation of the diaclyglycerol/protein kinase C pathway and induces contraction of vascular smooth muscle (Griendling *et al.*, 1989; Lee *et al.*, 1989; Sugiura *et al.*, 1989). Kodama *et al.* (1989) showed that ET induced contractions in Ca^{2+}-depleted muscle strips from porcine coronary artery, pretreated with caffeine and histamine in Ca^{2+}-free buffer, without intracellular Ca^{2+} mobilization and the contraction was inhibited by an inhibitor of protein kinase C.

To elucidate the mechanism responsible for the vasoconstrictor effects of ET in Ca^{2+}-free buffer, we examined the interactions between the contractile responses of helical strips of rat thoracic aorta induced by ET-1 and those induced by other agents, such as noradrenaline, caffeine, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDB), urotensin II and calyculin-A, all of which cause contractions, through different mechanisms, in the absence of extracellular Ca^{2+}.

Methods

Recording of contractile activity

Male Wistar rats (150–250 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.: Abbott Laboratories, North Chicago, U.S.A.) and decapitated to obtain the descending thoracic aorta. Helically cut strips, 1.2 mm in width and 12 mm in length, were prepared from the aorta after removal of connective tissues and were suspended vertically in a 5 ml organ bath under 1.2 g tension in Krebs Henseleit solution of the following composition (in mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. The bath medium was maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The vascular strips were allowed to equilibrate for 90 min before starting experiments. During the equilibration period the bath solution was replaced every 20 min. Changes in muscle tension were recorded isometrically through force displacement transducer (Nihon Kohden, TB-651T). Ca^{2+}-free Krebs Henseleit solution was made by adding 1 mM ethylene glycol-*bis* (beta-aminoethylether) N,N'-tetraacetic acid (EGTA) instead of 2.5 mM $CaCl_2$. Endothelial cells were removed by repeatedly rubbing the aortic strips with intimal surface down over a sheet of filter paper moistened with Krebs Henseleit solution (Furchgott & Zawadzki, 1980). Each strip was subjected only to one ET-1 treatment. All responses are expressed as

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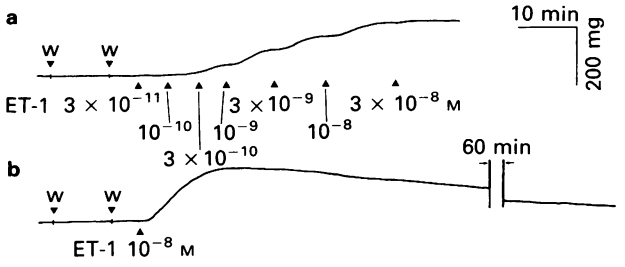


Figure 1 Chart records showing typical effects of endothelin-1 (ET-1) on rat thoracic aorta in the absence of extracellular Ca^{2+}. Aortic strips were preincubated with Ca^{2+}-free buffer containing 1 mM EGTA for 15 min and then ET-1 was added. (a) Contraction induced by cumulative additions of ET-1 in Ca^{2+}-free buffer. (b) ET-1 10^{-8} M-induced contraction of aortic strips in Ca^{2+}-free buffer; ($n = 6$).

means ± s.e. The data were analysed by use of unpaired Student's t test for two means; a value of $P < 0.05$ was considered statistically significant.

Reagents

Endothelin-1 (ET-1) was purchased from Peptide Institute, Osaka, Japan. Urotensin II was generously provided by Dr J. Rivier (Salk Institute, La Jolla, Ca, U.S.A.). Indomethacin, ibuprofen, tetrodotoxin, nitroprusside, prazosin, phorbol 12-myristate 13-acetate (PMA) and EGTA were purchased from Sigma Chemical Co. Noradrenaline (free base), atropine sulphate monohydrate, acetylcholine chloride, papaverine hydrochloride, caffeine, phorbol 12,13-dibutyrate (PDB) and calyculin-A were purchased from Wako Pure Chemical Industries, Ltd. O-2'-dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cyclic AMP) was donated by Daiichi Pharmaceutical Corp. Tokyo, Japan. Ketanserin tartarate was donated by Kyowa Hakko Kogyo Co. Ltd Tokyo, Japan. All other chemicals were of reagent grade or better. The solvent solutions were as follows: 0.25% acetic acid for ET-1 and urotensin II; 100% dimethyl sulphoxide (DMSO) for PMA (10^{-3} M), indomethacin (5×10^{-1} M), ibuprofen (1 M) and calyculin-A (10^{-3} M). The final concentration of DMSO in the muscle bath was never >0.1% at which concentration it caused no significant effect on the tension.

Results

Endothelin-1-induced contractile response in the absence of extracellular Ca^{2+} and effect of endothelial cells on the contraction

The cumulative contractile effects of ET-1 on an isolated strip of rat descending thoracic aorta in Ca^{2+}-free buffer containing 1 mM EGTA are illustrated in Figure 1a. The minimum effective concentration of ET-1 was 10^{-10} M and the maximum contractile responses was attained at a concentration of 10^{-8} M. In the absence of extracellular Ca^{2+}, 10^{-8} M ET-1 induced a slowly developing contraction and reached maximum tension (170.8 ± 17.8 mg, $n = 7$) within 25 min, but the tension gradually declined. In the presence of extracellular Ca^{2+}, 10^{-8} M ET-1 induced a sustained contraction (636.0 ± 22.6 mg, $n = 7$, data not shown). Table 1 summarizes the effects of acetylcholine on noradrenaline-precontracted strips and the ET-1-induced contractions in the absence and presence of endothelial cells. The effects of ET-1 on rubbed or unrubbed aortic strips were not significantly different.

Effects of various agents on endothelin-1-induced contractions

Table 2 summarizes the effects of preincubation for 5 min with various agents on the contraction induced by 10^{-8} M ET-1 in Ca^{2+}-free buffer. In each experiment, we used two strips which were contracted by 10^{-7} M noradrenaline in normal Krebs-Henseleit solution. One strip was then preincubated with one of the agents for 5 min in Ca^{2+}-free buffer and the other strip was used as a control. The two strips were equally reactive to noradrenaline. Nitroprusside, papaverine and dibutyryl cyclic AMP (which modify the level of cyclic nucleotides in smooth muscle cells) decreased the contractile response to ET-1. High concentrations of indomethacin (5×10^{-4} M), a non-steroidal anti-inflammatory agent, also inhibited the ET-1-induced contraction. However, another non-steroidal anti-inflammatory drug, ibuprofen (even at the high concentration of 10^{-3} M) had no effects on the contraction. Atropine (10^{-6} M), tetrodotoxin (2×10^{-6} M), ketanserin (10^{-6} M), prazosin (10^{-6} M) and verapamil (10^{-5} M) did not change the contractile response to 10^{-8} M ET-1 in Ca^{2+}-free buffer (data not shown).

Effects of endothelin-1 on Ca^{2+}-depleted aortic strips

After 15 min incubation in Ca^{2+}-free buffer, 10^{-2} M caffeine induced a biphasic contraction, which consisted of a small transient contraction followed by relaxation (Figure 2a). Washing with Ca^{2+}-free buffer caused recovery of the strip to the resting tension. The second and third applications of caffeine to the strip in Ca^{2+}-free buffer produced only relaxations. Noradrenaline (10^{-6} M) induced a typical phasic contraction and the second and third application of nor-

Table 2 Inhibition by various agents of 10^{-8} M endothelin-1 (ET-1)-induced contraction in Ca^{2+}-free buffer

Agents	n	*Inhibition* (%)
Nitroprusside (10^{-7} M)	5	75.6 ± 3.5**
Papaverine (3×10^{-5} M)	4	63.7 ± 2.6***
Dibutyryl cyclic AMP (10^{-4} M)	4	54.7 ± 3.6***
Indomethacin (5×10^{-4} M)	4	35.3 ± 6.9*
Ibuprofen (10^{-3} M)	4	5.1 ± 3.2 NS

Values are means ± s.e. NS, no significant difference.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (compared with the responses in the absence of agents). The 10^{-8} M ET-1-induced maximum contractile tension in the absence of the agents was 172.0 ± 13.3 mg.

Table 1 Effects of removal of endothelial cells on endothelin-1 (ET-1)-induced contraction in helical strips of rat thoracic aorta in the absence of extracellular Ca^{2+}

Endothelial cells	n	*Effects of acetylcholine* (10^{-6} M) *on noradrenaline* (10^{-7} M)*-precontracted strips*	*ET-1-induced contraction* EC_{50} (nM)	*Maximum tension* (mg)
Present	6	% relaxation (74.7 ± 5.5)	2.7 ± 0.6	145.5 ± 13.2
Absent	6	No relaxation	3.3 ± 0.1	160.0 ± 17.3

n, number of determinations. EC_{50}, concentration of ET-1 producing half maximum contraction. Values are means ± s.e. Noradrenaline (10^{-7} M)-induced responses in the presence and absence of endothelial cells were 480.8 ± 34.1 and 496.1 ± 24.2 mg, respectively. Endothelial cells were removed by rubbing aortic strips with intimal surface down, over a sheet of filter paper moistened with Krebs-Henseleit solution.

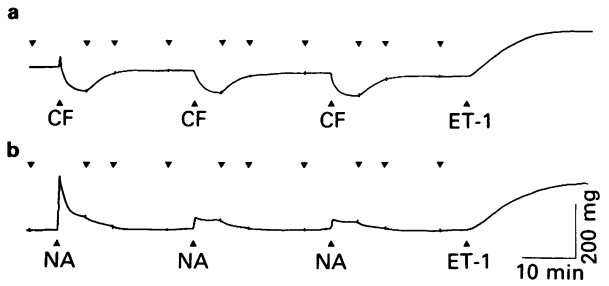


Figure 2 Typical chart recordings of endothelin-1 (ET-1, 10^{-8} M)-induced contraction in Ca^{2+}-depleted strips after three repeated applications of 10^{-2} M caffeine (CF) (a) or 10^{-6} M noradrenaline (NA) (b) in Ca^{2+}-free buffer; (▼) washed with Ca^{2+}-free buffer.

adrenaline in Ca^{2+}-free buffer produced only small and tonic contractions (Figure 2b). Such Ca^{2+}-depleted strips were also contracted by 10^{-8} M ET-1 in the same manner as untreated strips in the absence of extracellular Ca^{2+} (Figure 2 and Table 3).

Interactions between the agonists which induce contraction in the absence of extracellular Ca^{2+}

In the absence of extracellular Ca^{2+}, 10^{-7} M PMA induced a small but sustained contraction within 60 min. Precontraction induced by 10^{-7} M PMA did not change the 10^{-8} M ET-1-induced contraction, but decreased the contractile tension induced by 5×10^{-8} M PDB or 10^{-7} M PMA. Furthermore, pretreatment with 10^{-7} M PMA potentiated 10^{-8} M urotensin II-induced contraction. The results of these experiments are shown in Figure 3 and Table 4. The maximum contractions induced by 10^{-7} M PMA or 10^{-8} M urotensin II in the 10^{-8} M ET-1-precontracted strips were not significantly different from the response of untreated strips, but the maximum contractions produced by PMA or urotensin II in the ET-1-treated strips were somewhat lower than those in the untreated strips (Figure 4).

Calyculin-A a potent inhibitor of phosphatase, induced contraction in the absence of extracellular Ca^{2+}. Contraction induced by 10^{-7} M and 3×10^{-8} M calyculin-A required about 30 min and 110 min, respectively, to reach maximum tension (Figure 5a,b). The 3×10^{-8} M calyculin-A-induced increase in tension of aortic strips precontracted with 10^{-7} M PMA or 10^{-8} M ET-1 in Ca^{2+}-free buffer was lower than that developed in untreated strips (Figure 5 and Table 5). Conversely, preincubation with 3×10^{-8} M calyculin-A decreased the 10^{-8} M ET-1- or 10^{-7} M PMA-induced contraction (Table 6). Table 7 summarizes the effects of 10^{-7} M calyculin-A on Ca^{2+}-depleted muscle strips, prepared by three applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+}-free buffer. The maximum contractile tensions and the times to reach the maximum contractions obtained with 10^{-7} M calyculin-A in the caffeine or noradrenaline pretreated strips were not significantly different from those in the untreated strips.

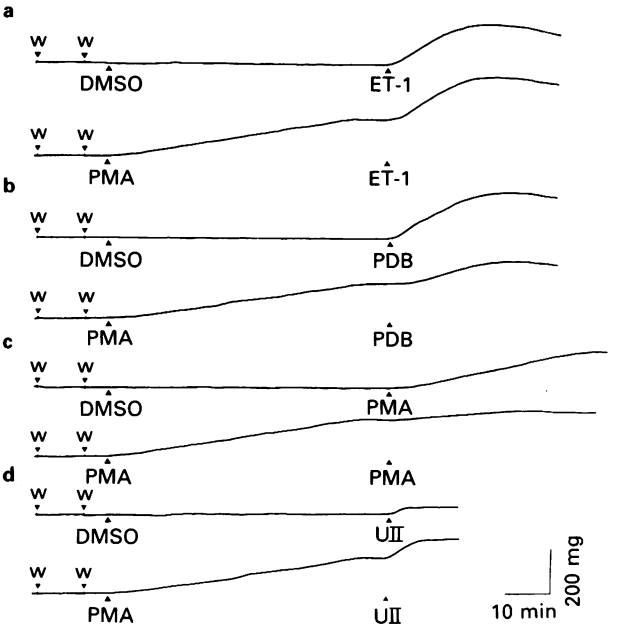


Figure 3 Chart records showing the effects of cumulative applications of endothelin-1 (ET-1, 10^{-8} M, $n = 6$) (a); phorbol 12,13-dibutyrate (PDB, 5×10^{-8} M, $n = 6$) (b); phorbol 12-myristate 13-acetate (PMA, 10^{-7} M, $n = 4$) (c); or urotensin II (UII, 10^{-8} M, $n = 5$) (d) on aortic strips precontracted with 10^{-7} M PMA. DMSO, 0.01% dimethyl sulphoxide. W (▼), washed with Ca^{2+}-free buffer.

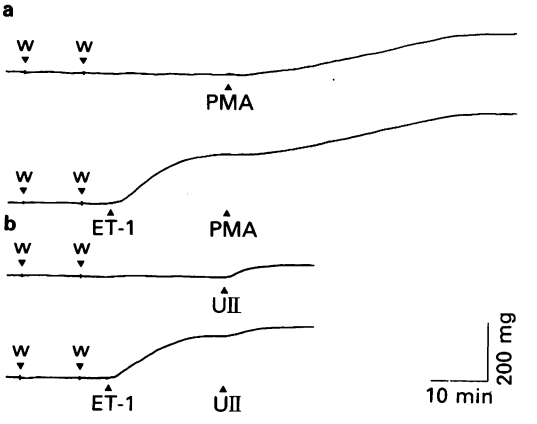


Figure 4 Typical chart records of the effects of the cumulative addition of phorbol 12-myristate 13-acetate (PMA 10^{-7} M) (a) or urotensin II (UII 10^{-8} M) (b) on aortic strips precontracted with endothelin-1 (ET-1 10^{-8} M). PMA 10^{-7} M-induced contractile tension in the non-treated strips and in the ET-1-precontracted strips were 165 ± 20.6 mg ($n = 5$) and 122.4 ± 26.2 mg ($n = 6$), respectively. 10^{-8} M Urotensin II-induced contractile tension in the untreated strips and in the ET-1-precontracted strips were 36.8 ± 5.3 mg ($n = 6$) and 26.0 ± 3.0 mg ($n = 6$), respectively. W (▼), washed with Ca^{2+}-free buffer.

Table 3 Endothelin-1 (ET-1 10^{-8} M)-induced contraction of Ca^{2+}-depleted muscle strips

Treatments	n	*Time to reach maximum contraction* (min)		ET-1 (10^{-8} M)-*induced maximum contractile tension* (mg)	
Preincubation for 15 min in Ca^{2+}-free buffer	6	21.1 ± 1.3	NS	185.0 ± 12.8	NS
Repeated applications of 10^{-2} M caffeine in Ca^{2+}-free buffer	7	24.1 ± 0.7	NS	172.0 ± 15.8	NS
Repeated applications of 10^{-6} M noradrenaline in Ca^{2+}-free buffer	6	21.6 ± 1.0		172.8 ± 18.0	

n, number of experiments. Values are means ± s.e. NS, no significant difference.

Table 4 Effects of endothelin-1 (ET-1), phorbol 12,13-dibutyrate (PDB), phorbol 12-myristate 13-acetate (PMA) or urotensin II on aortic strips precontracted with 10^{-7} M PMA

	Tension (mg)			
Treatments	ET-1 (10^{-8} M)	PDB (5×10^{-8} M)	PMA (10^{-7} M)	Urotensin II (10^{-8} M)
Control	170.8 ± 17.8 (6) NS	190.8 ± 13.0 (6) ***	146.1 ± 20.1 (4) ***	29.1 ± 2.0 (6) **
10^{-7} M PMA	193.1 ± 8.8 (6)	111.6 ± 11.0 (6)	28.7 ± 7.1 (4)	71.5 ± 9.8 (6)

Aortic strips were preincubated with Ca^{2+}-free buffer containing 1 mM EGTA and then 0.01% DMSO or 10^{-7} M PMA was added to the bath. DMSO 0.01% had no effect on the strips and 10^{-7} M PMA induced a sustained contraction (161.3 ± 12.5 mg, $n = 22$) within 60 min. After 60 min of the pretreatment, 10^{-8} M ET-1, 5×10^{-8} M PDB, 10^{-7} M PMA or 10^{-8} M urotensin II were added cumulatively. NS, no significant difference. ** $P < 0.01$; *** $P < 0.001$, compared with control strips. Values are means ± s.e. Number of determinations in parentheses.

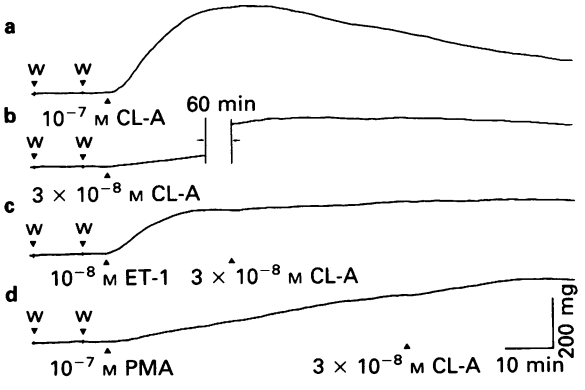


Figure 5 Typical chart records of the effects of calyculin-A (CL-A) on untreated aortic strips (a, $n = 5$; b, $n = 6$); on phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) precontracted strips (d, $n = 5$) or on endothelin-1 (ET-1, 10^{-8} M)-precontracted strips (c, $n = 4$) in the absence of extracellular Ca^{2+}. W (▼), washed with Ca^{2+}-free buffer.

Table 5 Effects of calyculin-A (3×10^{-8} M) on aortic strips precontracted with phorbol 12-myristate 13-acetate (PMA) (10^{-7} M) or endothelin-1 (ET-1) (10^{-8} M)

Treatments	n	*Calyculin-A* (3×10^{-8} M)-*induced contraction* (mg)		
Control	6	198.3 ± 23.0	***	***
PMA (10^{-7} M)	4	10.0 ± 6.3		
ET-1 (10^{-7} M)	5	58.0 ± 4.8		

After 10^{-8} M ET-1- or 10^{-7} M PMA-induced contraction reached maximum tension, 3×10^{-8} M calyculin-A was added. Values are means ± s.e. *** $P < 0.001$, compared with the response of untreated strips.

Table 6 Effects of endothelin-1 (ET-1, 10^{-8} M) or phorbol 12-myristate 13-acetate (PMA, 10^{-7} M) on aortic strips precontracted with calyculin-A (3×10^{-8} M)

		Tension (mg)	
Treatments	n	*ET-1* (10^{-8} M)	*PMA* (10^{-7} M)
Control	5	190.0 ± 14.8 **	19.0 ± 9.2 **
Calyculin-A	4	75.0 ± 19.3	77.0 ± 18.7

Aortic strips were preincubated with Ca^{2+}-free buffer containing 1 mM EGTA for 15 min and then 0.03% DMSO (control) or 3×10^{-8} M calyculin-A was added to the bath. DMSO 0.03% had no effect on the strips and 3×10^{-8} M calyculin-A induced a maximum contraction within 110 min. After 110 min of the pretreatment, 10^{-8} M ET-1 or 10^{-7} PMA was added cumulatively. ** $P < 0.01$, compared with control strips. Values are means ± s.e. n, number of determinations.

Discussion

In the present study, we examined the effects of ET-1 on rat thoracic aorta strips, focusing on the mechanisms of ET-1-induced contraction in the absence of extracellular Ca^{2+}. It has been reported that caffeine and noradrenaline release Ca^{2+} from intracellular stores of vascular smooth muscle through different mechanisms, and thus induce contractions in the absence of extracellular Ca^{2+} (Itoh *et al.*, 1983). Moreover, an overlap between an ET-sensitive intracellular Ca^{2+} store and caffeine-sensitive pool in vascular smooth muscle has been shown (Kai *et al.*, 1989). It has also been shown that ET, like noradrenaline, stimulates phospholipase C to produce inositol triphosphate and to mobilize intracellular Ca^{2+} in vascular smooth muscle (Marsden *et al.*, 1988; Van Renterghem *et al.*, 1988; Sugiura *et al.*, 1989; Takuwa *et al.*, 1990). However, our findings have shown that the Ca^{2+}-depleted muscle strips, after three repeated applications of caffeine or noradrenaline in Ca^{2+}-free buffer, were contracted by 10^{-8} M ET-1 in the same manner as untreated strips (Table 3 and Figure 2). These results suggest that the ET-1-induced contraction in Ca^{2+}-free buffer may not require an increase in intracellular Ca^{2+} concentration or, alternatively, it may be induced by intracellular Ca^{2+} from noradrenaline- and caffeine-insensitive Ca^{2+} sources. Kodama *et al.* (1989) reported that ET evoked a contraction of porcine coronary artery strips in Ca^{2+}-free buffer not only with intracellular Ca^{2+} mobilization but also without an increase in intracellular Ca^{2+} concentration, as determined by front surface fluorometry in arterial strips loaded with fura-2. Therefore, the ET-1-induced contraction of the Ca^{2+}-depleted strips from rat thoracic aorta may not be due to an intracellular Ca^{2+} mobilization.

Griendling *et al.* (1989) and Lee *et al.* (1989) reported that ET caused a pronounced stimulation of diacylglycerol and activation of protein kinase C in cultured vascular smooth muscle cells. Furthermore, ET-induced contraction of vascular smooth muscle in Ca^{2+}-free buffer was inhibited by H-7, a protein kinase C inhibitor (Kodama *et al.*, 1989; Sugiura *et al.*, 1989). Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, was shown to cause small and sustained contractions of rat thoracic aorta strips in Ca^{2+}-free buffer (Itoh & Lederis, 1987). These findings suggest that an activation of protein kinase C is involved in the ET-induced contraction in Ca^{2+}-free buffer. In the course of our study, however, interesting differences became apparent between ET-1 and PMA. The preincubation with 10^{-7} M PMA for 60 min in Ca^{2+}-free buffer did not affect the 10^{-8} M ET-1-induced contraction, but decreased the 5×10^{-8} M phorbol 12,13-dibutyrate (PDB) or another 10^{-7} M PMA-induced contraction (Table 4 and Figure 3a,b,c). Moreover, preincubation with 10^{-8} M ET-1, which causes maximum contraction in Ca^{2+}-free buffer, did not change the contraction induced by 10^{-8} M urotensin II, whereas preincubation with 10^{-7} M PMA potentiated the urotensin II-induced contraction (Table 4, Figure 3d, and Figure 4b). It is likely that the contractions induced by cumulative applications of two agents with different mechanisms may be additive, whereas in the case of contractions induced by cumulative applications of two agents with similar mechanisms, the first agent-induced precontraction may influence the second agent-induced contraction. Therefore, ET-1 and active phorbol esters may induce contraction of vascular strips in Ca^{2+}-free buffer through differ-

Table 7 Calyculin-A-induced contraction of Ca^{2+}-depleted muscle strips

Treatments	n	*Time to reach maximum contraction* (min)		*Calyculin-A* (10^{-7} M) *induced maximum contractile tension* (mg)	
Preincubation for 15 min in Ca^{2+}-free buffer	5	28.2 ± 1.1	NS	399.2 ± 38.7	NS
Repeated applications of 10^{-2} M caffeine in Ca^{2+}-free buffer	4	30.5 ± 3.2	NS	399.7 ± 55.8	NS
Repeated applications of 10^{-6} M noradrenaline in Ca^{2+}-free buffer	4	31.7 ± 2.6		423.5 ± 48.6	

Ca^{2+}-depleted muscle strips were pretreated with three repeated applications of 10^{-2} M caffeine or 10^{-6} M noradrenaline in Ca^{2+}-free buffer. *n*, number of experiments. Values are means ± s.e. NS, no significant difference.

ent mechanisms. Since H-7 inhibits not only protein kinase C but also other protein kinases, such as cyclic GMP-dependent protein kinase, cyclic AMP-dependent protein kinase and myosin light chain kinase (Hidaka *et al.*, 1984), attention should be paid to the interpretation of the effects induced by H-7. The urotensin II-induced contraction in the absence of extracellular Ca^{2+} consisted of two components: a relatively rapid contraction through intracellular Ca^{2+} mobilization from noradrenaline-sensitive Ca^{2+} store sites and a slow tonic contractile phase after the treatment with noradrenaline (Itoh *et al.*, 1987). The mechanism of the PMA-induced potentiation of the slow tonic contraction induced by urotensin II remains to be determined.

Calyculin-A, a potent phosphatase inhibitor, induces contraction of rat aortic strips in the absence of extracellular Ca^{2+} without an increase in cytoplasmic Ca^{2+} concentration, suggesting that the contraction is due to a Ca^{2+}-independent phosphorylation of myosin light chain and other proteins, by an inhibition of phosphatase activity (Ishihara *et al.*, 1989). In our experiments, the Ca^{2+}-depleted muscle strips were also contracted by calyculin-A. In the absence of extracellular Ca^{2+}, the pretreatments with 10^{-8} M ET-1 and 10^{-7} M PMA decreased contraction induced by 3×10^{-8} M calyculin-A (Figure 5, Table 5). The preincubation of the strips with 3×10^{-8} M calyculin-A also decreased the contractions induced by 10^{-8} M ET-1 and 10^{-7} M PMA (Table 6). The interactions between calyculin-A and ET-1 were similar to the interactions between calyculin-A and PMA, which activates protein kinase C and induces physiological actions through phosphorylation of some proteins by the kinase (Nishizuka, 1984). Therefore, it is possible that the ET-1-induced contraction in Ca^{2+}-free buffer may also involve the phosphorylation of unknown proteins.

Papaverine, dibutyryl cyclic AMP and nitroprusside, all of which influence postreceptor events mainly by modulating the levels or actions or cyclic nucleotides in vascular smooth muscle, inhibited the contractile response to ET-1. A high concentration of indomethacin (5×10^{-4} M), a cyclo-oxygenase inhibitor, decreased the contraction induced by ET-1 in Ca^{2+}-free buffer, whereas ibuprofen, another cyclo-oxygenase inhibitor, did not. Because indomethacin has also been shown to inhibit phosphodiesterase like papaverine (Flores *et al.*, 1972), the inhibitory effect of indomethacin on the ET-1-induced contraction may be due to its inhibitory action of phosphodiesterase, but not to that of cyclo-oxygenase.

Recent reports indicate that ET stimulates the release of endothelium derived relaxant factor (EDRF) and cyclo-oxygenase products from endothelial cells (Warner *et al.*, 1987; Folta *et al.*, 1989). However, in the present experiments the presence or absence of endothelial cells did not change the response to ET-1 in Ca^{2+}-free buffer (Table 1). Since the production of EDRF is dependent on extracellular Ca^{2+} (Griffith *et al.*, 1986), the ET-1-induced contraction in the absence of extracellular Ca^{2+} might not be affected by endothelial cells.

The phosphorylation of 20 kDa myosin light chain (MLC) is considered to lead to an interaction of actin with myosin which underlies the initiation of the contractile response (Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981). In many forms of smooth muscle, however, both the agonist-induced rise in intracellular Ca^{2+} concentration and the increase in the content of phosphorylated MLC are transient (Hartshorne & Siemankowski, 1981; Silver & Stull, 1984). Murphy and coworkers (Dillon *et al.*, 1981) have proposed that stress maintenance depends upon the formation of a so-called latch bridge between actin and myosin, being independent of an increase in myoplasmic Ca^{2+} concentration and MLC phosphorylation. Therefore, the Ca^{2+}-independent contractile responses induced by several agents such as ET-1, phorbol esters, urotensin II, calyculin-A may be appropriate models to analyze the latch bridge state of smooth muscle.

Recently, two independent groups (Arai *et al.*, 1990; Sakurai *et al.*, 1990) reported the structure of two different receptors for endothelins. Each is a rhodopsin-like receptor coupled to a G protein. One shows high specificity for ET-1 and is widely distributed in the central nervous system, the heart and the lung. The other is a non-selective ET receptor for the three ET isopeptides which is coupled through a G protein to phospholipase C, leading to transient increases in intracellular Ca^{2+}, but its messenger RNA is not detected in rat aorta. Therefore, the selective ET-1 receptor may be responsible for the contraction of rat thoracic aorta in Ca^{2+}-free buffer.

In summary, the present results indicate that ET-1 can cause phosphorylation of an unknown protein without an increase in myoplasmic Ca^{2+} through a different mechanism from phorbol esters, activators of protein kinase C, and induce a contractile response of rat thoracic aorta in Ca^{2+}-free buffer. Further work will be required to establish Ca^{2+}-independent phosphorylation of smooth muscle.

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Effects of Org 7797 on early, late and inducible arrythmias following coronary artery occlusion in rats and dogs

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1 The class Ic steroidal antiarrhythmic agent, Org 7797, was compared with two other Ic agents, flecainide and propafenone for intravenous activity against ischaemia-related cardiac arrhythmias and for electrophysiological actions *in vivo*. In addition the haemodynamic effects of Org 7797 were assessed in greyhounds.

2 Org 7797 (0.5 mg kg^{-1}) significantly reduced the expected incidence of early ischaemia-induced ventricular fibrillation (VF) in rats and greyhound dogs and at doses of 0.5–1.0 mg kg^{-1} antagonized reperfusion-induced arrhythmias. Comparative studies in rats showed Org 7797 to be 2–4 times more potent than flecainide or propafenone.

3 Org 7797 (0.5 mg kg^{-1}) slowed intracardiac conduction in anaesthetized beagles and again was at least 2–4 times more potent than flecainide or propafenone.

4 Org 7797 (0.5 and 2.0 mg kg^{-1}), flecainide (1.0 and 2.0 mg kg^{-1}) or propafenone (0.5 and 2.0 mg kg^{-1}), did not significantly prevent induction of tachyarrhythmias (VT) in dogs with 5–6 day old myocardial infarcts although all 3 drugs appeared to prevent induced VF. All 3 drugs (notably fecainide) did however reduce the VT rate.

5 All 3 drugs (1–2 mg kg^{-1}) suppressed spontaneous tachyarrhythmias in conscious beagle dogs with 1–2 day old infarcts. Propafenone was the least effective.

6 In an antifibrillatory dose (0.5 mg kg^{-1}), the major haemodynamic effect of Org 7797 was a 10% increase in peripheral vascular resistance. Stroke volume, cardiac output and coronary blood flow were unchanged. In therapeutic doses, Org 7797 was also less negatively chronotropic than flecainide.

7 It was concluded that Org 7797 is a potent antifibrillatory agent which is haemodynamically well tolerated. Higher doses are required to suppress late ischaemia-induced tachyarrhythmias which suggest that its antifibrillatory effects are the consequence of an action other than, or in addition to, sodium channel block.

Keywords: Org 7797; Ic drugs; myocardial infarction; arrhythmias; inducibility; haemodynamics; electrophysiology

Introduction

Org 7797 [(16α,17β)-17-methylamino-oestra-1,3,5(10-triene-3,16-diol(z)-2-butenedioate] is a new steroidal antiarrhythmic agent currently undergoing clinical evaluation. This compound has been shown to be effective against aconitine-induced and ischaemia-induced arrhythmias in rodents and to inhibit the fast inward sodium current in guinea-pig atria (Campbell *et al.*, 1986). Org 7797 has been further subclassified as a Ic agent on the basis of the kinetics of onset of rate-dependent sodium channel block in porcine isolated cardiac tissue (Winslow *et al.*, 1989a) and on its electrophysiological actions *in vivo* (Campbell *et al.*, 1991). The aim of the present study was to explore the antiarrhythmic potential of this compound against ischaemia-related arrhythmias in more detail and to compare its activity with that of two other Ic agents, flecainide and propafenone. The ability of Org 7797 to antagonize the development of early arrhythmias evoked by acute coronary artery ligation in rats and dogs, to prevent reperfusion-induced arrhythmias, to suppress spontaneous late arrhythmias and to prevent electrical induction of arrhythmias in dogs with 5–6 day old myocardial infarcts was therefore assessed together with its haemodynamic actions. In addition, the electrophysiological actions of Org 7797 *in vivo* were compared with those of flecainide and propafenone. Part of this work has already been published in abstract form (Winslow *et al.*, 1989b).

Methods

Early ischaemia-induced and reperfusion-induced arrhythmias in rats

Male Wistar rats (235–350 g) obtained from Olac and fed on Labsure CRMX pellets were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and prepared for coronary artery ligation as described by Clark *et al.* (1980). Arterial blood pressure and a limb lead II electrocardiogram (ECG) were recorded and the animals allowed to stabilize. Drugs (in 0.1 ml distilled water per 100 g body weight) or saline were given via the left femoral vein 15 min before occluding the main left descending coronary artery. Arrhythmias arising during the subsequent 30 min period were classified and analysed according to the Lambeth Conventions (Walker *et al.*, 1988). Rectal temperature was maintained at 37.0 ± 0.5°C.

To study reperfusion-induced arrhythmias, the procedure used was similar to that described above except that the ligature was tied around a small length of polythene tubing and 5 min after occlusion, the ligature released by sliding a scalpel blade over the tubing. The incidence of ventricular premature beats (VPBs), ventricular tachycardia (VT) and ventricular fibrillation (VF) consequent upon reperfusion were noted.

Haemodynamic measurements and early ischaemia-induced and reperfusion-induced arrhythmias in greyhound dogs

Experiments were carried out on greyhounds of either sex weighing between 21 and 35 kg. Anaesthesia was induced with intravenous sodium pentobarbitone (30–40 mg kg^{-1}) and maintained with additional barbiturate as required. Following endotracheal intubation, the dogs were artificially ventilated with room air using a positive pressure ventilation pump and stroke volume and rate adjusted to maintain arterial Po_2 between 90 and 100 mmHg (1 mmHg = 1.33 mbar) and blood pH between 7.35 and 7.45 pH units. Pancuronium bromide (3–4 mg) was given intramuscularly to prevent reflex muscular movements during surgery. Arterial blood pressure (BP), left

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ventricular pressure (LVP), cardiac output (CO) and left circumflex coronary blood flow (CBF) were recorded as described by Marshall & Muir (1981). A lead II ECG, from which heart rate (HR) was obtained, was recorded from adhesive limb electrodes.

Derived parameters were LV dP/dt P^{-1} (the rate of LVP development at a LVP of 40 mmHg), stroke volume (SV), peripheral vascular resistance (PVR) and external cardiac work (ECW).

A left thoracotomy was performed to expose the anterolateral aspect of the heart and the left anterior descending coronary artery (LAD) prepared for subsequent occlusion with a Diffenbach clamp at a point just distal to the septal branch. After a 30–40 min stabilization period, Org 7797 (in 10 ml distilled water) or 10 ml saline were given via the right femoral vein over a 2 min period and the LAD occluded 15 min later. Arrhythmias seen during the subsequent 30 min period of ischaemia were analysed as described for the rat. At the end of the occlusion period, reperfusion in surviving dogs (non-fibrillators) was accomplished by removing the clamp and gently stroking the artery. Reperfusion arrhythmias were assessed as described above. At the end of each experiment, blue dye was injected down the coronary artery at the level of the occlusion to assess the left ventricular 'area at risk'.

Statistics

In both the rat and dog studies, a Chi-square test was used to determine the significance of differences between the incidences of arrhythmias. A paired t test was used to detect significant drug-induced haemodynamic effects. All other comparisons were made with Student's t test.

Late ischaemia-induced arrhythmias in beagle dogs

Male or female beagle dogs (9–14 kg) were anaesthetized with halothane (1–2%) in N_2O/O_2 (1:1). Aseptic techniques were used to isolate the left carotid artery and a Fogarty 2F embolectomy catheter was manoeuvered into the LAD or circumflex coronary artery under fluorescopic control. The catheter balloon was inflated to occlude the artery for 90 min after which time the catheter was removed and the neck wound closed. Details of the method are given by Winslow *et al.* (1990).

Spontaneous arrhythmias were recorded from conscious animals on the first or second day after surgery for a 30 min control period. Test drugs were then infused over 10 min periods in a volume of 6 ml distilled water via a brachial vein and the ECG continuously monitored until at least 50 min after the last dose of test compound was given. Each dog received only one drug. The number of sinus beats recorded during successive 2 min intervals was expressed as a percentage of the total number of beats recorded during the same period (% SB) and compared, by a paired t test, with the mean % SB obtained during the control period.

Electrophysiological recording and programmed electrical stimulation in beagles

Five or six days after induction of myocardial infarction, dogs (10–14 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). Quadripolar and bipolar intracardiac electrodes (6F) were positioned in the right atrium and right ventricle respectively, via the left jugular vein. A bipolar electrode was positioned at the aortic arch via the left femoral artery for His bundle recording.

Electrophysiological measurements were made during normal sinus rhythm (NSR). The HV interval was measured from the start of the largest His deflection to the start of the ventricular electrogram. RR, QRS and QT intervals were measured from the lead II ECG and effects on repolarization assessed from JTc where

$$JTc = (QT - QRS) \div \sqrt{RR}$$

The ventricular effective refractory period (ERP) was determined during simultaneous atrial and ventricular pacing with a basic cycle length 20 ms shorter than the spontaneous sinus cycle length.

All measurements were made immediately before and 10 min after intravenous drug administration at which time programmed electrical stimulation (PES) was started. A BBC microcomputer, specially designed software and a Grass S88 stimulator were used to deliver up to 3 premature stimuli during normal sinus rhythm and/or during simultaneous atrial and ventricular pacing at a basic cycle length of 250 ms. The PES protocol is described in detail by Winslow *et al.* (1990).

The duration of the premature stimuli was 1 ms and the voltage was twice threshold (1–4 V). The PES protocol was invoked until sustained ventricular tachycardia (SVT) or VF was induced or until the protocol was exhausted. Non-sustained VT (NSVT) was defined as VT terminating spontaneously within 20 s. Termination of SVT sometimes required overdrive pacing or direct current (d.c.) conversion. If d.c. conversion was required, dogs were allowed to recover for at least 1 h before further challenge.

A paired t test was used to compare pre- and post-drug electrophysiological measurements.

Results

Early ischaemia-induced arrhythmias in anaesthetized rats

Results are summarized in Table 1. At doses of 2 mg kg^{-1}, Org 7797, flecainide and propafenone all abolished the development of VF and reduced the number of VPBs contributing to VT. However, unlike flecainide and propafenone, significant antifibrillatory effects were also seen at a dose of 0.5 mg kg^{-1} Org 7797 and the higher dose additionally reduced the number of single VPBs and salvos.

Table 1 Early ischaemia-induced arrhythmias in anaesthetized rats during the first 30 min of occlusion

			VPBs					
Treatment	(mg kg^{-1})	n	*Single*	*Salvo*	*VT*	*Total*	*% VF*	*% death*
Saline		17	150 ± 24	64 ± 17	397 ± 142	612 ± 171	53	29
Org 7797	0.5	10	197 ± 68	36 ± 20	182 ± 127	415 ± 205	10*	10
	2.0	10	55 ± 37*	5 ± 3**	123 ± 10*	72 ± 41**	0*	0
Flecainide	0.5	10	178 ± 52	78 ± 39	494 ± 217	751 ± 280	40	10
	2.0	10	276 ± 99	66 ± 29	90 ± 39*	431 ± 143	0*	0
Propafenone	0.5	9	152 ± 36	82 ± 28	1055 ± 473	1289 ± 495	33	22
	2.0	10	227 ± 80	116 ± 75	111 ± 43*	453 ± 175	0*	0

Each result under the VPB heading is the mean ± s.e.mean of results obtained from surviving animals in each group.
n refers to the total number of animals (survivors and non-survivors) included in each group.
* $P < 0.05$ and ** $P < 0.01$ denote significant differences from the appropriate control values.

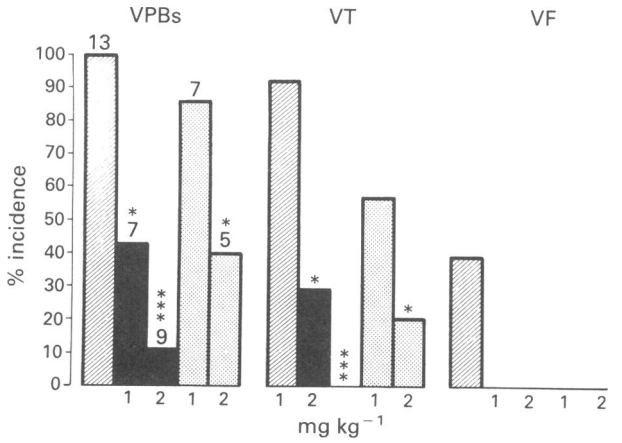


Figure 1 Effects of saline (hatched columns), Org 7797 (solid columns) and propafenone (stippled columns) on the % incidence of reperfusion-induced ventricular premature beats (VPBs), ventricular tachycardia (VT) and ventricular fibrillation (VF) in anaesthetized rats. * $P < 0.01$ and *** $P < 0.001$ denote significant differences from the appropriate control group. The number of animals in each group is shown in the VPB columns.

No sustained changes in blood pressure (115 ± 8/95 ± 7 mmHg in controls) were seen following administration of any of the 3 drugs. However, significant ($P < 0.01$) decreases in heart rate were observed following 2 mg kg^{-1} Org 7797, flecainide and propafenone (from 419 ± 9, 402 ± 17 and 415 ± 13 beats min^{-1} respectively to 402 ± 11, 370 ± 15 and 384 ± 15 15 min after administration) and following the lower dose of flecainide (from 441 ± 10 to 412 ± 12 beats min^{-1}).

Reperfusion-induced arrhythmias in anaesthetized rats

Both Org 7797 and propafenone (1–2 mg kg^{-1}) dose-dependently reduced the expected incidences of reperfusion-induced VF, VT and VPBs although Org 7797 was approximately twice as potent as propafenone (Figure 1).

Ischaemia-induced and reperfusion-induced arrhythmias in anaesthetized greyhound dogs

During the 30 min occlusion period, 9 out of 18 control animals developed VF whereas none of the 7 animals pretreated with 0.5 mg kg^{-1} Org 7797 fibrillated ($P < 0.05$) (Figure 2). Analysis of arrhythmias seen in non-fibrillating

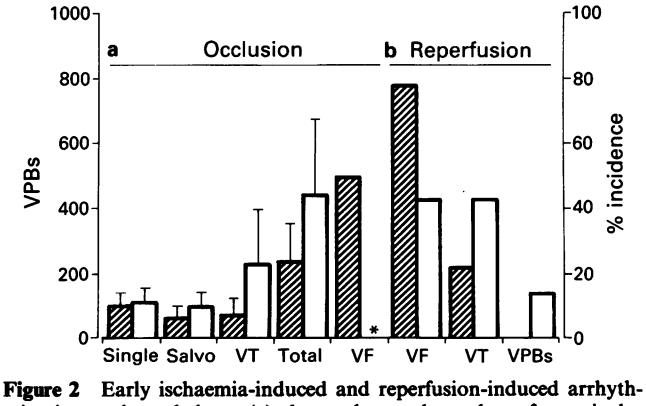


Figure 2 Early ischaemia-induced and reperfusion-induced arrhythmias in greyhound dogs: (a) shows the total number of ventricular premature beats (VPBs) and the % incidence of ventricular fibrillation (VF) recorded from animals pretreated with either 0.5 mg kg^{-1} Org 7797 (open columns) or 10 ml saline (hatched columns). Total VPBs have also been subdivided into single VPBs, salvos or ventricular tachycardia (VT) and the results expressed as the mean (s.e.mean shown by vertical bars) of 9 control dogs and 7 drug-treated dogs. (b) Shows the % incidence of single VPBs, VT and VF consequent upon reperfusion at the end of the 30 min occlusion period.
* $P < 0.05$ denotes a significant difference between Org 7797 and saline-treated dogs.

dogs showed no significant differences between the control and drug-treated groups. Of the 9 surviving control animals, 7 developed VF and 2 developed VT following reperfusion. Of the 7 Org 7797-treated dogs, 3 developed VF, 3 developed VT and 1 responded with only a few premature beats on reperfusion. However, these differences were not statistically significant. The area at risk was similar in control and drug-treated animals (33.9 ± 1.0 and 34.5 ± 1.4% of the left ventricle respectively).

Late-ischaemia-induced arrhythmias in conscious beagles

Org 7797, flecainide and propafenone (1 followed by 2 mg kg^{-1}) all significantly increased the number of sinus beats compared to pretreatment values (Figure 3). Quantitatively similar responses were obtained to Org 7797 and flecainide both of which appeared more potent and longer acting than equivalent doses of propafenone.

Inducibility of arrhythmias in anaesthetized beagles with 5–6 day old myocardial infarcts

None of the three drugs (Org 7797, 0.5 mg kg^{-1}; flecainide, 1.0 mg kg^{-1}; propafenone, 0.5 mg kg^{-1}) was particularly effective in this model (Figure 4). VT or VF could still be induced in 4 out of 6 dogs following Org 7797, in 4 out of 5 dogs following propafenone and in 6 out of 6 dogs following flecainide. However, sustained VT/VF in one dog in each of the

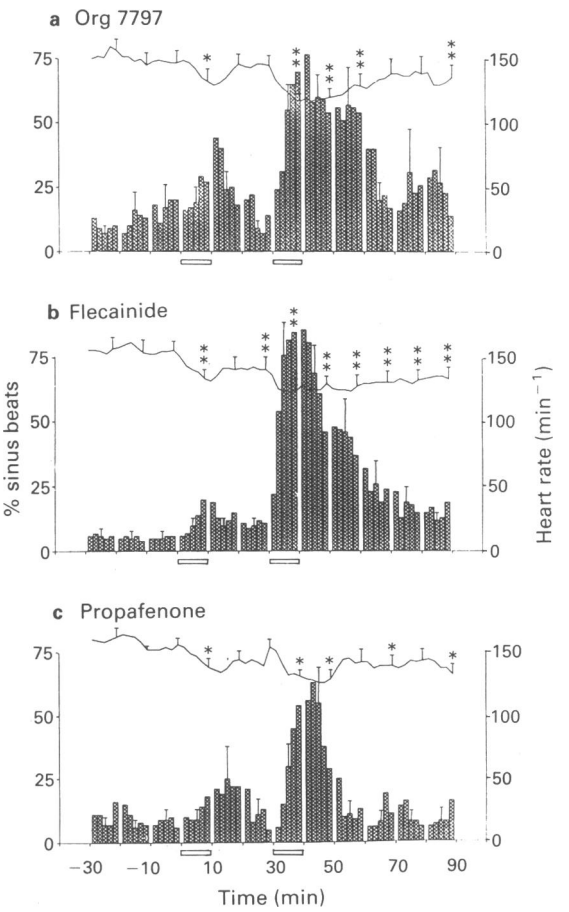


Figure 3 Effects of intravenous infusion of Org 7797 (a), flecainide (b) or propafenone (c) on the percentage of sinus beats (columns) assessed over successive 2 min intervals in conscious beagles 1 to 2 days after myocardial infarction. Heart rate is shown by the horizontal line. The horizontal bars denote the period during which 1.0 followed by 2.0 mg kg^{-1} of each drug was infused. Each result is the mean of 6 to 7 observations; vertical bars show s.e.mean.
* $P < 0.05$ and ** $P < 0.01$ denote significant differences from pretreatment values.

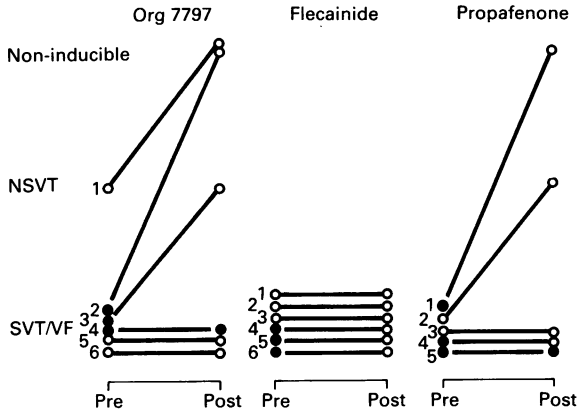


Figure 4 Arrhythmias induced by programmed electrical stimulation before (Pre) and after (Post) administration of Org 7797 (0.5 mg kg^{-1}), flecainide (1.0 mg kg^{-1}) and propafenone (0.5 mg kg^{-1}) to each of 5–6 anaesthetized beagles. The y-axis denotes the severity of the induced arrhythmia. NSVT, SVT and VF are nonsustained ventricular tachycardia, sustained tachycardia and fibrillation respectively; (●) and (○) denote the type of arrhythmia induced (VF or VT respectively).

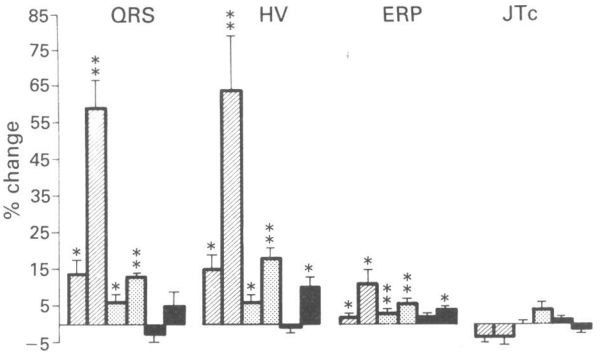


Figure 5 Electrophysiological effects of Org 7797 (0.5 and 2.0 mg kg^{-1}) (hatched columns), flecainide (1.0 and 2.0 mg kg^{-1}) (stippled columns) and propafenone (0.5 and 2.0 mg kg^{-1}) (solid columns) 10 min after administration to anaesthetized beagles. Each result is the mean of the % change from pre-drug values in 5–6 animals; vertical bars show s.e.mean.
* $P < 0.05$ and ** $P < 0.01$ denote significant changes from pretreatment values.

Org 7797 and propafenone groups was converted to non-sustained VT whilst VF in 3 out of 3 flecainide-treated, 2 out of 3 Org 7797-treated and 2 out of 3 propafenone-treated animals was prevented. Higher doses (2 mg kg^{-1}) of Org 7797 or flecainide did not confer additional protection against inducibility of arrhythmias whilst dog 2 (see Figure 4) in the propafenone group became non-inducible and the response in dog 4 was converted to non-sustained VT.

The induced VT rate was reduced by the lower dose of flecainide (from 410 ± 33 to 370 ± 27 beats min^{-1}, $P < 0.05$) but not by the lower doses of either Org 7797 or propafenone. Flecainide, Org 7797 and propafenone, all at 2 mg kg^{-1}, significantly ($P < 0.05$) reduced VT rate from 410 ± 33, 418 ± 27 and 412 ± 38 beats min^{-1} respectively to 338 ± 14, 370 ± 26 and 329 ± 31 beats min^{-1}.

Electrophysiological effects

Table 2 gives mean control values of RR, QRS, HV, ERP and JTc obtained before drug administration.

The RR interval was unchanged by propafenone (0.5 or 2 mg kg^{-1}) or the lower doses of Org 7797 (0.5 mg kg^{-1}) and flecainide (1.0 mg kg^{-1}) whilst 2 mg kg^{-1} Org 7797 and flecainide modestly but significantly ($P < 0.05$) prolonged RR by 9 ± 2 and 10 ± 3% respectively. Org 7797 and flecainide dose-dependently prolonged QRS and HV and to a much lesser extent prolonged ERP whilst JTc was unaltered (Figure 5). The lower dose of propafenone was without significant electrophysiological effects whilst the higher dose failed to prolong QRS significantly. However, HV and ERP were lengthened whilst again JTc was unchanged. The effects of Org 7797 on conduction were much more prominent than those of either flecainide or propafenone.

Haemodynamic effects of Org 7797 in anaesthetized greyhounds

The immediate haemodynamic effects of a bolus injection of Org 7797 (0.5 mg kg^{-1}) were small but significant. These were decreases in heart rate (5%) and LV dP/dt P^{-1} (11%) and increases in left ventricular end-diastolic pressure (LVEDP) and peripheral vascular resistance of 23% and 7% respectively ($P < 0.05$). These effects were sustained for at least 10 min at which time systolic blood pressure was also modestly but significantly increased (Table 3). Cardiac output, stroke volume and coronary blood flow were not, significantly altered.

Table 2 Baseline values (ms) for electrophysiological parameters recorded from beagle dogs pre-drug administration

	n	*RR*	*QRS*	*HV*	*JTc*	*ERP*
Org 7797	6	393 ± 9	45 ± 2	27 ± 3	275 ± 9	158 ± 2
Propafenone	5	398 ± 34	45 ± 1	24 ± 1	266 ± 11	162 ± 9
Flecainide	6	347 ± 5	35 ± 0	25 ± 0	279 ± 11	144 ± 2

Values are the mean ± s.e.mean of *n* observations.

Discussion

Results of the present study have shown that Org 7797 has potent antifibrillatory properties in myocardial infarction. At an intravenous dose of 0.5 mg kg^{-1}, Org 7797 significantly prevented early ischaemia-induced VF in anaesthetized rats and greyhound dogs and prevented electrical induction of VF in 2 out of 3 beagle dogs with established infarcts. This dose also halved the expected incidence of reperfusion-induced VF in the greyhound (from 78 to 43%) although the result did not reach statistical significance whilst a higher dose (1 mg kg^{-1}) abolished the development of reperfusion-induced VF in the rat. Comparative antiarrhythmic studies in rats suggest that Org 7797 is approximately 2 to 4 times more potent than either flecainide or propafenone. The superior potency of Org 7797 was confirmed in electrophysiological studies in anaesthetized beagles in which conduction in both the His-Purkinje system and the ventricular myocardium (reflected in the HV and QRS intervals) was significantly slowed following administration of 0.5 mg kg^{-1} whereas 2.0 mg kg^{-1} flecainide was required to induce qualitatively similar changes in these parameters. Electrophysiologically, propafenone appeared the least potent of the 3 drugs and indeed QRS lengthening was not statistically significant at the higher dose (2.0 mg kg^{-1}).

Despite the apparently greater potency of Org 7797 compared to propafenone, 0.5 mg kg^{-1} of the latter drug appeared at least as effective as Org 7797 against inducible arrhythmias in dogs with established infarcts whilst, if anything, the higher dose was more effective than Org 7797 (or flecainide). This discrepancy might be explained by the known β-adrenoceptor blocking actions of propafenone (Brown *et al.*, 1986; Burnett *et al.*, 1988) since β-blockers e.g. (propranolol, timolol or atenolol) suppress inducible arrhythmias in canine models similar to that employed in our laboratory (Gang & Bigger, 1984; Uprichard & Harron, 1989). In contrast, selective sodium channel block by Ic (or Ib) agents generally only prevents inducible VT/VF in 10 to 30 percent of animals (e.g. Sullivan *et al.*, 1990) a success rate similar to that found in the present study with Org 7797 (33%). Flecainide (1 or 2 mg kg^{-1}) did not prevent induction of VT in any animal but at both doses reduced the VT rate, an action expected when

Table 3 Haemodynamic changes induced by Org 7797 (0.5 mg kg^{-1}, i.v.) 10 min after administration to anaesthetized greyhounds

	Pre Org 7797	*10 min after Org 7797*	*% change*
Systolic blood pressure (mmHg)	163 ± 7	177 ± 6**	+8.6
Diastolic blood pressure (mmHg)	118 ± 6	127 ± 7	+7.6
Heart rate (beats min^{-1})	148 ± 4	144 ± 3*	−2.7
Cardiac output (litres min^{-1})	3.09 ± 0.32	2.96 ± 0.29	−4.2
Stroke volume (ml beat^{-1})	21.0 ± 2.1	20.7 ± 2.0	−1.4
Left ventricular pressure (mmHg)	158 ± 6	163 ± 7	+3.2
Left ventricular end diastolic pressure (mmHg)	9.1 ± 0.91	10.9 ± 1.1**	+19.8
Left ventricular dP/dt P^{-1} (s^{-1})	54.4 ± 4.8	48.9 ± 4.1	−10.1
External cardiac work (kgm min^{-1})	5.72 ± 0.60	5.72 ± 0.48	0
Peripheral vascular resistance (dynes s cm^{-3})	37.3 ± 3.8	40.9 ± 4.2**	+9.7
Coronary blood flow (ml min^{-1})	43.4 ± 5.4	45.3 ± 7.1	+4.4

Each result is the mean ± s.e.mean of 7 observations.
* $P < 0.05$ and ** $P < 0.01$ denote significant differences between pre- and post-drug values.

drug-induced slowing of conduction in the presence of anatomical block exceeds prolongation of refractoriness (Wellens *et al.*, 1984). Slowing of the VT rate was also seen following the higher doses of Org 7797 and propafenone but not following the lower doses (0.5 mg kg^{-1}) even though inducible arrhythmias were prevented in 20–33% of the animals. These differences between the three drugs may indicate differences in their mechanism(s) of action.

Org 7797 (and propafenone) also differed from flecainide with regard to effects on sinus rate relative to antifibrillatory potency. Whereas, in the rat, significant bradycardia was induced by subthreshold antifibrillatory doses of flecainide and therapeutic doses of propafenone, therapeutic doses of Org 7797 (0.5 mg kg^{-1}) failed to alter sinus rate. This difference between Org 7797 and flecainide was also observed in the beagle.

Although Org 7797 was potent against early ischaemia-induced VF, it was surprisingly less potent against spontaneous tachyarrhythmias arising 24–48 h after myocardial infarction and indeed was only equipotent with flecainide and propafenone. Since these arrhythmias result mainly from enhanced automaticity of surviving but 'electrically-depressed' sub-endocardial Purkinje fibres (Scherlag *et al.*, 1970; Lazzara *et al.*, 1974) and are sensitive to sodium channel blocking agents (reviewed by Marshall & Winslow, 1982), these observations would suggest that arrhythmias of automatic origin are less sensitive to Org 7797 than those arising from reentry, the major mechanism underlying early ischaemia-induced fibrillation (Janse & Wit, 1989). On the other hand, although all three drugs have class Ic actions, as shown in the present study by slowing of intracardiac conduction, unchanged repolarization and only small increases in refractory periods (Harrison, 1985), it is possible that different or additional drug properties are responsible for the marked antifibrillatory actions of Org 7797 seen at lower doses in both rats and dogs. In this context it may be relevant that the Ic agents, lorcainide and flecainide (and also a number of Ib agents) have often been found to increase, rather than decrease, susceptibility to early ischaemia-induced VF in large animals (Gerin & Kulbertus, 1978; Zipes & Troup, 1978; Lederman *et al.*, 1989). These pro-arrhythmogenic effects can be attributed to further slowing of conduction during reentry with a consequent decrease in wavelength (the distance an impulse travels during its own refractory period) thereby facilitating reentry (Wellens *et al.*, 1984). In contrast, Kirchhof *et al.* (1991) have shown that supraventricular antifibrillatory doses of Org 7797 in conscious dogs diminish physiological rate-dependent shortening of refractoriness resulting in a prolongation of wavelength during maximal heart rates. In addition, Janse *et al.* (1990) using pig isolated perfused hearts found that in concentrations required to prevent ischaemia-induced VF, Org 7797 substantially prolonged both the shortest possible pacing interval and the corresponding refractory period, attenuated or prevented shortening of the refractory period during subsequent application of premature stimuli and prolonged the average interval between depolarizations during electrically induced VF in non-ischaemic hearts (from 87 to 150 ms). An equimolar concentration of flecainide had little, if any, effect on the shortest pacing interval, induced significantly less lengthening of the VF interval and unlike Org 7797, failed to prevent VF induced by direct current during ischaemia.

Taken together, the results from the above studies strongly suggest that the antifibrillatory action of Org 7797 can be explained by prolongation of wavelength at very short cycle lengths due to prolongation of refractoriness outweighing slowing of conduction. This action could involve blockade of potassium channels as has recently been demonstrated for flecainide (Follmer & Colatsky, 1990).

However in comparison to flecainide, and perhaps other class Ic drugs, this property of Org 7797 might become manifest at low doses which have relatively less effect on sodium channels. This could explain the 4 fold difference between the dose required to prevent VF (due to reentry) and the dose required to suppress VT arising from abnormal sodium currents in depressed Purkinje tissue.

In an antifibrillatory dose, Org 7797 exerted minimal haemodynamic effects in anaesthetized greyhound dogs. The only significant effects were an almost negligible decrease in heart rate (3%), a 10% increase in peripheral vascular resistance together with a 9% increase in systolic blood pressure and a 20% increase in LVEDP accompanied by a 10% decrease in LV dP/dt P^{-1}. However, these effects on cardiac contractility were not sufficient to reduce either stroke volume or cardiac output. Coronary blood flow was preserved. Both propafenone and flecainide are known to be cardiodepressant (reflected by a reduced left ventricular ejection fraction) in antiarrhythmic doses (reviewed by Nestico *et al.*, 1988) whilst PVR is either unchanged by either drug, decreased by propafenone or increased by flecainide.

In conclusion, Org 7797 is a potent antifibrillatory agent which, in higher doses, suppresses late ischaemia-induced arrhythmias of automatic origin. Unlike flecainide and propafenone, therapeutic doses of Org 7797 exert minimal negative inotropic effects. In antifibrillatory doses its major haemodynamic effect is to increase modestly systemic vascular resistance whilst coronary blood flow is preserved. Although suppression of tachyarrhythmias arising from abnormal automaticity can be attributed to a class Ic action, the mechanism underlying its antifibrillatory action is not clearly understood but may be a consequence of attenuation (or indeed lengthening) of refractory periods at fast frequencies resulting in a prolongation of wavelength.

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The mechanism of LTE_4-induced histamine hyperresponsiveness in guinea-pig tracheal and human bronchial smooth muscle, *in vitro*

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1 Preincubation of guinea-pig tracheal smooth muscle with leukotriene E_4 (LTE_4) *in vitro* increased its subsequent responsiveness to histamine.

2 LTE_4 pretreatment of guinea-pig tracheal strips did not affect the subsequent responsiveness to either the contractile agents, carbachol and KCl, or to the relaxant β-adrenoceptor agonist, isoprenaline.

3 LTE_4-induced airway histamine hyperresponsiveness was blocked by indomethacin (5 μM), GR32191 (3 μM), atropine (1 μM) and tetrodotoxin (1 μM).

4 U46619, a stable thromboxane A_2-analogue, at a non-contractile concentration of 4 nM, increased tracheal smooth muscle sensitivity to histamine.

5 Both LTE_4 and U46619 pretreatment increased the contractile response of tracheal smooth muscle to electrical field stimulation.

6 Preincubation of human bronchial spirals with LTE_4 *in vitro* increased its subsequent responsiveness to histamine.

7 LTE_4-induced histamine hyperresponsiveness of human bronchus was inhibited by GR32191 (3 μM) and atropine (1 μM).

8 It is proposed that LTE_4 induces guinea-pig airway smooth muscle hyperresponsiveness to histamine via a facilitation of cholinergic neurotransmission, which is dependent upon the secondary generation of prostanoid mediator(s) acting on TP-receptors situated on cholinergic nerve terminals. In addition, it is suggested that LTE_4 may induce histamine hyperresponsiveness of human bronchus *in vitro* by a similar mechanism as to that seen in guinea-pig central airway smooth muscle.

Keywords: Leukotriene E_4; asthma; histamine; hyperresponsiveness; nerves, acetylcholine; neurotransmission; prostaglandin

Introduction

The sulphidopeptide leukotrienes, leukotriene C_4 (LTC_4), LTD_4 and LTE_4, comprise the activity previously recognized as slow-reacting substance of anaphylaxis. LTC_4, LTD_4 and LTE_4 are potent bronchoconstrictors of respiratory smooth muscle (Dahlen *et al.*, 1980), and may play a role in the pathophysiology of bronchial asthma.

In vitro studies have demonstrated that LTE_4 induces histamine hyperresponsiveness in guinea-pig tracheal spirals, but not parenchymal strips (Lee *et al.*, 1984). The enhancement of histamine responsiveness was dependent upon both the concentration of LTE_4 and the time of preincubation with the smooth muscle and was inhibited by the cyclo-oxygenase inhibitor, indomethacin, suggesting the secondary generation of a prostanoid. The production of histamine hyperresponsiveness by LTE_4 was critically dependent upon the configuration of the molecule (Lee *et al.*, 1987), particularly the structural integrity of the carboxyl group at C-1 and the nature of the peptide side-chain. Pretreatment of tracheal smooth muscle with LTC_4 or LTD_4 did not affect histamine responsiveness. Indeed, Creese & Bach (1983) have reported that preincubation of guinea-pig tracheal smooth muscle with LTD_4 enhanced tissue responsiveness to histamine only in the presence of low extracellular calcium ions.

We have now extended the previous studies to investigate further the mechanism of LTE_4-induced hyperresponsiveness to histamine *in vitro* in guinea-pig and human airway smooth muscle.

Methods

Guinea-pig tracheal strips

Guinea-pig tracheal strips, three cartilage rings thick, were prepared from Dunkin-Hartley male guinea-pigs (Charles River, UK. 300–350 g) for *in vitro* recording of isometric contraction with Grass FTO3D transducers. The strips were suspended in 5 ml organ baths containing modified Krebs solution (composition, mM: NaCl 118, KH_2PO_4 1.2, KCl 4.7, $NaHCO_3$ 15.4, glucose 5.5, $MgCl_2$ 1.2 and $CaCl_2$ 1.3). The tissues were aerated with 95% O_2; 5% CO_2 and maintained at 37°C. Each tissue was loaded with an initial tension of 2 g. The tissues were allowed to equilibrate for 1 h and were washed every 15 min over this period of time.

Once a stable baseline tension had been established (approximately 1.5 g), each tissue was exposed to logarithmically increasing concentrations of histamine (Sigma UK) over the range 1 nM to 100 μM in order to construct a cumulative histamine dose-response curve. The degree of contraction elicited by 100 μM histamine was assigned the value of 100% and all the subsequent contractile responses on that tissue were expressed as a percentage of this reference value. After completion of the initial histamine dose-response curve, the tissues were washed with Krebs solution every 15 min, for 1 h. During this time, the tension of each tissue returned to that recorded before histamine exposure.

Determination of the reproducibility of histamine dose-response curves in paired guinea-pig tracheal preparations

After the washout period and the re-establishment of the initial baseline tension, a second histamine dose-response curve was constructed. First and second histamine dose-response curves, on the same tissue, were compared in order to determine the within-tissue reproducibility of the smooth muscle response to histamine. The second histamine curves, from paired preparations from the same trachea, were compared in order to determine the between-tissues reproducibility of the histamine response.

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The effect of leukotriene E_4 pretreatment on histamine responsiveness of guinea-pig tracheal smooth muscle

In selected experiments, after completion of the first histamine curve, paired tissues were washed with Krebs solution every 15 min, for 1 h, until the tensions returned to their initial baseline values. Test tissues were then exposed to either 4 nM, 15 nM or 29 nM LTE_4 and control tissues to buffer for an incubation time of 15 min. This incubation time proved to be optimal in a study of the kinetics of the effect of LTE_4 on histamine responsiveness (results not shown). These concentrations of LTE_4 were selected as representing 10%, 30% and 50% respectively of the contractile response seen with 100 μM histamine. After incubation, the tissues were washed every 15 min for 60 min, in order to re-establish initial baseline tension values. A second histamine dose-response curve was then established.

LTE_4 was prepared by chemical synthesis as previously described (Lewis *et al.*, 1980). Concentrations of leukotrienes were determined by ultraviolet absorbance at 280 nm assuming a molar extinction coefficient (Σ) of 40 000 cm^{-1} M^{-1}.

Agonist selectivity of leukotriene E_4-induced hyperresponsiveness of guinea-pig tracheal smooth muscle

LTE_4-induced hyperresponsiveness of guinea-pig tracheal smooth muscle to agonists other than histamine was investigated. The effect of a 15 min preincubation with 4 nM or 15 nM LTE_4 on the responsiveness of tracheal smooth muscle to either the muscarinic agonist, carbachol (Sigma), or to the non-specific agonist, potassium chloride (KCl), was investigated. Concentration-effect curves were constructed to carbachol over the range 0.1 nM to 10 μM and to KCl over the range 1 mM to 60 mM. In addition, the ability of LTE_4 to influence tracheal responsiveness to a relaxant agonist was studied. After pretreatment with LTE_4, tracheal strips were contracted with 100 μM histamine and isoprenaline (Sigma) dose-response curves were then constructed over the dose range 0.1 nM to 10 μM. Control tissues were used to determine the decay of the histamine-induced contraction with time.

Pharmacological modulation of the effect of leukotriene E_4 pretreatment on histamine responsiveness of guinea-pig tracheal smooth muscle

The ability of various inhibitors or antagonists to block the effect of LTE_4 pretreatment on histamine responsiveness was investigated in separate experiments.

The cyclo-oxygenase inhibitor, indomethacin (Sigma), at a concentration of 5 μM was added to the Krebs solution at the beginning of the experiment for both the test (LTE_4-treated) and the control (buffer-treated) tissues. The effect of indomethacin on histamine responsiveness of tracheal smooth muscle was first established: histamine dose-response curves were constructed in the absence or presence of 5 μM indomethacin. The addition of indomethacin resulted in a drop in baseline tension, which was corrected by readjusting back to the initial baseline value.

The other inhibitors/antagonists used were the muscarinic antagonist, atropine (Sigma) 1 μM; the sodium-channel blocker, tetrodotoxin (Sigma) 1 μM; and the TP-receptor antagonist, GR32191 {[1R-[1α(Z), 2β, 3β, 5δ]]-(+)-7-[5-([1,1'-biphenyl] - 4 - ylmethoxy) - 3 - 3 - hydroxy - 2 - (1 - piperidinyl) cyclopentyl-4-heptenoic acid, hydrochloride} (Glaxo Group Research, UK) 3 μM. The method of investigation adopted for these drugs was identical to that used to assess the effect of indomethacin on LTE_4-induced histamine hyperresponsiveness. Atropine, tetrodotoxin or GR32191 caused no change in the smooth muscle baseline tension. All substances were diluted in 9 g $litre^{-1}$ saline solution.

Using a similar protocol to that employed for studying the effect of LTE_4 pretreatment, the thromboxane A_2-mimetic, U46619 {11α,9α-epoxymethano-PGH_2} (Glaxo Group Research, UK), was tested for its capacity to enhance smooth muscle responsiveness. Test tissues were incubated with a non-contractile concentration of U46619 (4 nM), for 15 min and paired control tissues were exposed to buffer for the same period of time. Histamine responsiveness was assessed 60 min after washing out the U46619 when the basal tone had been re-established.

Electrical field stimulation on guinea-pig tracheal strips

Tracheal strips were set up for electrical field stimulation (EFS) as described previously (Belvisi *et al.*, 1990). By use of a Grass S88 stimulator, frequency-response curves were constructed over the frequency range 1–50 Hz for each tissue. The following parameters were used: voltage, 20 V; pulse width, 0.1 ms; length of stimulation was 10 s with a 2 min rest period. At least three EFS responses were obtained and the mean of these values was taken. The degree of contraction produced by 50 Hz stimulation, on the first curve, was assigned the value of 100% contractile response. All tissues were incubated with 1 μM propranolol, in order to antagonise the effect of released sympathetic neurotransmitters. The contractile response to EFS could be blocked by tetrodotoxin (1 μM), showing that the response was of a neural origin.

LTE_4 (4 nM) was incubated with the test tissues for 15 min and then washed out. Control tissues were treated with buffer. After the preparations had regained initial basal tone levels, and having been washed for 1 h, a second frequency-response curve was constructed. In separate experiments, in the presence of 5 μM indomethacin, test tissues were exposed to 4 nM U46619 (a non-contractile dose) and control tissues to buffer 30 min before a second frequency-response curve was constructed.

Human bronchus

Macroscopically normal sections of human lung were obtained from patients undergoing surgery for carcinoma of the lung. The age range of these patients was 55–78 years. Bronchial tissue was dissected free from the surrounding parenchymal tissue and blood vessels. The diameter of these bronchial segments was 3–4 mm. The segments were cut spirally to a length of 15 mm and attached with cotton loops, through the cartilage, to the transducer and secured at the bottom of the organ bath.

The same conditions were used as for guinea-pig tracheal smooth muscle and histamine dose-response curves were similarly constructed.

The effect of leukotriene E_4 pretreatment on histamine responsiveness of human bronchus

The concentrations of LTE_4 eliciting 10% and 30% contraction of human bronchus were determined from cumulative dose-response curves constructed over the concentration range 0.01 nM to 0.3 μM. Control tissues were incubated with buffer and paired test tissues were treated with concentrations of LTE_4 that elicited either 10% or 30% contraction. The incubation time was 15 min. The tissues were subsequently washed every 15 min for 60 min and allowed to return to baseline tension and a second histamine dose-response curve was then constructed.

Pharmacological modulation of the effect of leukotriene E_4 pretreatment on histamine responsiveness of human bronchus

The ability of both GR32191 and atropine to block the effect of LTE_4 on histamine responsiveness was investigated. Either GR32191, at a concentration of 3 μM, or atropine, at a concen-

tration of 1 μM, was added to the bathing solution at the start of selected experiments.

Statistics

The concentration of histamine that elicited a 50% maximum contraction (EC_{50}) was interpolated from the second histamine dose-response curve for each tissue. The results are expressed as the change in EC_{50} concentration values (mean $\pm$ s.d.) for the paired tissues. For the analysis of data, Student's t test for paired data was used.

Results

Reproducibility of histamine dose-response curves on guinea-pig tracheal strips

Reproducibility of histamine dose-response curves was assessed both within (Figure 1a) and between tissues (Figure 1b) over the concentration range 1 nM to 100 μM. The threshold concentration for eliciting contraction was found to be 100 nM and was the same for both the first and second dose-effect curves to histamine. The concentrations of histamine elicting 50% contraction (EC_{50}) on the first and second histamine curves in the same tissue were 3.5 $\pm$ 1.4 μM and 3.3 $\pm$ 2 μM (mean $\pm$ s.d., n = 40), respectively. Randomly selected, paired tissues, taken from the same trachea, had EC_{50} values on the second histamine dose-response curve of 2.8 $\pm$ 2.1 μM and 3.0 $\pm$ 2.5 μM (n = 17), respectively.

The effect of leukotriene E_4 pretreatment on histamine responsiveness on guinea-pig tracheal strips

LTE_4 dose-response curves were constructed on guinea-pig tracheal strips over the concentration range 1 mM to 1 μM.

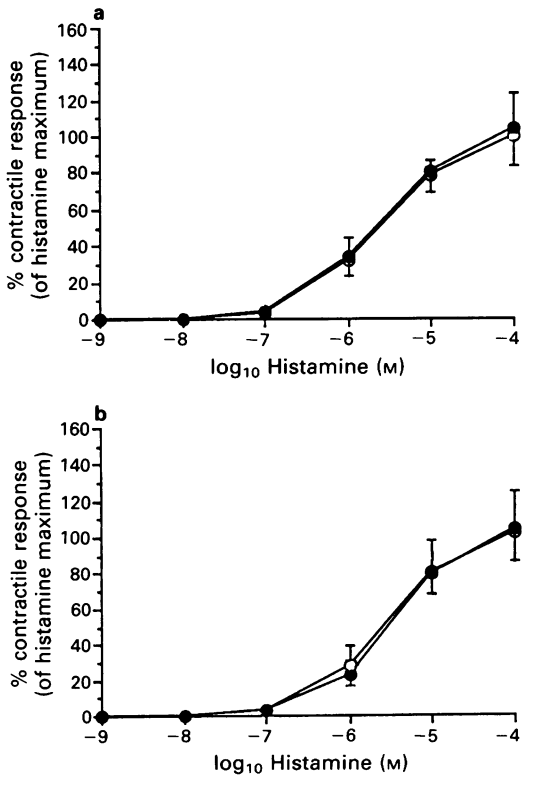


Figure 1 (a) Reproducibility of dose-response curves to histamine on the same guinea-pig tracheal strips. First histamine curve (○) and second histamine curve (●) (mean with s.d. shown by vertical bars, n = 40). (b) Reproducibility of curves to histamine between paired tissues (○, ●) from guinea-pig trachea (mean with s.d. shown by vertical bars, n = 17).

Concentrations of LTE_4 elicting 10%, 30% and 50% contraction were 4 $\pm$ 2.1 nM, 15 $\pm$ 3.8 nM and 29 $\pm$ 7 nM (n = 4), respectively. The EC_{50} concentration for LTE_4 on tracheal smooth muscle was approximately 100 fold less than that for histamine on this preparation. No further studies were carried out with 29 nM LTE_4 because it proved difficult to wash out the leukotriene and return the tissues to baseline tension. Thus, tissues were pretreated with either buffer, 4 nM or 15 nM LTE_4 for an incubation time of 15 min. The threshold concentration of histamine (100 nM) that elicited contraction of the tracheal smooth muscle was unaffected by pretreatment with either 4 nM or 15 nM LTE_4. The decrease in histamine EC_{50} values of tissues treated with LTE_4 (4 nM) compared to tissues treated with buffer, for 15 min, was 12 $\pm$ 4.4 fold (n = 6), respectively (Figure 2). This represented a significant ($P < 0.005$) increase in histamine sensitivity. Similarly, there was a significant ($P < 0.02$) 4.5 $\pm$ 1.9 fold (n = 5) increase in tissue sensitivity to histamine after pretreatment with 15 nM LTE_4 (Figure 2).

There was no significant difference between buffer- or LTE_4-treated tissues in terms of the maximal response to histamine (100 μM). The maximum contractile response for LTE_4 (4 nM)-treated tissues and their corresponding control preparations was 121 $\pm$ 9% and 102 $\pm$ 8% (n = 6), respectively; and for the 15 nM LTE_4-treated tissues and their controls was 120 $\pm$ 8% and 103 $\pm$ 19% (n = 5), respectively.

Agonist selectivity of leukotriene E_4-induced hyperresponsiveness of guinea-pig tracheal strips

LTE_4 pretreatment did not affect tracheal smooth muscle responsiveness to either carbachol (Figure 3a) or KCl (Figure 3b). There was no change in the carbachol EC_{50} values of control tissues and tissues treated with either 4 nM or 15 nM LTE_4. Similarly, LTE_4 pretreatment of tracheal strips was without effect on the maximal response to carbachol (10 μM); the maximal responses of control tissues and tissues treated with either 4 nM or 15 nM LTE_4 were 190 $\pm$ 26%, 176 $\pm$ 29% and 192 $\pm$ 34% (n = 4), respectively.

There was no change in the EC_{50} values for KCl of control tissues and those tissues preincubated with either 4 nM or 15 nM LTE_4 (n = 4). Additionally, LTE_4 preincubation of tracheal strips did not alter the maximal response to KCl (60 mM); the maximal responses of control tissues and tissues treated with either 4 nM or 15 nM LTE_4 were 140 $\pm$ 28%, 126 $\pm$ 28% and 134 $\pm$ 20% (n = 4).

LTE_4 pretreatment also did not affect tracheal smooth muscle responsiveness to isoprenaline. There was no change in the concentrations of isoprenaline producing 50% inhibition (IC_{50}) of histamine-induced contraction of control tissues pretreated with buffer and tissues pretreated with either 4 nM or 15 nM LTE_4. Additionally, there was no alteration in the

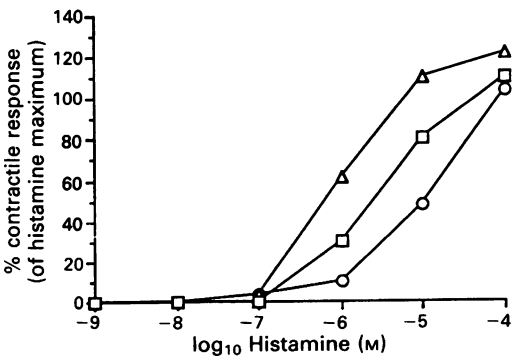


Figure 2 A representative experiment showing histamine dose-response curves constructed on guinea-pig tracheal smooth muscle pretreated with buffer (○); leukotriene E_4 4 nM (△) or 15 nM (□), for 15 min. Similar results were obtained in 6 experiments.

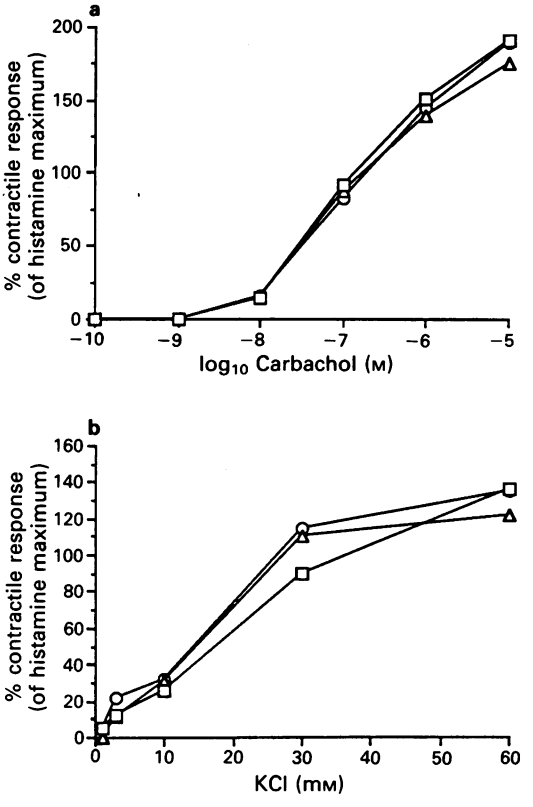


Figure 3 (a) A representative experiment showing dose-response curves to carbachol constructed on guinea-pig tracheal smooth muscle pretreated with buffer (○), leukotriene E_4 (LTE_4) 4 nM (△) or 15 nM (□), for 15 min. Similar results were obtained in 4 experiments. (b) A representative experiment showing dose-response curves to KCl constructed on guinea-pig tracheal smooth muscle pretreated with buffer (○), LTE_4 4 nM (△) or 15 nM (□), for 15 min. Similar results were obtained in 4 experiments.

maximal relaxation to 10 μM isoprenaline. The maximal relaxations of control, 4 nM LTE_4- and 15 nM LTE_4-treated tissues were 93 ± 4, 91 ± 23 and 97 ± 3% ($n = 4$), respectively. There was an approximate 17% decay in the histamine-induced contraction over the time period taken to construct an isoprenaline dose-response curve, as observed in parallel control tissues.

Pharmacological modulation of the effect of leukotriene E_4 on histamine responsiveness on guinea-pig tracheal strips

The enhanced responsiveness to histamine after pretreatment with LTE_4 (4 nM) was inhibited by the presence of either the cyclo-oxygenase inhibitor, indomethacin (5 μM); the TP-receptor antagonist, GR32191 (3 μM); atropine (1 μM) or the nerve conduction blocker, tetrodotoxin (TTX) (1 μM) (Figure 4a–d).

Indomethacin Addition of indomethacin (5 μM) to tissues did not affect the sensitivity to histamine. There was no change in the histamine EC_{50} values for buffer- and indomethacin-treated tissues. However, there was a significant ($P < 0.05$) increase in the maximal contraction to histamine (the values for buffer- and indomethacin-treated tissues were 108 ± 17% and 151 ± 18% ($n = 4$), respectively).

Incubation of tissues with indomethacin (5 μM) inhibited histamine hyperresponsiveness elicited by LTE_4 (Figure 4a). There was no change in the histamine EC_{50} values for control tissues and tissues treated with 4 nM and 15 nM LTE_4.

GR32191 Addition of GR32191 (3 μM) did not affect tissue sensitivity to histamine. Incubation of tissues with GR32191 (3 μM) inhibited LTE_4-induced histamine hyperresponsiveness (Figure 4b).

Atropine Addition of atropine (1 μM) to tissues did not affect their sensitivity to histamine. Incubation of tissues with atropine (1 μM) inhibited LTE_4-induced histamine hyperresponsiveness (Figure 4c). As a positive control, tissues were pretreated with either buffer or 4 nM LTE_4 in the absence of atropine. There was a significant ($P < 0.02$) 10 ± 4.6 fold change ($n = 4$) in the EC_{50} values for buffer-treated and LTE_4-treated tissues.

Tetrodotoxin There was no apparent effect of TTX (1 μM) on the sensitivity of tracheal strips to histamine. TTX inhibited LTE_4-induced histamine hyperresponsiveness (Figure 4d).

The effects of U46619 pretreatment on smooth muscle responsiveness

The EC_{50} of U46619 on tracheal strips was approximately 40 ± 18 nM ($n = 3$) indicating that U46619 is about 100 fold more potent than histamine as a contractile agonist. A non-contractile concentration, 4 nM, was selected for further study. Cumulative histamine concentration-effect curves were constructed on tracheal strips after preincubation with either buffer or U46619 for 15 min (Figure 5). After pretreatment of tissues with 4 nM U46619, there was a significant ($P < 0.05$) decrease in histamine EC_{50} compared to control tissues of 6.27 ± 4.07 fold ($n = 4$). U46619 (4 nM) did not change the maximal response to 100 μM histamine.

The effects of leukotriene E_4 or U46619 pretreatment on electrical field stimulation (EFS) of guinea-pig tracheal strips

The capacity of LTE_4 to influence guinea-pig tracheal response to EFS was investigated. Pretreatment of tissues with LTE_4 (4 nM), for 15 min, significantly ($P < 0.05$) increased the contractile response to EFS (Figure 6a).

Tissues pretreated with U46619 (4 nM) also significantly ($P < 0.02$) increased the EFS-induced contraction of guinea-pig trachea (Figure 6b).

Reproducibility of histamine dose-response curves on human bronchial spirals

Paired tissues from the same human bronchus were assessed for between tissue variability of the histamine response. Tissues were selected at random and the second histamine dose-response curves from each were compared. The histamine EC_{50} on paired human bronchial spirals was 5.8 ± 0.4 μM and 6.2 ± 0.3 μM ($n = 4$). The maximal responses of these tissues of 100 μM histamine were 99 ± 11 and 107 ± 17%, respectively ($n = 4$).

The effect of leukotriene E_4 pretreatment on histamine responsiveness of human bronchial spirals

Cumulative LTE_4 dose-response curves were constructed over the range 0.01 nM to 0.3 μM LTE_4. The concentrations of LTE_4 eliciting 10% and 30% contraction were 0.8 ± 0.2 nM and 4.8 ± 2.1 nM ($n = 4$), respectively. The decreases in histamine EC_{50} values for tissues pretreated with either 0.8 nM or 4.8 nM LTE_4, for 15 min, compared to control tissues were 2 ± 1.2 and 4 ± 0.9 fold ($n = 4$), respectively (Figure 7). The 4 fold leftward shift in tissues treated with 4.8 nM LTE_4 was significant ($P < 0.02$) when compared with control tissues.

Pharmacological modulation of the effect of leukotriene E_4 on histamine responsiveness of human bronchial spirals

There was no change in the histamine EC_{50} values of control tissues and tissues preincubated with LTE_4 (4.8 nM) in the pre-

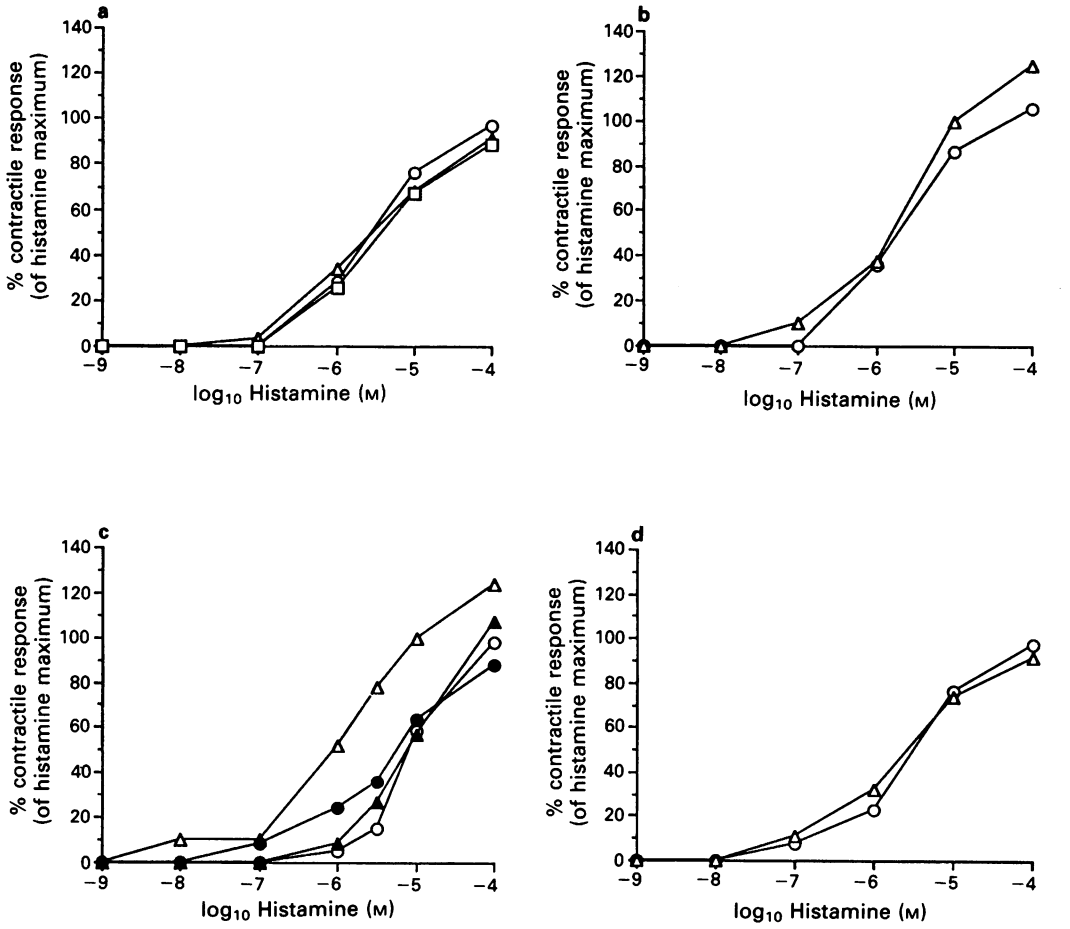


Figure 4 (a) A representative experiment showing histamine dose-response curves constructed, in the presence of indomethacin 5 μM, on guinea-pig tracheal smooth muscle pretreated with buffer (○), leukotriene E_4 (LTE_4) 4 nM (△) or 15 nM (□), for 15 min. Similar results were obtained in 4 experiments. (b) A representative experiment showing histamine dose-response curves constructed, in the presence of GR32191 3 μM, on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or LTE_4 4 nM (△), for 15 min. Similar results were obtained in 4 experiments. (c) A representative experiment showing histamine dose-response curves constructed, either in the absence (open symbols) or presence (closed symbols) of atropine 1 μM, on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or LTE_4 4 nM (△), for 15 min. Similar results were obtained in 4 experiments. (d) A representative experiment showing histamine dose-response curves constructed, in the presence of tetrodotoxin 1 μM, on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or LTE_4 4 nM (△), for 15 min. Similar results were obtained in 4 experiments.

sence of GR32191 (3 μM). Thus, GR32191 antagonized LTE_4-induced histamine hyperresponsiveness (Figure 8a).

There was no change in the histamine EC_{50} values of control tissues and tissues preincubated with LTE_4 (4.8 nM) in the presence of atropine (1 μM). Atropine therefore also inhibited LTE_4-induced histamine hyperresponsiveness (Figure 8b).

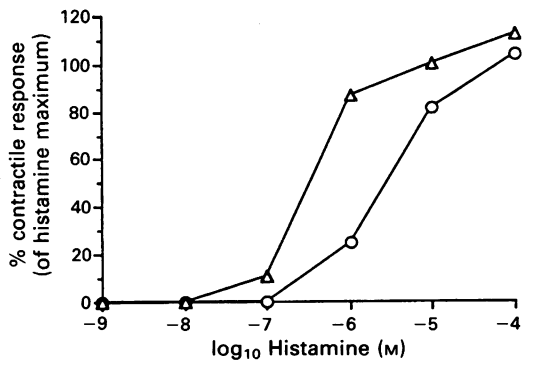


Figure 5 A representative experiment showing histamine dose-response curves constructed on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or U46619 4 μM (△), for 15 min. Similar results were obtained in 4 experiments.

Discussion

Previous work has established that preincubation of guinea-pig tracheal smooth muscle with LTE_4 enhances the subsequent contractile response to histamine. The effect was dose- and time-related with 15 min preincubation time being optimal (Lee *et al.*, 1984). The results described in this study confirm that hyperresponsiveness to histamine can be induced by LTE_4 in guinea-pig central airway. LTE_4 was able to induce airway histamine hyperresponsiveness that was sustained for at least 60 min after the LTE_4 had been removed. This suggests that the phenomenon was a long-lived response that may have been maintained at a post-receptor level. LTE_4 shifted the dose-response curve to the left, with a decrease in the EC_{50} value but there was no significant change in the maximal response to histamine. Between-tissue variability studies show that histamine responses of isolated tracheal smooth muscle are reproducible. LTE_4 pretreatment has been shown to enhance histamine responsiveness of guinea-pig tracheal smooth muscle by approximately 10 fold when compared to control tissues treated with buffer. Since LTE_4 pretreatment of guinea-pig tracheal smooth muscle did not affect its responsiveness to either the muscarinic agonist, carbachol, or to the non-specific agonist, KCl, LTE_4-induced hyperresponsiveness appeared to be relatively selective towards histamine. Previous work has shown that concentrations of LTC_4 and LTD_4, which produced the same degree of

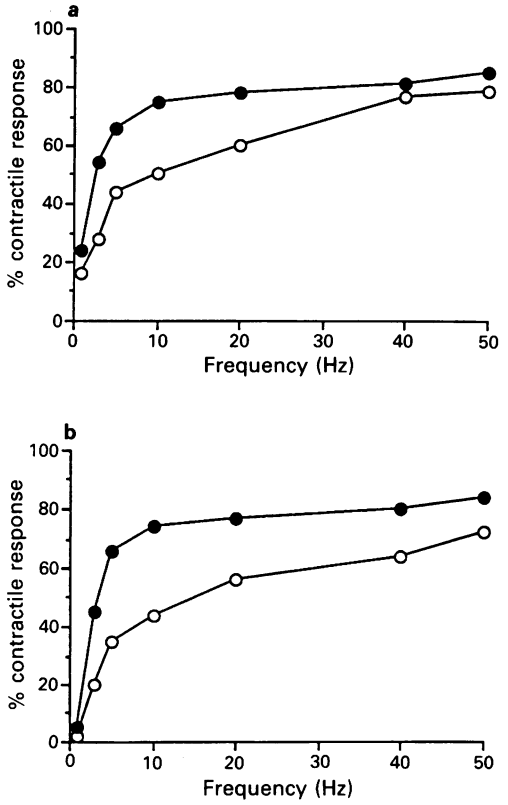


Figure 6 (a) A representative experiment showing frequency-response curves constructed on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or leukotriene E_4 (LTE_4) 4 nM (●), for 15 min and then subjected to electrical field stimulation. Similar results were obtained in 4 experiments. (b) A representative experiment showing frequency-response curves constructed on guinea-pig tracheal smooth muscle pretreated with either buffer (○) or U46619 4 nM (●), for 15 min and then subjected to electrical field stimulation. Similar results were obtained in 4 experiments.

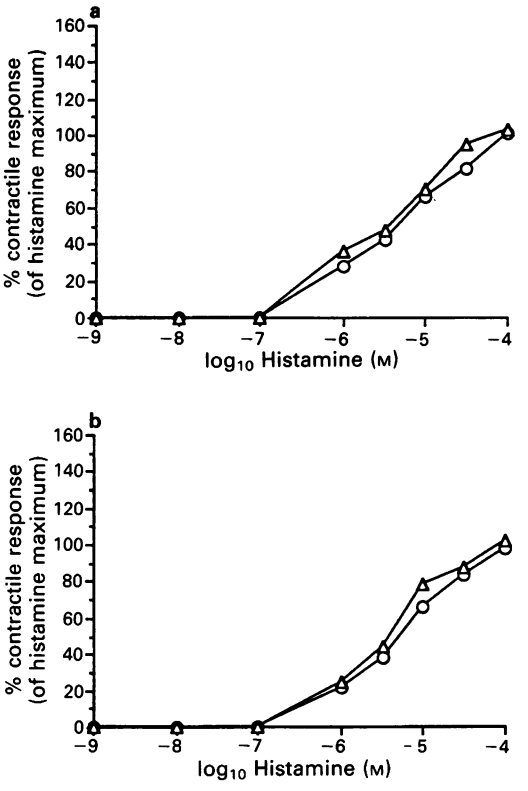


Figure 8 (a) A representative experiment showing histamine dose-response curves constructed on human bronchial smooth muscle pretreated with either buffer (○) or leukotriene E_4 (LTE_4) 4.8 nM (△) in the presence of GR32191 3 μM, for 15 min. Similar results were obtained in 4 experiments. (b) A representative experiment showing histamine dose-response curves constructed on human bronchial smooth muscle pretreated with either buffer (○) or LTE_4 4.8 nM (△) in the presence of atropine 1 μM, for 15 min. Similar results were obtained in 4 experiments.

contraction of guinea-pig tracheal strips as 4 nM and 15 nM LTE_4, did not enhance histamine responsiveness (Lee *et al.*, 1984). Thus, leukotriene-induced hyperresponsiveness to histamine of guinea-pig tracheal smooth muscle can be dissociated from leukotriene-induced contraction.

Lee *et al.* (1984) and the present results have shown that indomethacin did not alter the contraction of guinea-pig tracheal smooth muscle produced by LTE_4, but inhibited the increased reactivity to histamine after LTE_4 pretreatment. This suggests that LTE_4-elicited contraction and LTE_4-induced hyperresponsiveness of guinea-pig tracheal strips may be produced via different mechanisms. Previous and present findings suggest that the induction of hyper-

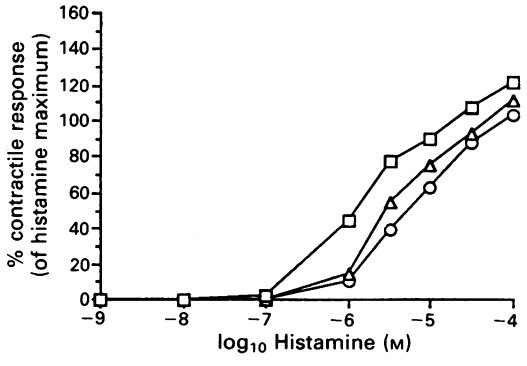


Figure 7 A representative experiment showing histamine dose-response curves constructed on human bronchial smooth muscle pretreated with buffer (○), leukotriene E_4 (LTE_4) 0.8 nM (△) or 4.8 nM (□), for 15 min. Similar results were obtained in 4 experiments.

responsiveness is caused by the release of prostanoid mediators. Steel *et al.* (1979) showed that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and PGE_2 were the principal prostaglandins released from guinea-pig trachea and thromboxane A_2 (TxA_2) has been implicated in several studies, both *in vivo* and *in vitro*, in the induction of hyperresponsiveness (Aizawa *et al.*, 1985; Chung *et al.*, 1986; O'Byrne & Fuller, 1989). We have confirmed these earlier reports by showing that pretreatment of tissues with U46619, a stable TxA_2-analogue, significantly increased the sensitivity and the maximal response to histamine. According to the classification of prostanoid receptors proposed by Coleman *et al.* (1984), TxA_2 is the most potent agonist at TP-receptors; $PGF_{2\alpha}$ and PGD_2 can also act at this receptor site, but are less potent (Coleman *et al.*, 1984; 1985). GR32191 is a selective TP-receptor antagonist (Humphrey *et al.*, 1990). GR32191 inhibited LTE_4-induced hyperresponsiveness to histamine in a concentration range which is known to inhibit TP-receptor-mediated contraction of this tissue (Lumley *et al.*, 1989), suggesting that LTE_4 is mediating its effects via prostanoids acting at the TP-receptor.

Previous work by Kikuchi *et al.* (1984) has shown that histamine may stimulate vagally-mediated bronchoconstriction by an H_1-receptor. We therefore tested the hypothesis that LTE_4-induced hyperresponsiveness may involve enhancement of a histamine-neuronal interaction. Atropine inhibited the LTE_4-induced hyperresponsiveness in both human bronchial and guinea-pig tracheal smooth muscle supporting the view that a cholinergic mechanism may be involved. The finding that TTX, a nerve conduction blocker, also inhibited LTE_4-induced hyperresponsiveness confirmed the involvement of a neurogenic mechanism.

Electrical field stimulation (EFS) was used to elicit endogenous neurotransmitter release from nerves within the tracheal

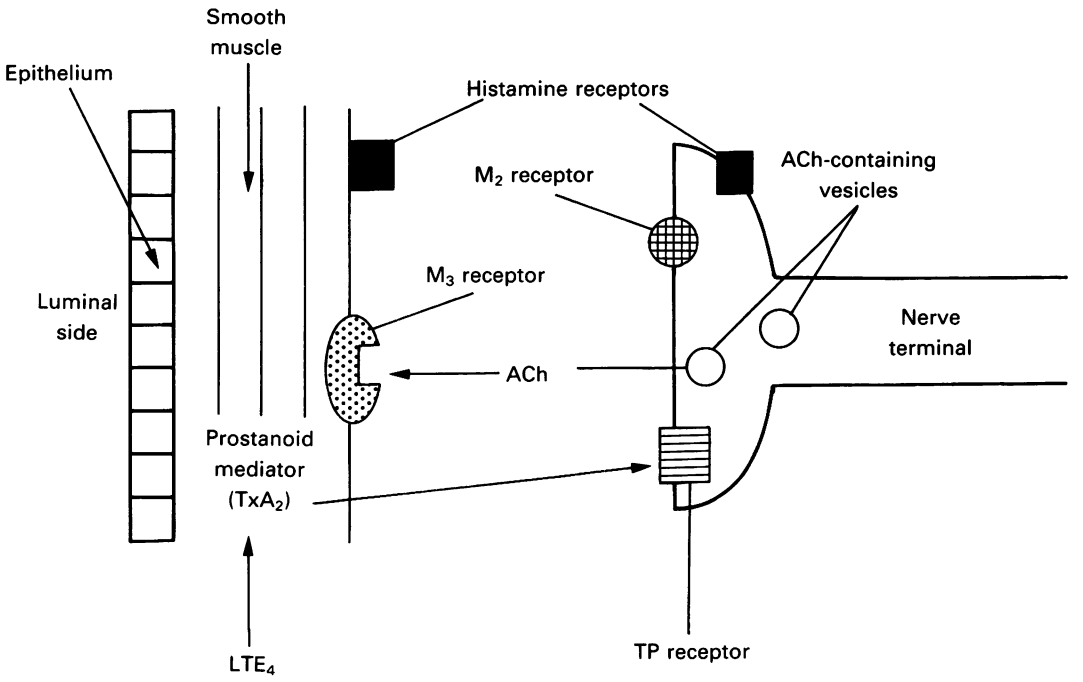


Figure 9 A proposed mechanism for leukotriene E_4 (LTE_4)-induced histamine hyperresponsiveness of central airway smooth muscle. It is suggested that LTE_4 stimulates the production of a prostanoid mediator which interacts with the TP-receptor, which is located on the cholinergic nerve terminal, to 'prime' neuronal activity. Histamine interacts with neuronal histamine receptors to elicit the release of acetylcholine (ACh). Released ACh then interacts with postjunctional muscarinic M_3 receptors.

smooth muscle preparation. Pretreatment with LTE_4 resulted in an enhancement of the contractile response to EFS. Tissues that were incubated with U46619 were also significantly more responsive to EFS, confirming the findings of other groups (Chung *et al.*, 1985; Munoz *et al.*, 1986). The results of Munoz *et al.* (1986) were similar to those described here in that the phenomenon of induced-hyperresponsiveness was relatively long-lasting (at least 1 h) and, additionally, was without effect on an exogenously applied muscarinic agonist.

Arm *et al.* (1988) have demonstrated that LTE_4 enhances histamine airways responsiveness *in vivo* in asthmatic subjects. We have therefore performed a limited number of studies to ascertain whether the mechanism of LTE_4-induced hyperresponsiveness in human airways is similar to that in guinea-pig tissue. Histamine responses between smooth muscle preparations from the same human lung were highly reproducible, thereby allowing comparison of histamine dose-response curves. Pretreatment of bronchial smooth muscle with LTE_4 resulted in a 4 fold decrease in histamine EC_{50} values. The phenomenon was inhibited by GR32191 and atropine suggesting the involvement of the TP- and muscarinic receptors, as in guinea-pig airways.

In conclusion, we propose that LTE_4 induces guinea-pig tracheal hyperresponsiveness to histamine via a facilitation of cholinergic neurotransmission, as previously suggested by Palmer *et al.* (1981) for the enhancement of neuronal firing in Purkinje fibres. The mechanism by which LTE_4 induces this phenomenon appears prostanoid-dependent, probably acting at TP-receptors. These receptors may be located on cholinergic nerve terminals and serve to 'prime' or partially depolarize nerves without causing neuronal firing. Neuronal firing is elicited by the action of histamine and the enhanced responsiveness is due to a combined action of histamine, acting at H_1-receptors, and acetylcholine, which acts postjunctionally at muscarinic M_3-receptors, on the smooth muscle cell (Figure 9).

The finding that smooth muscle responsiveness to carbachol was not affected by LTE_4 pretreatment would be explained by the fact that carbachol acts on muscarinic 'autoreceptors' (M_2), located on cholinergic nerve terminals (MacLagan & Barnes, 1989), resulting in a possible inhibition of the release of neuronally-derived ACh. KCl contracts smooth muscle by direct depolarization and would bypass any neuronal mechanism. Thus, LTE_4 would not be expected to augment KCl responses in this postulated model (Figure 9).

The findings that both GR32191 and atropine blocked LTE_4-induced hyperresponsiveness to histamine in human bronchial smooth muscle, suggests that a similar mechanism may operate in both human and guinea-pig airway tissue.

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An analysis of the mechanism of the inotropic action of some milrinone analogues in guinea-pig isolated atria

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1 It has been reported previously that the milrinone analogues, ethyl 5-cyano-1,6-dihydro-2-methyl-6-oxo-3 pyridine carboxylate (I) and ethyl 5-cyano-1,6-dihydro-2-ethyl-6-oxo-3 pyridine carboxilate (II) exert a positive inotropic effect (EC_{50} = 15.6 ± 0.2 μM and 40.3 ± 0.1 μM) both on spontaneously beating and on electrically driven atria from reserpine-treated guinea-pigs. In the present study the mechanism of the inotropic action of these two agents was investigated.

2 In electrically driven left atrium from resperpine-treated guinea-pigs the EC_{50} values for inotropic activity for compounds (I) and (II) corresponded to that of milrinone (EC_{50} = 25 ± 0.1 μM) but compound (I) induced a greater maximum effect. This corresponded to a percentage increase in developed tension over control of 63 ± 0.3 whereas the maximum inotropic effect of milrinone was 48 ± 0.3 and that of compound (II) was 47 ± 0.2.

3 The inotropic activity of compounds (I) and (II) (10–100 μM) was resistant to propranolol (0.1 μM), thus excluding the involvement of β-adrenoceptors.

4 Since the inotropism induced by compounds (I) and (II) was not reduced by carbachol (1 nM–0.5 μM), an action involving changes in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) can be excluded.

5 The inotropic action of compounds (I) and (II) was blocked selectively by 8-phenyltheophyline (10 μM) or adenosine deaminase (2 u ml^{-1}).

6 Both (I) and (II) inhibited, in an apparently competitive manner, the negative inotropic effect induced by N^6-(L-phenylisopropyl) adenosine (L-PIA), a stable adenosine agonist. The pA_2 values for (I) and (II) were 4.79 and 4.36, respectively.

7 In rat brain compounds (I) and (II) inhibited the specific binding of N^6-cyclohexyl[^{3}H]-adenosine-([^{3}H]-CHA) with an IC_{50} of 0.18 ± 0.01 mM and 0.25 ± 0.02 mM, respectively, which were similar to their IC_{50} values for blocking the PIA-induced negative inotropic effect and which are also in the range of concentrations that are effective in inducing positive inotropism in guinea-pig atria.

8 The results from the present study suggest that antagonism of endogenous purines causes positive inotropism without affecting intracellular cyclic AMP levels.

Keywords: Guinea-pig atria; milrinone analogues; adenosine; inotropism

Introduction

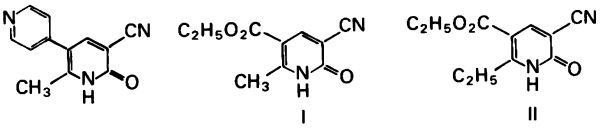
Modulation of the inotropic state of the heart occurs in a multifaceted manner and, as a consequence, contractility may be increased via multiple biochemical pathways (Colucci *et al.*, 1986). Most pharmacological interventions ultimately increase intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels, which in turn augment Ca^{2+} uptake and availability by contractile proteins. New cardiotonic drugs such as amrinone, milrinone, pimobendan and sulmazole stimulate cyclic AMP accumulation by at least three different mechanisms: (1) inhibition of cyclic AMP phosphodiesterase (PDEase) (Meisheri *et al.*, 1980; Kariya *et al.*, 1982; Endoh *et al.*, 1982; 1985; 1986), (2) antagonism towards endogenous adenosine at R_i inhibitory receptors (Dorigo & Maragno, 1986; Earl *et al.*, 1986; Parson *et al.*, 1988; Dorigo *et al.*, 1990) and (3) stimulation of adenylate cyclase by functional block of the inhibitory guanine nucleotide regulatory protein G_i (Parson *et al.*, 1988). However the extent to which each of these mechanisms is involved in the inotropic effects remains to be determined. In an attempt to obtain compounds with a more specific therapeutic indication, milrinone was taken as the parent drug for the synthesis of various analogues (Mosti *et al.*, 1989). The esters (Figure 1) ethyl 5-cyano-1,6-dihydro-2-methyl-6-oxo-3 pyridine carboxylate (I) and ethyl 5-cyano-2-ethyl-1,6-dihydro-6-oxo-3-pyridine carboxylate (II) were the most effective inotropic agents in spontaneously beating or in electrically driven atria from reserpine-treated guinea-pigs (Mosti *et al.*, 1989). The aim of this study was to elucidate the mechanism of their inotropic action.

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Methods

Isolated atrial preparations

Male guinea-pigs (300–500 g) were killed by a blow to the head followed by exsanguination and the atria were separated from the ventricles and kept in a physiological salt solution (composition mM: NaCl 120, KCl 2.7, $MgCl_2$ 0.09, NaH_2PO_4 0.4, $CaCl_2$ 1.37, $NaHCO_3$ 11.9, glucose 5.5). Left atria were mounted in a bath containing 30 ml of the same solution maintained at 29°C and bubbled vigorously with a mixture of



Milrinone

Esters of 2-substituted-5-cyano-1,6-dihydro-6-oxo-3 pyridincarboxylic acids

Figure 1 Chemical structures of milrinone and its analogues (I) and (II).

95% O_2 and 5% CO_2 which produced a pH of 7.5. The left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated at a frequency of 1.5 Hz by square wave electrical pulses of 3 ms duration and a voltage 10% to 20% greater than threshold, delivered by a Grass stimulator (Mod. 24KR). The control developed tension ranged from 0.09 to 0.20 mN. Changes in developed tension were recorded by a writing oscillograph (Basile Unirecord System, Mod. 7050). The electrical stimulation was performed in order to eliminate any influence on contractile activity due to variations in frequency rate.

Inotropic activity

The experiments were performed on atria obtained from reserpine-treated animals. Reserpine (2 mg kg^{-1}, i.p.) was given 48 and 12 h before the animals were killed, in order to eliminate the influence of noradrenaline which might be released from sympathetic nerve terminals (Temma *et al.*, 1977). Noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (10 μmol) before starting the experiments. Experiments were performed only in preparations unresponsive to tyramine. After 90 min of equilibration 0.1 μM noradrenaline was added repeatedly until two reproducible responses were obtained. Inotropic agents were added cumulatively and the inotropic effect was recorded for 5 min after it reached its maximum before adding a higher concentration. Where indicated, propranolol and carbachol were added to the bathing fluid 10 min before the inotropic agents whereas adenosine deaminase and 8-phenyltheophylline were added 20 min before the inotropic agents. The basal and the stimulated contractile activity was determined by measuring the amplitude of the developed tension. The data are expressed as % variation from the control (atria incubated without drugs).

N^6-cyclohexyl[^3H]-adenosine ([^3H]-CHA) binding assay

For receptor binding assays male Wistar rats (150–200 g) were decapitated and the brain (minus brainstem and cerebellum) was dissected on ice. The tissue was homogenized in a Polytron (setting 5) in 20 vol of 50 mM Tris-HCl pH 7.4. The homogenate was centrifuged at 48,000 *g* for 10 min and the pellet was suspended in buffer, centrifuged and resuspended in Tris-HCl containing 2 iu of adenosine deaminase per ml. After 30 min incubation at 37°C, membranes were centrifuged and the pellet was stored at −70°C. Binding experiments with [^{3}H]-CHA (13.5 Ci mmol^{-1}), (Bruns *et al.*, 1980), were performed in 1 ml of buffer which contained 1 nM [^{3}H]-CHA, membranes from 15 mg (wet weight) of tissue and the compounds to be tested. After 120 min incubation at 23°C, separation of bound from free ligand was performed by rapid filtration through Whatman GF/B filters which were washed 3 times with ice-cold buffer, dried and counted in 5 ml of acidified Instagel. Non-specific binding was defined as binding in the presence of 10 μl N^6-(L-phenylisopropyl) adenosine (L-PIA) and was < 10% of total binding. Six different concentrations of the test compound were assayed in triplicate and the IC_{50} was calculated by probit analysis.

Materials

The drugs used and their sources of supply were as follows: milrinone (Sterling Winthrop), 8-phenyltheophylline (Calbiochem-Behring), noradrenaline bitartrate, propranolol hydrochloride, carbachol chloride, adenosine deaminase, (−)-isoprenaline hydrochloride, tyramine (Sigma). [^{3}H]-CHA (NEN Research Products), Instagel (Packard), L-PIA (RBI). Milrinone analogues I and II were synthesized by L. Mosti in the Institute of Pharmaceutical Sciences, Genova University, Italy.

Calculations

Data are shown as mean ± standard error of the mean (s.e.mean). Statistical significance of the differences was calculated by Student's *t* test for paired data. Values were considered to be statistically different when *P* was lower than 0.05.

The concentration of an agonist giving 50% of maximal inotropism (EC_{50}) was estimated by interpolation on the curve drawn through the mean data points for contractility as a function of log concentration.

The pA_2 values for compounds (I) and (II) were calculated by Schild regression analysis.

Results

Effect of compounds (I) and (II) on contractile activity: comparison with milrinone

In electrically driven left atrium, esters (I) and (II) induced a concentration-dependent increase of contractile force. As previously observed (Mosti *et al.*, 1989), (I) was more active than milrinone. The maximum percentage increase over control obtained with (I) was higher (+63%) than that caused by milrinone (+48%) (Figure 2). Compound (II) and milrinone showed similar activity. As the concentration of both (I) and (II) was increased, the positive inotropic effect was followed and then masked by a negative phase of inotropism that, at equal concentrations, was more pronounced than that

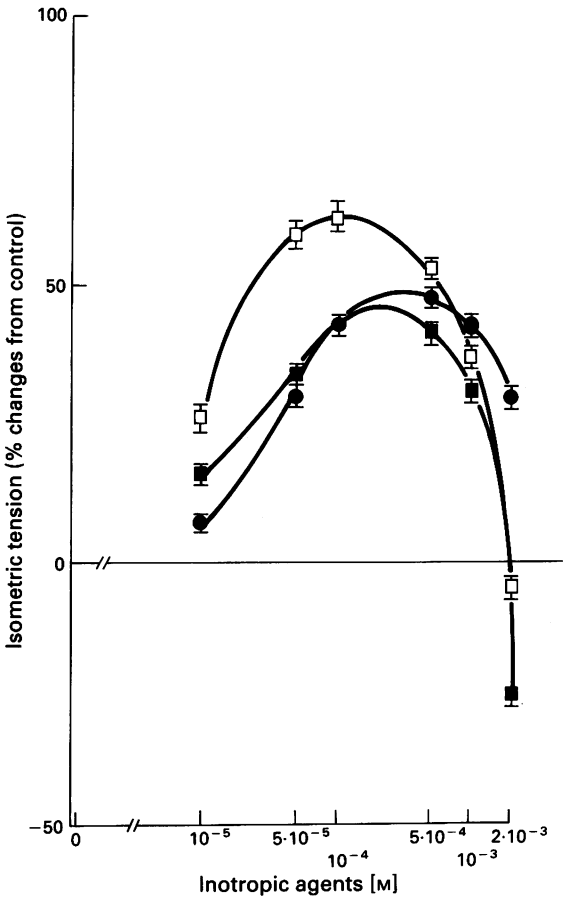


Figure 2 Inotropic effect of compounds (I) and (II): comparison with milrinone. Left atrium was isolated from reserpine-treated guinea-pigs and electrically driven as explained under Methods: (●) milrinone; (□) (I), (■) (II). Each point is mean of six to nine determinations from nine different experiments; s.e. shown by vertical bars.

induced by milrinone. These effects were completely reversible by washing.

Inotropic response to (I) and (II) in the presence of propranolol and carbachol

Propranolol (0.1 μM), which completely inhibited the maximum inotropic effect induced by 30 nM isoprenaline (data not shown) did not modify the inotropic activity of (I) and of (II) (Table 1). Inotropism induced by (I) and (II) and the respective EC_{50}s were not reduced by carbachol at a concentration, (10 nM), that does not modify basal activity of the atria. At 0.5 μM, a concentration that inhibits the contractility of the atria almost completely, carbachol enhanced the inotropic activity of (I) and (II) (Table 1) but this effect may in part be due to a suppressed baseline. In the same concentration-range carbachol inhibited the inotropic response to isoprenaline (Table 2).

Involvement of endogenous adenosine in the contractile activity of (I) and (II)

In atria incubated in the presence of adenosine deaminase (2 u ml^{-1}), the enzyme that inactivates endogenous adenosine

Table 1 Effect of propranolol and of carbachol (CCh) on the contractile activity of compounds (I) and (II) in electrically driven left atrium from reserpine-treated guinea-pigs

	Maximum isometric tension (% increase over control)				
Compounds		*Propranolol* (0.1 μM)	*CCh* (10 nM)	*CCh* (0.5 μM)	
(I)	77.85 ± 0.91	75.94 ± 0.21 NS	73.50 ± 2.53 NS	85.38 ± 2.96	$P < 0.001$
(II)	55.00 ± 1.74	55.71 ± 0.34 NS	55.33 ± 1.76 NS	62.87 ± 2.31	$P < 0.001$
			EC_{50} (μM)		
(I)	21.50 ± 0.51	21.50 ± 0.33	22.00 ± 0.18	48.00 ± 0.47	
(II)	34.00 ± 0.62	34.50 ± 0.35	34.00 ± 0.43	45.00 ± 0.61	

Each result is mean ± s.e.mean of five to eight assays from eight different experiments. The statistical significance of the changes induced by propranolol and by carbachol was calculated by Student's *t* test. *P* values were calculated vs respective control (atria perfused without propranolol or carbachol). NS = not statistically significant ($P > 0.05$).

Table 2 Effect of carbachol (CCh) on the contractile activity of isoprenaline in electrically driven left atrium from reserpine-treated guinea-pigs

		Isometric tension (% increase over control)		
Isoprenaline (nM)		*CCh* (10 nM)	*CCh* (0.1 μM)	*CCh* (0.3 μM)
—	—	−0.25 ± 0.01	−45.21 ± 1.87	−79.63 ± 2.31
0.3	28.48 ± 0.78	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
1	64.64 ± 0.88	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
3	109.97 ± 2.56	41.11 ± 0.96*	0.00 ± 0.00*	0.00 ± 0.00*
10	140.27 ± 3.09	68.91 ± 1.78*	23.15 ± 1.01*	0.00 ± 0.00*
30	193.48 ± 2.26	94.95 ± 1.88*	39.64 ± 1.18*	0.60 ± 0.05*
100	203.16 ± 2.73	118.40 ± 5.19*	45.11 ± 1.07*	9.07 ± 0.46*

Each result is mean ± s.e.mean of four assays from four different experiments. The statistical significance of the changes induced by carbachol was calculated by Student's *t* test. *P* values were calculated vs respective controls (atria perfused without carbachol). * = $P < 0.001$.

Table 3 Effect of adenosine deaminase (ADA) on the contractile activity of compounds (I) and (II) in electrically driven left atrium from reserpine-treated guinea-pigs

		Isometric tension (% increase over control)			
Compounds (μM)		*ADA* (1 u ml^{-1})	*% inhib.*	*ADA* (2 u ml^{-1})	*% inhib.*
—	—	32.71 ± 1.26*		52.02 ± 1.23*	
I 10	27.10 ± 1.19	15.22 ± 0.65*	(70%)	0.00 ± 0.00*	(100%)
II 10	9.37 ± 1.16	3.95 ± 0.14*	(58%)	0.00 ± 0.00*	(100%)
I 30	39.71 ± 1.16	21.97 ± 0.49*	(60%)	0.00 ± 0.00*	(100%)
II 30	19.65 ± 1.26	9.81 ± 0.70*	(51%)	0.00 ± 0.00*	(100%)
I 50	50.61 ± 1.02	17.10 ± 0.79*	(66%)	0.00 ± 0.00*	(100%)
II 50	29.11 ± 0.93	13.20 ± 0.60*	(55%)	0.00 ± 0.00*	(100%)
I 70	63.04 ± 1.33	21.95 ± 0.84*	(65%)	0.00 ± 0.00*	(100%)
II 70	32.29 ± 1.05	16.24 ± 0.41*	(50%)	0.00 ± 0.00*	(100%)
I 100	79.55 ± 2.40	22.72 ± 0.74*	(71%)	0.00 ± 0.00*	(100%)
II 100	47.83 ± 0.91	17.95 ± 0.96*	(63%)	0.00 ± 0.00*	(100%)

Each result is mean ± s.e.mean of four assays from four different experiments. The statistical significance of the changes induced by adenosine deaminase was calculated by Student's *t* test. *P* values were calculated vs respective control (atria perfused without adenosine deaminase). * $P < 0.001$; (...%) = percentage inhibition vs control (atria perfused without adenosine deaminase).

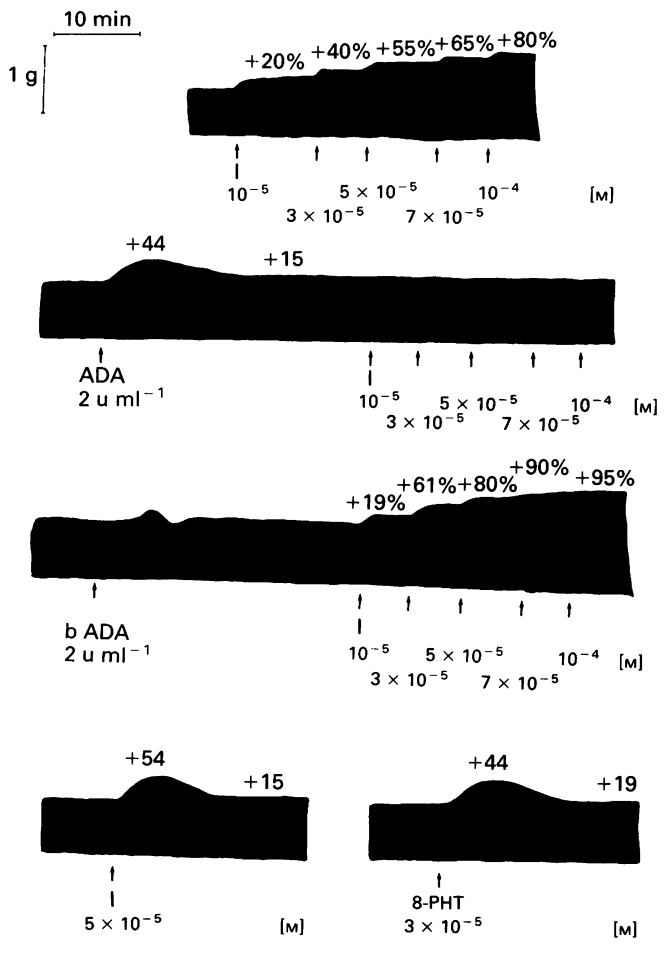


Figure 3 Effect of boiled adenosine deaminase (bADA) on the inotropic action of compound (I) and comparison of the inotropic responses induced by adenosine deaminase (ADA), 8-phenyltheophylline (8-PHT) and compound (I). Left atrium was isolated from reserpine-treated guinea-pigs and electrically driven as explained under Methods. The experiment reported is representative of six different experiments with similar results. bADA = boiled adenosine deaminase; I = compound (I); +...% = percentage increase in developed tension over control.

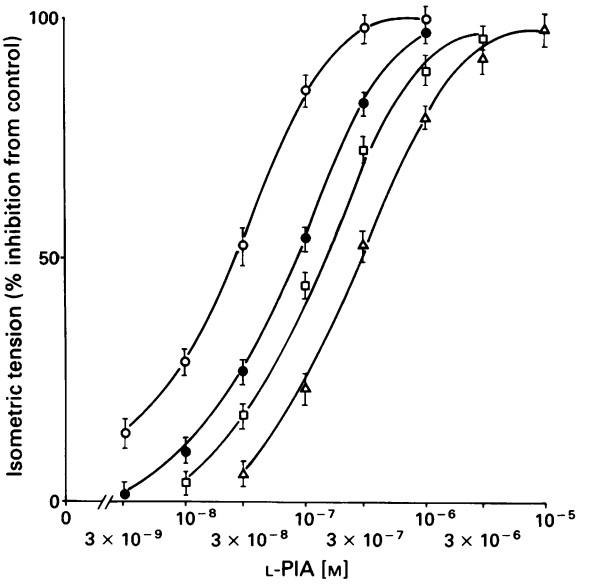


Figure 4 Effect of compound (I) on N^6-(L-phenylisopropyl) adenosine (L-PIA)-induced negative inotropic response. Left atrium was isolated from reserpine-treated guinea-pigs and electrically driven as explained under Methods: (○) L-PIA; (●) L-PIA plus 3×10^{-5} M (I); (□) L-PIA plus 7×10^{-5} M (I); (△) L-PIA plus 10^{-4} M (I).

by metabolizing it to inosine, both (I) and (II) lost their positive inotropic effect (Table 3, Figure 3). As shown in Table 3 the inhibition exerted by adenosine deaminase was concentration-dependent. The addition of the enzyme ($2\,u\,ml^{-1}$) evoked by itself a sustained increase in tension that lasted for 15–20 min and that left the heart preparation stabilized at a higher (+15%) contractile activity than the control (Figure 3). The boiled enzyme, on the contrary, induced only a transient increase in contractile force and did not influence the inotropic response of guinea-pig left atrium to (I) and (II) (Figure 3). The adenosine receptor antagonist, 8-phenyltheophylline (30 μM) exerted an inotropic effect similar to that induced by adenosine deaminase (Figure 3). The increase in contractile force induced by adenosine deaminase and by 8-phenyltheophyline as well as that induced by compounds (I) and (II) (data not shown) declined after reaching its maximum (Figure 3), thus suggesting that the interference of some unknown control mechanism in the inotropic activity. In left atria preincubated with increasing concentrations of 8-phenyltheophylline, a progressive inhibition of the positive inotropic effect induced by (I) (Table 4) and (II) (not shown) was evident, while the negative response was amplified (Table 4). The potent agonist at R_i adenosine receptors, L-PIA, caused concentration-dependent decrease of cardiac contractility (Figures 4 and 5). This negative influence on the heart was inhibited in an apparently competitive manner both by (I) and by (II), which caused a progressive rightward and parallel shift of the log concentration response curve for L-PIA. The pA_2 values calculated by the Schild regression analysis for (I) and (II) were 4.79 and 4.36, respectively. To confirm the ability

Table 4 Effect of 8-phenyltheophylline (8-PHT) on the contractile activity of compound (I) in electrically driven left atrium from reserpine-treated guinea-pigs

	Isometric tension (% increase over control)				
8-PHT		*Compound (I)*			
(μM)		10 μM	50 μM	100 μM	2 mM
—	—	21.64 ± 1.29	49.52 ± 0.99	70.13 ± 1.58	−27.27 ± 1.18*
1	15.78 ± 1.35	4.72 ± 0.53*	22.91 ± 0.46*	44.63 ± 1.01*	−51.44 ± 1.48*
3	26.68 ± 0.73	0.00 ± 0.00*	12.84 ± 1.42*	34.11 ± 0.81*	−68.12 ± 0.80*
10	33.82 ± 0.59	0.00 ± 0.00*	6.71 ± 0.27*	15.99 ± 0.92*	−81.81 ± 1.10*
30	44.21 ± 1.19	0.00 ± 0.00*	−0.96 ± 0.02*	−4.34 ± 1.22*	−92.57 ± 1.40*

Each result is mean ± s.e.mean of four to six assays from six different experiments. The statistical significance of the changes induced by 8-phenyltheophylline was calculated by Student's *t* test. *P* values were calculated vs respective controls (atrium perfused with compound I alone). * $P < 0.001$.

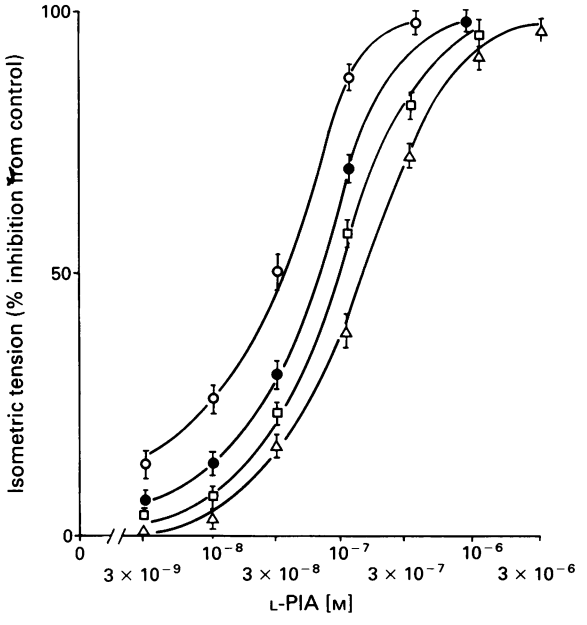


Figure 5 Effect of compound (II) on N^6-(L-phenylisopropyl) adenosine (L-PIA)-induced negative inotropic response. Left atrium was isolated from resperpine-treated guinea-pigs and electrically driven as explained under Methods: (○) L-PIA; (●) L-PIA plus 5×10^{-5} M (II); (□) L-PIA plus 10^{-4} M (II); (△) L-PIA plus 5×10^{-4} M (II).

of (I) and (II) to displace endogenous adenosine from its receptor, the influence of (I) and (II) on [^{3}H]-CHA binding to R_i receptors of the rat brain was studied. [^{3}H]-CHA is a specific agonist for these receptors (Lewis *et al.*, 1981) and its binding parameters in rat brain can be related to physiological responses in the heart (Oei *et al.*, 1988). From the binding experiments the affinity of milrinone analogues for R_i receptors was evaluated on the bases of the IC_{50} that were 0.18 ± 0.01 mM for (I) and 0.25 ± 0.02 mM for (II). These concentrations were similar to those that increased inotropic activity of guinea-pig left atria and to the EC_{50}s that antagonized the negative effect of L-PIA on atrial contractility.

Discussion

The bipiridine derivative, milrinone, is a potent inotropic and vasodilator agent (Alousi *et al.*, 1983a,b). Amrinone and milrinone are generally referred to as inhibitors of PDEase III, thus implying that their inotropic and chronotropic actions may be sustained by an increase in cyclic AMP levels (Honerjäger *et al.*, 1981; Endoh *et al.*, 1982; Silver *et al.*, 1987; Ito *et al.*, 1988). However, recent data indicate that other mechanisms may be responsible for this increase, e.g. an antagonism towards endogenous adenosine at R_i receptors (Dorigo & Maragno, 1986; Earl *et al.*, 1986; Parson *et al.*, 1988) and a G protein-dependent stimulatory action on adenylate cyclase that is abolished by pretreatment with pertussis toxin (Parson *et al.*, 1988).

In the present study two milrinone analogues, esters of pyridine carboxylic acids, induced a significant increase in contractile force of guinea-pig left atria that was not related to a release of endogenous catecholamines, as the animals were treated with reserpine, nor to an interaction with β-adrenoceptors, as propranolol did not influence the observed increase in developed tension. Carbachol is known to abolish selectively the elevation of heart contractility and of cyclic AMP levels induced either by adenylate cyclase stimulation or by PDEase inhibition in different heart preparations (Endoh, 1979). Also in the guinea-pig left atrium carbachol (1 nM–0.5 μM) inhibited in a concentration-dependent manner the cyclic AMP-mediated inotropic response to isoprenaline, but not the positive inotropic effect of compounds (I) and (II). These findings suggest that the involvement of cyclic AMP in the effect of compounds (I) and (II) is unlikely.

When the heart preparations were treated with adenosine deaminase, the enzyme that inactivates endogenous adenosine, or with 8-phenyltheophylline, a competitive adenosine antagonist (Smellie *et al.*, 1979; Burns, 1981) a significant increase of developed tension was observed. The mechanism underlying the secondary decrease of this effect and the stabilization of inotropism at a level higher than the basal one is unknown.

In atria pretreated with adenosine deaminase, compounds (I) and (II) did not evoke any positive inotropic effect. Similarly, in atria exposed to 8-phenyltheophylline the positive inotropic response to compounds (I) and (II) was markedly inhibited while the negative inotropic response was potentiated. This observation suggests the possible existence of a complex mechanism of action for these milrinone analogues. At relatively low concentrations compounds (I) and (II) might competitively antagonize the binding of endogenous adenosine to its receptor in the cardiac cells whereas, at higher concentrations, they might activate a cellular system directly responsible for a negative influence on contractile activity.

This cellular system appears to be independent of the adenosine one, since the negative influence exerted by (I) and (II) is still present in atrial preparations preincubated with adenosine deaminase (data not shown). The apparently competitive antagonism exerted by the tested compounds towards the negative inotropic effect of the adenosine derivative, L-PIA, together with finding that compounds (I) and (II) inhibit the specific binding of [^{3}H]-CHA to R_i receptors in rat brain, further shows that the two milrinone analogues tested exert a positive inotropic effect by preventing the negative influence of endogenous adenosine on contractile activity. This mechanism was previously found to account for the increase in atrial contractility caused by amrinone and by milrinone (Dorigo & Maragno, 1986; Dorigo *et al.*, 1987). In guinea-pig atria the negative inotropic and chronotropic action of adenosine is not necessarily related to changes in the cyclic AMP and/or cyclic GMP content (Endoh *et al.*, 1983; Bohm *et al.*, 1984). Moreover Schutz & Tuisle (1981) and Schmitz *et al.* (1981) suggested that in guinea-pig heart, adenosine receptors are not functionally related to adenylate cyclase. On the other hand the negative effects exerted by adenosine on the guinea-pig atria appear to be related to a reduction in the action potential duration in response to a direct inhibition of Ca^{2+} channels (Belardinelli *et al.*, 1983; Bohm *et al.*, 1984; Cerbai *et al.*, 1988; Prostran & Varagic, 1988) and/or to a reduction in Ca^{2+} flux into the cell due to an increased K^+ conductance and hyperpolarization of cell membrane (Jochem & Nawrath, 1983; Bohm *et al.*, 1984; Visentin *et al.*, 1990). Thus, the observed interaction between milrinone analogues and endogenous adenosine may well result in a significant inotropic response mediated by an increased intracellular Ca^{2+} uptake and availability of Ca^{2+} for contraction, possibly unrelated to changes in cyclic AMP levels that the two compounds might still cause by affecting adenylate cyclase and/or phosphodiesterase activity. In cardiac muscle, a correlation between the cyclic AMP elevation mediated by PDE III inhibition and inotropic responses has been demonstrated (Kobylarz *et al.*, 1985; Silver, 1989). In this respect Weishaar *et al.* (1987) stressed that in guinea-pig heart, PDEase III, the isoenzyme that is selectively inhibited by amrinone and milrinone, may be either biochemically uncoupled from myocardial contractile proteins or may be compartmentalized in the cytosol so that increases in cyclic AMP concentrations do not necessarily cause increases in contractility. The lack of an effect of carbachol on the inotropic action of the milrinone analogues offers only preliminary, indirect evidence for the lack of cyclic AMP involvement in the effect of these compounds. Further studies are needed to support this view.

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Novel kainate derivatives: potent depolarizing actions on spinal motoneurones and dorsal root fibres in newborn rats

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1 Neuropharmacological actions of several kainate derivatives (kainoids) were examined for electrophysiological effects in the isolated spinal cord and the dorsal root fibre of the newborn rat.

2 Some kainoids caused depolarization of the motoneurone much more effectively than kainic acid or domoic acid and others were weaker. The rank order of the depolarizing activities of the kainoids tested here is as follows: 4-(2-methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (MFPA) > acromelic acid A > domoic acid ≧ 4-(2-hydroxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (HFPA) ≧ acromelic acid B > kainic acid.

3 In the isolated dorsal root fibre, domoic acid caused the most significant depolarization. There were distinct differences with regard to the rank order of the depolarizing activity between the motoneurone and the dorsal root fibre. The rank order in the dorsal root fibre is domoic acid > acromelic acid B > 5-bromowillardiine ≧ MFPA > acromelic acid A > HFPA > kainic acid.

4 Significant desensitization of kainate receptors was observed in the isolated dorsal root fibre during prolonged application of L-glutamate, kainate and its derivatives. Cross desensitization was also observed among these excitatory amino acids. Receptors desensitized by kainate did not respond to MFPA, HFPA and acromelic acids, suggesting that these kainate derivatives activated common kainate receptors in the dorsal root fibre.

5 In both motoneurones and dorsal root fibres, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) effectively depressed the depolarization induced by kainoids, and neither 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP) nor picrotoxin blocked or affected the depolarization, but there were some differences in pharmacological potencies of glutamate antagonists between both preparations.

6 MFPA, HFPA and acromelic acids should provide valuable pharmacological tools for analysis of physiological functions of excitatory amino acids, in particular, as specific agonists for some subtypes of kainate receptors.

Keywords: Kainate derivatives; excitatory amino acid; depolarizing activity; spinal motoneurone; dorsal root C fibre; desensitization; electrophysiology

Introduction

Kainic acid is one of the most potent excitants in the mammalian central neurone and its powerful excitatory actions gave rise to the excitotoxic concept that glutamate destroys neurones by excessive activation of excitatory receptors. A potent kainate analogue, acromelic acid A, isolated from a poisonous mushroom, *Clitocybe acromelalga* (Konno *et al.*, 1983; 1988), causes depolarization more markedly than kainic acid or domoic acid in the newborn rat spinal motoneurone (Ishida & Shinozaki, 1988), and is also one of the most potent excitatory amino acids in both vertebrates and invertebrates (Shinozaki *et al.*, 1986; 1991; Shinozaki, 1988; 1991; Shinozaki & Ishida, 1988a,b). Acromelic acid has two structural isomers, acromelic acids A and B. From the structural and pharmacological similarities of acromelic acids to kainic acid or domoic acid, it seems reasonable to predict kainate-type excitotoxic actions of acromelate in the mammalian central nervous system. However, single systemic injection of acromelic acid A caused behavioural and pathological effects quite different from those seen after systemic administration of kainate in the rat (Shinozaki *et al.*, 1989a; 1991; Kwak *et al.*, 1990; 1991). It is of great interest to examine these pharmacological differences between kainate and acromelate. In the course of the study on chemical synthesis of acromelic acids and their derivatives (Shirahama *et al.*, 1988; Hashimoto *et al.*, 1990; Hashimoto & Shirahama, 1990), we were able to obtain some kinds of kainate related compounds (kainoids), which were expected to provide a useful tool for analysing the mechanism underlying kainate functions.

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At present, highly specific antagonists for kainate receptors are not yet available, therefore, it is difficult to classify electrophysiologically these derivatives as pure kainate agonists, but it has been reported that dorsal root C fibres of immature rats are directly depolarized by kainic acid, whereas quisqualate and (±)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) are much less active than kainate, and N-methyl-D-aspartate (NMDA) does not cause depolarization of the dorsal root even at higher concentrations (Davies *et al.*, 1979; Agrawal & Evans, 1986; Huettner, 1990), although the pharmacological properties of the dorsal root C fibre depolarization have been reported to be not always identical to those of spinal motoneurones (Evans *et al.*, 1987; Shinozaki, 1991). Therefore, we have compared the neuropharmacological properties of depolarizations induced by kainoids in spinal motoneurones and dorsal root fibres of the newborn rat.

Methods

The methods used for the electrophysiological experiments in the isolated newborn rat spinal cord were essentially similar to those described previously (Shinozaki *et al.*, 1989b). The spinal cords of 1–7 day old Wistar rats were used for the experiments. Under ether anaesthesia, the spinal cord below the thoracic part was isolated, hemisected sagitally and placed in a 0.15 ml bath perfused at a fixed flow rate of 5–6 ml min^{-1} with artificial cerebrospinal fluid (in mM: NaCl 138.6, KCl 3.4, $CaCl_2$ 1.3, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0) which was oxygenated with a gas mixture of 95% O_2 and 5% CO_2. Tetrodotoxin (TTX, 0.5 μM) was added to the bathing solution in order to block spontaneous depolarization and indirect

drug effects. In some cases, $MgCl_2$ was added to the perfusing fluid in a concentration of 1.0 mM to block the depolarizing action of N-methyl-D-aspartate (NMDA)-type agonists. The potential changes generated in the motoneurones were recorded extracellularly from the L_3-L_5 ventral root with a suction electrode. For the recording of depolarization induced in the isolated dorsal root fibre (L_3-L_5), the central end was placed inside the suction electrode which was positioned 3–4 mm from the peripheral end bathed with Ringer and drug solutions. Excitatory amino acids and other test compounds were applied to the preparation either by perfusion or by brief-pulse injection into the perfusion system at a constant duration. The temperature of the perfusing fluid was kept at 27°C.

Drugs

The following compounds were used: acromelic acids A and B generously donated by Prof. H. Shirahama, Hokkaido University), N-acetylkainate (a generous gift from the late Prof. T. Takemoto), (±)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Tocris), 5-bromowillardiine and willardiine (Tocris), 3-[(±)-2-carboxypiperazine-4-yl]propyl-1-phosphonic acid (CPP, Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris), dihydroxykainic acid (Tocris), domoic acid (Sigma), sodium L-glutamate mono (Wako), L-α-kainic acid (Sigma), N-methyl-D-aspartic acid (NMDA, Sigma), picrotoxin (Tokyo Kasei), L-quisqualic acid (purity >99.7%) isolated from the plant, *Quisqualic indica L.*

The following 4-substituted derivatives of 2-carboxy-3-pyrrolidineacetic acid were tested: 4-(2-carboxypyridine)-2-carboxy-3-pyrrolidineacetic acid (CPPA), 4-(2-carboxypyridine N-oxide)-2-carboxy-3-pyrrolidineacetic acid (CNOPA), 4-(2-hydroxymethylpyridine)-2-carboxy-3-pyrrolidineacetic acid (HMPPA), 4-(2-hydroxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (HFPA), 4-(2-methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (MFPA), 4-methylketone-2-carboxy-3-pyrrolidineacetic acid (MKPA, a generous gift from the late Prof. T. Takemoto), 4-(2-methylpyridine)-2-carboxy-3-pyrrolidineacetic acid (MPPA) and 4-phenyl-2-carboxy-3-pyrrolidineacetic acid (FPA). These derivatives except for MKPA were all generous gifts from Prof. H. Shirahama, Hokkaido University.

Results

Various depolarizing activities of kainate derivatives

Spinal motoneurones When kainate derivatives tested in the present study (Figure 1) were added to the bathing solution in various concentrations, almost all derivatives demonstrated a depolarization of the spinal motoneurones with a large variation in their depolarizing activities. Some derivatives caused

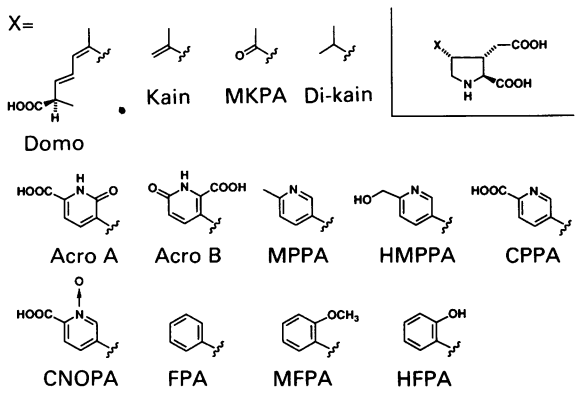


Figure 1 Chemical structure of kainate derivatives tested in the present study. Acro A: acromelic acid A; Acro B: acromelic acid B; Domo: domoic acid; Di-kain: dihydrokainic acid; Kain: kainic acid; for other abbreviations, see text.

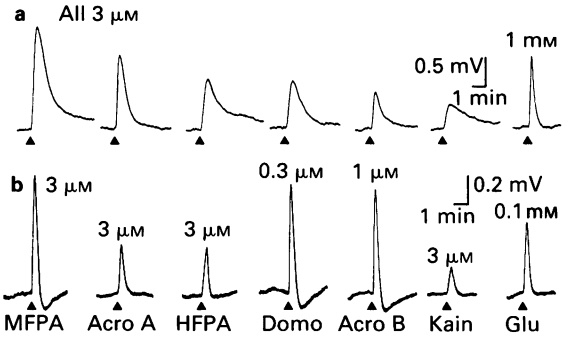


Figure 2 (a) Sample records of depolarizing responses to various kainate derivatives (3 μM) and L-glutamate (1 mM) in the newborn rat spinal motoneurone. (b) Sample records of the depolarization of the dorsal root fibre. The concentration is shown near each trace. Test samples were applied at the point of solid triangles for a period of 10 s. MFPA: 4-(2-methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid; HFPA: 4-(2-hyrodoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid; Acro A: acromelic acid A; Acro B: acromelic acid B; Domo: domoic acid; Kain: kainic acid; Glu: L-glutamate.

the depolarization much more effectively than kainic acid. 4-(2-Methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (MFPA) was the most potent among the kainoids tested here, when they were compared in terms of peak amplitudes of their depolarizing responses at the same concentration (Figure 2a). The depolarizing responses to excitatory amino acids were generally reproducible in the present spinal preparation, and could be roughly divided into two groups showing fast and slow responses (Figure 2a). Kainic acid, domoic acid, 4-2-hydroxyphenyl)-2-carboxy-3-pyrrolidineacetic acid (HFPA) and MFPA produced the relatively prolonged time course of action (slow responses), but acromelic acids A and B, quisqualate and L-glutamate gave relatively fast responses. The time course of depolarizing responses to MFPA closely resembled that of kainic acid and domoic acid. Thus, kainate derivatives tested in the present study did not always show slow responses. Figure 3 shows dose-response curves for the kainoids, and relative potencies were tentatively calculated from concentrations producing the same depolarization as kainate 5 μM (Table 1). The rank order was as follows: MFPA > acromelic acid A > domoic acid ≧ (HFPA) ≧ acromelic acid B > HMPPA ≒ CPPA ≒ kainic acid > MPPA ≒ FPA > CNOPA > MKPA. This order did not change even when the duration of drug application was prolonged to 2 min. It was difficult to determine the relative potency of excitation induced by these kanoids and excitatory amino acids in terms of the peak amplitude, because the slopes of dose-response curves for these excitatory amino acids differed significantly (Figure 3), and the peak amplitudes of depolarizing responses to excitatory amino acids sometimes varied in accordance with the duration of drug application (Shinozaki *et al.*, 1989b). This could lead to an underestimate of the more slowly developing responses relative to the fast responses.

Dorsal roots The isolated dorsal root fibre of the newborn rat demonstrated a large variety of depolarizing responses to the kainoids tested in the present study (Figure 2b). These depolarizing responses were concentration-dependent, and almost all the kainoids tested here and L-glutamate had a very short time course of action; this may be attributed in part to rapid development of receptor desensitization (see below). For the experiment shown in Figure 4, peak amplitudes of depolarizing responses to these kainoids were plotted against their concentrations under conditions quite similar to those of Figure 3. It was possible to determine the relative potency of their depolarizing activities in the dorsal root fibre, because dose-response curves had almost similar slopes, except for L-glutamate, in which peak amplitudes did not increase further at concentrations greater than 100 μM (Figure 4), probably

Table 1 Mean equimolar potency ratios of various kainate derivatives in the dorsal root fibres and motoneurones of the newborn rat

	Dorsal root fibres		*Motoneurones*	
Compound	Mean ± s.e.mean	n	Mean ± s.e.mean	n
Domoate	34 ± 2.4	8	2.2 ± 0.08	7
Acro B	13 ± 0.7	5	1.4 ± 0.04	5
Br-Willardiine	5.9 ± 0.57	4	2.3 ± 0.08	4
MFPA	4.2 ± 0.29	4	3.8 ± 0.12	6
Acro A	1.7 ± 0.08	8	2.7 ± 0.1	10
HFPA	1.3 ± 0.13	6	1.9 ± 0.12	7
Kainate	1.0	10	1.0	12
CPPA	0.91 ± 0.12	6	0.82 ± 0.04	6
HMPPA	0.45 ± 0.04	6	0.91 ± 0.04	6
CNOPA	0.29 ± 0.06	7	0.22 ± 0.02	6
FPA	0.22 ± 0.03	5	0.45 ± 0.03	5
MKPA	0.11 ± 0.02	4	0.15 ± 0.02	5
Glutamate	0.11 ± 0.01	10	0.02 ± 0.003	12
MPPA	0.045 ± 0.006	4	0.57 ± 0.05	4
Quis	0.035 ± 0.005	6	2.5 ± 0.11	8
AMPA	0.014 ± 0.005	4	1.2 ± 0.05	6
DihydroKA	<0.01	4	<0.01	4
N-acetylKA	<0.01	4	<0.01	4
NMDA	<0.01	4	0.42 ± 0.04	8
Willardiine	<0.01	4	0.37 ± 0.04	4

Molar potency ratios, relative to kainate, for depolarization of dorsal root fibres and motoneurones of the newborn rat were determined on at least four preparations for each test compound. The parallel displacement of responses from the line plotted for kainate 5 μM on a log concentration scale gave the potency ratios. For full names see Figure 3 legend.

because of the development of receptor desensitization. The rank order of depolarizing activities in the dorsal root fibres was different from that in the spinal motoneurones. Domoic acid and acromelic acid B demonstrated the highest potency

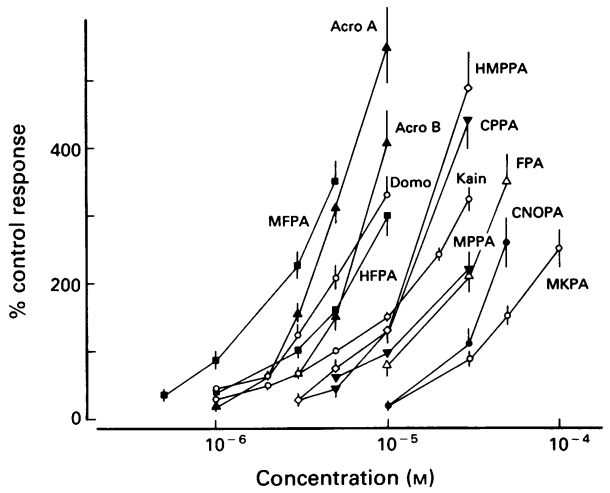


Figure 3 Concentration-depolarization relationships for various kainate derivatives in the newborn rat spinal motoneurone. Peak amplitudes of depolarizing responses to agonists (10 s application) were plotted against their concentrations. Responses from individual preparations have been normalized, so that results are expressed as a percentage of the control depolarization to 5 μM kainic acid. Vertical lines represent standard error of means (s.e.mean; *n* at least 4), MFPA: 4-(2-methoxyphenyl)-2-carboxy-3-pyrrolidineacetic acid; Acro A: acromelic acid A; Acro B: acromelic acid B; Domo: domoic acid; HFPA: 4-(2-hydroxyphenyl)-2-carboxy-3-pyrrolidineacetic acid; HMPPA: 4-(2-hydroxymethylpyridine)-2-carboxy-3-pyrrolidineacetic acid; CPPA: 4-(2-carboxypyridine)-2-carboxy-3-pyrrolidineacetic acid; Kain: kainic acid; MPPA: 4-(2-methylpyridine)-2-carboxy-3-pyrrolidineacetic acid; FPA: 4-phenyl-2-carboxy-3-pyr-rolidineacetic acid; CNOPA: 4-(2-carboxypyridine N-oxide)-2-carboxy-3-pyrroli-dineacetic acid; MKPA: 4-methylketone-2-carboxy-3-pyrroli- dineacetic acid.

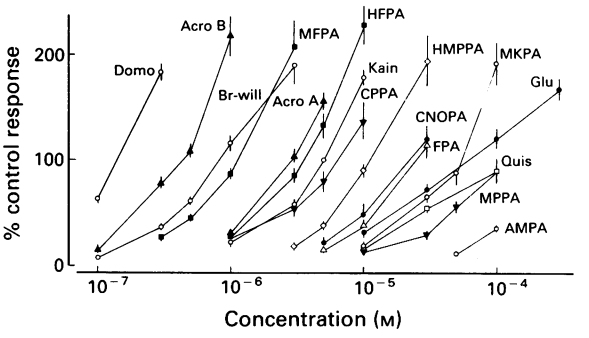


Figure 4 Concentration-depolarization relationships for various kainate derivatives in the dorsal root fibre of the newborn rat. Peak amplitudes of depolarizing responses to agonists (10 s application) were plotted against their concentrations (results were normalized to that of kainate 5 μM). Vertical lines represent s.e.mean (*n* at least 4). Abbreviations and experimental conditions are the same as those in Figure 3.

in the dorsal root fibre. AMPA was much less active than kainic acid in causing depolarization of the dorsal root fibre, and NMDA did not cause any depolarization even at a high concentration of 100 μM. Similar results in the rat dorsal root fibre have been obtained by Agrawal & Evans (1986) with AMPA and NMDA. The rank order of the depolarizing activity in the dorsal root fibre is: domoic acid > acromelic acid B > 5-bromowillardiine ≧ MFPA > acromelic acid A > HFPA > kainic acid > CPPA > HMPPA > CNOPA ≒ FPA ≧ MKPA = L-glutamate > MPPA. Table 1 presents their relative depolarizing activities in the dorsal root fibre as compared with those in spinal motoneurones.

When L-glutamate and the kainoids tested here were added to the perfusion fluid for a period of more than 30 s, the amplitude of their depolarizing responses was not maintained, but rapidly declined despite the presence of these excitatory amino acids. The decrease in amplitude of depolarizing responses to these kainoids during their application appears to be due to desensitization. Significant desensitization seemed to be one of the characteristics of kainate receptors on the dorsal root fibres of the newborn rat. L-Glutamate induced desensitization of the receptor more markedly than kainate and other kainoids and onset of desensitization induced by L-glutamate seemed more rapid than that by the kainoids tested here (Figure 5). On the other hand, in the newborn rat spinal motoneurones, desensitization was hardly detectable even after prolonged application of high concentrations of kainic acid or L-glutamate. After prolonged application of kainic acid to the spinal motoneurones, the amplitude of depolarization induced by acromelic acid A did not decrease but rather increased in an additive manner.

Cross desensitization between kainate derivatives

Since significant desensitization of kainate receptors developed in the dorsal root fibres after prolonged application of L-glutamate or kainate derivatives, it seemed relatively easy to test the development of cross desensitization. Cross desensitization induced by various excitatory agonists would provide evidence that they activate common receptors. As shown in Figure 5, cross desensitization was observed between L-glutamate (1 mM) and kainic acid (5 μM), and between kainic acid (100 μM) and acromelic acid A (5 μM). Similar results were obtained between domoic and kainic acids and between MFPA and kainic acid; however, even after the development of receptor desensitization induced by kainate or L-glutamate, γ-aminobutyric acid (GABA) (5 μM) still caused normal responses of the dorsal root fibre. At high concentrations, quisqualate and AMPA caused a slight depolarization of the dorsal root fibre, but after the receptor was desensitized by

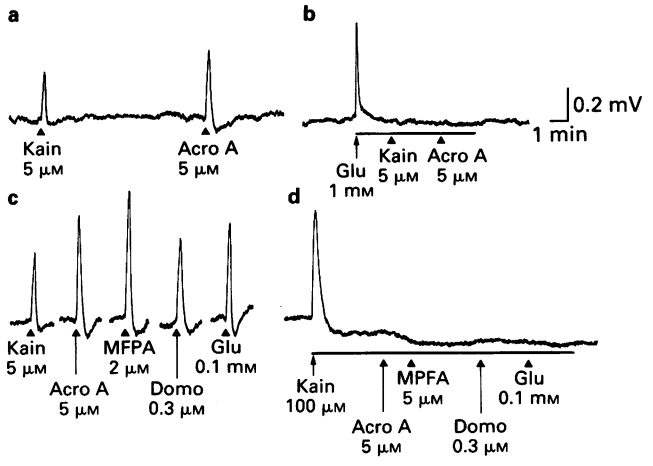


Figure 5 Desensitization of receptors on the dorsal root fibre. (a) Control responses to kainic acid and acromelic acid A (10 s application, 5 μM). (b) No further depolarization occurred by kainate or acromelic acid A after desensitization induced by L-glutamate. (c) Control responses to various kainate derivatives and L-glutamate (10 s application). (d) No responses after development of kainate-induced desensitization. The concentration was shown near each response. Abbreviations are the same as in Figure. 3.

kainate, they did not cause any further depolarization. Therefore, quisqualate, AMPA and all kainoids seemed to act at the same receptor on the dorsal root fibre. When MFPA or HFPA was applied immediately after development of kainate-induced desensitization of kainate receptors, further additional responses were not observed suggesting that MFPA and HFPA activated receptors in common with kainate in the dorsal root fibre.

Depression of kainoid-induced depolarization by CNQX but not by CPP

It has been shown that CNQX depresses depolarizing responses to kainate- and quisqualate-type agonists (Honore *et al.*, 1988), and CPP is a selective NMDA antagonist (Davies *et al.*, 1986). Therefore, it was predicted that the kainoid-induced depolarization would not be affected by CPP but depressed effectively by CNQX. In fact, CPP, in concentrations ranging from 2 to 100 μM, hardly depressed amplitudes of depolarization induced by the kainoids tested here in the newborn rat spinal motoneurones, and magnesium ions (1 mM) also did not affect the response to these kainoids. On the other hand, CNQX was quite effective in reducing

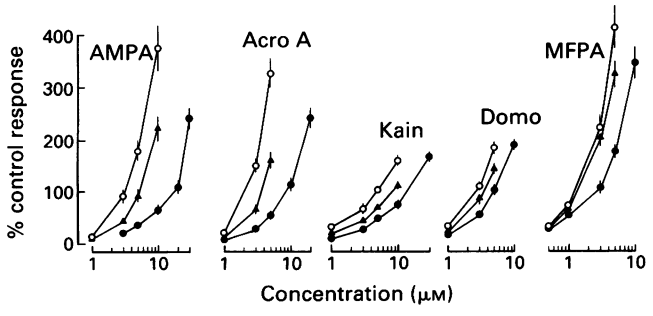


Figure 6 Dose-response curves for various kainate derivatives in the absence and presence of CNQX in the newborn rat spinal motoneurones. Peak amplitudes of depolarizing responses to AMPA, kainate, acromelic acid A, domoic acid and MFPA were plotted against their logarithmic concentrations in the absence and presence of 1 and 5 μM CNQX (results were normalized to that of kainate 5 μM). Vertical lines represent s.e.mean (n at least 4). Control (○); CNQX 1 μM (▲); CNQX 5 μM (●). Abbreviations are the same as in Figure 3.

kainoid-induced depolarization (Figure 6), but there were some differences in the inhibitory action of CNQX on depolarizing responses to the kainoids, particularly in the case of relatively low concentrations of CNQX. CNQX at a concentration of 1 or 5 μM depressed more effectively depolarizing responses to acromelic acids A and B, AMPA and 5-bromowillardiine than those to kainic acid, domoic acid, MFPA and HFPA in the newborn rat spinal motoneurone (Figure 6). The order of depression of agonist-induced responses with CNQX as an antagonist was as follows: AMPA (1) $\fallingdotseq$ 5-bromowillardiine (1) > acromelate A (1.2) > acromelate B (2.0) > kainate (2.3) > MFPA (3.2) $\fallingdotseq$ HFPA (3.4) $\geqq$ domoate (3.7). The numbers in parentheses represent the dose of CNQX, relative to AMPA, for causing the same depression of depolarization (N at least 4, calculated from the pA_2 value). On the other hand, the dorsal root fibre, depolarizing responses to 5-bromowillardiine and the kainoids tested here were almost equally depressed by CNQX, but quite insensitive to selective NMDA blockers, suggesting a single receptor type on dorsal root fibres.

Discussion

In the present study, both HFPA and MFPA showed potent depolarizing activities in the dorsal root fibre and the spinal motoneurone. In our previous examinations, the rank order of IC_{50} for displacement of [^{3}H]-kainate binding in the adult rat spinal cord was as followed: domoate > HFPA > kainate $\fallingdotseq$ MFPA > quisqualate > acromelic acid B > L-glutamate > acromelic acid A $\gg$ NMDA = AMPA, and that of [^{3}H]-AMPA was quisqualate > AMPA > MFPA $\fallingdotseq$ acromelic acid A > L-glutamate > HFPA > domoate > kainate $\gg$ NMDA. MFPA and HFPA demonstrated high binding affinities for kainate receptors in the rat spinal cord, substantially comparable to kainate or domoate, but acromelic acid A showed a considerably lower affinity for kainate receptors than kainate, and it was of particular interest that acromelic acid A and MFPA possessed valuable affinities for AMPA receptors. The AMPA receptor may exist in two forms, one of which has high affinity for AMPA and quisqualate, while the other has higher affinity for kainate but is distinct from the kainate receptor (Watkins *et al.*, 1990a).

The difference in depolarizing activities between dorsal root C fibres and motoneurones of the newborn rat would provide some useful information for elucidating the mechanism of activation of kainate receptors (Agrawal & Evans, 1986; Evans *et al.*, 1987; Shinozaki, 1991). Dorsal root fibres of the newborn rat are depolarized selectively by kainate and its derivatives (Agrawal & Evans, 1986; Shinozaki, 1991). Some excitatory amino acids, however, that are not related structurally to kainate, including 5-bromowillardiine (Agrawal & Evans, 1986) and some derivatives of 2-(carboxycyclopropyl)glycine (CCG) (Ishida *et al.*, 1991), also cause considerable depolarization of the dorsal root fibre, and 5-bromowillardiine is much more potent than kainate in causing depolarization of the dorsal root fibre. Therefore, they have been regarded as kainate-type agonists (Watkins *et al.*, 1990a; Ishida *et al.*, 1991).

The present work revealed that MFPA was so far the most potent among the kainoids in the newborn rat spinal motoneurones; however, it was not superior to domoic acid in the dorsal root fibre. The depolarizing activity of MFPA in the dorsal root fibre was almost equal to that of 5-bromowillardiine. Acromelic acid B was significantly more potent than acromelic acid A in the dorsal root fibre unlike the spinal motoneurone. Domoic acid and MFPA were almost equally sensitive to CNQX in both the dorsal root fibre and the motoneurone, and their pharmacological properties seemed quite similar in quality in both preparations. However, there was a difference in the depolarizing activity of the kainoids between both preparations; depolarizing

responses to acromelic acids A and B were significantly depressed by CNQX in the spinal motoneurone in a manner similar to that of AMPA, while kainate and domoate were a little more resistant to CNQX. Watkins *et al.* (1990b) have obtained similar results using acromelic acid A, AMPA, kainate and domoate in the neonatal rat spinal motoneurones. Significant desensitization of kainate receptors was observed in the dorsal root fibre (Agrawal & Evans, 1986) and the dorsal root ganglion (Huettner, 1990) while it did not occur in the spinal motoneurone. Therefore, the difference in degree of development of receptor desensitization between both preparations may play a key role for a difference in the rank order of the depolarizing activity, but the existence of different types of kainate receptors is also strongly suggested. Multiplicity of kainate receptors based on their permeability to Ca^{2+} is also proposed from electrophysiological evidence on cultured rat hippocampal neurones (Iino *et al.*, 1990). In addition, recent studies on cDNA for glutamate receptors suggest the presence of a family of kainate receptor subunits with regional difference in the rat brain and with differential potencies of some non-NMDA agonists (Boulter *et al.*, 1990; Keinanen *et al.*, 1990; Sommer *et al.*, 1990; Egebjerg *et al.*, 1991; Hollmann *et al.*, 1991; Werner *et al.*, 1991).

Systemic administration of acromelic acid A to the rat causes persistent spastic paraplegia after demonstrating tonic extension of the hindlimbs followed by transient flaccid paralysis, without limbic seizures or 'wet-dog-shakes' (WDS), and induces specific lesions of interneurones in the lower spinal cord with little or no damage in the hippocampal neurones (Shinozaki *et al.*, 1989a; 1991; Shinozaki, 1991; Kwak *et al.*, 1990; 1991). Kainate never causes tonic extension of the hindlimbs or spastic paraplegia. Thus, acromelic acid A and kainic acid demonstrate quite distinct behavioural signs and regional differences in neuronal damage when administered systemically. In our preliminary experiments in the rat, MFPA induced interesting behavioural signs, both tonic extension of the hindlimbs and limbic seizures, which were characteristic of acromelic acid A and kainic acid respectively, suggesting at least two types of kainate receptors (Shinozaki *et al.*, 1990; 1991). Therefore, the kainoids tested here would be expected to provide a useful addition to known kainoids as a valuable tool for examination of supposed subtypes of kainate receptors.

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Staurosporine inhibits the extent of acetylcholine receptor recovery from carbachol-induced desensitization in snake twitch fibres

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1 The effect of the protein kinase inhibitor, staurosporine, on the extent and time course of recovery following carbachol-induced desensitization was studied in snake twitch-muscle fibres maintained in an isotonic potassium propionate solution and voltage-clamped to +30 mV.

2 Pretreatment with staurosporine (0.5 μM) decreased the extent of recovery of spontaneous miniature endplate current (m.e.p.c.) amplitudes following desensitization by a sustained application of 540 μM carbachol. Recovery was inhibited by approximately 50% without altering the time course of m.e.p.c. recovery.

3 Staurosporine also produced a concentration-dependent (10 nM to 0.5 μM) decrease in the amplitude of a second carbachol-induced current, following a wash period, as compared to the amplitude of the current produced by the initial carbachol application. Pretreatment with 0.5 μM K252a, another wide spectrum protein kinase inhibitor, also decreased the extent of recovery of the response to a second carbachol application following desensitization.

4 Staurosporine pretreatment (0.5 μM) had no effect on either the kinetics of receptor-channel gating or the initial endplate sensitivity to agonist. This was determined by comparing the amplitude of the carbachol (540 μM)-induced currents and the amplitude and decay rate of m.e.p.cs in control and staurosporine-treated fibres.

5 Staurosporine had no effect on the time course of desensitization onset produced during the initial application of 540 μM carbachol or the depth of desensitization produced by the end of a 2–3 min exposure to 540 μM carbachol.

6 Elevation of the external calcium concentration from 1 to 10 mM during the 540 μM carbachol application completely antagonized the decreased extent of recovery of m.e.p.c. amplitude produced by pretreatment with 0.5 μM staurosporine.

7 We suggest that phosphorylation of a population of acetylcholine receptors is required for complete recovery from desensitization, and that staurosporine inhibits the protein kinases responsible for this phosphorylation.

8 We further propose that a transient increase in intracellular calcium, produced by an increase in calcium influx through agonist-activated endplate channels, stimulates additional protein kinase activity, which in turn, antagonizes the effect of staurosporine-treatment on recovery.

Keywords: Protein kinase inhibitor; carbachol; nicotinic receptor; protein phosphorylation; calcium

Introduction

Desensitization of the nicotinic acetylcholine receptor (ACh receptor) is the decrease in receptor chemosensitivity produced during prolonged exposure to cholinoceptor agonists (Katz & Thesleff, 1957). Although desensitization is an intrinsic property of the ACh receptor, many environmental factors can alter the rate of desensitization onset, including membrane voltage (Magazanik & Vyskocil, 1970; Fiekers *et al.*, 1980; Cachelin & Colquhoun, 1989), calcium (Manthey, 1966; Nastuk & Parsons, 1970; Fiekers *et al.*, 1980), neurotransmitters and other drugs (Ochoa *et al.*, 1989) and receptor phosphorylation (Huganir & Greengard, 1990).

In contrast, much less is known about the possible factors regulating the recovery from desensitization. In particular, no previous studies have investigated the role of phosphorylation in recovery.

Consequently, this study was undertaken to determine if known inhibitors of protein kinases altered the time course or extent of receptor recovery from carbachol-induced desensitization. In these initial studies, the inhibitor staurosporine was chosen because it is known to inhibit both protein kinase C and adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase in numerous tissues (Tamaoki *et al.*, 1986). Both of these kinases are known to phosphorylate the acetylcholine receptor molecule (Huganir & Greengard, 1987; 1990) and phosphorylation by these kinases appears to increase the rate of desensitization onset in many preparations including chick myotubes (Eusebi *et al.*, 1987a), isolated *Torpedo* receptors (Huganir *et al.*, 1986; Hopfield *et al.*, 1988) and rat soleus muscle endplates (Middleton *et al.*, 1986).

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Methods

General

All experiments were done on voltage-clamped twitch fibre endplates in the costocutaneous muscle of the garter snake (*Thamnophis*). The method of dissection and the criteria used for identification of twitch fibres were similar to those published previously (Connor *et al.*, 1984; Fiekers *et al.*, 1987). The muscle preparations were dissected in a HEPES-buffered physiological salt solution (composition in mM: NaCl 159, KCl 2.5, $CaCl_2$ 1.0, $MgCl_2$ 4.2 HEPES (N-2-hydroxy-

ethylpiperazine-N'-2-ethanesulphonic acid) 1.0; pH 7.2) and then bathed in an isotonic HEPES-buffered potassium propionate solution consisting of (in mM): potassium propionate 161, $CaCl_2$ 1.0, $MgCl_2$ 4.2, $CsCl_2$ 5.0, HEPES 1.0, pH 7.2; 20–23°C. The advantage of the potassium-depolarized preparation is that during exposure to agonist, muscle contraction is eliminated in adjacent fibres which are not voltage-clamped. Further, we have shown that depolarized muscle fibres can be voltage-clamped over a wide range of potentials with only one species of monovalent cation (potassium) contributing significantly to the miniature endplate current (m.e.p.c.) and carbachol (CCh)-induced current ($e.p.c._{CCh}$). We also have shown that the kinetics of receptor channel gating and desensitization are similar to those found in preparations maintained in a sodium containing solution (Dionne & Parsons, 1981; Connor *et al.*, 1984).

The endplate region of an individual muscle fibre was voltage clamped by use of a two microelectrode voltage-clamp system similar to that employed in previous studies (Fiekers *et al.*, 1980; Connor *et al.*, 1984; Fiekers *et al.*, 1987). Carbachol (540 μM, Sigma Chemical Co., St Louis, MO, U.S.A.) and rinsing solutions were applied locally to the endplate region of individual voltage-clamped fibres by hydrostatically controlled microperfusion (Fiekers *et al.*, 1980; Connor *et al.*, 1984). Because the neuromuscular junction of the snake twitch fibre is very compact, agonist concentration equilibrates quickly and effective voltage control can be maintained over the entire endplate region during agonist application (Connor *et al.*, 1984).

In most experiments, m.e.p.cs or agonist-induced currents were recorded from muscle fibres voltage-clamped to +30 mV for the following reasons. First, at positive membrane potentials, the peak carbachol-induced current ($e.p.c._{CCh}$) amplitude is less than that produced at negative potentials which allows for better voltage control over a wider range of agonist concentrations (Connor *et al.*, 1984; Colquhoun & Ogden, 1988). Second, the fast rate of desensitization (time constant in seconds) is slowed at positive membrane potentials (Fiekers *et al.*, 1980; Cachelin & Colquhoun, 1989). This is important because the concentration change at the receptor with the microperfusion application technique is diffusion limited. Therefore, any truncation of the maximum response occurring because some receptors are being desensitized while others are being activated should be minimized. Lastly, high concentrations of agonist were necessary to promote rapid and complete desensitization and to avoid prolonged periods of voltage clamping for an individual endplate. High concentrations of agonist are known to cause channel blockade (Sine & Steinbach, 1984). However, because channel blockade is a voltage-dependent process, it does not occur as readily at positive membrane potentials and therefore should not be a factor complicating the interpretation of these results.

Muscles were pretreated with the protein kinase inhibitors, staurosporine or K252a (Calbiochem, San Diego, CA, U.S.A.) for 10–15 min in most experiments. The inhibitors were first dissolved in dimethyl sulphoxide (DMSO) and then diluted to the final concentration with potassium propionate solution. The drug was then added directly to the bathing solution. In a few experiments, staurosporine was perfused directly onto the endplate to test for immediate effects of the inhibitor on m.e.p.c. characteristics. Control experiments were done with 0.2% DMSO added to the bathing solution.

Measurement of spontaneous miniature endplate currents

M.e.p.c. amplitude and decay time constant were determined in some experiments. An individual endplate was voltage-clamped to either +30 mV or −100 mV and the m.e.p.cs recorded and collected on a FM recorder for subsequent analysis. Records were analyzed by the computer programme SCAN kindly provided by John Dempster, University of Strathclyde. Currents were averaged in time blocks of 5–30 s

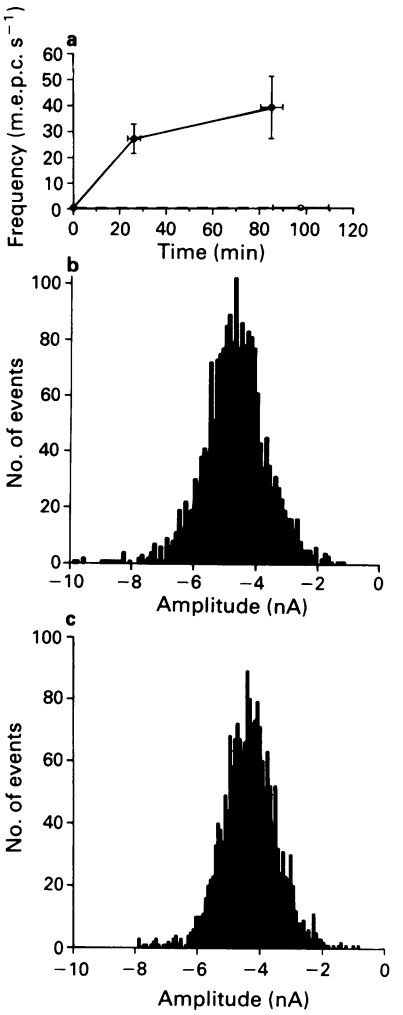


Figure 1 Results showing that m.e.p.c. frequency increased during exposure to the isotonic potassium propionate solution containing 1 mM calcium and 4.2 mM magnesium, but that the m.e.p.c. amplitude distribution remained similar to that obtained in the normal sodium containing solution: (a) shows the averaged frequency of m.e.p.cs recorded at different endplates (indicated by the solid line) during sustained exposure to the potassium propionate solution. The dashed line gives the averaged m.e.p.c. frequency obtained from control fibres over the same time period. The values represent mean for both the frequency and time of recording; bars show s.e.mean. The error bar for the control points was within the circle. The fibres were voltage-clamped to −150 mV. (b and c) Show the amplitude distribution of m.e.p.cs recorded at a control endplate maintained in the normal sodium solution (b) and another fibre kept in the isotonic potassium propionate solution for 60 min (c). The m.e.p.cs were recorded with the fibres voltage-clamped to −150 mV.

to determine the peak m.e.p.c. amplitude. The estimation of the time constant for the decay of the m.e.p.c. was determined by the method previously described (Connor *et al.*, 1984) at negative voltages, where both the amplitude and the duration of the m.e.p.c. are increased, allowing more accurate kinetic analysis.

The frequency of m.e.p.cs was increased in the potassium-depolarized preparations (Figure 1a) above that occurring in preparations maintained in control sodium solution ($0.38 \pm 0.09\,s^{-1}$). The elevated frequency made it possible to record many m.e.p.cs during short time periods. However, because 4.2 mM magnesium and only 1 mM calcium was present in the solution, the increased frequency was not high enough to deplete the terminals (Coniglio & Parsons, unpublished electron microscopic study). Further, the amplitude distribution of m.e.p.cs recorded after 1–2 h in the isotonic potassium solution containing 4.2 mM magnesium and 1.0 mM

calcium and voltage-clamped to −150 mV was the same as that found for m.e.p.cs recorded in the normal sodium solution (Figure 1b). Consequently, under the conditions of these experiments, there was no evidence of a population of small-mode m.e.p.cs (Searl *et al.*, 1990).

Measurement of the rate of desensitization onset and the recovery of sensitivity

Individual endplates were voltage-clamped to +30 mV and perfused with 540 μM carbachol for 2–3 min. During exposure to agonist at +30 mV, a transient outward current developed which peaked within a few seconds and then slowly decayed toward baseline. The peak value of the $e.p.c._{CCh}$ was used to estimate the extent of activation (Fiekers *et al.*, 1980; Connor *et al.*, 1984) and the time course of decline of the current from the peak towards the baseline was taken to indicate the time course of desensitization onset (Fiekers *et al.*, 1980; Connor *et al.*, 1984). The time constants for the rate of desensitization onset were determined by digitizing the current trace, normalizing to the peak current value, and fitting the decay portion of the curve using a non-linear curve fitting programme, to give the best fit with either a single or double exponential expression (Connor *et al.*, 1984).

Recovery was determined in two different ways. In the first method, the time course of recovery of m.e.p.c. amplitude was determined following desensitization (Magleby & Pallotta, 1981). To do this, an endplate was clamped to +30 mV and m.e.p.cs collected to obtain a mean amplitude. The endplate was then perfused with 540 μM carbachol for 2–3 min to allow desensitization to proceed. When the $e.p.c._{CCh}$ had plateaued, indicating desensitization had reached a steady state level (no m.e.p.cs were observed during this period), the endplate was then perfused with agonist-free, potasssium propionate solution and the return of m.e.p.cs continuously collected for many minutes.

In the second method, at different time intervals ranging from 5 to 15 min following the initiation of the wash period, the endplate was then re-perfused with 540 μM carbachol and the activation-desensitization sequence recorded again (Scubon-Mulieri & Parsons, 1977; 1978). The extent of recovery was expressed as the peak current recorded in the second agonist exposure ($e.p.c._{CCh2}$) as a percentage of the peak current recorded in the initial exposure to carbachol ($e.p.c._{CCh1}$).

Estimate of the depth of desensitization produced at the end of the carbachol application

The equilibrium level of desensitization reached at the end of a sustained agonist application was determined by a double perfusion technique (Scubon-Mulieri & Parsons, 1978; Fiekers *et al.*, 1980). Individual fibres, voltage clamped to +30 mV, were perfused with 540 μM carbachol for 2–3 min, at which time the current had reached a plateau value, suggesting that desensitization had reached an equilibrium level. The endplate was then perfused with 5.4 mM carbachol, a concentration which should activate any nondesensitized, activatable receptors (Connor *et al.*, 1984). The resulting current was expressed as a percentage of the current amplitude reported previously for 5.4 mM carbachol in the absence of prior desensitization (Connor *et al.*, 1984). This value indicated the percentage of ACh receptors which were not desensitized at the end of the few minute exposure to carbachol.

Statistical analyses

All results are expressed as the mean ± s.e.mean obtained in a number of different fibres. Statistical significance was determined by Student's *t* test or analysis of variance with a $P < 0.05$ considered significant.

Results

Staurosporine decreased the extent of recovery from carbachol-induced desensitization

Our initial experiments demonstrated that pretreatment with staurosporine significantly decreased the extent of receptor recovery from carbachol-induced desensitization. In these experiments, endplate sensitivity was determined by measuring m.e.p.c. amplitude before and following a prolonged exposure to 540 μM carbachol, a concentration known to desensitize twitch fibre endplates within a few minutes exposure (Connor *et al.*, 1984). M.e.p.cs were collected from endplates voltage-clamped to +30 mV, prior to perfusion with 540 μM carbachol for 2–3 min and then continuously, following the initiation of a local application of carbachol-free potassium propionate solution. The m.e.p.cs were rapidly and completely abolished during the exposure to carbachol. In untreated endplates, m.e.p.c. amplitude recovered to approximately 90% of the pre-carbachol amplitude within 30–60 s following termination of carbachol application (Figure 2a). Similar experiments were done using preparations pretreated

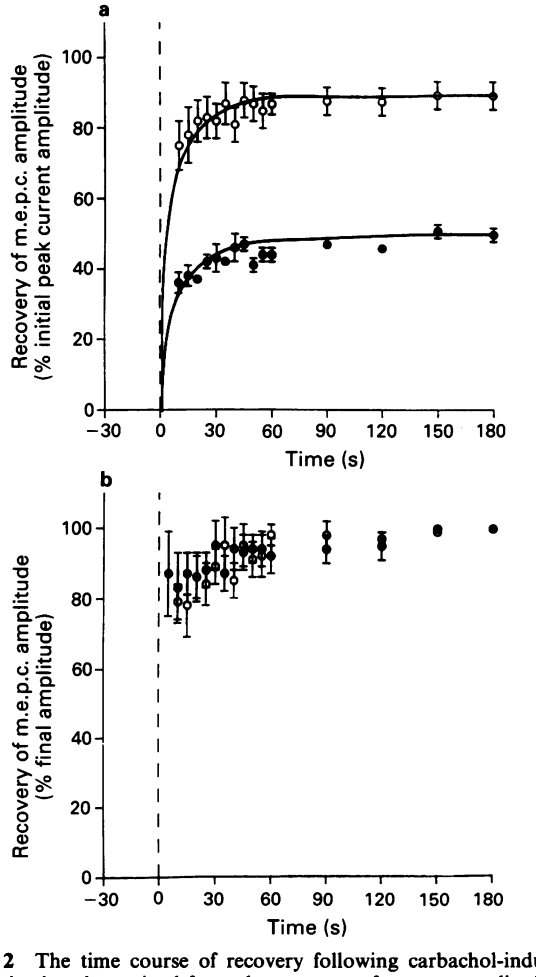


Figure 2 The time course of recovery following carbachol-induced desensitization determined from the recovery of m.e.p.c. amplitude at control and 0.5 μM staurosporine pretreated endplates. Preparations were pretreated with either potassium propionate solution containing 0.2% dimethylsulphoxide (○) or 0.5 μM staurosporine (●) for 10–15 min. Individual endplates were voltage clamped to +30 mV and m.e.p.cs recorded prior to perfusion with 540 μM carbachol and then following the initiation of perfusion with carbachol-free potassium propionate solution (onset indicated by the dashed vertical line). In (a) averaged values are shown which are expressed as a percentage of the pre-perfusion amplitude obtained for each individual endplate. The values are the mean from 3–5 endplates; vertical bars show s.e.mean. In (b) the time course of recovery is shown when the values are expressed as a percentage of the final amplitude of recovery.

with 0.5 μM staurosporine for 10–15 min and then voltage-clamped to +30 mV. At the staurosporine-treated endplates, m.e.p.c. amplitude also recovered rapidly following removal of the carbachol. However, the extent of the amplitude recovery was only 46% of the pre-prefusion value within 30–60 s (Figure 2a) and the amplitude of the m.e.p.cs did not increase any further at longer intervals up to 10 min (data not shown).

Although the final extent of recovery was less at the staurosporine-treated endplates, there was no marked difference in the time course of the recovery from desensitization to its final extent between control and staurosporine-treated endplates. This was evident when the time course of recovery of m.e.p.c. amplitude was expressed as a percentage of the amplitude at 3 min (Figure 2b).

The decrease in m.e.p.c. amplitude during recovery at staurosporine-treated fibres was not a presynaptic effect

Experiments were done to demonstrate that the effect of staurosporine on the recovery of the m.e.p.c. amplitude was not due to a presynaptic effect of staurosporine which reduced quantal size. In these experiments, individual endplates from control preparations or preparations pretreated with 0.5 μM staurosporine were voltage-clamped to +30 mV and then locally perfused with 540 μM carbachol. The carbachol-induced current ($e.p.c._{CCh1}$) developed within 1–3 s, peaked and then declined gradually to a plateau level. After 2–3 min, the carbachol application was terminated and the endplate washed by local perfusion with carbachol-free potassium propionate solution for 5 to 15 min to allow recovery to occur. After 5, 10 or 15 min, the current generated during a second application of 540 μM carbachol ($e.p.c._{CCh2}$) was determined. The extent of recovery was estimated from the ratio $e.p.c._{CCh2}/e.p.c._{CCh1}$. In the control fibres, the peak amplitude of $e.p.c._{CCh2}$ reached approximately 90% of the peak amplitude of $e.p.c._{CCh1}$ by 10 min of recovery and remained at this value at longer times (Figure 3). In contrast, in the staurosporine-treated fibres, after approximately 5 min of recovery, the amplitude of $e.p.c._{CCh2}$ was approximately 45% of that produced in the initial application and did not increase further with longer periods of recovery (Figure 3).

We used this dual application technique to determine the concentration-dependence of the staurosporine-induced decrease in extent of recovery. Preparations were pretreated with concentrations of staurosporine ranging from 10 nM to 0.5 μM for 10–15 min. Endplates were then voltage-clamped to +30 mV, perfused with 540 μM carbachol, washed for 10 min, and re-perfused with carbachol. The extent of recovery was expressed as the ratio of $e.p.c._{CCh2}$ to $e.p.c._{CCh1}$. The results presented in Figure 4 show that staurosporine inhibited the extent of recovery in a concentration-dependent manner.

Additional experiments using the dual application procedure were done to test whether another widely used protein kinase inhibitor, K252a, could decrease the extent of recovery from desensitization. In four experiments, we found that in

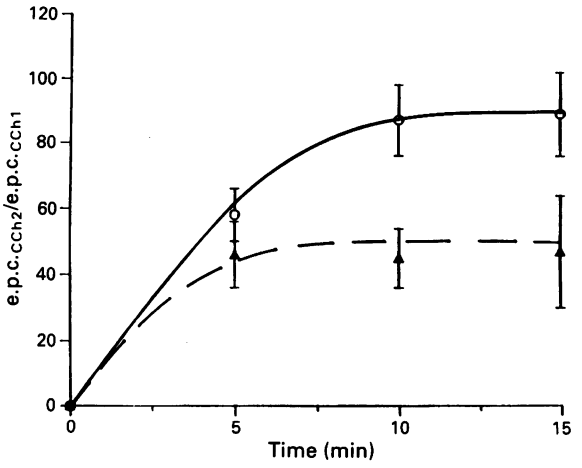


Figure 3 Comparison of the extent and time course of recovery following carbachol-induced desensitization at control fibres (○) and fibres pretreated with 0.5 μM staurosporine (▲). Individual endplates were voltage-clamped to +30 mV, perfused with 540 μM carbachol for 1–2 min, washed with agonist-free potassium propionate solution for times ranging from 5 to 15 min, and then re-perfused with 540 μM carbachol. The extent of recovery is expressed as the ratio of $e.p.c._{CCh2}$ to $e.p.c._{CCh1}$. Values represent the mean of 4 to 8 fibres; s.e.mean shown by vertical bars.

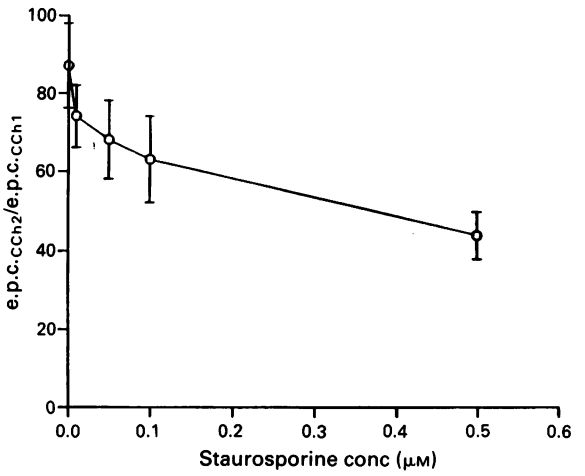


Figure 4 Results demonstrating the concentration-dependence of staurosporine-induced inhibition of the extent of recovery following carbachol-induced desensitization. Preparations were pretreated with concentrations of staurosporine ranging from 10 nM to 0.5 μM for 10–15 min. Endplates were voltage-clamped to +30 mV, perfused with 540 μM carbachol, washed with potassium propionate solution for 10 min, and then re-perfused with carbachol. The extent of recovery is expressed as the ratio of $e.p.c._{CCh2}$ to $e.p.c._{CCh1}$. Values shown are the mean of 5–8 fibres; vertical bars show s.e.mean.

Table 1 Effects of 0.5 μM staurosporine on peak endplate current, miniature endplate current amplitude and the time constant for miniature endplate current decay in snake twitch fibres

	+30 mV		−100 mV	
	$e.p.c._{CCh1}$ (nA)	m.e.p.c. (nA)	m.e.p.c. (nA)	τ (ms)
Control	269 ± 21 (21)	1.31 ± 0.13 (9)	−5.35 ± 0.28 (5)	1.61 ± 0.11 (5)
Staurosporine	247 ± 31 (15)	1.20 ± 0.17 (11)	−5.91 ± 0.65 (5)	1.38 ± 0.08 (5)

Preparations were pretreated with either potassium propionate solution containing 0.2% DMSO (control), or potassium propionate containing 0.5 μM staurosporine. Values represent the mean ± s.e.mean for *n* fibres (shown in parentheses). There were no significant differences between control and staurosporine-treated fibres for any of the parameters measured.
$e.p.c._{CCh1}$: peak amplitude of the carbachol-induced endplate current at +30 mV.
m.e.p.c: peak amplitude of the average miniature endplate current at the voltage indicated.
τ: time constant for the exponential decay of the average miniature endplate current.

fibres pretreated with 0.5 μM K252a there was a 53.2 ± 14.4% reduction in the extent of recovery of $e.p.c._{CCh2}$ after a 10 min wash following the 540 μM carbachol-induced desensitization.

The decrease of recovery was not due to an effect of staurosporine on receptor activation

Neither the amplitude of the initial carbachol-induced current nor the amplitude or kinetics of m.e.p.cs recorded in the absence of carbachol were altered by staurosporine treatment. The peak amplitude of m.e.p.cs and $e.p.c._{CCh}$ recorded at endplates voltage-clamped to +30 mV were not significantly different in control fibres or fibres pretreated with 0.5 μM staurosporine (Table 1).

We also found that the local perfusion of endplates with 0.5 μM staurosporine had no effect on the amplitude or decay characteristics of m.e.p.cs recorded at −100 mV (Table 1). M.e.p.c. duration was too brief at endplates voltage-clamped to +30 mV to determine accurately the decay rate so that these experiments were done with fibres voltage-clamped to negative potentials.

The staurosporine-induced decrease in extent of recovery was not due to a change in desensitization time course or the depth of desensitization produced by the sustained exposure to carbachol

We have also analyzed the time course of decline of the $e.p.c._{CCh}$ at control and staurosporine-treated endplates to test whether desensitization onset during the initial exposure to carbachol was altered by drug treatment. The carbachol-induced currents recorded in different fibres were digitized and normalized to the peak amplitude for each current. These normalized records were averaged and then fit either as a single or double exponential function to estimate the time constant(s) for current decay, which was used to indicate the time course of desensitization onset (Fiekers *et al.*, 1980; Connor *et al.*, 1984). The decay time course in approximately 50% of the control cells was adequately fitted as a single exponential function whereas, in the remaining control cells, two exponentials were required for an adequate fit. In contrast, in all of the staurosporine-treated cells, the decay was adequately fitted as a single exponential function. Because of this difference in fitting requirements, it was not possible simply to compare the time constant values for the current decay in the control and staurosporine-treated fibres. Therefore, we averaged the digitized, normalized decay phase of both the control currents and currents recorded from staurosporine-treated fibres. For these averaged currents from both the control fibres and staurosporine-treated fibres, voltage-clamped to +30 mV, the current decay during the initial sustained carbachol perfusion could be adequately fitted as a single exponential function. The estimated time constant for the decay phase of the averaged currents was not markedly different for the 12 control fibres (20.5 s) and the 16 fibres pretreated with staurosporine (25.0 s) (Figure 5). These results indicated that the time course of desensitization produced by this method of carbachol application was not different at control and staurosporine-treated fibres.

The decrease in the extent of recovery at staurosporine-treated endplates also was not due to a difference in the depth of desensitization produced at the end of the 540 μM carbachol application. To estimate the extent of desensitization produced by a 2–3 min exposure to 540 μM carbachol, control endplates and staurosporine-treated endplates, voltage-clamped to +30 mV, were locally perfused with carbachol until the current plateaued (2–3 min for both groups). The endplates were then perfused with 5.4 mM carbachol to activate any non-desensitized receptors. The amplitude of the current produced by 5.4 mM carbachol was similar at control endplates and staurosporine-treated endplates. To obtain an estimate of the number of non-desensitized, activatable receptors available after the 2–3 min exposure to 540 μM carbachol, we compared these responses with the $e.p.c._{CCh}$ amplitudes produced by 5.4 mM carbachol in the absence of prior desensitization (Connor *et al.*, 1984). The results summarized in Table 2 indicate that following pretreatment with 0.5 μM staurosporine, there was a slight, but non-significant, difference in the depth of desensitization produced by sustained exposure to 540 μM carbachol.

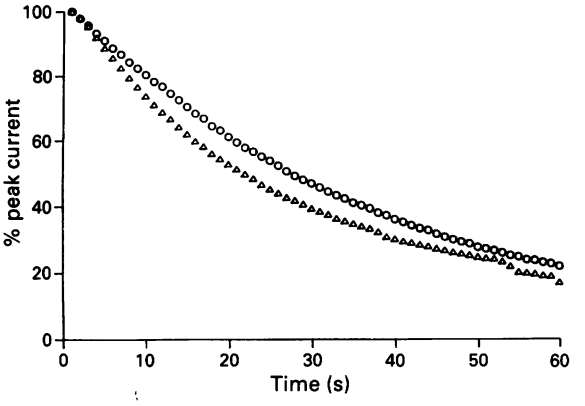


Figure 5 Results showing the curve fits for the decay phase of averaged 540 μM carbachol-induced currents obtained at 12 control (○) and 16 staurosporine-treated (△) endplates. The averaged records were obtained from individual records which were digitized and normalized to their peak current amplitude. In both cases, the decay phase of the averaged current could be adequately fitted as a single exponential function. The time constant for the decay phase of the averaged currents was 20.5 s for the control fibres and 25.0 s for the 0.5 μM staurosporine-treated endplates.

Elevation of calcium during carbachol application antagonizes staurosporine-induced decrease in the extent of recovery of m.e.p.c. amplitude following desensitization

We found that an increase in the concentration of calcium in the carbachol-containing solution which was microperfused onto the endplate reversed the inhibition of recovery by staurosporine pretreatment. As in the first series of experiments, m.e.p.cs were recorded before, and then after, a 2–3 min local perfusion of the endplate region with 540 μM carbachol. However, in these experiments, the muscle preparations were bathed in potassium propionate solution containing 1 mM calcium, but the carbachol perfusing solution contained 10 mM calcium so that an elevated external calcium concentration was present during the carbachol application. During the recovery period, the endplate was perfused with the potassium

Table 2 Extent of desensitization by carbachol is unchanged with staurosporine treatment in snake twitch fibres

	$e.p.c._{5.4\,mM}$ (nA)	*Estimated % activatable receptors*
Control	34.6 ± 5.6 (5)	3.8%
Staurosporine 0.5 μM	20.2 ± 4.0 (5)	2.2%

Preparations voltage-clamped to +30 mV were perfused with 540 μM carbachol until the current plateaued and the endplate was then perfused with 5.4 mM carbachol. The estimated % of activatable receptors was determined by comparing the current produced following desensitization to that produced by 5.4 mM carbachol in the absence of prior desensitization. There was no significant difference in the extent of desensitization in preparations pretreated with 0.5 μM staurosporine. Results shown are the mean ± s.e.mean of *n* fibres (shown in parentheses).

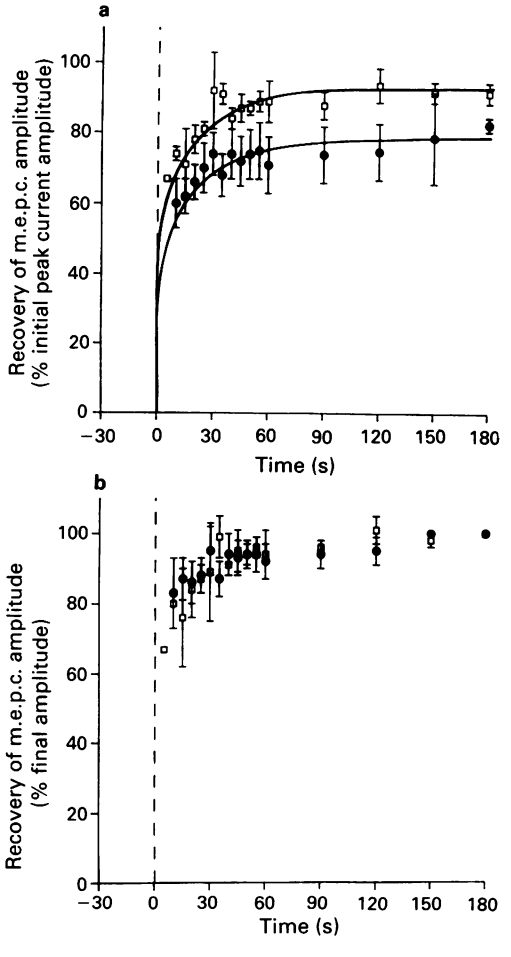


Figure 6 Results showing the antagonism of the staurosporine-induced decrease in recovery produced when the extracellular calcium concentration was raised to 10 mM during the application of 540 μM carbachol: (a) shows the time course of recovery of m.e.p.c. amplitude from carbachol-induced desensitization estimated at control fibres and staurosporine-treated (0.5 μM) fibres. The individual endplates were voltage-clamped to +30 mV. Results obtained when 10 mM calcium was present during the 540 μM carbachol application at untreated endplates (●); m.e.p.c. recovery when 10 mM calcium was included during the carbachol perfusion to fibres pretreated with 0.5 μM staurosporine (□). M.e.p.cs were recorded prior to perfusion with 540 μM carbachol and then following the initiation of perfusion with agonist-free solution (onset indicated by the dashed lines). In (a), averaged values are shown which are expressed as a percentage of the pre-carbachol m.e.p.c. amplitude. The values are the mean from 3–5 endplates; vertical bars show s.e.mean. In (b), the time course of recovery is shown with the values expressed as a percentage of the m.e.p.c. amplitude recorded at the end of the recovery period.

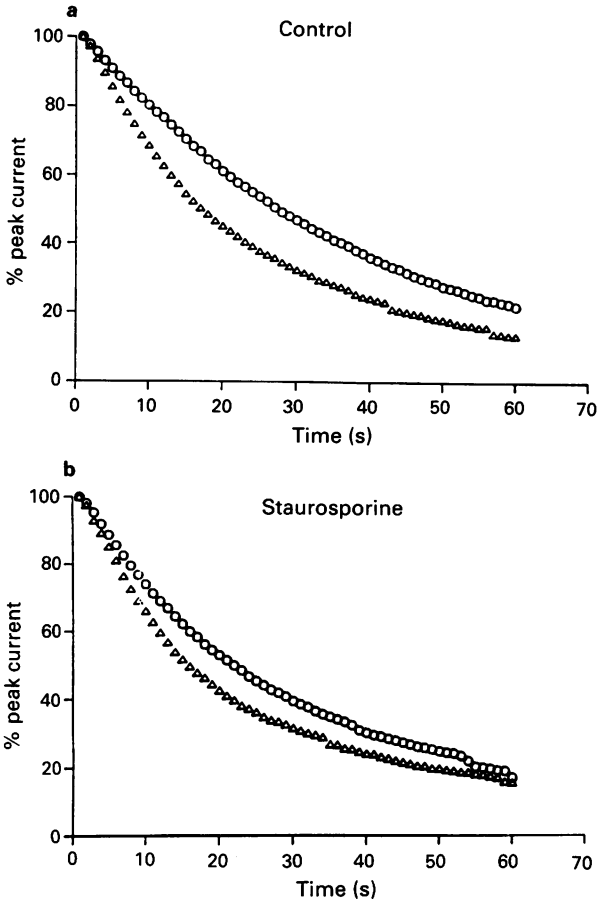


Figure 7 Results demonstrating that elevating extracellular calcium from 1 to 10 mM during carbachol application increased the rate of decay of the carbachol-induced current at both control and staurosporine-treated endplates. Records represent averaged, digitized, individual records which were normalized to their peak current amplitude. In (a) and (b), (○): results obtained when the calcium concentration was 1 mM (n = 12 for control (a); n = 16 for staurosporine-treated (b) and (△): results obtained when the calcium concentration was 10 mM (n = 16 for controls (a); n = 9 for staurosporine-treated (b)).

propionate solution containing 1 mM calcium. In the absence of staurosporine-pretreatment, elevation of calcium during the carbachol perfusion did not noticeably influence the extent or time course of recovery of m.e.p.c. amplitudes following desensitization. At endplates pretreated with 0.5 μM staurosporine, the m.e.p.c. amplitude also recovered fully following desensitization when the calcium concentration of the carbachol containing solution was 10 mM. In fact, the extent of recovery of the m.e.p.c. amplitude was slightly greater at staurosporine-treated endplates than in control fibres exposed to the elevated calcium concentration although the time course of recovery of the m.e.p.c. amplitudes was not different at control or staurosporine-pretreated fibres. These results are summarized in Figure 6.

Raising calcium accelerates desensitization (Manthey, 1966; Nastuk & Parsons, 1970). We found that elevation of the external calcium concentration from 1 mM to 10 mM during the carbachol application, increased the rate of desensitization onset to an equivalent degree in both control and staurosporine-treated fibres (Figure 7).

Discussion

The results of the present study demonstrate that pretreatment with the protein kinase inhibitor, staurosporine, reduced the extent of recovery of m.e.p.c. amplitude following carbachol-induced desensitization. The decrease in the extent of m.e.p.c. amplitude recovery occurred without any obvious change in the rate of recovery. Other results showed that staurosporine also decreased the extent of recovery of carbachol-induced currents. The decrease in extent of recovery from desensitization was not due to a staurosporine-induced alteration in either the time course of desensitization onset or the equilibrium level of desensitization reached at the end of the carbachol application. Further, staurosporine had no direct effect on either the agonist sensitivity or the kinetics of receptor-channel gating.

We found that the final extent of recovery of m.e.p.c. amplitude or the amplitude of the current produced by a second application of carbachol (Figures 2 and 3) was comparably inhibited by staurosporine pretreatment. However, the time course of recovery was markedly different. In the first instance, when m.e.p.c. amplitudes were used, the final level of recovery

was reached within approximately 90 s. In contrast, when carbachol-induced current amplitude was used, recovery was apparently not complete until 10 min. With the microperfusion application technique, the agonist concentration equilibrates over the endplate region much more slowly than that occurring during the generation of a m.e.p.c. Consequently, with the dual application method of testing recovery, the carbachol-induced currents produced by the second application of agonist reach their peak value after a few seconds. Therefore, the peak amplitude is a function of both receptor activation and desensitization (Dilger & Brett, 1990). Because the rate of desensitization is accelerated during the recovery period (Scubon-Mulieri & Parsons 1977; 1978; Wagoner & Pallotta, 1988), this would be expected to truncate the peak amplitude of $e.p.c._{CCh2}$ and produce an apparent slower rate of recovery. Consequently, we suggest that the second method cannot be used to estimate the kinetics of recovery.

The simplest explanation of the effect of staurosporine on recovery is that full recovery from desensitization requires phosphorylation of the ACh receptor or a closely associated protein. The observation that pretreatment with another protein kinase inhibitor, K252a, also reduced the extent of recovery of $e.p.c._{CCh2}$ does provide indirect support for our suggestion. K252a inhibits protein kinase C and cyclic nucleotide-dependent protein kinases, but via a different mechanism of action from staurosporine (Kase *et al.*, 1987). However, we have not directly measured phosphorylation in this study. Consequently, we cannot exclude the possibility that this effect is a direct modulation of receptor function rather than the result of protein kinase inhibition.

Several investigators have proposed that following phosphorylation of the ACh receptor, the rate of desensitization is accelerated (Huganir *et al.*, 1986; Eusebi *et al.*, 1987b; Hopfield *et al.*, 1988). However, some of these conclusions have been questioned by other investigators (Wagoner & Pallotta, 1988; Cachelin & Colquhoun, 1989). In the present study, we found that the apparent rates of desensitization and equilibrium level of desensitization were not affected by staurosporine pretreatment. These observations suggest that phosphorylation of the receptor by a staurosporine-sensitive protein kinase, following carbachol-induced activation, does not regulate the development of desensitization at snake twitch endplates. However, with the microperfusion technique, agonist equilibration over the endplate region takes 1–2 s so that any very fast (ms time constant) phase of desensitization onset could not be measured (Dilger & Brett, 1990). Therefore, if a very fast component of desensitization were regulated in our preparation by phosphorylation, we would not have been able to detect it. Cachelin & Colquhoun (1989) have argued that there probably is not much fast desensitization in the millisecond range. Further, these authors found that the development of desensitization at frog endplates is not dependent on phosphorylation (Cachelin & Colquhoun, 1989). Lastly, in muscle preparations from other species, the component of desensitization accelerated following phosphorylation of the ACh receptor had a time constant of seconds (Huganir & Greengard, 1990). Consequently, we suggest that if phosphorylation of the snake ACh receptor following activation, was required for desensitization to occur, then inhibition of phosphorylation by staurosporine should have produced a marked change in the time course of desensitization onset. We conclude, therefore, that phosphorylation by a staurosporine-sensitive kinase does not regulate the rate of desensitization at snake twitch fibre endplates, but does determine the extent of recovery from desensitization.

We were not able to compare directly the time course of desensitization at individual control and staurosporine-treated endplates because the current decay could not be consistently fitted either to a single or double exponential function. Consequently, we compared curves that were generated using averaged values for the current decay from many control and staurosporine-treated fibres. Under these conditions, there was no significant difference in time course of desensitization at control or staurosporine-treated preparations. However, two exponentials were required more often in individual control fibres adequately to describe the time course of desensitization than in the drug-treated fibres. The time course of desensitization commonly has two components (Connor *et al.*, 1984; Fiekers *et al.*, 1987; Cachelin & Colquhoun, 1989). The initial component is voltage-dependent so that the time constant of the fast phase of current decay can approach that of the slower component at depolarized potentials. Therefore, in fibres voltage-clamped to +30 mV, the values of the two time constants were not sufficiently different in some experiments clearly to require two exponentials for an adequate fit. The observation that more of the records from staurosporine-treated endplates could be adequately fitted as a single exponential than at control endplates may indicate that staurosporine slowed the initial phase of desensitization to a small extent. However, even if this occurred, we suggest that such a small change would not change the basic conclusions of this study.

Elevation of extracellular calcium increased the rate of desensitization at both control fibres and fibres pretreated with staurosporine. Although the mechanism by which calcium enhances desensitization onset is unknown, one plausible mechanism is that calcium stimulates protein kinase activity which in turn promotes phosphorylation of the receptor-channel complex at sites which regulate the rate of development of desensitization. However, if this mechanism is involved, then the kinase(s) activated by calcium must be staurosporine-insensitive.

The following scheme is proposed as one plausible mechanism by which staurosporine might inhibit the extent of recovery from desensitization. We suggest that during sustained exposure to concentrations of carbachol which rapidly desensitize essentially all of the activatable ACh receptors, some ACh receptors become irreversibly inactivated. Under these conditions, new receptors must replace the irreversibly inactivated ACh receptors for complete recovery to occur. Further, we suggest that these new receptors come from a readily available pool of nonfunctional ACh receptors which must be phosphorylated by a staurosporine-sensitive protein kinase to become activatable. The fact that full recovery can occur within 60–90 s indicates that the process does not require the synthesis of new receptors. Staurosporine pretreatment would decrease the final extent of recovery by blocking the conversion of these nonfunctional ACh receptors to activatable ACh receptors. This scheme is not unprecedented. Studies on ciliary ganglion cells have demonstrated that agents which promote phosphorylation increase the number of functional ACh receptors by converting a pool of nonfunctional receptors to activatable ACh receptors (Margiotta *et al.*, 1987a,b; Berg *et al.*, 1989). Synthesis of new receptors is not required for the increased number of functional receptors. Rather, it is proposed that the increased number of activatable ACh receptors results from the conversion of a pool of nonfunctional receptors which are already in the postsynaptic membrane and become activatable following phosphorylation (Berg *et al.*, 1989).

Elevating the extracellular calcium concentration during the period of carbachol application essentially eliminated the decreased extent of recovery from desensitization produced by staurosporine pretreatment. We have proposed that phosphorylation of the acetylcholine receptor by a staurosporine-sensitive protein kinase may be required for full recovery of endplate sensitivity following a sustained exposure to a desensitizing concentration of carbachol. If this conclusion is valid, then potentiation of protein kinase activity might be expected to antagonize the inhibition of the extent of recovery by staurosporine. Intracellular calcium is a co-factor for protein kinase C activation and promotes activation of protein kinase A (Rasmussen, 1986). Both of these kinases phosphorylate the ACh receptor (Huganir & Greengard, 1987; 1990). Further, acetylcholine-gated endplate channels are permeable to calcium (Takeuchi, 1963). Therefore, calcium influx occurs at

the endplate during exposure to nicotinic agonists, which in turn causes an elevation of the intracellular ionized calcium concentration near the inner surface of the postsynaptic membrane (Parsons & Nastuk, 1969; Manthey, 1974). Elevation of the extracellular calcium concentration increases the amount of calcium entering the fibre (Parsons & Nastuk, 1969). Consequently, we propose that the reversal by calcium of the staurosporine-induced inhibition of recovery may be due to a calcium-stimulated increase in kinase activity (Rasmussen, 1986).

In conclusion, these experiments demonstrate for the first time that nicotinic acetylcholine receptor recovery following agonist-induced desensitization may be regulated by protein phosphorylation, such that inhibition of this phosphorylation decreases the extent of recovery without altering the rate of recovery.

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Analysis of the 5-HT receptors mediating contractions in the rabbit isolated renal artery

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1 Using a number of agonist and antagonist compounds, we have attempted to characterize the responses and receptors involved in the effects of 5-hydroxytryptamine (5-HT) in the rabbit isolated renal artery.

2 In vessel segments precontracted with the thromboxane-mimetic agent, U46619 (100 nM), neither 5-HT (10^{-8} to 10^{-4} M) nor 5-carboxamidotryptamine (5-CT; 10^{-8} to 3×10^{-4} M) caused relaxations like those observed with methacholine. Both 5-HT and 5-CT further increased the tone of the vessels, with pD_2 values of 7.1 and 7.9, respectively.

3 In the absence of U46619, both 5-HT (10^{-7} to 3×10^{-3} M) and 5-CT (10^{-7} to 10^{-3} M) contracted the rabbit renal artery, but with reduced potencies. The contractions to 5-HT were reproducible and the rank order of potency (pD_2) of the agonists was: α-methyl-5-HT (5.7), sumatriptan (5.3), 5-HT (5.1), 8-hydroxy-2(di-n-propylamino)tetralin (5.0), 5-CT (4.7) and 5-methoxytryptamine (4.3). 1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane, flesinoxan and RU 24969 elicited either only small contractions or none at all.

4 The contractile effect of 5-HT was unaffected by MDL 72222 (10^{-6} M) and metergoline (10^{-8} and 10^{-7} M), was weakly antagonized by ketanserin and phentolamine (pK_B: 6.6 and 6.8, respectively), but was effectively antagonized by methiothepin (pK_B: 8.6). Responses to 5-CT and sumatriptan were affected by ketanserin, phentolamine and methiothepin similarly to 5-HT-induced responses.

5 Ketanserin was ineffective against noradrenaline-induced contractions, which were antagonized by phentolamine with a pK_B of 7.3. The pK_B values of phentolamine against 5-HT, 5-CT or sumatriptan were about half a log unit lower than against noradrenaline.

6 In vascular preparations treated with cocaine (3×10^{-5} M), the potency (pK_B) of phentolamine as an antagonist of the responses to noradrenaline (7.6) and 5-HT (6.7) did not differ significantly from the values in untreated preparations. However, the difference between the pK_B values of phentolamine against the two agonists was now about one log unit.

7 Pretreatment of the vascular strips with 6-hydroxydopamine (1.5×10^{-3} M) did not significantly affect responses to 5-HT or 5-CT, but almost eliminated those to tyramine. Cocaine (3×10^{-5} M) slightly potentiated noradrenaline-induced contractions, but did not significantly affect those induced by 5-HT.

8 These data suggest that: (i) 5-HT receptors mediating vasodilatation are not present in the rabbit renal artery smooth muscle or endothelium; (ii) the contractile effect of 5-HT does not involve the release of noradrenaline from sympathetic nerve stores; (iii) the 5-HT receptor in the rabbit renal artery is not of the 5-HT_2, 5-HT_3 or 5-HT_4 type. The pharmacological properties of this receptor most closely resemble those described for the heterogeneous 5-HT_1-like category.

Keywords: 5-Hydroxytryptamine, 5-hydroxytryptamine receptors; rabbit, renal artery; sumatriptan; 5-carboxamidotryptamine; 8-hydroxy-2(di-n-propylamino)tetralin; U46619 interactions; ketanserin; phentolamine

Introduction

There is a wide variation in the renal vascular response to 5-hydroxytryptamine (5-HT; serotonin) with investigators reporting either no change (Caldicott *et al.*, 1970), increases (McCubbin *et al.*, 1962), decreases (Vyden *et al.*, 1974; Saxena *et al.*, 1978; Wright & Angus, 1987) or even bi- or multiphasic changes (Blackshear *et al.*, 1986; Wright & Angus, 1989) in renal blood flow. Moreover, there are conflicting claims about the 5-HT receptors which mediate the effects of 5-HT in the renal vasculature. In the canine renal vasculature, Blackshear *et al.* (1986) reported 5-HT_1-like receptor-mediated vasodilator and 5-HT_2 receptor-mediated vasoconstrictor components, while Shoji *et al.* (1989) concluded that 5-HT_2 receptors mediate vasodilatation and 5-HT_1-like receptors are involved in vasoconstriction. In the rabbit, notwithstanding that 5-carboxamidotryptamine (5-CT), a selective agonist at 5-HT_1-like receptors (Bradley *et al.*, 1986; Saxena & Villalón, 1990), decreases renal blood flow, Wright & Angus (1987) have suggested that renal vasodilatation and vasoconstriction in this species is mediated by 5-HT_1-like and 5-HT_2 receptors, respectively. The vasoconstrictor effect of 5-CT has been explained by it causing release of 5-HT from blood platelets (Wright & Angus, 1989). In view of this and other complicating factors operating in intact animals, such as prostaglandin release (Blackshear *et al.*, 1986) and sympathetic neuroinhibition (Charlton *et al.*, 1986), the present study was performed in the isolated renal artery of the rabbit. Using a number of agonist and antagonist compounds, we have attempted to characterize the nature of 5-HT receptors in this tissue.

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Methods

Tissue preparation

New Zealand white rabbits (ENKI, Helmond, The Netherlands), weighing 2.0–2.8 kg, were killed by injections of pentobarbitone sodium (Nembutal; 60 mg kg^{-1}) into a marginal ear vein. The right and left renal arteries were cleaned of fat and connective tissue *in vitro*, and placed in Krebs solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 8.3; pH 7.4). Arteries were either used on the same day or after overnight storage at 4°C in the Krebs solution. The maximum effect and pD_2 value of 5-HT were unaffected by storage.

Ring segments (3–5 mm in length) of rabbit renal artery were placed in organ bath chambers filled with 10 ml of Krebs solution maintained at 37°C and bubbled constantly with a mixture of 95% O_2 and 5% CO_2. The segments were carefully suspended between two stainless steel wire hooks (0.2 mm diameter) inserted into the lumen to record changes in isometric tension. No attempt was made to remove the endothelium. Tension was measured with a Harvard isometric transducer. Preparations were maintained at an initial resting tension of 2 g. During the equilibration period, the Krebs solution was changed at 15 min intervals and the vascular segments were re-stretched to the resting tension. This procedure was repeated three times. After equilibration, contractile responses to 30 mM KCl were ascertained and the preparations washed; the responses to KCl and subsequent washings were repeated once again. Concentration-effect curves were then established for 5-HT and other agonists.

The relaxant effects of 5-HT (in the presence of 10^{-5} M ketanserin to avoid 5-HT_2 receptor-mediated responses) and 5-CT were studied in vascular segments precontracted to a near maximal level with 100 nM U46619. The contractile effects of 5-HT and other agonists were investigated in quiescent vessels (i.e. without precontraction with U46619). In all vessel segments precontracted with U46619, and in about one third of the experiments on quiescent vessels, 10^{-7} M methacholine was added in the bath at the end of the experiment to verify endothelial function. In all cases, a relaxant response to methacholine was observed thus showing the viability of the endothelium.

Determination of agonist potency

5-HT was added to the fluid bathing the renal artery preparations by a cumulative concentration schedule, allowing sufficient time for the effects of each concentration to become fully established before adding the next concentration, until a maximum response (E_{max}) was obtained or relaxation started to occur. After frequent washing, tissues were left for at least 30 min before re-challenging with either 5-HT or another agonist. On each preparation, one or two concentration-effect curves to 5-HT were obtained; preliminary experiments showed that at least three consecutive concentration-effect curves to 5-HT were reproducible. One preparation was used as a control in which the concentration-effect curve to 5-HT was repeated, while concentration-effect curves to various other agonists were constructed on other preparations from the same animal. Not more than two agonists were tested on one preparation.

Determination of antagonist potency

Concentration-effect curves to 5-HT were established in the rabbit renal artery as described. One preparation was used as a control and a single concentration of antagonist was added to the other organ baths. An antagonist equilibration time of 30 min was allowed before agonist concentration-effect curves were re-determined.

Acute sympathetic denervation and uptake blockade

In vitro sympathetic denervation of the renal artery segments was achieved by the method described by Aprigliano & Hermsmeyer (1976) with certain modifications. Briefly, a solution (composition in mM: NaCl 112, KCl 5, $CaCl_2$ 1.25, $MgSO_4$ 1.2, glucose 11.5 and glutathione 0.1; pH 4.9) was supplemented with 1.5×10^{-3} M 6-hydroxydopamine. After incubation in this solution for 15 min at 37°C, the renal artery segments were washed several times and equilibrated in Krebs solution for a further 60 min. Paired experiments were performed on preparations treated either with 6-hydroxydopamine or the vehicle alone. The effectiveness of the sympathetic denervation was checked by measuring contractions elicited by 10^{-4} M tyramine. After washing, concentration-effect curves were constructed for 5-HT and 5-CT in both control and 6-hydroxydopamine-treated segments.

The effect of sympathetic neuronal uptake blockade on the responses to 5-HT and noradrenaline were studied in experiments conducted in the continuous presence of cocaine (3×10^{-5} M).

Analysis of the data

The concentration-effect curves for the agonists were analysed by means of a computerized curve fitting technique described by De Lean *et al.* (1978). The parameters thus obtained (i.e. pD_2 and E_{max}) were averaged for the respective agonists. In the case of antagonists, the shifts in the agonist-induced concentration-effect curves were calculated from the pD_2 values in each individual preparation before and after addition of an antagonist. pK_B values were calculated from equations described by Kenakin (1987), with the assumption that the antagonism was competitive. All data are presented as mean ± s.e.mean. Student's *t* test was used to evaluate the significance of the differences in pD_2 and E_{max} values.

Materials

The compounds used in this study were kindly supplied by, or purchased from, the companies mentioned: 5-carboxamidotryptamine maleate (gift: Glaxo, Ware, U.K.), cocaine (Sigma Chemical Co., St Louis, U.S.A.), 1-(2,5-dimethoxy-4-iodo-phenyl)-2-aminopropane hydrochloride (DOI) (Research Biochemicals Inc., Natic, U.S.A.), flesinoxan hydrochloride (gift: Duphar B.V., Weesp, The Netherlands), glutathione (E. Merck, Amsterdam, The Netherlands), 6-hydroxydopamine hydrochloride (Sigma Chemical Co., St Louis, U.S.A.), 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Co., St Louis, U.S.A.), ketanserin tartrate (gift: Janssen Pharmaceutica, Beerse, Belgium), metergoline (gift: Farmitalia, Milan, Italy), methacholine bromide (Janssen Chimica, Beerse, Belgium), methiothepin methane sulphate (gift: Hoffman La Roche, Mijdrecht, The Netherlands), 5-methoxytryptamine hydrochloride (Research Biochemicals Inc., Natic, U.S.A.), α-methyl-5-hydroxytryptamine maleate (gift: Glaxo, Ware, U.K.), MDL 72222 ([1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate]; gift: Marion Merrell Dow Research Institute, Strasbourg, France), noradrenaline hydrochloride (gift: Fluka AG, Switzerland), 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT); gift: Marion Merrell Dow Research Institute, Strasbourg, France), phentolamine hydrochloride (gift: Ciba-Geigy, Basel, Switzerland), RU 24969 ([5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl-)-1H-indole succinate]; gift: Roussel Laboratories, Hoevelaken, The Netherlands), sumatriptan (GR 43175; gift: Glaxo, Ware, U.K.), tyramine hydrochloride (gift: Fluka AG, Switzerland) and U46619 ([9,11-dideoxy-11α,9α-epoxymethano- prostaglandin $F_{2\alpha}$]; The Upjohn Chemical Company, Kalamazoo, U.S.A.).

Results

Investigation of vasodilator responses

The possibility that 5-HT and 5-CT may elicit vasodilatation in the rabbit isolated renal artery was investigated in vessels precontracted with 100 nM of the thromboxane receptor agonist, U46619, which increased the vessel tone submaximally by 2.4 ± 0.3 g ($n = 11$). As illustrated in Figure 1, neither 5-HT (10^{-8} to 10^{-4} M; in vessels treated with ketanserin to avoid 5-HT_2, receptor-mediated responses) nor 5-CT (10^{-8} to 3×10^{-4} M) relaxed precontracted renal artery segments, which, as expected, responded to 10^{-7} M methacholine with a decrease in tone. Instead, both 5-HT and 5-CT caused further contractions. In these vessel segments precontracted with U46619, the pD_2 and E_{max} values were 7.1 ± 0.3 and

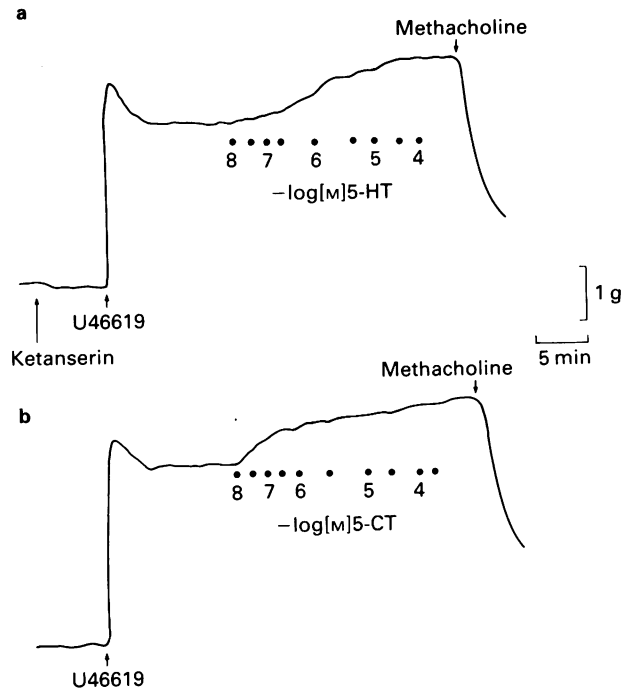


Figure 1 Effects of different concentrations of 5-hydroxytryptamine (5-HT) (a) and 5-carboxamidotryptamine (5-CT) (b) on rabbit isolated renal artery segments precontracted with U46619 (10^{-7} M). Ketanserin (10^{-5} M) was added to the segment exposed to 5-HT to antagonize 5-HT_2 receptors. At each dot (•) increasing (half log molar sequence) concentrations of 5-HT (10^{-8} to 10^{-4} M) or 5-CT (10^{-8} to 3×10^{-4} M) were added. Neither compound elicited vasorelaxation; instead contractile responses were observed. Administration of methacholine (10^{-7} M) resulted in relaxation of the tissues.

1.7 ± 0.5 g ($n = 6$), respectively for 5-HT and 7.9 ± 0.2 and 1.0 ± 0.2 g ($n = 5$), respectively, for 5-CT.

Investigation of contractile responses

Agonist studies In the absence of U46619, 5-HT caused contractions of the rabbit renal artery, but with a reduced potency (pD_2; 5.1 ± 0.1; E_{max} 3.6 ± 0.2 g; $n = 20$). As illustrated in Figure 2, the 5-HT-induced concentration-effect curves were reproducible; the E_{max} ($109.5 \pm 2.5\%$ of the first curve) and pD_2 (5.2 ± 0.1) values of the second concentration-effect curves were not significantly different from those of the first curves. The 5-HT receptor agonists DOI, flesinoxan and RU 24969 elicited either small or no contractions but α-methyl-5-HT, 5-CT, 8-OH-DPAT, sumatriptan and 5-methoxytryptamine all caused concentration-dependent contractions (Figure 2). The pD_2 and E_{max} values for each agonist are shown in Table 1. The rank order of potency was α-methyl-5-HT > sumatriptan = 5-HT = 8-OH-DPAT > 5-CT > 5-methoxytryptamine. The E_{max} developed by sumatriptan was significantly ($P < 0.05$) less than that of the other agonists.

It should be noted that, in the absence of U46619, the pD_2 values were lower and the E_{max} values were higher than those obtained for 5-HT and 5-CT in the presence of U46619. While the differences in the pD_2 values may represent an increase in the affinity of 5-HT and 5-CT in the presence of U46619 (see Discussion), the decrease in the E_{max} may just be due to the fact that the tissues probably cannot contract further.

Table 1 The pD_2 and E_{max} values of a number of 5-hydroxytryptamine (5-HT) receptor agonists calculated from concentration-effect curves obtained on the rabbit isolated renal artery

Agonist	pD_2	E_{max}
α-Methyl-5-HT	5.7 ± 0.2	109 ± 4*
Sumatriptan	5.3 ± 0.2	52 ± 13*
5-HT 1st curve	5.1 ± 0.1	100
5-HT 2nd curve	5.2 ± 0.2	109 ± 3*
8-OH-DPAT	5.0 ± 0.3	87 ± 6*
5-CT	4.7 ± 0.2	90 ± 2*
5-Methoxytryptamine	4.3 ± 0.2	117 ± 14
DOI	Inactive	
Flesinoxan	Inactive	
RU 24969	Inactive	

For abbreviations, see Materials.
The pD_2 value and maximal response (E_{max}) were calculated by a computerized analysis of the individual curves according to De Lean *et al.* (1978). Values are mean ± s.e.mean ($n = 4$–6). The maximal response is expressed as percentage of the initial E_{max} obtained for 5-HT. * $P < 0.05$ vs. 5-HT 1st curve.

Effects of antagonists and of cocaine on contractile responses to 5-HT, 5-CT, sumatriptan and noradrenaline

The 5-HT receptor antagonists methiothepin, ketanserin, metergoline, MDL 72222 and the α-adrenoceptor antagonist,

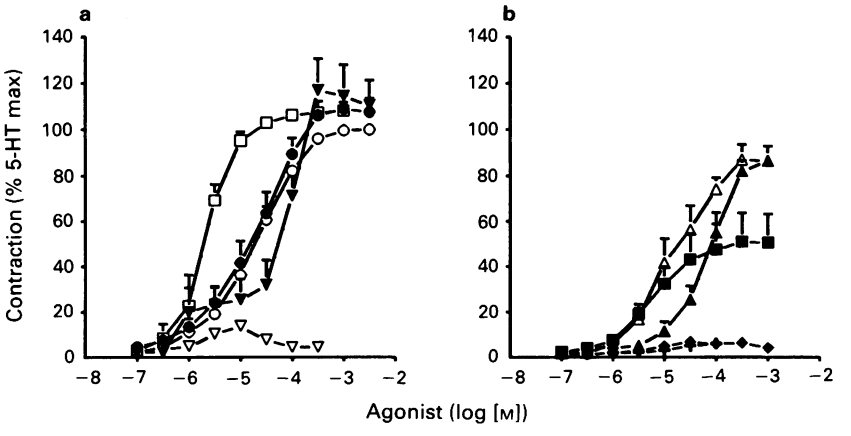


Figure 2 Rabbit isolated renal artery: concentration-effect curves to: 5-hydroxytryptamine (5-HT) 1st curve (○); 5-HT, 2nd curve (●); 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (▽); α-methyl-5-HT (□); and 5-methoxytryptamine (▼)(a) and to: 8-hydroxy-2(di-n-propylamino)tetralin (△); 5-carboxamidotryptamine (▲); sumatriptan (■); flesinoxan (◆) and RU 24969 (+) (b). Points represent mean (with s.e.mean shown by vertical bars) ($n = 4$–6) of the increase in force expressed as a percentage of the initial maximal responses to 5-HT.

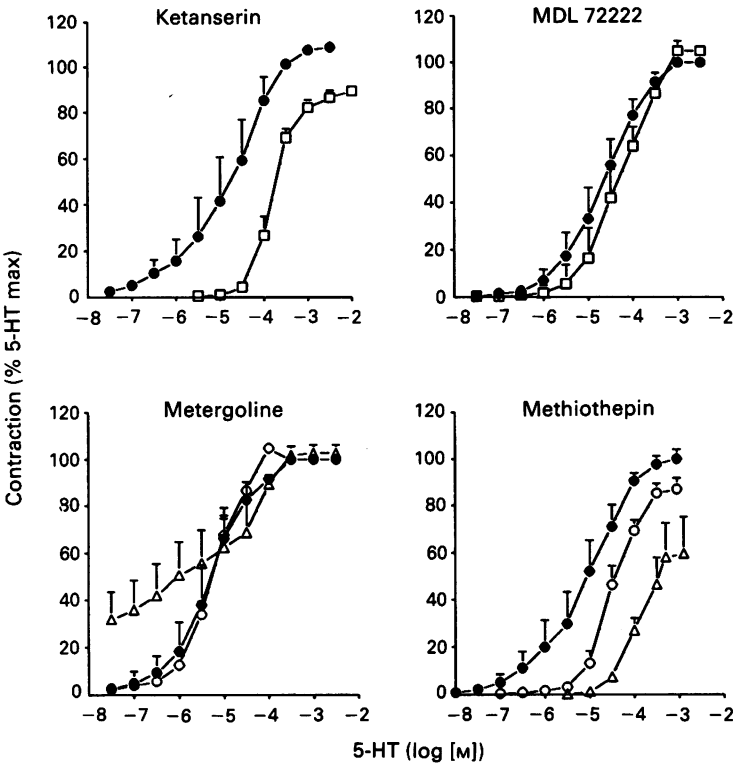


Figure 3 Rabbit isolated renal artery: concentration-effect curves to 5-hydroxytryptamine (5-HT) in the absence (●) and presence (open symbols) of ketanserin, MDL 72222, metergoline or methiothepin. The concentrations of the antagonists used were 10^{-8} (○), 10^{-7} (△) and 10^{-6} (□) M. Points represent mean (with s.e.mean shown by vertical bars) ($n = 4$–6) of the increase in force expressed as a percentage of the initial maximal responses to 5-HT.

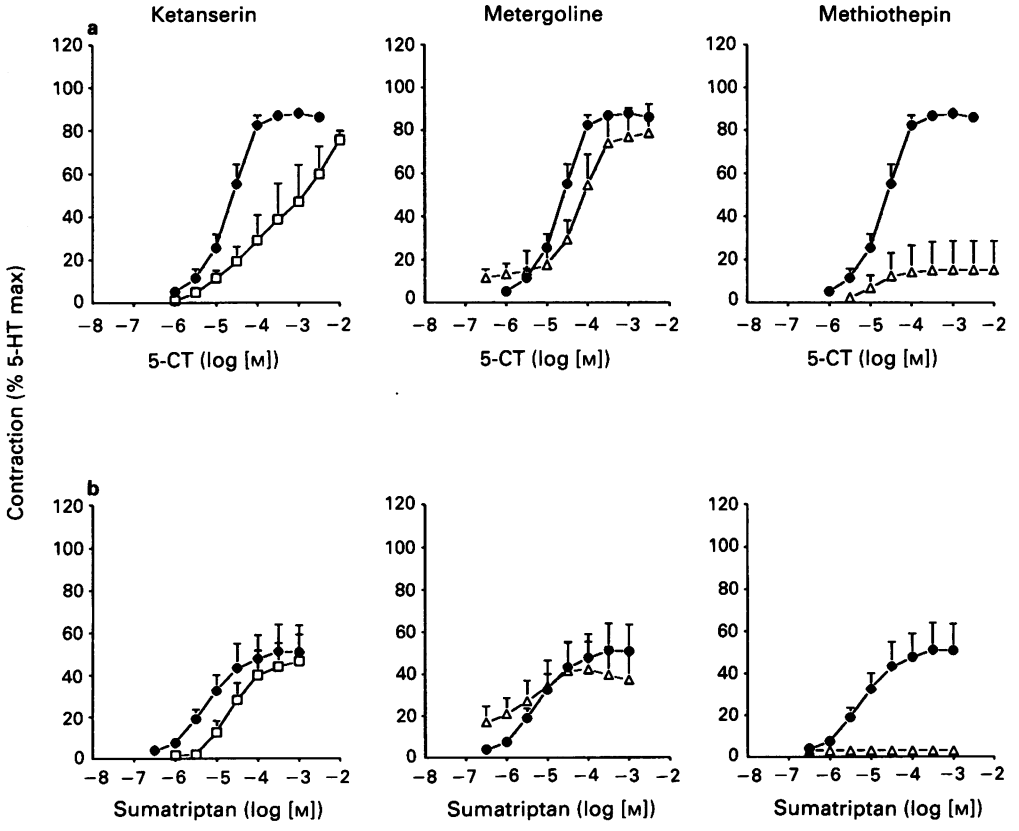


Figure 4 Rabbit isolated renal artery: concentration-effect curves to 5-carboxamidotryptamine (5-CT, a) and sumatriptan (b) in the absence (●) and presence (open symbols) of ketanserin, metergoline or methiothepin. The concentrations of the antagonists used were 10^{-7} (△) and 10^{-6} (□) M. Points represent mean (s.e.mean shown by vertical bars) ($n = 4$–6) of the increase in force expressed as a percentage of the initial maximal responses to 5-HT.

phentolamine, were investigated as antagonists of the contractile responses elicted by 5-HT, 5-CT and sumatriptan as well as by noradrenaline in the rabbit isolated renal artery. MDL 72222 (10^{-6} M) and metergoline (10^{-8} and 10^{-7} M) did not antagonize the effects of 5-HT, but metergoline had some contractile activity at 10^{-7} M. Both ketanserin (10^{-6} M) and methiothepin (10^{-8} and 10^{-7} M) antagonized 5-HT-induced contractile responses (Figure 3).

The responses to 5-CT and sumatriptan were not significantly affected by the presence of 10^{-7} M metergoline, but in the presence of 10^{-6} M ketanserin they were shifted significantly to the right. Responses to the agonists were non-competitively antagonized by 10^{-7} M methiothepin (Figure 4).

Phentolamine (10^{-6} M) produced small rightward shifts of the concentration-effect curves of 5-HT, 5-CT and sumatriptan (Figure 5).

The effects of methiothepin (10^{-7} M) and ketanserin (10^{-6} M) on noradrenaline-induced contractions are shown in Figure 6. Ketanserin did not significantly affect the responses to noradrenaline, but methiothepin antagonized the responses, producing a parallel shift to the right of the concentration-effect curve.

Since the concentration-effect curves to 5-HT, 5-CT and sumatriptan were obtained over the range 10^{-6} to 10^{-4} M, the antagonistic activity of most compounds had to be restricted to determination of shifts in concentration-effect curves produced by a single concentration of the antagonist. Therefore, where applicable, only antagonist affinities (pK_B) were calculated (Kenakin, 1987) from the results and these are listed in Table 2. Ketanserin, being equiactive against 5-HT, 5-CT and sumatriptan, was less potent than methiothepin. The pK_B values for phentolamine against 5-HT, 5-CT and sumatriptan were also similar, but they were slightly less (half a log unit) than that found when noradrenaline was the agonist. The effects of phentolamine on the responses to 5-HT and noradrenaline were also studied in the presence of cocaine (3×10^{-5} M). Whether cocaine was used or not, the pK_B

Table 2 The pK_B values for the antagonism of 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), sumatriptan and noradrenaline by ketanserin, methiothepin and phentolamine in the rabbit isolated renal artery

		Antagonist	
Agonist	*Ketanserin*	*Methiothepin*	*Phentolamine*
In the absence of cocaine			
5-HT	6.6 ± 0.1	8.6 ± 0.4	6.8 ± 0.1*
5-CT	6.7 ± 0.4	NC	6.9 ± 0.1
Sumatriptan	6.6 ± 0.2	NC	6.8 ± 0.1*
Noradrenaline	Inactive	8.1 ± 0.2	7.3 ± 0.1
In the presence of cocaine (3×10^{-5} M)			
5-HT	—	—	6.7 ± 0.1*
Noradrenaline	—	—	7.6 ± 0.4

The pK_B values were calculated by an analysis of the individual curves according to Kenakin (1987). Values are mean ± s.e.mean (n = 4–6). NC; Not calculated as there was a near complete suppression of responses in the presence of 10^{-7} M of the antagonist.
* $P < 0.05$ vs. corresponding pK_B values against noradrenaline.

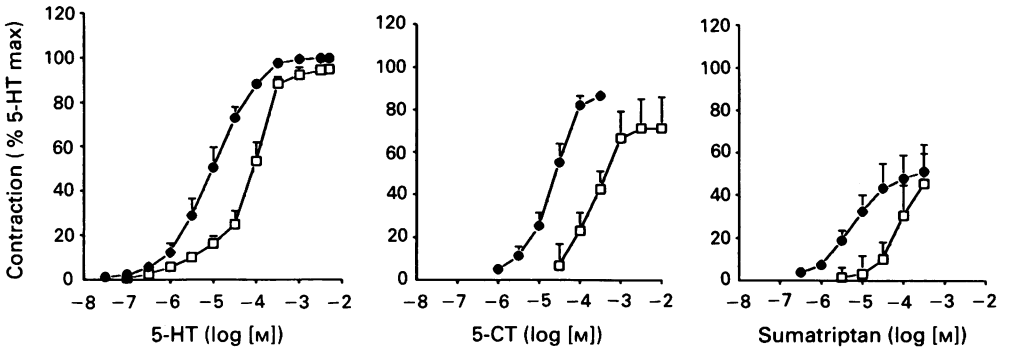


Figure 5 Rabbit isolated renal artery: concentration-effect curves to 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT) and sumatriptan in the absence (●) and presence of phentolamine (10^{-6} M; □). Points represent mean (s.e.mean shown by vertical bars) (n = 4–6) of the increase in force expressed as a percentage of the initial maximal responses to 5-HT.

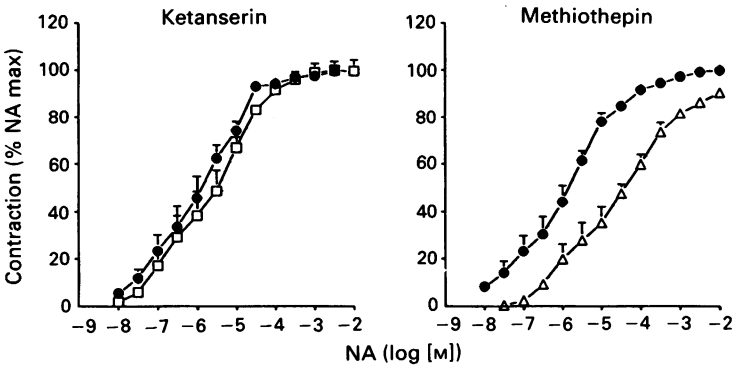


Figure 6 Rabbit isolated renal artery: concentration-effect curves to noradrenaline (NA) in the absence (●) and presence (open symbols) of ketanserin and methiothepin. The concentrations of the antagonists used were 10^{-7} (△) and 10^{-6} M (□). Points represent mean (s.e.mean shown by vertical bars) (n = 4–6) of the increase in force expressed as a percentage of the initial maximal responses to noradrenaline.

Table 3 The responses to 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), tyramine and noradrenaline observed in rabbit isolated renal artery segments, with or without treatment with 6-hydroxydopamine (1.5×10^{-3} M) and cocaine (3.0×10^{-5} M)

		pD_2	E_{max} (g)
6-Hydroxydopamine			
5-HT	Untreated ($n = 8$)	5.8 ± 0.3	5.1 ± 0.4
	Treated ($n = 4$)	5.8 ± 0.3	4.4 ± 0.3
5-CT	Untreated ($n = 6$)	4.9 ± 0.2	4.6 ± 0.7
	Treated ($n = 4$)	4.7 ± 0.4	4.2 ± 0.6
Tyramine	Untreated ($n = 12$)	—	4.4 ± 0.4§
	Treated ($n = 8$)	—	0.6 ± 0.1§
Cocaine			
5-HT	Before ($n = 6$)	5.1 ± 0.1	3.5 ± 0.6
	After ($n = 4$)	5.0 ± 0.1	3.7 ± 0.8
Noradrenaline	Before ($n = 7$)	6.0 ± 0.1	4.7 ± 0.1
	After ($n = 4$)	6.8 ± 0.1*	5.6 ± 0.5

The pD_2 values and maximal responses (E_{max}) were calculated by a computerized analysis of the individual curves according to De Lean *et al.* (1978). Values are mean ± s.e.mean. Number of observations in parentheses.
* Significantly different ($P < 0.05$) from the corresponding values before treatment.
§ Response to a single concentration (10^{-4} M) of typramine.

values of phentolamine against 5-HT (6.7 ± 0.1 and 6.8 ± 0.1, respectively) and noradrenaline (7.6 ± 0.4 and 7.3 ± 0.1, respectively) were not statistically different. However, the difference between the pK_B values of phentolamine against the two agonists was more marked (about one long unit) in the presence of cocaine than in its absence (Table 2).

Effects of 6-hydroxydopamine and cocaine on the contractile responses to 5-HT, 5-CT, tyramine and noradrenaline

Table 3 presents the pD_2 and E_{max} values of agonists acting on the rabbit isolated renal artery before and after treatment with either 6-hydroxydopamine or cocaine. Treatment with 6-hydroxydopamine markedly attenuated the effects of tyramine, but responses to 5-HT and 5-CT were not significantly affected. The potency of noradrenaline but not that of 5-HT, was significantly increased by cocaine.

Discussion

The aim of the present study was to examine the effects of 5-HT, and the receptors involved in mediating them, in the isolated renal artery of the rabbit. Unlike the results obtained in whole animals (McCubbin *et al.*, 1962; Blackshear *et al.*, 1986; Wright & Angus, 1989; Shoji *et al.*, 1989) or in the perfused isolated kidney (Verbeuren *et al.*, 1990), no vasodilatation was observed with either 5-HT or 5-CT when the renal artery segments were precontracted with U46619. This suggests that the receptors mediating vasodilator responses to 5-HT are predominantly located on arterioles and not on the large conduit renal artery. On the other hand, as observed in many investigations *in vivo* (Vyden *et al.*, 1974; Saxena *et al.*, 1978; Blackshear *et al.*, 1986; Wright & Angus, 1987; Shoji *et al.*, 1989), 5-HT did elicit a contractile response. This response was further analysed by comparing the effects of 5-HT with those of a series of potent 5-HT receptor agonists (5-CT, 8-OH-DPAT, RU 24969, sumatriptan, flesinoxan, α-methyl-5-HT, 5-methoxytryptamine and DOI) and of noradrenaline, and by examining the antagonist properties of ketanserin, methiothepin, MDL 72222, metergoline and phentolamine.

The nature of the 5-HT receptor mediating renal artery contractions

The scheme proposed by Bradley *et al.* (1986) has served as a framework for the classification of 5-HT receptors. MDL 72222 (Fozard, 1984) and ketanserin (Feniuk *et al.*, 1985) are highly potent antagonists at 5-HT_3 and 5-HT_2 receptors, respectively. In the present experiments we found that MDL 72222 was inactive and that ketanserin was only a weak antagonist of responses to 5-HT. The resistance to blockade by MDL 72222 suggests that 5-HT_3 receptors are not involved. The weak antagonism by ketanserin (pK_B: 6.6 against about 8.5 at 5-HT_2 receptors) and the inactivity of the selective 5-HT_2 receptor agonist DOI (Richardson & Hoyer, 1990; Saxena & Villalón, 1990) suggests that 5-HT_2 receptors are also not involved. Though another 5-HT_2 receptor agonist, α-methyl-5-HT (Hoyer, 1989; Saxena & Villalón, 1990), contracted renal artery, this compound is also a potent 5-HT_1-like receptor agonist (Feniuk *et al.*, 1985; Ismaiel *et al.*, 1990). Indeed, the mimicking of the contractile effect of 5-HT by other 5-HT_1-like receptor agonists (5-CT, 8-OH-DPAT, 5-methoxytryptamine and sumatriptan) and the potent antagonist action of methiothepin not only rule out the involvement of putative 5-HT_4 receptors (see Saxena & Villalón, 1990), but also suggest that 5-HT_1-like receptors may mediate the 5-HT-induced contractions of rabbit renal artery.

However, it must be pointed out that the effects of the 5-HT receptor agonists are not entirely in keeping with the participation of 5-HT_1-like receptors in 5-HT-induced contractions of the rabbit isolated renal artery. The pD_2 values of the different 5-HT_1-like receptor agonists in the quiescent vessels (i.e. without precontraction with U46619) were substantially lower (4.3 to 5.7; Table 1) than might be expected from their affinity at 5-HT_1 binding sites (see Hoyer *et al.*, 1985; Hoyer, 1989; Saxena & Villalón, 1990). Moreover, in contrast to the accepted criteria (Bradley *et al.*, 1986), 5-CT (pD_2; 4.7) proved to be less potent than 5-HT (pD_2: 5.1). Surprisingly however, in the vessels precontracted with U46619 (used primarily for the purpose of detecting vasodilatation), the pD_2 values of 5-CT (7.9) and 5-HT (7.1) were nearer to those that are usually reported for 5-HT_1-like receptor-mediated responses (Bradley *et al.*, 1986; Hoyer, 1989; Saxena & Villalón, 1990). The reasons for the changes in pD_2 values of 5-CT and 5-HT are not known, but a very similar phenomenon has recently been described for the 5-HT_1-like receptors in the guinea-pig isolated iliac artery where 5-HT, 5-CT and sumatriptan were virtually ineffective in the absence of tone, but generated appreciable force when tissues were precontracted with threshold concentrations of prostaglandin $F_{2\alpha}$ (Sahin-Erdemli *et al.*, 1991).

The antagonism by ketanserin of the responses to 5-HT, 5-CT and sumatriptan could reflect a low affinity of this drug for the 5-HT_1-like receptors present in the rabbit renal artery. Ketanserin does attenuate 5-HT_1-like receptor-mediated contractions in some other vascular tissues, such as the rabbit saphenous vein (Martin & MacLennan, 1990; Van Heuven-Nolsen *et al.*, 1990) and porcine cranial arteriovenous anastomoses (Saxena *et al.*, 1989; Den Boer *et al.*, 1991). These vessels are contracted by 5-HT, 5-CT and sumatriptan and the effects of these agonists are effectively antagonized by methiothepin, but only weakly by ketanserin.

Does the putative 5-HT_1-like receptor in the renal artery resemble any of the known 5-HT_1 receptor subtypes?

Bradley *et al.* (1986) have pointed out that 5-HT_1-like receptors are heterogeneous and several subtypes, some having a corresponding binding site (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} or 5-HT_{1D}), have been described (see Saxena & Villalón, 1990). In order to characterize further the 5-HT_1-like receptor in the rabbit renal artery, experiments were carried out with compounds that are considered relatively selective for the putative

subtypes: 8-OH-DPAT and flesinoxan (5-HT_{1A}; Middlemiss & Fozard, 1983; Richardson & Hoyer, 1989), RU 24969 (5-HT_{1A} and 5-HT_{1B}; Hoyer *et al.*, 1985), sumatriptan (5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D}; Peroutka & McCarthy, 1989; Schoeffter & Hoyer, 1989; Sumner & Humphrey, 1989), DOI (5-HT_{1C} and 5-HT_2; Hoyer, 1989), α-methyl-5-HT (low affinity for all four subtypes; Hoyer, 1989; Ismaiel *et al.*, 1990) and metergoline (high affinity for all four subtypes; Hoyer, 1989). A contribution by 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} receptors to responses to 5-HT can be excluded since RU 24969, flesinoxan and DOI were inactive and several of the agonists effective in this study (5-CT, 8-OH-DPAT and sumatriptan) have very low affinities for 5-HT_{1C} binding sites. The ineffectiveness of metergoline against the renal vascular contractions elicited by 5-HT, 5-CT and sumatriptan tends to rule out the involvement of the 5-HT_{1D} subtype as well. There are several examples of vascular locations of 5-HT_1-like receptors not entirely resembling any of the above subtypes: the dog and rabbit saphenous vein (Feniuk *et al.*, 1985; Humphrey *et al.*, 1988; Van Heuven-Nolsen *et al.*, 1990), human basilar artery (Parsons *et al.*, 1989) or porcine cranial arteriovenous anastomoses (Saxena *et al.*, 1989; Den Boer *et al.*, 1991). Though the rank order of potency (5-CT > 5-HT > α-methyl-5-HT > sumatriptan > 8-OH-DPAT = RU 24969) found at the dog saphenous vein 5-HT_1-like receptor differs from that (α-methyl-5-HT > sumatriptan = 5-HT = 8-OH-DPAT > 5-CT > 5-methoxytryptamine; RU 24969 inactive) found at the 5-HT_1-like receptor in the rabbit renal artery in the absence of tone, the relative potencies of 5-HT and 5-CT were similar in precontracted vessels.

Does the renal artery 5-HT receptor differ from α-adrenoceptors?

In view of the fact that the contractile effects of 5-HT in some blood vessels of the rabbit may be mediated by a direct action on α-adrenoceptors (Apperley *et al.*, 1976; Black *et al.*, 1981; Feniuk, 1984), we have attempted to distinguish between the effects of noradrenaline and 5-HT receptor agonists. Several observations indicate that noradrenaline and 5-HT contract the rabbit isolated renal artery by activating different receptors: firstly the pK_B values of phentolamine against 5-HT, 5-CT and sumatriptan (6.8–6.9) were somewhat lower than that against noradrenaline (7.3); secondly, though the potency of phentolamine against noradrenaline and 5-HT did not differ significantly in vascular preparations in the absence or presence of cocaine, the difference between the pK_B values against the two agonists became more marked after cocaine treatment (Table 2); thirdly, ketanserin (10^{-6} M) did not significantly affect noradrenaline-induced renal artery contractions, but significantly affected responses to 5-HT, 5-CT and sumatriptan with pK_B values around 6.6; however, noradrenaline may still act via α_2-adrenoceptors in the presence of ketanserin; and, fourthly, not only 5-HT, but several other 5-HT_1-like receptor agonists, which are not known to activate α-adrenoceptors, contracted the rabbit renal artery in the present study.

The antagonism by phentolamine of 5-HT-, 5-CT- and sumatriptan-induced responses (pK_B values of 6.8–6.9) suggests that phentolamine may also antagonize the 5-HT_1-like receptor in the rabbit isolated renal artery. Indeed, it is known that phentolamine is an antagonist with low potency at 5-HT receptors (Hoffman & Lefkowitz, 1990), including the 5-HT_1-like receptors in the human isolated basilar artery (Parsons *et al.*, 1989).

Is the 5-HT-induced contraction of the renal artery mediated by noradrenaline release?

It has been reported that high concentrations of 5-HT can have indirect sympathomimetic actions by releasing noradrenaline (McGrath, 1977; Saxena & Villalón, 1990). Since relatively high concentrations of 5-HT, 5-CT and other agonists were needed to elicit renal artery contractions, it is possible that, like tyramine, these agents may release noradrenaline from sympathetic nerve endings. However, this is unlikely since the contractile responses to 5-HT and 5-CT were unaltered upon treatment of tissues with 6-hydroxydopamine, which clearly inhibited tyramine-induced contractions (see Table 3). *In vitro* treatment with 6-hydroxydopamine has been shown to produce noradrenergic denervation of vascular tissues (Aprigliano & Hermsmeyer, 1976). Furthermore, the response to 5-HT was not altered by cocaine, which moderately potentiated contractile responses to noradrenaline.

In conclusion, the present study shows that 5-HT contracts the rabbit isolated renal artery by activation of a 5-HT_1-like receptor. This effect was mimicked by a number of agonists having affinity for 5-HT_1-like receptors, but DOI, flesinoxan and RU 24969 were inactive. Methiothepin was a potent antagonist, while metergoline, ketanserin and MDL 72222 showed little or no antagonist activity. These results suggest that the renal vasoconstrictor effects of 5-CT in the conscious rabbit may not be entirely due to 5-HT_2 receptor activation following the release of 5-HT from blood platelets by 5-CT (Wright & Angus, 1989), but could be mediated, partly or entirely, by a 5-HT_1-like receptor on renal vessels. Such a 5-HT_1-like receptor mediating a short-lasting renal vasoconstriction has also recently been described in the anaesthetized dog (Cambridge *et al.*, 1991).

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Selective inhibition of K^+-stimulation of Na,K-ATPase by bretylium

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1 The effects of bretylium were investigated on purified Na,K-ATPase from guinea-pig heart and on the Na/K pump in trout erythrocytes, with a view to further identifying the mechanism(s) associated with its antiarrhythmic effects.

2 Na,K-ATPase activity of the thiocyanate-dispersed enzyme was determined by the measurement of inorganic phosphate produced by ATP hydrolysis.

3 When the concentrations of each of the Na,K-ATPase activating components were varied in turn, bretylium (1–5 mmol l^{-1}) exhibited competitive-type effects against K^+ with a K_i of 1.4 mmol l^{-1} and noncompetitive-type effects against Na^+, Mg^{2+} and ATP.

4 In K^+ influx studies in trout erythrocytes with $^{86}Rb^+$ used as the marker, the inhibition of total influx observed with bretylium (5 and 10 mmol l^{-1}) was attributable to the bretylium cation selectively inhibiting the Na/K pump-mediated influx with the associated tosylate anion inhibiting Na/K cotransport.

5 The observed inhibition kinetics indicated that the bretylium cation (2–15 mmol l^{-1}) competitively inhibited K^+ stimulation of the Na/K pump at 6 and 1.25 mmol l^{-1} external K^+ with a mean K_1 of 2.3 mmol l^{-1}.

6 The effects demonstrated on the functioning Na/K pump in erythrocytes confirmed the Na,K-ATPase findings, with bretylium selectively inhibiting K^+ stimulation of the pump mechanism in both cases.

7 It is suggested that Na,K-ATPase inhibition may contribute to the antiarrhythmic and positive inotropic effects of bretylium with the cardiac accumulation of bretylium also possibly being a further important factor.

Keywords: Bretylium; Na,K-ATPase; antiarrhythmic agents; guinea-pig heart; trout erythrocytes

Introduction

Bretylium (tosylate) is a quaternary ammonium compound with a Class III antiarrhythmic action used in the treatment of ventricular fibrillation and tachycardias resistant to first-line agents like lignocaine or procainamide (Koch-Weser, 1979; Anderson, 1985). Its prominent electrophysiological effects observed *in vitro* at concentrations in the 10^{-5}–10^{-4} mol l^{-1} range, are prolongation of action potential duration (APD) and effective refractory period (ERP) without any slowing of conduction (Wit *et al.*, 1970; Bigger & Jaffe, 1971; Cardinal & Sasyniuk, 1978; Heissenbutel & Bigger, 1979). Although such effects may be of some relevance in explaining its salutary antifibrillatory action, little is known about the underlying cellular/biochemical mechanisms involved (Bacaner *et al.*, 1986; Kopia & Lucchesi, 1987).

It has been suggested that K^+ channel blockade is the basis for the effectiveness of bretylium in raising the ventricular fibrillation threshold resulting in increased cellular refractoriness (Bacaner *et al.*, 1986; Bkaily *et al.*, 1988), with similarities having been implicated (Edwards, 1982) between the K^+ binding sites on the voltage-dependent-K^+ channel and on the Na,K-ATPase enzyme. The latter represents the Na/K pump mechanism in a variety of cells and species (Bonting *et al.*, 1961; Bonting & Caravaggio, 1963; Tosteson, 1963), with both the enzyme and the pump being specifically inhibited by ouabain and other cardiac glycosides as a principal characteristic (Glynn, 1968). Tetraethylammonium (TEA) and its derivatives exhibit both K^+ channel blocking (Armstrong, 1971) and Na/K pump inhibitory properties (Sachs, 1967; Sachs & Conrad, 1968; Kropp & Sachs, 1977; Klein & Ellory, 1980). Initial evidence was obtained from previous work in this laboratory of TEA and other small molecular weight quaternary ammonium compounds including, dimethylquaternary propranolol and bretylium, preferentially inhibiting the K^+ stimulation of brain Na,K-ATPase (Abdul-Cader *et al.*, 1973; 1977; 1984). Amiodarone, another Class III antiarrhythmic agent (Mason, 1987), was reported to inhibit guinea-pig heart Na,K-ATPase with competition against ATP being involved (Broekhuysen *et al.*, 1972), suggesting that modulation of Na,K-ATPase function might be associated with its antiarrhythmic effects.

In the present work, the kinetics of bretylium inhibition of Na,K-ATPase were investigated to ascertain whether it selectively inhibited K^+ activation of cardiac enzyme by use of a thiocyanate dispersed enzyme preparation obtained from guinea-pig heart. Comparable studies were also performed in trout erythrocytes, which exhibit an advantageously high K^+ influx rate with an appreciable Na/K pump component (Bourne & Cossins, 1984), to see if bretylium inhibited K^+ stimulation of the Na/K pump in a functioning situation.

Methods

Preparation of Na,K-ATPase

A 20% w/v homogenate was prepared in Solution A by the Shirachi *et al.* (1970) procedure from three guinea-pig hearts (approx. 4 g tissue) taken from male animals (300–400 g) and the 35,000 *g* fraction obtained by the Akera & Brody (1968) method, using Solution B in the second 35,000 *g* centrifugation. Subsequent purification was similar to that described by Broekhuysen *et al.* (1972) with LiBr replaced by NaSCN (Skou, 1974) as the enzyme dispersing agent. The 35,000 *g* pellet was suspended in Solution C containing 1 mol l^{-1} NaSCN and left at room temperature for 1 h with occasional swirling. After a 60 min 100,000 *g* centrifugation and three further centrifugations (30 min at 100,000 *g* in Solution D), the pellet was suspended in Solution E giving the thiocyanate-dispersed enzyme and 0.25 ml aliquots stored frozen at −40°C. The protein content of the enzyme preparation (yield approx. 20 ml) was 165 μg ml^{-1} as determined by the Lowry *et al.* (1951) method.

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Determination of Na,K-ATPase activity

The enzyme was thawed and diluted (1 in 2) at 5°C with 250 mmol l^{-1} sucrose adjusted to pH 7.5 with Tris base. It was kept at 5°C prior to incubation (30 min at 37°C; 26.1 μg enzyme ml^{-1}, volume 30 μl) with ATP/$MgCl_2$/NaCl/KCl/Tris-HCl buffer pH 7.5 mixture at the concentrations required. The reaction was started by the addition of enzyme (10 μl) and inorganic phosphate produced by ATP hydrolysis determined by the Atkinson *et al.* (1973) method, using 30 μl Solution F to stop the reaction; 15 min later when at room temperature, 130 μl Solution G was added and the absorbance measured at 820 nm after 10 min to allow maximal colour development. Additions of Solutions F and G were followed by thorough mixing. Each determination was made in triplicate and the mean absorbance used in calculating the results. Reagent blanks and KH_2PO_4 (10 nmol) standards were treated similarly. When ATP concentration was varied, the incubation volume was increased to 90 μl to minimize % substrate converted. Bretylium was included where indicated in the text. Na/K-ATPase activities were determined as the difference between total (Na + K + Mg)- and Mg-dependent ATPase activities observed in the absence and presence of ouabain (3 mmol l^{-1}) respectively. Mg-ATPase accounted for < 10% of the total activity measured under optimal conditions. Na,K-ATPase activities were expressed as μmol ATP hydrolysed per mg enzyme (protein) per hour (μmol mg^{-1} h^{-1}) and as percentages of the activity determined under optimal conditions in the presence of (mmol l^{-1}): ATP 5, $MgCl_2$ 5, NaCl 110, KCl 10, Tris 20, HCl buffer pH 7.5.

Determination of K^+ influx

K^+ influx was determined in rainbow trout (*Salmo gairdneri*) erythrocytes by the method of Bourne & Cossins (1984) based upon $^{86}Rb^+$ uptake measurement, with a 10 min incubation period at 10°C and 1% bovine serum albumin present to prevent haemolysis. Before K^+ influx assay, the separated erythrocytes were equilibrated for 10–18 h in Solution H to ensure they were not in a catecholamine-stimulated condition (Bourne & Cossins, 1982). This was followed when external K^+ concentration was lowered, by 30 min further equilibration in a solution of equal osmolality containing an increased (<4%) NaCl concentration. Mean results of duplicate determinations in the absence or presence of bretylium were expressed as mmol K^+ transported per litre (packed cell volume; p.c.v.) per hour. The Na/K pump-mediated component (A) of K^+ influx was determined by use of frusemide (1 mmol l^{-1}) to inhibit the Na/K cotransport component (B) and subtracting the residual component (C) measured in the presence of ouabain (1 mmol l^{-1}) plus frusemide (1 mmol l^{-1}). Similarly, (B) was determined as the difference between ouabain (1 mmol l^{-1})-insensitive influx and (C). At 6 mmol l^{-1} K^+, (A), (B) and (C) represented approximately 44, 48 and 8% respectively of total influx measured.

Kinetic and statistical methods

Woolf S/v against S plots (linear regressions) were obtained from the Na,K-ATPase data, where v = % Na,K-ATPase activity (initial velocity) and S = substrate or activator concentration (Webb, 1963), with S varied in the presence and absence of fixed inhibitor (bretylium) concentrations (I). When competitive kinetics were indicated, K_i (inhibitor dissociation constant) was determined as 1/slope of the K_m ratio minus 1 against I plot (linear regression), analogous to the dose-ratio minus 1 against antagonist concentration plot used in quantitative pharmacology (Arunlakshana & Schild, 1957), where K_m ratio = ratio of activator concentrations giving half-maximal velocity in presence/absence of inhibitor. In the erythrocyte experiments, inhibition kinetics of K^+ stimulation of the Na/K pump were investigated by varying I at fixed S concentrations, to avoid having to preincubate cells with a range of K^+ concentrations in the same experiment. In this case I/i against I plots (linear regressions) were used, where i = % inhibition. Since competitive and mixed type inhibition cannot be distinguished from the appearance of these plots by the curves being parallel in both cases with slopes = 1/maximal inhibition, competition was inferred when closely agreeing K_i values were obtained from the IC_{50} values (−x intercepts) using equation (I) below. This was indicative of the data fitting equation (II) as a linear transformation of the hyperbolic function (III) which applies under competitive conditions (Webb, 1963).

$$\text{(I)}\ K_i = \frac{IC_{50}}{1 + S/K_m} \qquad \text{(II)}\ \frac{I}{i} = I + K_i\left[1 + \frac{S}{K_m}\right]$$

$$\text{(III)}\ i = \frac{1}{1 + K_i/I[1 + S/K_m]}$$

Data based on mean %v or %i values (10–90%) obtained from 4 or 5 (n) experiments with different aliquots of enzyme or erythrocytes from different fish were used in the linear regressions. The coefficients of variation of the observed individual v and i values were <0.26 with the majority (>95%) in the 0.08–0.16 range. In all cases, the regressions (r = 0.989–0.999) were significant ($P < 0.001$) as determined by F ratio (within regression variance/residual variance) tests. Both the unpaired Student's t test and the nonparametric Wilcoxon Rank Sum test (two tailed) were used to assess the significance of the difference between K^+ influx rates observed under different conditions, based on the acceptance level of $P < 0.05$. The P values quoted cover the results of both tests.

Materials

Solution A containing (mmol l^{-1}): sucrose (250), L-histidine (5), EDTA (5) and Na deoxycholate (0.2% w/v) at pH 6.8 was modified to give Solutions B-E by including the following in addition to sucrose 250: B, L-histidine (5) and EDTA (1) at pH 7.0; C, L-histidine (5), EDTA (1) and NaSCN (1000) at pH 7.5; D, L-histidine (30) at pH 7.5; E, EDTA (1) at pH 7.5. Each solution was adjusted to the required pH with HCl or NaOH.

Solution F, 5% w/v Cirrasol/2% w/v ammonium molybdate in 1.8 mol l^{-1} H_2SO_4/H_2O (2:5:3 v/v).

Solution G, 5% w/v L-ascorbic acid/2.5% w/v Na dodecyl sulphate in 5 mmol l^{-1} Na_4 EDTA/H_2O (2:5:3 v/v).

Solution H, isosmotic trout saline prepared by mixing appropriate volumes of 120 and 170 mmol l^{-1} NaCl solutions containing (mmol l^{-1}); KCl (6), D-glucose (5) and imidazole (15), HCl pH 7.6 to give the same osmolality as the original plasma. Deionised twice distilled water was used throughout.

Tris ATP used in the determinations of Na,K-ATPase activity was prepared from Na_2-ATP by ion exchange (Schwartz *et al.*, 1962). Na_2-ATP (adenosine 5'-triphosphate disodium salt, vanadate free), bovine serum albumin, Cirrasol (Lubrol W) and ouabain octahydrate were obtained from Sigma Chemical Co. (Poole, Dorset), sucrose and Tris (Ultra Pure Reagents) from Bethesda Research Laboratories, PO Box 6009, Gaithersburg, MD 20877, U.S.A. and ^{86}rubidium chloride (170–360 mCi mmol $^{-1}$) from Amersham International UK (Aylesbury, Bucks). All other chemicals were either British Drug Houses (Dagenham, Essex) or Fisons Ltd., (Loughborough, Leics.) A.R. grade with frusemide (Hoechst, Hounslow, Middlesex) and bretylium tosylate (Wellcome, Beckenham, Kent) obtained as indicated.

Results

Na,K-ATPase studies

A preliminary enzyme characterization programme was performed in order to establish optimal conditions for the determination of Na,K-ATPase activity. This involved (i) varying the concentration of each activation component (Na^+, K^+,

Mg^{2+} and ATP) independently, (ii) checking the linearity of the velocity/time and velocity/enzyme concentration relationships and (iii) observing the ouabain sensitivity (IC_{50} 4 μmol l^{-1}). Subsequently, the effects of bretylium were observed against K^+ by varying the K^+ concentration (0.5–15 mmol l^{-1}) with fixed concentrations (mmol l^{-1}) of Na^+ (110), Mg^{2+} (5) and ATP (5) in the presence and absence of drug, and effects against the other activation components observed using a similar protocol in each case. The K_m values obtained from the S/v against S plots for K^+, Na^+, Mg^{2+} and ATP under control conditions when no bretylium was present (Figures 1–4) were 1.8, 12, 1.7 and 0.32 mmol l^{-1} respectively.

The parallel appearance of the Woolf plot (Figure 1) indicated that bretylium (1–5 mmol l^{-1}) inhibited K^+ activation of Na,K-ATPase in a competitive manner from the graded increases observed in K_m (−x intercept) without any apparent change in maximal velocity (V_m = 1/slope). The K_m ratio − 1 against I plot (Inset Figure 1) gave further evidence of competition by going through the origin, yielding a K_i for bretylium of 1.4 mmol l^{-1}. In contrast, bretylium exhibited noncompetitive-type effects against Na^+, Mg^{2+} and ATP (Figures 2–4) when tested at the highest concentration (5 mmol l^{-1}) used in the varied K^+ concentration experiments. In each case, bretylium decreased the V_m by approximately 20% as calculated from the difference in slopes with K_m being unchanged. Bretylium had no demonstrable effect on Mg-ATPase activity under the conditions used.

K^+ influx studies

In preliminary experiments with trout erythrocytes bretylium (1–10 mmol l^{-1}) inhibited total K^+ influx more effectively at 2 mmol l^{-1} K^+ than at 6 mmol l^{-1} K^+, suggesting that some element of competition against K^+ was involved. The inhibi-

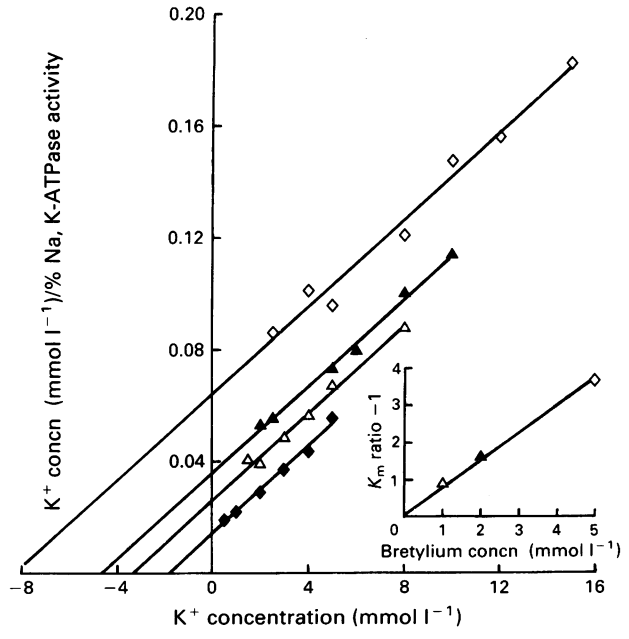


Figure 1 Woolf plots (linear regressions) for K^+ activation of guinea-pig heart Na,K-ATPase observed in the absence (◆) and presence of 1 (△), 2 (▲) and 5 (◇) mmol l^{-1} bretylium. Na,K-ATPase activity determined at varied KCl concentrations (1–15 mmol l^{-1}) with fixed concentrations (mmol l^{-1}) of ATP (5), $MgCl_2$ (5), NaCl (110), Tris (20)-HCl buffer pH 7.5 and enzyme (26.1 μg protein ml^{-1}) after 30 min incubation (30 μl volume) at 37°C. 100% Na,K-ATPase activity = rate measured (14.1 ± 0.34 μmol mg^{-1} h^{-1}; ±s.e.mean, n = 17) at 10 mmol l^{-1} K^+ when no bretylium present. Each point represents mean data from 4–5 experiments.

Inset: K_m ratio −1 against bretylium concentration plot (linear regression) where K_m ratio = ratio of −x intercepts of main plots obtained in presence/absence of bretylium.

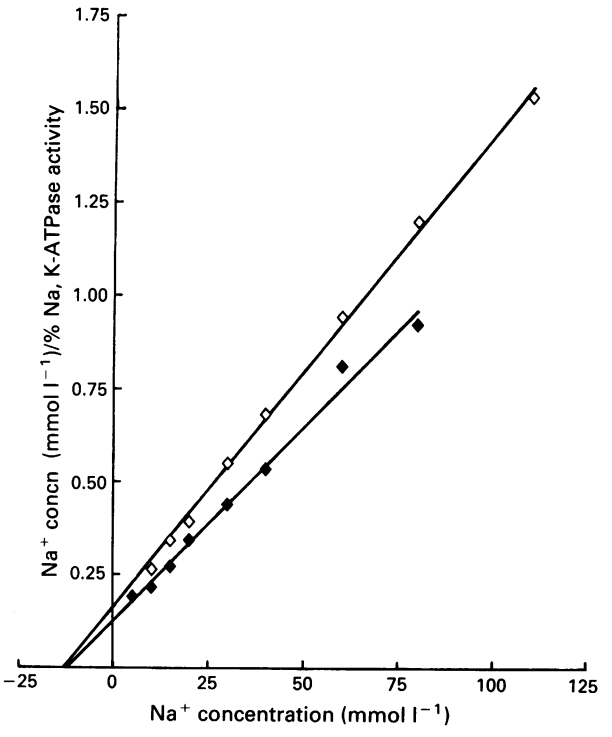


Figure 2 Woolf plots (linear regressions) for Na^+ activation of guinea-pig heart Na,K-ATPase observed in the absence (◆) and presence of 5 mmol l^{-1} bretylium (◇). Na,K-ATPase activity determined at varied NaCl concentrations (5–110 mmol l^{-1}) with fixed concentrations (mmol l^{-1}) of ATP (5), $MgCl_2$ (5), KCl (10), Tris (20)-HCl buffer pH 7.5 and enzyme (26.1 μg protein ml^{-1}) after 30 min incubation (30 μl volume) at 37°C. 100% Na,K-ATPase activity = rate measured (14.2 ± 0.41 μmol mg^{-1} h^{-1}, ±s.e.mean, n = 9) at 110 mmol l^{-1} Na^+ when no bretylium present. Each point represents mean data from 4–5 experiments.

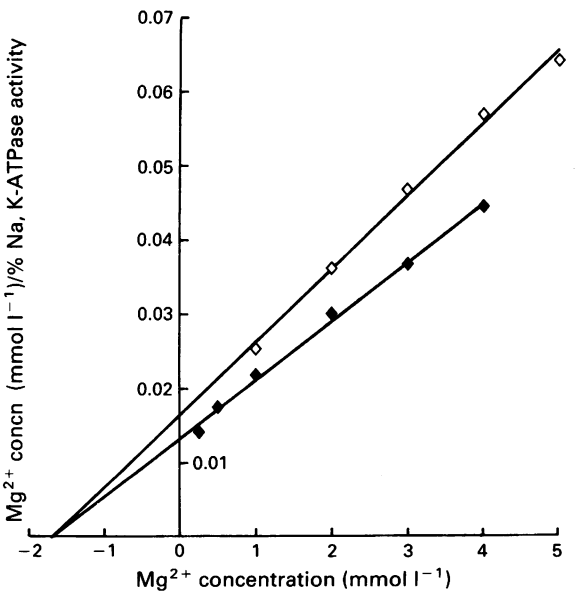


Figure 3 Woolf plots (linear regressions) for Mg^{2+} activation of guinea-pig heart Na,K-ATPase observed in the absence (◆) and presence of 5 mmol l^{-1} bretylium (◇). Na,K-ATPase activity determined at varied $MgCl_2$ concentrations (0.25–5 mmol l^{-1}) with fixed concentrations (mmol l^{-1}) of ATP (5), NaCl (110), KCl (10), Tris (20)-HCl buffer pH 7.5 and enzyme (26.1 μg protein ml^{-1}) after 30 min incubation (30 μl volume) at 37°C. 100% Na,K-ATPase activity = rate measured (13.9 ± 0.44 μmol mg^{-1} h^{-1}; ±s.e.mean, n = 8) at 5 mmol l^{-1} Mg^{2+} when no bretylium present. Each point represents mean data from 4 experiments.

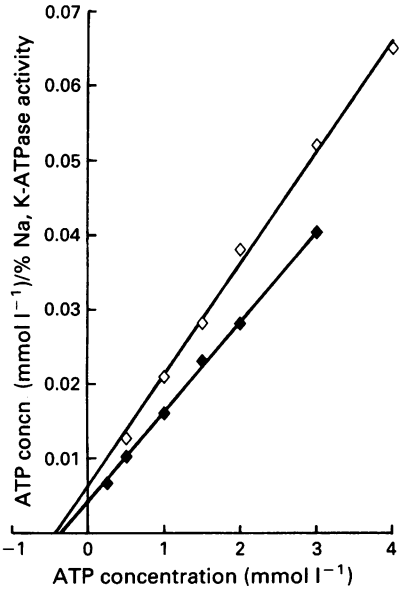


Figure 4 Woolf plots (linear regressions) for ATP activation of guinea-pig heart Na,K-ATPase observed in the absence (◆) and presence (◇) of 5 mmol l^{-1} bretylium. Na,K-ATPase activity determined at varied ATP concentrations (0.25–5 mmol l^{-1}) with fixed concentrations (mmol l^{-1}) of $MgCl_2$ (5), NaCl (110), KCl (10), Tris (20)-HCl buffer pH 7.5 and enzyme (26.1 μg protein ml^{-1}) after 30 min incubation (90 μl volume) at 37°C. 100% Na,K-ATPase activity = rate measured (14.6 ± 0.07 μmol mg^{-1} h^{-1}, ±s.e.mean, $n = 8$) at 5 mmol l^{-1} ATP when no bretylium present. Each point represents mean data from 4 experiments.

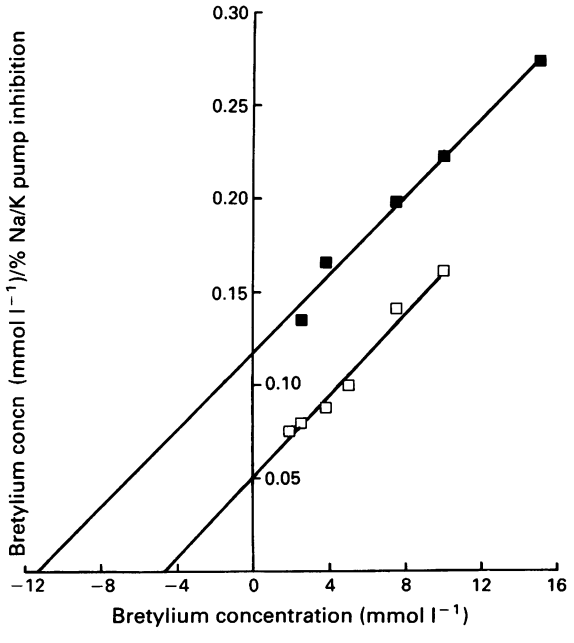


Figure 5 I/i against I plots (linear regressions) for inhibition (i%) of Na/K pump-mediated K^+ influx in trout erythrocytes produced by 1–15 mmol l^{-1} bretylium at 6 (■) and 1.25 (□) mmol l^{-1} K^+, with $^{86}Rb^+$ used as the marker for K^+. Influx measured in the presence of 1 mmol l^{-1} frusemide and residual (ouabain plus frusemide-insensitive) influx subtracted. Erythrocytes (2–5% p.c.v.) were incubated (1 ml volume) with $^{86}RbCl$ (0.5–2 μCi), KCl (6 or 1.25 mmol l^{-1}), NaCl (147–152 mmol l^{-1}), D-glucose (5 mmol l^{-1}), imidazole (15 mmol l^{-1})-HCl buffer pH 7.6 and BSA (1% w/v) for 10 min at 10°C. Each point represents mean data from 4 experiments. When no bretylium present, Na/K pump-mediated K^+ influx rates (±s.e.mean) measured at 6 and 1.25 mmol l^{-1} K^+ = 8.1 ± 0.27 ($n = 20$) and 6.5 ± 0.18 ($n = 24$) mmol K^+ l^{-1} (p.c.v.) h^{-1} respectively.

tion was subsequently accounted for, when the effects of bretylium (tosylate) and Na tosylate (*p*-toluene sulphonic acid, sodium salt) were observed on the different influx components at 2 mmol l^{-1} K^+, by the bretylium cation selectively inhibiting the Na/K pump component and the associated tosylate anion inhibiting Na/K cotransport. Na tosylate (5 and 10 mmol l^{-1}) inhibited Na/K cotransport (±s.e.mean, $n = 5$) by $8.2 \pm 1.6\%$ ($P > 0.5$) and $24.4 \pm 5.6\%$ ($P < 0.01$) respectively without any evidence of it influencing Na/K pump-mediated influx or residual influx. When the reduction in total K^+ influx produced by 10 mmol l^{-1} bretylium tosylate was corrected for the toxylate effect on Na/K cotransport and expressed as a percentage of the inhibition produced by ouabain (1 mmol l^{-1}), the Na/K pump inhibition ($47.2 \pm 3.1\%$, ±s.e.mean, $n = 4$) observed was not significantly different ($P > 0.05$) from the inhibition ($41.6 \pm 2.4\%$, ±s.e.mean; $n = 4$) measured by determining Na/K pump activity in the presence of frusemide as described under Methods. In order to eliminate the complicating effect of the tosylate anion in Na/K cotransport, the latter method was used in the subsequent investigation of the inhibition kinetics of bretylium on the Na/K pump.

The parallel curves on the I/i against I plot (Figure 5) obtained at 6 and 1.25 mmol l^{-1} K^+ indicated that bretylium cation (1.9–15 mmol l^{-1}) competitively inhibited K^+ stimulation of the functioning Na/K pump. This was confirmed by the similarity in the respective K_i values of 2.2 and 2.4 mmol obtained for bretylium at 6 and 1.25 mmol l^{-1} K^+ using the K_m for K^+ (1.5 mmol l^{-1}) determined previously with the system (Bourne & Cossins, 1984). Although the mean K_i value (2.3 mmol l^{-1}) was higher than the K_i value (1.4 mmol l^{-1}) obtained from the inhibition of K^+ activation of Na,K-ATPase, the agreement seemed reasonable considering the differences in the two systems.

Discussion

Evidence was obtained of bretylium competitively inhibiting K^+ stimulation of purified Na,K-ATPase from guinea-pig heart while being noncompetitive against the other activators (Na^+ and Mg^{2+}) and substrate (ATP). A selective effect of bretylium was also observed on the Na/K pump-mediated component of K^+ influx in trout erythrocytes attributable to the bretylium cation, with competition against K^+ again being indicated. In both cases, the findings may be explained by the bretylium cation interacting with the external K^+ site or at a closely adjacent site modulating K^+ affinity. The similarity in the K_i values (1.4–2.3 mmol l^{-1}) obtained for bretylium and the K_m values (1.5–1.8 mmol l^{-1}) for K^+ suggests that bretylium may have approximately the same affinity as K^+ in the two systems. Recently reported IC_{50} values (Dzimiri & Almotrefi, 1990) for bretylium (2.45 mmol l^{-1}) and ouabain (1.9 μmol l^{-1}) on guinea-pig heart Na,K-ATPase are in general agreement with the present results.

The selective inhibition of K^+ stimulation of Na,K-ATPase and of the functioning Na/K pump was demonstrated with overall bretylium concentrations in the 1–15 mmol l^{-1} range. This is much higher than the plasma concentrations (1–6 μmol l^{-1}) achieved in antiarrhythmic therapy (Rapeport, 1985) and those (0.02–1 mmol l^{-1}) giving *in vitro* electrophysiological effects (Wit *et al.*, 1970; Waxman & Wallace, 1972; Namm *et al.*, 1975). As bretylium is extensively accumulated in the heart (Namm *et al.*, 1975), its antiarrhythmic actions apparently correlated better with myocardial concentrations than with plasma concentrations (Anderson *et al.*, 1980). Similar bretylium accumulation (approx. 30 fold) was observed (Namm *et al.*, 1975) in cardiac tissue from normal and immunosympathectomized rats indicating that it was not principally due to accumulation in the associated sympathetic neurones. The highest bretylium concentrations were found in subcellular fractions of tissue exhibiting the highest Na,K-ATPase activities. Thus, accumulated bretylium concentra-

tions in close proximity to the enzyme may conceivably affect Na/K pump function.

The Na/K pump regulates and maintains cellular electrolyte gradients, influencing responses to electrical and chemical stimuli (Glitsch, 1979). In addition to it being markedly sensitive to changes in cytosolic Na^+ concentration (Glitsch, 1982), the modulation of Na/K pump function can alter the electrophysiological properties of the cell. For example, sub-toxic cardiac glycoside concentrations giving partial inhibition of the pump, initially prolong and then shorten APD (Brinkman & Ravens, 1976) and decrease the resting membrane potential (RMP) (Miura & Rosen, 1978). These effects have been attributed to an increased K^+ concentration in the diffusion restricted area of the extracellular space cleft (Baumgarten & Isenberg, 1977) as possibly mediated by Na^+ activated K^+ channels (Kameyama *et al.*, 1984). Also, short term changes in Na/K pump function during a cardiac cycle may alter the course of repolarization, RMP and Ca^{2+} metabolism linked to pump function in Na-Ca exchange (Herzig & Lüllman, 1989). The outward current generated by active Na^+ efflux via the Na/K pump, is believed (Glitsch, 1982) to contribute to the RMP, resulting in hyperpolarization and a delay in slow diastolic depolarization (Vassalle, 1970) with shortening of the APD (Gadsby & Cranefield, 1979). Thus partial inhibition of the Na/K pump may cause APD prolongation as observed initially with cardiac glycosides (Brinkman & Ravens, 1976) and bretylium (Wit *et al.*, 1970; Waxman & Wallace, 1972). However, this does not preclude other mechanisms also operating with bretylium when sustained prolongation of APD and ERP occurs (Wit *et al.*, 1970; Cardinal & Sasyniuk, 1978; Anderson *et al.*, 1980).

The question which arises from these results is whether there might be two components involving Na,K-ATPase interactions in the bretylium cardiac effects. Under the conditions used when little time was available for bretylium accumulation to occur, competition against K^+ observed with the highly charged quaternary ammonium compound may be most readily explained by it interacting with external K^+ sites (Glynn, 1968; Robinson & Flashner, 1979). Later as accumulation proceeds and the drug penetrates the membrane, some further effects may be generated by interactions at other sites, with for example internal inhibitory K^+ sites (Bond & Hudgins, 1979) or 'occluded' K^+ sites (Post *et al.*, 1972) possibly being involved. Another possible implication in view of the reported K^+ channel blocking effects of bretylium (Bacaner *et al.*, 1986; Bkaily *et al.*, 1988), is whether K^+ channel function is also affected either directly or indirectly by the accumulated drug. These speculations are based on the hypothesis of bretylium perhaps being capable of interacting generally with K^+ sites and this being influenced by the accessibility of the drug to the sites.

It appears that the positive inotropic effect produced by high bretylium concentrations may be due to Na,K-ATPase inhibition. Although this has been attributed to transient catecholamine release (Papp & Vaughan Williams, 1969; Markis & Koch-Weser, 1971; Heissenbutel & Bigger, 1979), neither reserpine (Graham & Chandler, 1973) nor propranolol (Amsterdam *et al.*, 1970) prevented it appearing. This suggests that a more direct action such as Na,K-ATPase inhibition might be involved. From the results of the present work, bretylium would not be expected to exhibit any appreciable cardiotonic action as it only had approximately 1/1000 of the Na,K-ATPase inhibitory potency of ouabain, when added immediately prior to observing the effects. Further investigations are in progress to explore the possibility of Na/K pump inhibition being produced with lower bretylium concentrations (10^{-6}–10^{-4} mol l^{-1}) on cardiac tissue when sufficient time has been allowed for bretylium accumulation (Namm *et al.*, 1975) to occur.

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Inositol 1,4,5-trisphosphate- and guanosine 5′-O-(3-thiotriphosphate)-induced Ca^{2+} release in cultured airway smooth muscle

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1 The interaction between inositol 1,4,5-trisphosphate ($InsP_3$) and guanosine 5′-O-(3-thio triphosphate) (GTPγS) releasable calcium (Ca^{2+}) pools was examined using ^{45}Ca effluxes in permeabilized cultured airway smooth muscle cells from rabbit trachea.

2 Addition of $InsP_3$ or GTPγS caused a concentration-dependent release of intracellular Ca^{2+}. The release of Ca^{2+} by $InsP_3$ was much greater than with GTPγS. Pretreatment with maximally effective $InsP_3$ (10 μM) abolished the GTPγS-induced Ca^{2+} release, whereas pretreatment with 100 μM GTPγS reduced the $InsP_3$-induced Ca^{2+} release by 25%.

3 Ryanodine (100 μM), also gave a large release of intracellular Ca^{2+}. After pretreatment with 100 μM ryanodine, GTPγS did not induce Ca^{2+} release, and $InsP_3$-induced Ca^{2+} release was reduced by 76%.

4 Caffeine (50 mM), produced a slow release of intracellular Ca^{2+}. Pre-exposure to 50 mM caffeine had no effect on the GTPγS-induced Ca^{2+} release but reduced the $InsP_3$ releasable Ca^{2+} by 58%.

5 Pretreatment with ryanodine abolished the caffeine-induced Ca^{2+} release, and addition of caffeine before ryanodine reduced the ryanodine-induced Ca^{2+} release by 64.4%.

6 These results suggest that there are at least three pools of Ca^{2+} present within airway smooth muscle cells. The largest pool is released by $InsP_3$ or ryanodine, another is released either by a high concentration of $InsP_3$ or on application of GTPγS, and the third by $InsP_3$ alone. Ca^{2+} may be able to move from the GTPγS-sensitive pool into the $InsP_3$- and ryanodine-sensitive pool when this becomes depleted. In contrast, the opposite movement of Ca^{2+} cannot occur.

Keywords: Inositol 1,4,5-trisphosphate; guanosine 5′-O-(3-thio triphosphate) (GTPγS); caffeine; ryanodine; GTP; sarcoplasmic reticulum; smooth muscle; Ca^{2+} release

Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$) is an important second messenger linking agonist stimulation with release of Ca^{2+} from an intracellular store, believed to be the sarcoplasmic reticulum (SR) (Berridge & Irvine, 1984). Recently there has been great interest in the rôle of guanosine 5′-triphosphate (GTP) or non-hydrolysable GTP analogues such as guanosine 5′-O-(3-thio triphosphate) (GTPγS) in releasing intracellular Ca^{2+}, and in their relationship to $InsP_3$-induced Ca^{2+} release.

The release of Ca^{2+} in response to activation by GTP has been observed both in permeabilized cells of a neuronal cell line (Gill *et al.*, 1986) and in microsomal membrane fractions (Ueda *et al.*, 1986). GTPγS was also found to induce Ca^{2+} release in permeabilized guinea-pig vein smooth muscle (Somlyo *et al.*, 1988).

Chueh *et al.* (1987) suggested that three intracellular compartments exist in neuroblastoma cells; one sensitive to both GTP and $InsP_3$, another to GTP alone and a third not releasing Ca^{2+} in response to either agent. The GTP-releasable pool was found to be larger than the $InsP_3$-releasable pool.

The intracellular Ca^{2+} store can be investigated by use of the agents ryanodine and caffeine. Ryanodine is a naturally occurring plant alkaloid which has been found to act specifically on the SR in skeletal and cardiac muscle (Wier *et al.*, 1985; Sutko *et al.*, 1985). In rabbit aortic smooth muscle ryanodine was found to induce SR Ca^{2+} loss thereby depleting the noradrenaline-sensitive Ca^{2+} store (Hwang & van Breemen, 1987), while in guinea-pig taenia caecum, ryanodine stimulated a leak of Ca^{2+} from the caffeine-sensitive Ca^{2+} store (Hisayama & Takayanagi, 1988). The methylxanthine, caffeine, is also well established as releasing intracellular Ca^{2+} from smooth muscle cells (Leijten & van Breemen, 1984; Benham & Bolton, 1986). In airway smooth muscle, caffeine can evoke contraction by liberation of intracellular Ca^{2+} (Ito & Itoh, 1984; Small *et al.*, 1988).

The aim of this study was to investigate differing Ca^{2+} pools in airway smooth muscle, and the mechanism of action of Ca^{2+} translocation within them. This was examined by studying the effect of adding $InsP_3$ and GTPγS sequentially and also by investigating the effects of ryanodine and caffeine on $InsP_3$- and GTPγS-induced Ca^{2+} release. Part of this work has been communicated to the British Pharmacological Society (Chopra *et al.*, 1989a).

Methods

Cell culture

Adult male New Zealand White rabbits (2–3 kg in weight) were injected intravenously with a lethal dose (50–60 mg kg^{-1}) of pentobarbitone. The cylindrical trachea was removed immediately and under aseptic conditions dissected free of connective tissue. The cartilage rings surrounding the strip of smooth muscle could then be seen clearly. An incision was made longitudinally through the cartilage, and the trachea opened up to reveal the muscle. The epithelium was removed by scraping across the surface with a scalpel blade. The strip of muscle was then cut gently away from the cartilage and incubated for 30 min at 37°C in Hank's buffered salt solution (in mM: NaCl 136.8, KCl 5.4, $MgSO_4$ 0.8, $Na_2HPO_4 \cdot 7H_2O$ 0.4, $KH_2PO_4 \cdot 7H_2O$ 0.4, $CaCl_2 \cdot 2H_2O$ 1.3, $NaHCO_3$ 4.2, glucose 5.6, phenol red 20 μg ml^{-1}, Gibco) containing collagenase (type 1, Sigma) 1 mg ml^{-1} and elastase (type I, Sigma) 3.3 u ml^{-1} in an atmosphere of 5% CO_2 in air (pH 7.4). After this time, the smooth muscle could be separated from any

remaining connective tissue with the aid of a dissecting microscope. It was then cut into 1 mm square fragments and incubated for 150 min in Hank's solution containing collagenase 1 mg ml^{-1}, elastase 15 u ml^{-1} and 1% bovine albumin (adjusted to pH 7.4). The mixture of enzyme solution and tissue was separated by centrifugation in a sterile polypropylene tube at 130 g for 5 min at 22°C. The enzyme solution was removed and replaced with 2 ml of Dulbecco's modified Eagles Medium supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum, penicillin 100 u ml^{-1} and streptomycin (100 μg ml^{-1}). The tissue was dispersed into cell clumps and single cells by repeated pipetting for 2 min.

The cells were seeded at a density of 3.2×10^5 viable cells ml^{-1} into 35 mm dishes. Cell viability, assessed by trypan blue exclusion was >95%. The dishes were then incubated and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. The culture medium was replaced every 72–96 h. After 6–10 days the cells grew to confluency (4.1×10^5 cells/dish) and were subcultured by removing them from the base of the dish with a plastic scraper. They were dispersed by repeated pipetting and seeded at a lower density (0.8×10^5 cells/ml) into 25 cm^2 flasks. Subsequent passages reached confluency after 4–7 days (1.1×10^6 cells/flask) and were subcultured into 75 cm^2 flasks at the same lower density. On then reaching confluency (2.5×10^6 cells/flask) the cells were subcultured into 35 mm dishes (≈0.2 mg cell protein per dish at confluency) for experimental use and further 75 cm^2 flasks in order to continue the cell line. Subculturing was performed up to 10 passages, and experiments were performed on cells from between 5–10 passages. Preliminary experiments showed no loss of surface receptors at these passages.

Ca^{2+} efflux from permeabilized cells

All experiments were performed at room temperature (22°C). The tissue culture medium was removed from the dish and the cell monolayer was washed initially in physiological salt solution (PSS) (composition in mM: NaCl 140, KCl 5.0, $MgCl_2$ 1.0, glucose 10.0, N-2hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) 10.0, $CaCl_2$ 1.5, pH 7.4). The experiments were performed in solutions for permeabilized cells containing KCl 130, $MgCl_2$ 5, Tris-maleate buffer 20, ATP 3.15, pH 6.8, and either 0.1 or 4.0 mM ethylene glycol-*bis*(β-aminoethylether) N,N,N,N-tetraacetic acid (EGTA). After the initial wash in PSS the cells were washed in 4 mM EGTA solution for 2 min, and then permeabilized for 15 min with an identical solution but also containing 40 μM digitonin. After washing in a 0.1 mM EGTA solution for a further 2 min, the cells were incubated in ^{45}Ca-labelled solution (10 μCi ^{45}Ca ml^{-1}) with a free Ca^{2+} concentration of 1 μM for 30 min, by which time steady state uptake was achieved (Figure 1). A free Ca^{2+} concentration of 1 μM was achieved by adding the appropriate amount of $CaCl_2$ to the 0.1 mM EGTA-containing solution, calculated using 1×10^{-6} M^{-1} as the apparent binding constant of EGTA for Ca^{2+} at pH 6.8, 22°C (Saida & Nonomura, 1978).

The labelling solution was then removed and ^{45}Ca was allowed to efflux from the cells into 0.1 mM EGTA. The efflux solution was changed every min for 10 min, and then every 5 min for 55 min. Each aliquot was collected in a counting vial. Combinations of $InsP_3$, GTPγS, ryanodine and caffeine were added to the efflux solution for specific 5 min intervals during the 55 min period and the resultant effect on Ca^{2+} loss estimated.

Cells were harvested from the dishes at the end of the experiment in PSS containing collagenase (1 mg ml^{-1}) and trypsin (0.1 mg ml^{-1}). The radioactivity of the cells, of each vial and the labelling solution was estimated by liquid scintillation counting. The amount of Ca^{2+} in the intracellular store could be quantified by analysis of the efflux of ^{45}Ca from the cells. The intracellular Ca^{2+} store was assumed to be the most slowly effluxing compartment of the permeabilized cells. Extrapolation of this slow monoexponential loss of Ca^{2+} content back to the onset of the efflux procedure gives an estimate of the content of the intracellular store at the end of the Ca^{2+} uptake period (Yamamoto & van Breemen, 1986; Twort & van Breemen, 1989). Ca^{2+} content was expressed as nmol Ca mg^{-1} protein and Ca^{2+} loss as nmol Ca mg^{-1} protein min^{-1}. By measuring the decrease in Ca^{2+} content on addition of each agent, and correcting for normal efflux at this time point, values for Ca^{2+} release on addition of the agent could be calculated. Protein estimations were measured by the method of Lowry *et al.* (1951) on identical dishes cultured at the same time as the test dishes.

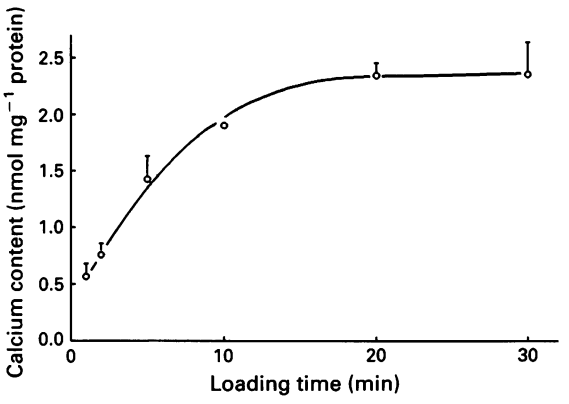


Figure 1 Time course of ^{45}Ca uptake by permeabilized cells. Loading was initiated at time 0. Steady state uptake was achieved by 20 min, and in all subsequent experiments cells were loaded for 30 min (n = 3).

Results are expressed as mean ± s.d. The difference between the results was compared by Student's t test for unpaired values. For clarity the majority of figures show the results of a single experiment representative of the series. Concentration-response curves were fitted by use of a commercial software package, (Sigmaplot 4, Jandel Scientific, U.S.A.).

Chemicals

$InsP_3$, GTPγS, GTP, GppNHp, caffeine, collagenase type I, elastase type I and trypsin type III were all purchased from Sigma. Ryanodine was purchased from Calbiochem. Tissue culture materials and antibiotics were purchased from Gibco.

Results

$InsP_3$- and GTPγS-induced Ca^{2+} release

Addition of $InsP_3$ or GTPγS caused a concentration-dependent release of intracellular Ca^{2+}. The concentration-response curve for $InsP_3$ is shown in Figure 2a, with an EC_{50} of 2.31 ± 0.41 μM. Maximally effective $InsP_3$ (10 μM) released 0.5 ± 0.27 nmol Ca^{2+} mg^{-1} cell protein. The EC_{50} for GTPγS-induced Ca^{2+} release was 61.5 ± 4.7 μM (Figure 2b). GTPγS, 100 μM, released 0.12 ± 0.03 nmol Ca^{2+} mg^{-1} protein, which was significantly less than that released by maximal $InsP_3$ (n = 5, $P < 0.05$). The concentration-response curve for GTPγS clearly does not reach a plateau at 100 μM GTPγS and the calculated plateau maximum was 0.15 ± 0.03 nmol Ca^{2+} mg^{-1} protein. Concentrations of GTPγS higher than 100 μM were however difficult to achieve in practice, due to the stock concentrations required.

Addition of neomycin (100 μM), which has been shown to inhibit phospholipase C (Cockcroft & Gomperts, 1985; Cockcroft *et al.*, 1987), for 15 min prior to, and during incubation with GTPγS showed no inhibition of response, indicating that there was no stimulation of the plasmalemma G protein involved in phosphoinositide turnover by GTPγS (n = 3).

As previously observed (Chopra *et al.*, 1989b), pretreatment with 10 μg ml^{-1} low molecular weight heparin was found to

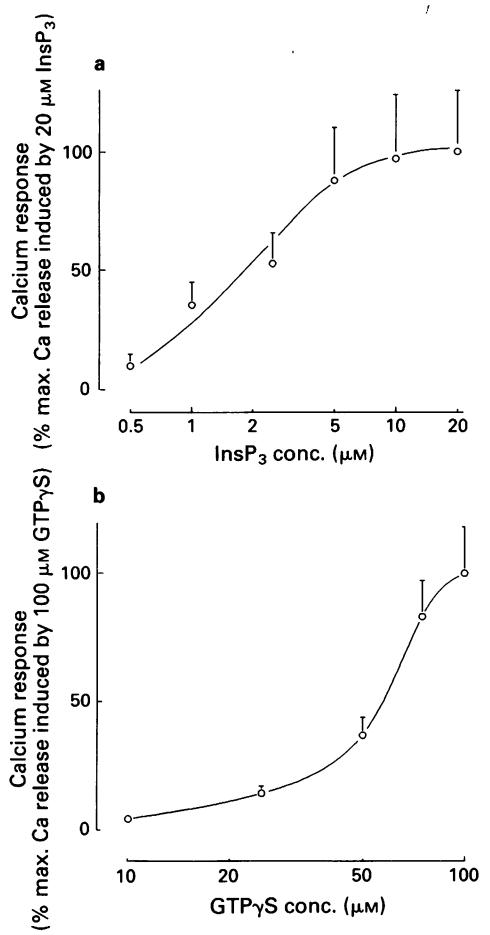


Figure 2 (a) Concentration-response of $InsP_3$-induced calcium release. Symbols represent the mean with s.e.mean shown by vertical bars, $n = 4$. (b) Concentration-response of GTPγS-induced calcium release. Symbols represent the mean with s.e.mean shown by vertical bars, $n = 4$.

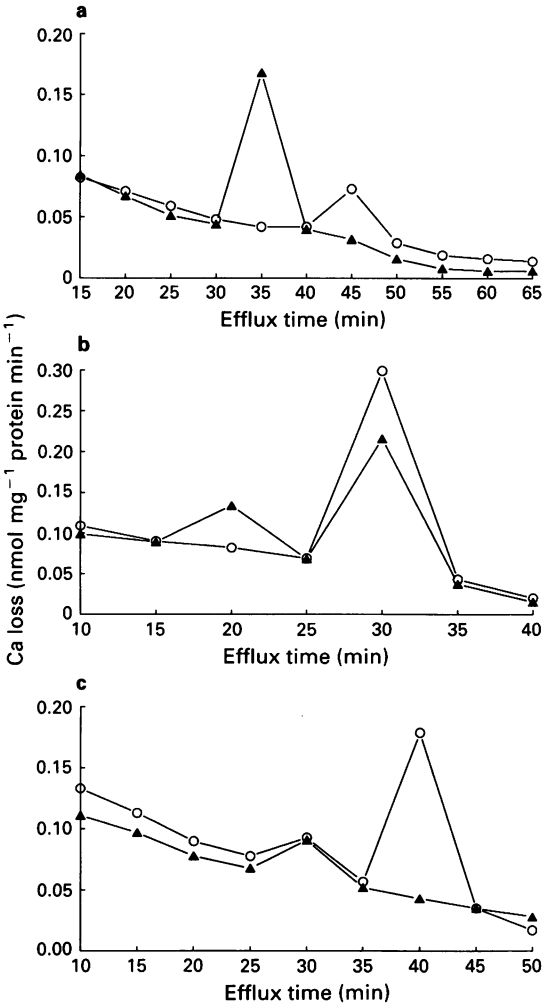


Figure 3 (a) (○) Increase in rate of Ca^{2+} loss induced by GTPγS; 100 μM GTPγS was added at 40 min for 5 min; (▲) increase in rate of Ca^{2+} loss induced by maximally effective $InsP_3$; 10 μM $InsP_3$ was added for 15 min at 30 min, and 100 μM GTPγS added for 5 min at 40 min. The figure shows that pretreatment with 10 μM $InsP_3$ abolished any subsequent GTPγS-induced Ca^{2+} release. The data shown are from a single representative experiment out of a series of three. (b) Effect of pretreatment with GTPγS: (▲) GTPγS (100 μM) was added at 15 min for 15 min, and $InsP_3$ (10 μM) at 25 min for 5 min. Pretreatment with GTPγS clearly reduced $InsP_3$ induced Ca^{2+} release; (○) control experiment where 10 μM $InsP_3$ was added at 35 min in the absence of GTPγS. The data shown are from a single representative experiment out of a series of three. (c) Effect of low molecular weight heparin on $InsP_3$ and GTPγS induced Ca^{2+} release. (○) GTPγS (100 μM) added at 25 min for 15 min, and $InsP_3$ (10 μM) at 35 min for 5 min. (▲) as (○) but 10 μg ml^{-1} heparin added from 10–50 min inclusive. Low molecular weight heparin blocked $InsP_3$ but not GTPγS induced Ca^{2+} release. The data shown are from a single representative experiment out of a series of three.

inhibit the $InsP_3$-induced Ca^{2+} release but not the GTPγS-induced release.

Sequential addition of $InsP_3$ and GTPγS

No release of Ca^{2+} was measured in response to 100 μM GTPγS following 10 μM $InsP_3$ (Figure 3a). However, following sub-maximal $InsP_3$ (2 μM) which released approximately 50% of the $InsP_3$-releasable calcium (Figure 2), 100 μM GTPγS released significantly less calcium than that released by 100 μM GTPγS alone (37.2% ± 7.6, $P < 0.01$, $n = 5$).

Addition of 100 μM GTPγS prior to maximally effective 10 μM $InsP_3$, reduced the $InsP_3$ response to 75.1% ± 8.9 ($n = 3$, $P < 0.05$) (Figure 3b). The GTPγS response corresponded to 30.0% ± 0.8 of the $InsP_3$-releasable pool. In the presence of heparin from 20–45 min, the secondary release of Ca^{2+} on addition of $InsP_3$ was abolished, $n = 3$ (Figure 3c).

Effect of ryanodine and caffeine on $InsP_3$- and GTPγS-induced Ca^{2+} release

It proved impossible to construct meaningful concentration-response curves for either caffeine or ryanodine. No further increases in Ca^{2+} release were found however for concentrations of ryanodine above 100 μM, or of caffeine above 50 mM.

Maximally effective ryanodine (100 μM) released 0.42 ± 0.13 nmol Ca^{2+} mg^{-1} protein ($n = 5$). Low molecular weight heparin (4000–6000) (10 μg ml^{-1}) could not block ryanodine-induced Ca^{2+} release, ($n = 3$). Pretreatment with 100 μM ryanodine reduced the subsequent $InsP_3$-induced Ca^{2+} release to 0.12 ± 0.09 nmol Ca^{2+} mg^{-1} (24%) which was significantly less than that induced by maximal $InsP_3$ ($n = 5$, $P < 0.02$), but still significantly greater than zero ($P < 0.05$, $n = 5$). Pretreatment with 100 μM ryanodine abolished the response to GTPγS, ($n = 4$) (Figure 4a,b).

Maximally effective caffeine (50 mM) released 0.11 ± 0.01 nmol Ca^{2+} mg^{-1}, and on pretreatment reduced subsequent $InsP_3$-induced release to 0.21 ± 0.01 nmol Ca^{2+} mg^{-1} (42%), which was significantly less than that induced by maximally effective $InsP_3$ alone ($n = 4$, $P < 0.01$) (Figure 5a). Pretreatment with caffeine did not alter subsequent GTPγS-induced Ca^{2+} release ($n = 5$, $P > 0.1$) (Figure 5b). The response to caffeine was noticeably slower than that to ryanodine.

Relationship between ryanodine- and caffeine-induced Ca^{2+} release

Sequential addition of 50 mM caffeine followed by 100 μM ryanodine caused two releases of Ca^{2+}. The release observed on

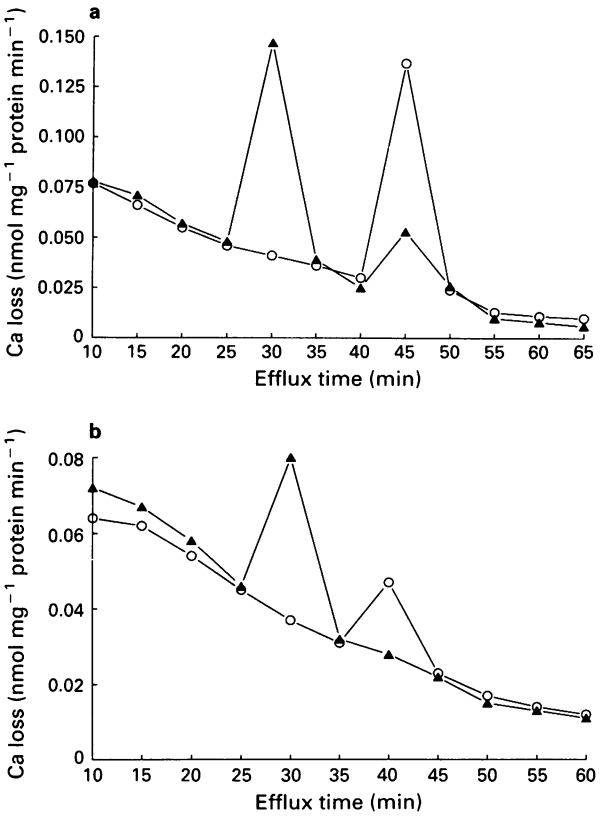


Figure 4 (a) Increase in rate of Ca^{2+} loss induced by ryanodine. (▲) Ryanodine (100 μM) added at 25 min for 20 min, and $InsP_3$ (10 μM) at 40 min for 5 min. Pretreatment with 100 μM ryanodine substantially reduced $InsP_3$-induced Ca^{2+} loss. (○) Control $InsP_3$-induced Ca^{2+} release without pretreatment with ryanodine. The data shown are from a single representative experiment out of a series of five. (b) Effect of ryanodine on GTPγS-induced Ca^{2+} release. (▲) Ryanodine (100 μM) added at 25 min for 15 min, and GTPγS (100 μM) at 35 min for 5 min. Pretreatment with ryanodine abolished the GTPγS-induced Ca^{2+} release. (○) Control GTPγS-induced Ca^{2+} release without ryanodine. The data shown are from a single representative experiment out of a series of four.

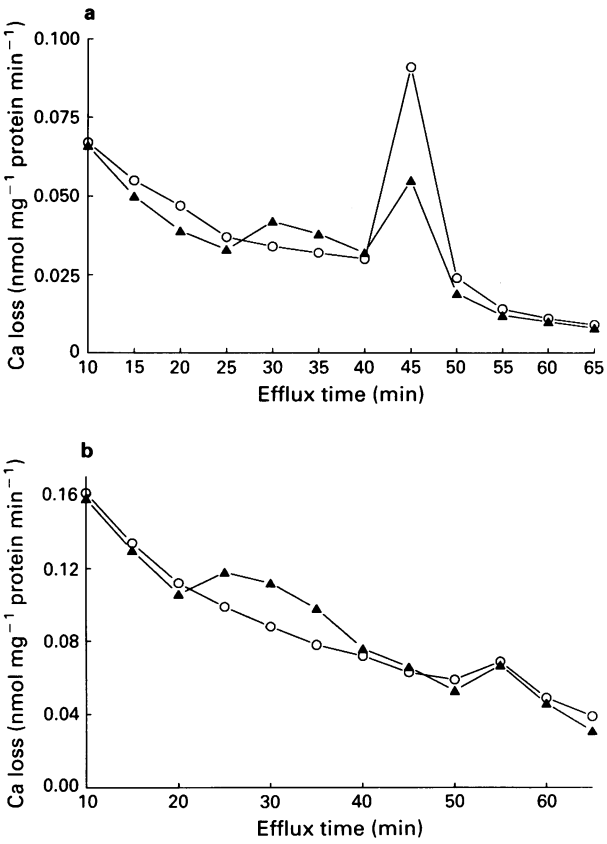


Figure 5 (a) Increase in rate of Ca^{2+} release induced by maximally effective caffeine. (▲) Caffeine (50 mM) added at 25 min for 20 min, and 10 μM $InsP_3$ at 40 min for 5 min. Pretreatment with caffeine reduced the $InsP_3$-induced Ca^{2+} release. (○) Control $InsP_3$-induced Ca^{2+} release without caffeine. The data shown are from a single representative experiment out of a series of four. (b) Effect of caffeine on GTPγS-induced Ca^{2+} release. (▲) Caffeine (50 mM) added at 20 min for 35 min, and GTPγS (100 μM) at 50 min for 5 min. Pretreatment with caffeine had no effect on GTPγS-induced Ca^{2+} release. (○) Control GTPγS-induced Ca^{2+} release without caffeine. The data shown are from a single representative experiment out of a series of five.

addition of ryanodine was markedly reduced to 35.6% ± 11.6 and significantly different from that of ryanodine alone, ($n = 3$, $P < 0.01$) (Figure 6a). However, sequential addition of 100 μM ryanodine followed by 50 mM caffeine caused only one release of Ca^{2+} which was that induced by ryanodine alone, ($n = 3$) (Figure 6b).

Discussion

We have demonstrated that both $InsP_3$ and GTPγS induce intracellular Ca^{2+} release within cultured airway smooth muscle cells. The GTPγS-releasable Ca^{2+} pool in airway smooth muscle cells may be similar to the GTP-sensitive Ca^{2+} pool described by other workers (Gill *et al.*, 1986; Ueda *et al.*, 1986; Somlyo *et al.*, 1988). This is in contrast with the findings of Dawson *et al.* (1986) who showed that in liver microsomes GTP or $InsP_3$ alone induced no intracellular Ca^{2+} release, but that GTP could promote the effectiveness of $InsP_3$-induced Ca^{2+} release. It also contrasts with the findings in vascular smooth muscle that another non-hydrolysable analogue of GTP, GppNHp, can substitute for GTP in the GTP-dependent $InsP_3$-induced Ca^{2+} release in skinned vascular smooth muscle, but cannot induce Ca^{2+} release itself (Saida & van Breemen, 1987).

Also of relevance are the observations by Gill *et al.* (1986) in neuroblastoma cells that GTPγS did not release intracellular Ca^{2+} but blocked GTP-stimulated release of Ca^{2+}, and that GppNHp had no effect either in releasing Ca^{2+}, or in blocking the action of GTP. We have found that GTP, GppNHp and GTPγS can all induce intracellular Ca^{2+} release. The relationship between the actions of these analogues is currently being investigated. It is debatable whether GTP hydrolysis is required for the $InsP_3$-independent Ca^{2+} release in airway smooth muscle. GTPγS may be acting at the same site as GTP in airway smooth muscle cells by a mechanism not requiring hydrolysis. However Chueh & Gill (1986) have suggested that since GTPγS can induce a slow release of Ca^{2+} in neuroblastoma cells, a slow hydrolysis of the γ-thiophosphate, or possible transfer of the phosphorothioate residue to a protein may be occurring. In preliminary experiments in airway smooth muscle we have discovered that guanosine 5'-diphosphate (GDP), or the non-hydrolysable analogue, guanosine 5'-O-(3-thiodiphosphate) (GDPβS) can block GTP- but not GTPγS-induced calcium release. This may therefore be an indication that GTP and GTPγS are acting by different mechanisms.

Our results suggest that there are at least two pools of Ca^{2+} present, one that is released by $InsP_3$ alone and a smaller pool which can be released by either GTPγS or a high concentration of $InsP_3$. They differ from those obtained by Chueh *et al.* (1987) in N1E-115 neuroblastoma cells, who showed that maximal GTP-induced Ca^{2+} release was greater than $InsP_3$-induced Ca^{2+} release. These workers also showed that after maximal $InsP_3$-induced Ca^{2+} release, addition of GTP released more Ca^{2+} so that the total released equalled that observed following addition of GTP in the absence of $InsP_3$. The relative effectiveness of $InsP_3$ and GTP would therefore appear to be reversed between these two tissues.

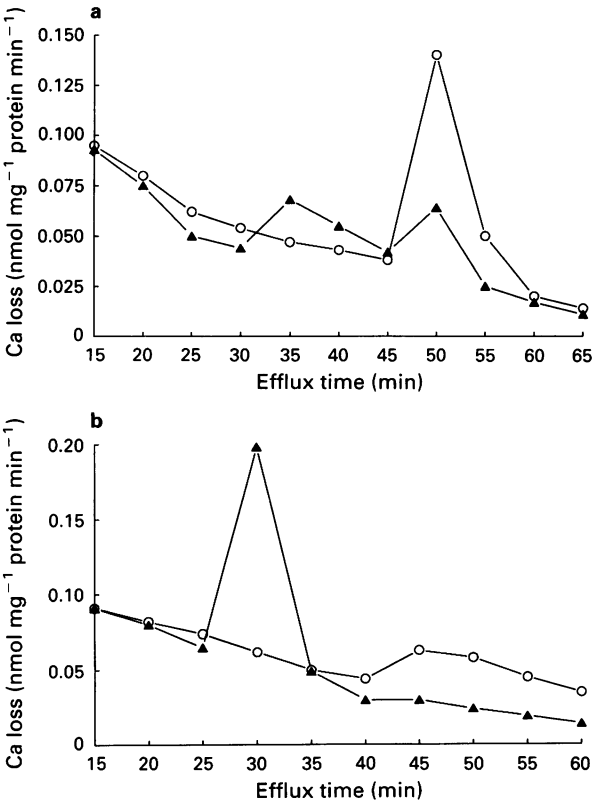


Figure 6 (a) Relationship between caffeine- and ryanodine-sensitive Ca^{2+} pools. (▲) Caffeine (50 mM) added at 30 min for 20 min, 100 μM ryanodine at 45 min for 5 min. Caffeine released part of the ryanodine-releasable Ca^{2+} pool. (○) Control ryanodine-induced Ca^{2+} release without caffeine. The data shown are from a single representative experiment out of a series of three. (b) (▲) Ryanodine (100 μM) added at 25 min for 30 min, and caffeine (50 mM) at 40 min for 15 min. Ryanodine abolished the caffeine releasable pool. (○) Control caffeine-induced Ca^{2+} release without ryanodine. The data shown are from a single representative experiment out of a series of three.

The discrepancy between the present results and those variously described above cannot be ascribed purely to differences in experimental technique, but must relate to an underlying diversity in tissue type.

Addition of maximally effective ryanodine produces a similar action to that of adding a high concentration of $InsP_3$. This is interesting since it has been discovered that the structure of the $InsP_3$ receptor, described by Furuichi *et al.* (1989) for mouse cerebellum, closely resembles that of the ryanodine receptor described by Takeshima *et al.* (1989) in skeletal muscle. However, since we have found that heparin does not block ryanodine-induced Ca^{2+} release, ryanodine and $InsP_3$ would seem to act at a similar but not identical class of sarcoplasmic reticulum receptor.

The action of ryanodine cannot however be explained in terms of the two compartment model described above, as a proportion of the $InsP_3$-sensitive pool is insensitive to ryanodine. A modified, three compartment model is shown in Figure 7. In this there are two connected compartments, one of which, the larger, is sensitive to $InsP_3$ and ryanodine (I_1), and the other sensitive to GTPγS (G_1). A separate compartment is sensitive to $InsP_3$ alone (I_2). It is important to note that these divisions are not necessarily meant to represent actual physical compartments.

Application of maximally effective GTPγS would cause emptying of G_1 only. It is unclear whether this would involve an SR G-protein, as experiments to investigate this have so far proved equivocal. Our results suggest that maximally effective $InsP_3$ results in Ca^{2+} release from all three compartments. However, following application of sub-maximal $InsP_3$ (2 μM), the remaining Ca^{2+} in the store can be released with GTPγS. This suggests either that $InsP_3$ then has a direct, albeit less efficacious action on G_1, or that a substantial reduction in $[Ca^{2+}]$ in I_1 can cause a release of Ca^{2+} from G_1 into I_1 which is then lost. A sub-maximal dose of $InsP_3$ would not entirely empty I_1 with the result that the transfer from G_1 would not be complete. It is conceivable that under such an arrangement the G_1 store could be involved with refilling of I_1.

The described effects of ryanodine, if it acted only on I_1, would be consistent with the above, and with the existence of an $InsP_3$-sensitive, but ryanodine-insensitive store, I_2. Because ryanodine can abolish any Ca^{2+} release by caffeine, yet caffeine has no effect on subsequent GTPγS-induced release, caffeine must act on I_1 alone. It is clear however, that caffeine is much less effective in promoting Ca^{2+} release than either ryanodine or $InsP_3$, and this may explain the lack of any effect on the GTPγS-releasable fraction; caffeine does not reduce the $[Ca^{2+}]$ in I_1 sufficiently for any transfer to occur from G_1 to I_1. The reason why caffeine is less effective in promoting Ca^{2+} release is unclear. It is possible that its much slower mode of action than either $InsP_3$ or ryanodine prevents the store emptying within the time available, although

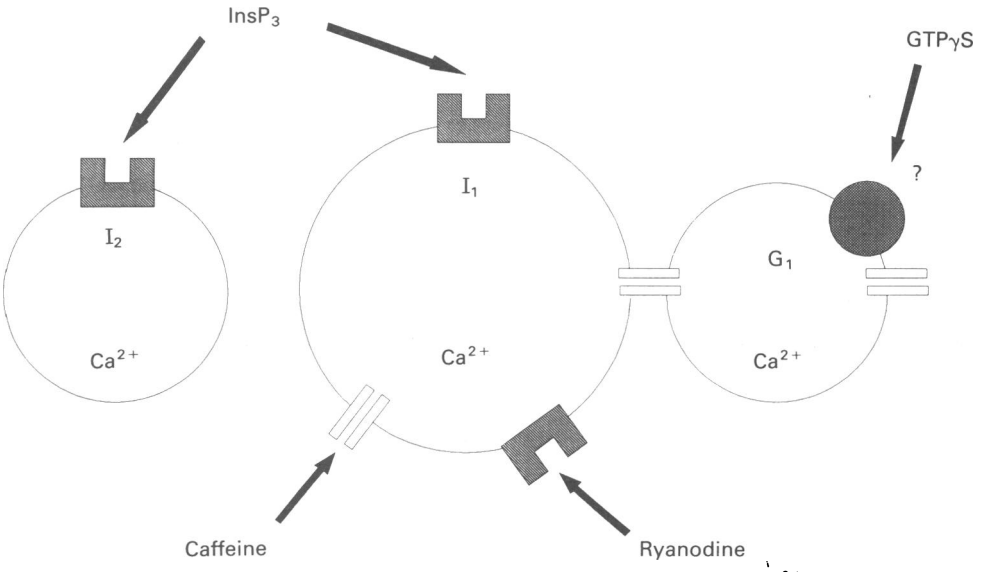


Figure 7 Putative three compartment model for $InsP_3$- and GTPγS-sensitive intracellular Ca^{2+} stores in airway smooth muscle. $InsP_3$ and ryanodine are shown acting at specific receptors, whereas caffeine probably acts via a non-receptor-operated mechanism. The mechanism by which GTPγS acts is unclear. (See text for explanation).

efflux does normally return to control levels within 20 min (see Figure 5b). Further experiments are required to clarify the mechanism of action of caffeine in this preparation.

The function of the GTPγS-releasable pool is unclear. It is possible that the size of the $InsP_3$ pool could be regulated by a GTP-dependent mechanism. It has been suggested by Ghosh *et al.* (1989) that the coordinated emptying of $InsP_3$-sensitive and -insensitive Ca^{2+} pools may have significance in the generation of effector-evoked cytosolic Ca^{2+} oscillations. Mullaney *et al.* (1987) has shown in the smooth muscle cell line DDT_1MF-2 that GTP activates Ca^{2+} uptake in the presence of oxalate. They proposed that GTP activates a transmembrane conveyance of Ca^{2+} between oxalate-permeable and impermeable compartments. If there is a pool of Ca^{2+} which accumulates in response to GTP, this could be responsible for the GTP-dependent $InsP_3$-induced Ca^{2+} release. The relationship between any such pool and those described here remains unclear.

As 100 μM GTPγS only approximates to the maximally effective concentration (Figure 2b), our results must underestimate the size of the GTPγS-sensitive Ca^{2+} pool by about 20%. In absolute terms, this underestimation is small compared to the size of the $InsP_3$-sensitive Ca^{2+} pool, and does not alter the model postulated in Figure 7.

In conclusion, we have shown that both $InsP_3$ and GTPγS cause release of Ca^{2+} from intracellular stores in airway smooth muscle of the rabbit. The GTPγS-sensitive pool is clearly separate from, but related to that sensitive to $InsP_3$. In addition, there is probably another $InsP_3$-sensitive, but ryanodine- and GTPγS-insensitive Ca^{2+} pool.

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Cromakalim (BRL 34915) counteracts the epileptiform activity elicited by diltiazem and verapamil in rats

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1 The effects of BRL 34915 (cromakalim), a potassium channel opener, have been tested on the epileptiform activity elicited by high doses/concentrations of some calcium antagonists in *in vivo* (diltiazem) and *in vitro* (diltiazem and verapamil) experiments in rats.

2 Diltiazem (150–300 mg kg^{-1}, i.p.) induced behavioural and electroencephalographic (EEG) seizures that were completely prevented by cromakalim (10 nmol/10 μl, i.c.v.). Whereas, pentobarbitone (5–10 mg kg^{-1}, i.p.) only prevented the behavioural component of the seizures.

3 In hippocampal slices, verapamil (1.5–2.0 mM) produced, within 30–60 min of perfusion, a CA1 epileptiform bursting in 80% of the experiments. This epileptiform activity was prevented by the cromakalim concentration (50 μM) that did not affect the control CA1 synaptic transmission *per se*. Pentobarbitone also prevented verapamil-induced epileptiform bursting only at the concentration (100 μM) that also reduced control CA1 synaptic transmission.

4 Diltiazem (1.5 mM) produced a biphasic excitatory-depressant effect within 60 min of perfusion. A CA1 epileptiform bursting appeared in 100% of the experiments within 30 min of perfusion. These excitatory effects were followed by a depression phase, characterized by a reduction of the magnitude of CA1 excitatory postsynaptic potentials (e.p.s.ps) and population spike.

5 The diltiazem-induced epileptiform bursting was prevented by cromakalim at a concentration (50 μM) that did not affect the control CA1 synaptic transmission *per se*. Pentobarbitone also prevented the diltiazem-induced epileptiform bursting only at a concentration (100 μM) that also reduced the control CA1 synaptic transmission. Both cromakalim (50 μM) and pentobarbitone (100 μM) failed to affect the depressant effects of diltiazem on CA1 hippocampal area. On the contrary, high (3.3 mM) calcium solutions prevented both the excitatory and the depressant effects of 1.5 mM diltiazem within 60 min.

6 These data indicate an involvement of potassium currents in the epileptiform activity elicited by high doses of diltiazem and verapamil.

Keywords: Diltiazem; verapamil; cromakalim; epilepsy

Introduction

Calcium ions are involved in the generation of epileptic phenomena, and calcium currents underlie the building up of paroxismal deporalization shifts (PDSs) elicited by different epileptogenic agents (Wong & Prince, 1978). In accordance with these findings, calcium antagonists show anticonvulsant effects on different models of focal and generalized seizures. Flunarizine and dihydropyridines block the tonic seizures due to pentylenetetrazol in rats, or to audiogenic stimulation in DBA/2 mice (De Sarro *et al.*, 1988; Popoli *et al.*, 1988). Verapamil and nimodipine reduce the interictal EEG spikes induced by penicillin in cats (Walden *et al.*, 1985), or by cefazolidine in rabbits (Morocutti *et al.*, 1986).

Calcium antagonists have also been shown to induce some proconvulsant phenomena. Nifedipine potentiates the EEG and behavioural seizures induced by 4-aminopyridine (Velos *et al.*, 1990) or by kainate and quisqualate (Vezzani *et al.*, 1988) in rats. Nimodipine and nitredipine potentiate the clonic convulsions and the mortality elicited by N-methyl-D-aspartate in rats (Dolin *et al.*, 1988). In addition, verapamil has a proconvulsant effect on the EEG seizures produced by pentylenetetrazol in rabbits (Ots *et al.*, 1987). Verapamil also has a biphasic effect on penicillin-induced epileptogenic foci in cats, where a brief period of increasing spike frequency precedes the period of suppression of the spiking activity (Walden *et al.*, 1985). Diltiazem and nimodipine potentiate the EEG spiking due to the dissociative anaesthetic phencyclidine in rats (Popoli *et al.*, 1990). Furthermore, in rat hippocampal slices, diltiazem, in the same way as the lowering of calcium ion concentrations does, alters CA1 paired pulse inhibition into paired pulse facilitation (Sagratella *et al.*, 1991). The mechanisms underlying the excitatory effects exerted by high doses of calcium antagonists are largely unknown. It has been reported that modifications of the cytosolic calcium levels lead to changes in the efficiency of potassium currents (Alger & Nicoll, 1980; Heinemann *et al.*, 1990; Hotson & Prince 1980). Thus, high doses of calcium antagonists may induce excitation *via* a blockade of potassium currents.

In the present paper, we show that high doses/concentrations of diltiazem or verapamil produce epileptogenic effects in *in vivo* and *in vitro* experiments in rats. The influence of the potassium channel opener BRL 34915 (cromakalim) on the effects elicited by verapamil or diltiazem has also been studied. Pentobarbitone, a well-known anticonvulsant drug having a different mechanism of action, was tested for comparison.

Methods

In vivo *studies*

Adult male Wistar rats (200–250 g) were adopted for this study. The animals were kept under standardized humidity, temperature and lighting conditions (on a constant 07 h 00 min to 19 h 00 min light-dark schedule), and they had free access to water and food. Under equithesin anaesthesia, four screw electrodes were permanently fixed with dental acrilic to the surface of the exposed skull over the bilateral sensorimotor and visual cortices. In some of these rats (randomly allotted to the cromakalim treatment) a cannula was permanently implanted into the right lateral cerebral ventricle, by use of the stereotaxic coordinates given in the atlas of de Groot (1959). The electroencephalographic and behavioural observations were started 5–6 days after implantation. The experiments were carried out in animals connected through long wires to

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an 8-channel EEG Galileo apparatus and placed in a round plexiglass container (35 cm diameter).

In vitro *studies*

Slice preparation Male Wistar rats (200–250 g) were killed by decapitation under ether anaesthesia, the skull was opened and the hippocampus rapidly removed. Slices of hippocampus (400 μm thick) were cut with a tissue chopper (McIlwain) and immediately placed in an interface recording chamber, where they were constantly perfused at a rate of 2–3 ml min^{-1} with an artificial cerebral spinal fluid (CSF) saturated with 95% O_2 and 5% CO_2. The composition of the artificial CSF was as follows (mM): NaCl 122, KH_2PO_4 0.4, KCl 3, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.3, glucose 10 (pH 7.3). The temperature of the perfusion chamber was maintained at 33 ± 1°C. An interval of 60–90 min elapsed between the cutting of the slices and the start of the recording session.

Recording Field potentials (f.ps) were recorded in the CA1 area of the stratum pyramidale, after electrical stimulation (0.1 Hz, 70 μs, 100–200 μA) of the Schaffer collaterals (stratum radiatum) through 3 M NaCl-filled glass microelectrodes (10–20 Mohms). Electrical potentials were amplified, monitored on oscilloscopes and recorded on tape (Racal 4DS) by conventional techniques. Before drug application a stable f.p. was achieved within 30–60 min. The slope of the excitatory postsynaptic potentials (e.p.s.ps, measured from the beginning to the maximum of the positive deflection), and the amplitude of the population spikes (p.ss, measured from the beginning to the maximum of the negative deflection), were digitized at 10 kHz and analyzed on-line, by means of adeguate software package on a PS2 IBM computer on averaged (five consecutive traces) records. One slice was used for each experiment.

Drugs

In vivo *studies* All drugs were dissolved in saline and injected intraperitoneally (i.p., diltiazem and pentobarbitone) or intracerebroventricularly (i.c.v., cromakalim). The volume of the i.c.v. injection was always 10 μl; the same volume of saline solution was injected in control animals. Drug doses refer to the weight of the base.

In vitro *studies* The drugs were directly added to the perfusion solutions. Before the application of the drugs a stimulus-response curve of the CA1 e.p.s.p. slope and p.s. amplitude was obtained in each experiment. An intermediate stimulation level (inducing a 50% of the maximal response) was selected to assess the effects of the drugs for each experiment. The effects of the drugs were assessed after 60 min of perfusion on (1) the number of population spikes (mean ± s.e.mean) per field potential (p.ss/f.p.), (2) the duration in ms (mean ± s.e.mean) of the field potentials and (3) the % changes (mean ± s.e.mean) from the values of control e.p.s.p. slope and p.s. amplitude.

Sources The compounds used were obtained from the following sources: diltiazem, BRL 34915 (cromakalim) and verapamil were kindly supplied by Sigma Tau (Italy), Beecham (Great Britain) and Knoll (Germany), respectively. Sodium pentobarbitone was purchased from Abbot (France).

Data analysis

One-way ANOVA test, Student's *t* test and Fisher's exact test were used to assess the statistical significance in the data analysis.

Results

In vivo *studies*

Convulsant effects of diltiazem The EEG and behavioural effects of low to intermediate doses of diltiazem have been described in a previous paper (Popoli *et al.*, 1990). Diltiazem, administered at doses ranging from 5 to 100 mg kg^{-1}, i.p., did not induce very marked EEG or behavioural effects in rats.

In the present work, the administration of higher doses of diltiazem (150 and 300 mg kg^{-1}, i.p.) were found to induce EEG and behavioural seizures (Figure 1).

At the dose of 150 mg kg^{-1}, diltiazem induced seizures in 7/8 animals (mean latency of seizure onset: 7.10 ± 1 min). The EEG seizures were characterized by high-voltage (400–600 μV), high-frequency (5–10 Hz) spikes, and spike-and-wave complexes paralleled by clonic movements of the head and forelimbs. Two to five min after this 'clonic phase', intermittent seizures characterized by a more pronounced tonic component were seen. The EEG tracing showed higher frequency spikes (15–20 Hz) followed by postictal depression. Behaviourally, the animals were lying on their sides and showed a marked dispnoea and tonic extension of the limbs. In 4/7 animals, these seizures were followed by death (mean latency of death occurrence: 30 ± 6.5 min, see Table 1). The dose of 300 mg kg^{-1} diltiazem induced seizures in 8/8 animals. The mean latency of seizure onset was 6.8 ± 0.5 min. These seizures presented the same characteristics of those induced by diltiazem 150 mg kg^{-1}. All the animals died (mean latency of death occurence: 16.5 ± 1.5 min).

Influence of pentobarbitone on diltiazem-induced seizures The influence of pentobarbitone (5 and 10 mg kg^{-1}, i.p.) on the behavioural and EEG seizures elicited by 150 mg kg^{-1} of diltiazem was studied in two groups of 8 rats each (Table 1). Four out of 8 rats treated with pentobarbitone 5 mg kg^{-1} plus diltiazem (15 min later) presented EEG and behavioural seizures. Two animals died. The four animals which did not show convulsions presented a marked EEG spiking paralleled by head twitching. This EEG spiking, characterized by the occurrence of numerous (2–3 Hz) high voltage (400–600 μV) spikes, lasted for 60–90 min. None of the rats receiving pentobarbitone 10 mg kg^{-1} plus diltiazem showed seizures. All these animals presented marked EEG spiking paralleled by behavioural head twitchings (Figure 2). Pentobarbitone, administered alone at the same doses tested against diltiazem (5–10 mg kg^{-1}, i.p.), induced EEG cortical synchronization and behavioural depression.

Influence of cromakalim on diltiazem-induced seizures The K^+ channel opener, cromakalim, was administered i.c.v. at doses of 5 and 10 nmol/10 μl just before the i.p. injection of diltiazem (Table 1).

Two out of eight rats treated with 5 nmol of cromakalim plus diltiazem showed EEG and behavioural seizures closely similar to those observed with diltiazem alone. The mean latency of seizure onset was 8 min; the two animals died (mean latency of death occurrence: 30 ± 5 min). Three rats showed a moderate degree of EEG spiking (1–2 min^{-1}) accompanied by head twitching, phenomena which lasted for less than 60 min. The remaining three animals only showed a tendency to EEG desynchronization and a slight excitation. Eight rats received 10 nmol of cromakalim plus diltiazem. These animals showed neither seizures nor EEG spiking. They only presented a tendency to EEG desynchronization (Figure 2) and a moderate degree of excitation (chewing, sniffing, increased locomotion). Cromakalim, administered alone at the same doses tested against diltiazem (5 and 10 nmol/10 μl i.c.v.), did not induce remarkable EEG or behavioural effects.

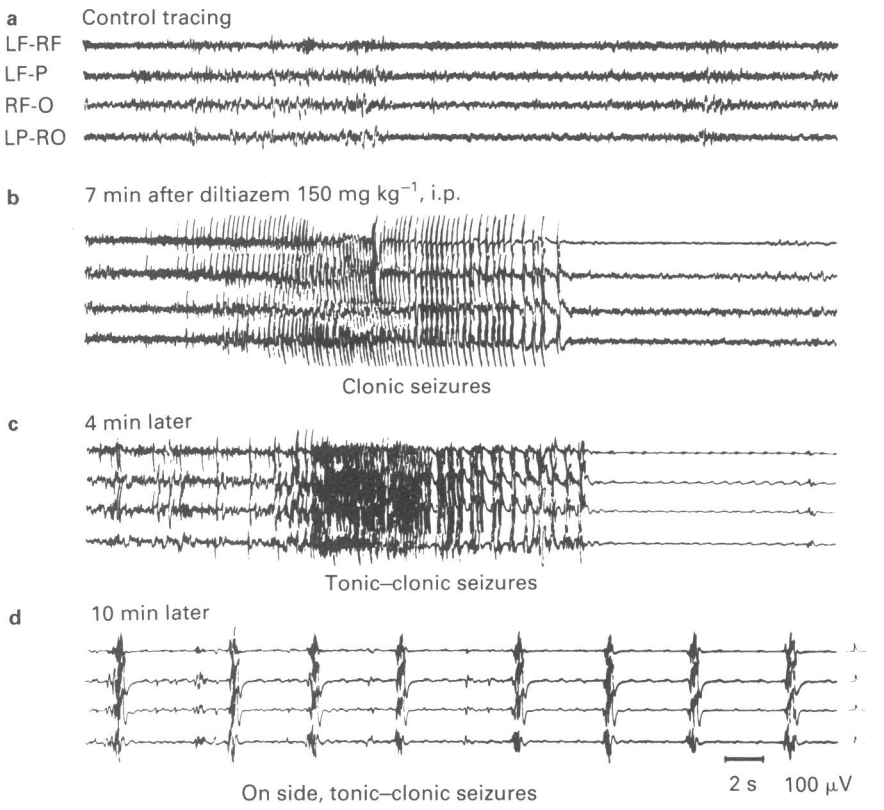


Figure 1 Convulsant effects of diltiazem in rats: the figure shows the convulsant response of diltiazem after the administration of 150 mg kg^{-1}, i.p. in the rat, which is characterized by three phases: clonic, tonic-clonic and postictal: (a) control EEG activity; (b) clonic phase of the diltiazem-induced seizures; (c) tonic-clonic phase of the convulsions; (d) postictal phase, characterized by a flattening of the EEG with superimposed high-voltage spikes.

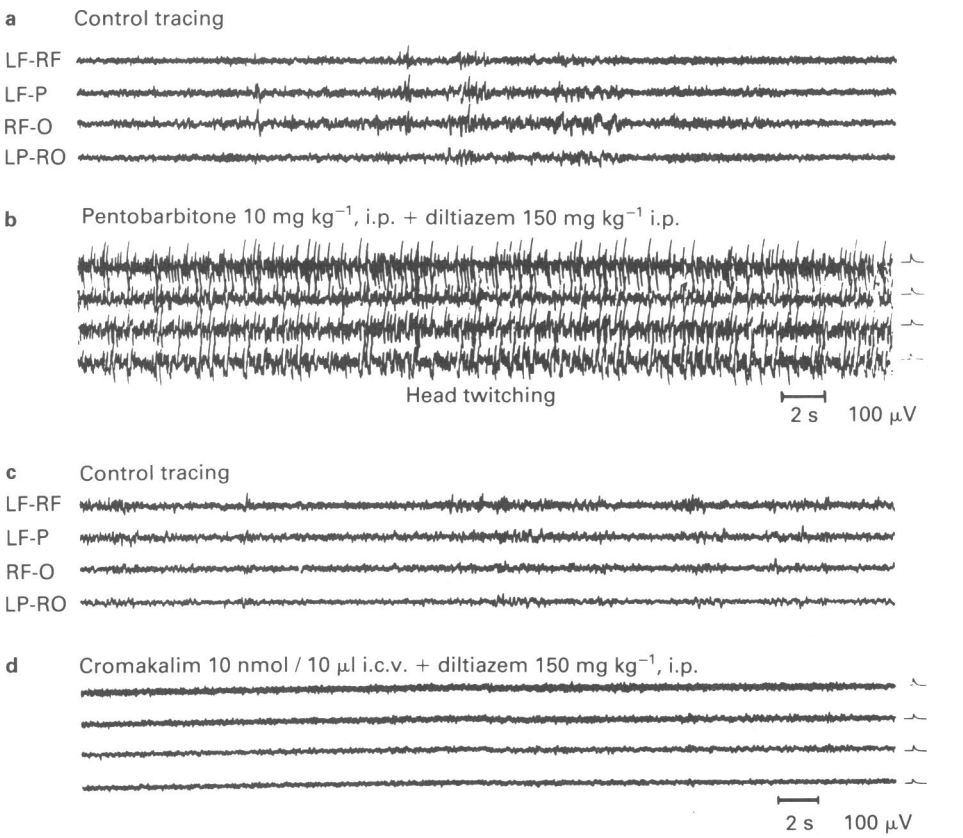


Figure 2 Influence of cromakalim and pentobarbitone on diltiazem-induced seizures in rats. The figure shows the antagonistic influence of cromakalim and pentobarbitone towards the convulsant effects induced by diltiazem (150 mg kg^{-1}, i.p.). In (b) pentobarbitone (10 mg kg^{-1}, i.p.) prevents the behavioural convulsions elicited by diltiazem leaving, however, a marked EEG spiking. In (d) cromakalim (10 nmol i.c.v.) completely prevents both EEG and behavioural seizures induced by diltiazem.

Table 1 Influence of BRL 34915 (cromakalim) and pentobarbitone on diltiazem-induced seizures

Treatments (+diltiazem 150 mg kg^{-1}, i.p.)	*Incidence of seizures*	*Mean latency of seizure onset*	*Incidence of death*	*Mean latency of death occurrence*
Saline	7/8	7.1 ± 1	4/8	30 ± 6.5
Pentobarbitone 5 mg kg^{-1}, i.p.	4/8	13.26 ± 4	2/8	40 ± 5
Pentobarbitone 10 mg kg^{-1}, i.p.	0/8**	—	0/8*	—
Cromakalim 5 nmol i.c.v.	2/8*	8.0 ± 0	2/8	30 ± 5
Cromakalim 10 nmol i.c.v.	0/8**	—	0/8*	—

Pentobarbitone was administered 15 min before diltiazem, while cromakalim was injected just prior to diltiazem. Each group was composed of 8 animals. The mean latency of seizure or death occurrence is expressed as min ± s.e.mean. *$P < 0.05$ and **$P < 0.001$ vs saline (Fisher's exact test) No differences were seen among the latency values (Student's t test).

In vitro *studies*

Effects on CA1 synaptic transmission The control CA1 f.p. consisted of a positive-going e.p.s.p. (0.8–1.2 mV ms^{-1}) and a single superimposed p.s. (2–5 ms). Slice perfusion with verapamil ($n = 6$) up to 1 mM did not significantly affect the CA1 e.p.s.p. slope (+10 ± 6%) or the p.s. amplitude (+12 ± 9%). Perfusion with higher concentrations (1.5 and 2 mM, $n = 6$ and $n = 10$, respectively) of verapamil induced a significant ($P < 0.01$) increase of the amplitude of the CA1 p.s. (40 ± 7% and 50 ± 9%, respectively) and the appearance of additional epileptiform p.ss (Table 2 and Figure 3) in 60% and 100% of the experiments, respectively.

Slice perfusion with diltiazem ($n = 6$) up to 0.5 mM did not significantly affect the CA1 e.p.s.p. slope (−6 ± 8%) or the PS amplitude (+4 ± 8%). Higher concentrations of diltiazem (1.5–2 mM, $n = 6$ and $n = 6$, respectively) induced a significant ($P < 0.01$) increase of the amplitude of the CA1 p.s. (32 ± 7% and 40 ± 6%, respectively), and the appearance of additional p.ss in 100% of the experiments (Table 3 and Figure 4). These excitatory effects of diltiazem lasted 30–45 min and were followed by a depression phase, during which the slope of CA1

Table 2 Antagonistic influence on the hippocampal epileptogenic activity of verapamil

Drug	*Conc* (mM)	n	*No.p.s./f.p.*	*f.p. duration* (ms)
Verapamil	1	6	1.1 ± 0.1	3.2 ± 0.3
Verapamil	2	10	2.8 ± 0.4(†)	12.3 ± 0.9(°)
Verapamil 2 + Pentobar 0.05		6	2.3 ± 0.7	10.5 ± 1.1
Verapamil 2 + Pentobar 0.1		6	1.3 ± 0.3(*)	4.5 ± 0.8(§)
Verapamil 2 + Cromakalim 0.05		6	1.2 ± 0.1(*)	3.4 ± 0.6(§)

The table shows the CA1 hippocampal epileptogenic activity of verapamil expressed as increase in the number of population spikes per field potential (No.p.s./f.p.) and field potential duration (f.p. duration). It also shows the antagonistic influence of pentobarbitone and cromakalim on the epileptiform activity of 2 mM verapamil.
Values are given ± s.e.mean, n = number of experiments.
Significantly different from 2 mM verapamil: *$P < 0.05$ according to Student's t test; significantly different from 2 mM verapamil: §$P < 0.05$ according to Student's t test; significantly different from 1 mM verapamil: †$P < 0.05$ according to Student's t test; significantly different from 1 mM verapamil: °$P < 0.05$ according to Student's t test.

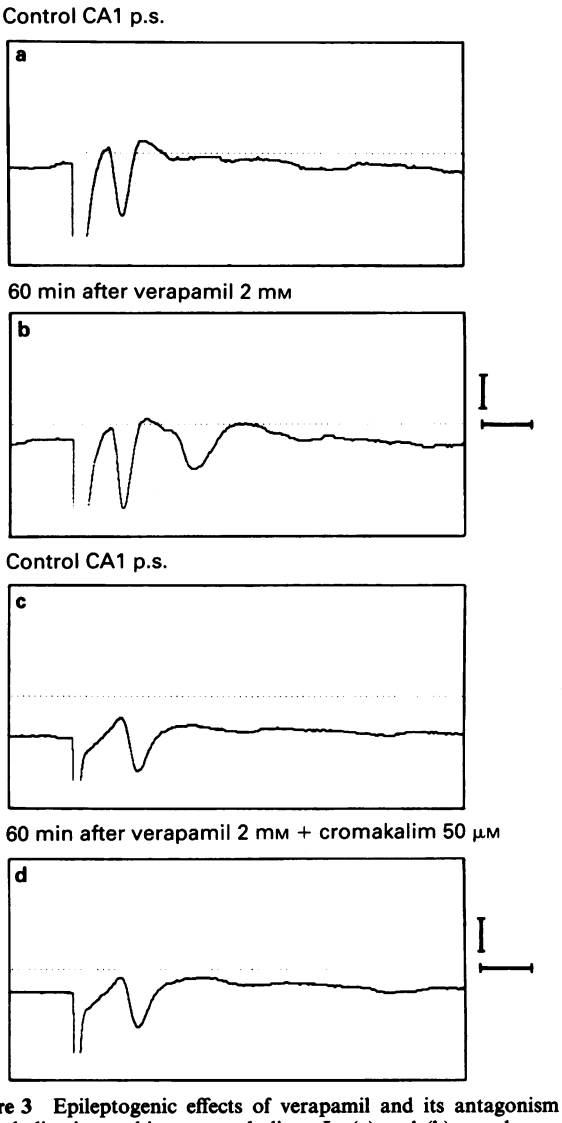


Figure 3 Epileptogenic effects of verapamil and its antagonism by cromakalim in rat hippocampal slices. In (a) and (b) are shown the epileptogenic responses (five averaged traces) elicited by high concentrations of verapamil in the CA1 region of hippocampal slices; (a) control CA1 field potential; (b) epileptiform bursting, consisting of additional population spikes, elicited 60 min after the perfusion with verapamil (2 mM). Calibrations: 5 ms, 2 mV. In (c) and (d) are shown the antagonistic influence of cromakalim on verapamil-induced effects (five averaged traces); (c) control CA1 field potential; (d) the co-application of cromakalim (50 μM) plus verapamil (2 mM) prevents, within 60 min, the excitatory effects of verapamil. Calibrations: 5 ms, 2 mV.

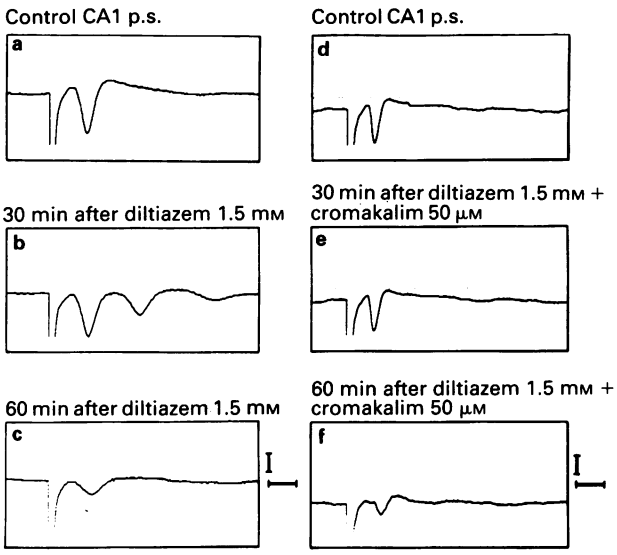


Figure 4 Excitatory-depressant effects of diltiazem and its antagonism by cromakalim in rat hippocampal slices. In (a), (b) and (c) are shown the biphasic response (five averaged traces) elicited by high concentrations of diltiazem in the CA1 region of hippocampal slices: (a) control CA1 field potential; (b) epileptiform bursting, consisting of additional population spikes, elicited by perfusion with diltiazem (1.5 mM), within 30 min; (c) depressant effects on CA1 field potential, elicited by perfusion with diltiazem (1.5 mM), within 60 min. Calibrations: 5 ms, 2 mV. In (d), (e) and (f) are shown the different actions of cromakalim on diltiazem-induced effects (five averaged traces): (d) control CA1 field potential; (e) the co-application of cromakalim (50 μM) plus diltiazem (1.5 mM) prevents, within 30 min, the excitatory effects of diltiazem; (f) the co-application of cromakalim (50 μM) plus diltiazem (1.5 μM) is unable to prevent, within 60 min, the depressant effects of diltiazem. Calibrations: 5 ms, 2 mV.

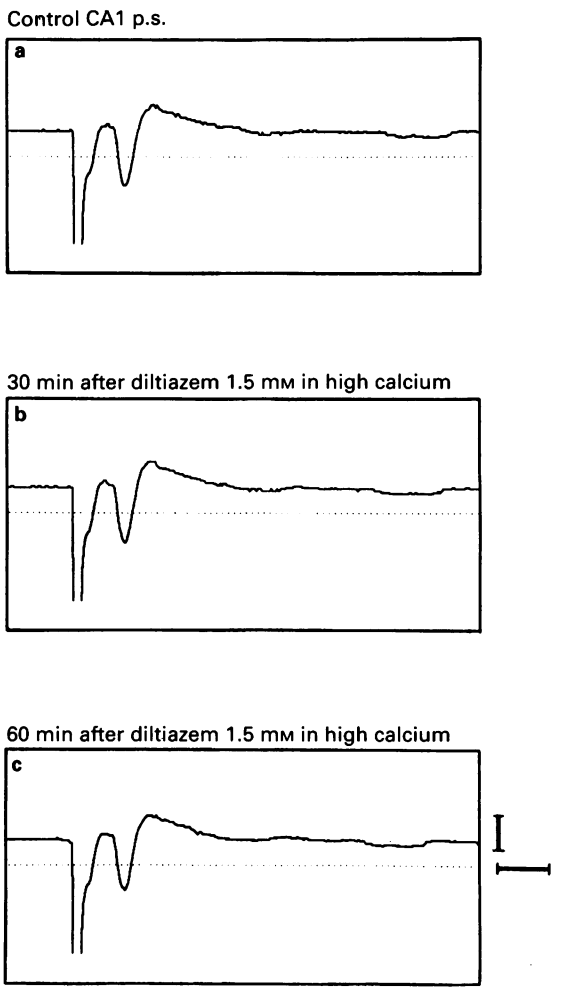


Figure 5 Influence of high calcium solutions on diltiazem-induced effects. The figure shows the antagonistic influence of increased calcium concentrations (3.3 mM) on the effects of diltiazem (five averaged traces). (a) Control CA1 field potential; (b) high (3.3 mM) calcium solutions prevent, within 30 min, the excitatory effects of diltiazem (1.5 mM); (c) high (3.3 mM) calcium solutions prevent, within 60 min, the depressant effects of diltiazem (1.5 mM). Calibrations: 5 ms, 2 mV.

Table 3 Antagonistic influence on the hippocampal epileptogenic activity of diltiazem

Drug	*Conc* (mM)	n	*No. p.s./f.p.*	*f.p. diration* (ms)
Diltiazem	1	6	1.1 ± 0.1	3.6 ± 0.8
Diltiazem	1.5	6	2.1 ± 0.1(†)	9.5 ± 0.5(°)
Diltiazem	2	6	2.6 ± 0.2(†)	13.5 ± 0.7(°)
Diltiazem + pentobarb	1.5 0.05	6	2.0 ± 0.3	10.6 ± 0.5
Diltiazem + pentobarb	1.5 0.1	6	1.3 ± 0.1(*)	4.3 ± 0.9(§)
Diltiazem + cromakalim	1.5 0.05	6	1.1 ± 0.1(*)	3.2 ± 0.5(§)
Diltiazem + high Ca^{2+}	1.5 3.3	6	1.2 ± 0.2(*)	3.4 ± 0.5(§)

The table shows the CA1 hippocampal epileptogenic activity of diltiazem expressed as increased in the number of population spikes per field potential (No.p.s./f.p.) and field potential duration (f.p. duration). It also shows that antagonistic influence of pentobarbitone (Pentobarb), high (3.3 mM) calcium solutions (high Ca^{2+}) and cromakalim on the epileptiform activity of 1.5 mM diltiazem.
Values are given ± s.e.mean. n = number of experiments.
Significantly different from 1.5 mM diltiazem: $^*P < 0.05$ according to Student's t test; significantly different from 1.5 mM diltiazem §$P < 0.05$ according to Student's t test; significantly different from 1 mM diltiazem †$P < 0.05$ according to ANOVA and two-tailed Student's t test; significantly different from 1 mM diltiazem °$P < 0.05$ according to ANOVA and two-tailed Student's t test.

e.p.s.p. and the amplitude of the population spike significantly ($P < 0.01$) decreased (85 ± 10% and 70 ± 7%, respectively) (Figure 4).

Slice perfusion with pentobarbitone ($n = 4$) up to 50 μM did not significantly affect the slope of the CA1 e.p.s.p. (+9 ± 6%) or the p.s. amplitude (+8 ± 7%). Higher concentrations of the drug (100 μM, $n = 4$), significantly ($P < 0.01$) decreased the slope of the CA1 e.p.s.p. (45 ± 8%) and the p.s. amplitude (56 ± 5%).

Slice perfusion with cromakalim ($n = 6$) up to 50 μM did not significantly affect the slope of the CA1 e.p.s.p. (+11 ± 8%) or the amplitude of the p.s. (+3 ± 6%).

Effects of pentobarbitone and cromakalim on the epileptogenic effects of calcium antagonists Co-application of 50 μM of pentobarbitone plus diltiazem (1.5 mM, $n = 6$) or verapamil (2 mM, $n = 6$) did not significantly prevent the excitatory effects elicited by the calcium antagonists (Tables 2 and 3). Co-application of 100 μM of pentobarbitone plus diltiazem (1.5 mM, $n = 6$) or verapamil (2 mM, $n = 6$) significantly ($P < 0.01$) prevented the excitatory effects of diltiazem and verapamil in 70% and 90% of the experiments, respectively (Tables 2 and 3).

Co-application of cromakalim (50 μM) plus diltiazem (1.5 mM, $n = 6$) or verapamil (2 mM, $n = 6$) significantly ($P < 0.01$) prevented the excitatory effects of diltiazem and

verapamil in 80% and 70% of the experiments, respectively (Figures 3 and 4, Tables 2 and 3).

Co-application of solutions of increased (from 1.3 to 3.3 mM) calcium concentrations plus 1.5 mM diltiazem completely and significantly ($P < 0.01$) prevented the excitatory effects of this drug in 100% of the experiments (Figure 5 and Table 3, $n = 6$). In the same series of experiments, the depressant effects of diltiazem were also significantly ($P < 0.01$) prevented ($-92 \pm 7\%$ of the reduction of the e.p.s.p. slope, $-94 \pm 5\%$ of the reduction of the CA1 p.s. amplitude) in 100% of the experiments. In contrast, co-application of pentobarbitone (100 μM, $n = 6$) or cromakalim (50 μM, $n = 6$) plus 1.5 mM diltiazem did not significantly prevent the depressive effects of the drug ($+8 \pm 9\%$ and $-12 \pm 7\%$ of the reduction of the CA1 p.s. amplitude, respectively) in 100% of the experiments.

Discussion

The lowering of the extracellular calcium concentration ultimately leads to blockade of synaptic transmission in brain slices (Heinemann *et al.*, 1990). Before the block of synaptic transmission, orthodromic and antidromic evoked potentials become epileptiform (Stringer & Lothman, 1988; Heinemann *et al.*, 1990). In fact, the lowering of the calcium concentration reduces i.p.s.ps more readily than e.p.s.ps, thus increasing excitation (Jones & Heinemann, 1987). In a previous paper, we showed that the calcium antagonist, diltiazem, shifted CA1 hippocampal paired pulse inhibition into paired pulse facilitation as did low-calcium solutions (Sagratella *et al.*, 1991). Now we show that high concentrations of diltiazem and verapamil induced epileptiform bursting in the CA1 area of hippocampal slices. In particular, in the present paper it has been shown that, like a lowering of calcium ions, diltiazem produces a biphasic excitatory-inhibitory effect on the synaptic transmission at the CA1 hippocampal area. The depressant effects of low-calcium solutions and high concentrations of diltiazem might be due to a specific blockade of N-type calcium currents. In fact the specific N-type calcium blocker, ω-conotoxin-GVIA, even at low concentrations blocked the CA1 synaptic potentials in rat hippocampal slices (Dutar *et al.*, 1989; Frank *et al.*, 1991). The epileptogenic properties of low-calcium solutions have been attributed to the reduction of both the surface charge screening (McLaughlin *et al.*, 1971) and the efficiency of calcium-dependent potassium currents (Heinemann *et al.*, 1990). In fact, modifications of the cytosolic calcium level lead to changes in the activation of potassium currents (Alger & Nicoll, 1980; Hotson & Prince, 1980). Thus, the blockade of calcium fluxes may eventually lead to increased excitability. In heart preparations, verapamil reduced the potassium currents (Kass & Tsien, 1975). In voltage-clamp studies various calcium antagonists reduced both calcium and potassium currents in single myocytes (Hadley & Hume, 1986).

Our *in vivo* studies have shown that high doses of diltiazem induced tonic-clonic seizures in rats. This finding is in accordance with a previous report by De Sarro *et al.* (1988), describing the convulsant effects of high doses of verapamil in mice.

In our experiments, pretreatment with anticonvulsant doses of pentobarbitone did not completely prevent diltiazem-induced seizures. In fact, although the rats presented no behavioural seizures, they still showed a marked EEG spiking. Unlike pentobarbitone, cromakalim, a potassium channel opener, completely antagonized both EEG and behavioural seizures.

In *in vitro* studies, pentobarbitone completely prevented calcium antagonist-induced CA1 epileptiform activity only at concentrations that also reduced control CA1 synaptic transmission. On the contrary, cromakalim completely prevented the CA1 hippocampal epileptiform bursting induced by diltiazem and verapamil at concentrations not affecting the control CA1 synaptic transmission. The fact that pentobarbitone did not completely prevent seizures *in vivo* and that it affected epilepsy *in vitro* only at depressant concentrations, clearly shows the lack of specificity of its anticonvulsant effects in this model. Conversely, cromakalim completely prevented both *in vivo* and *in vitro* epileptic phenomena at doses/concentrations that were without effect *per se*. This shows that the anti-epileptic effects of cromakalim are rather specific in this model, thus suggesting that the epileptic phenomena induced by high doses of diltiazem and verapamil may actually be attributed to a blockade of potassium currents. The activators of potassium channels enhance calcium influx as a consequence of enhanced potassium currents (Luckhoff & Busse, 1990). Thus, a marked reduction of the cytosolic level of calcium ions may underlie the epileptogenic effects of high doses of calcium antagonists. In agreement with this hypothesis, high-calcium solutions prevented the excitatory activity of diltiazem in rat hippocampal slices.

The fact that in our experiments diltiazem induced closely similar effects following both *in vitro* and *in vivo* administration, suggests that the concentrations which have been used in hippocampal slices are quite comparable with those achieved in the brain in our *in vivo* experiments. Data in the literature about the degree of penetration of systematically administered diltiazem into the brain (Naito *et al.*, 1986) as well as the difficulty of drug diffusion to cells in slice preparations (see Nicholson & Hounsgaard, 1983), particularly with slices maintained at an interface between the bathing solution and a humidified atmosphere (Gage & Robertson, 1985), further support this view.

The concentrations of verapamil we used in hippocampal slices, are over ten fold higher than those affecting the different calcium channels in cultured mammalian hippocampal neurones (Yaari *et al.*, 1987; Miller, 1987). At concentrations close to those active on hippocampal cultured neurones, only inorganic calcium antagonists (e.g. cadmium or nickel ions) are able to suppress completely the electrical synaptic response and the stimulus-induced changes in the calcium extracellular concentration in rat hippocampal slices. These changes were only partially affected by various organic calcium antagonists such as verapamil and nifedipine (Heinemann *et al.*, 1990). In particular, diltiazem (200 μM) induced a shift of paired pulse inhibition to paired pulse facilitation (Sagratella *et al.*, 1991) and verapamil (150 μM) induced a reduction of the amplitude of the slow afterhyperpolarization (Jones & Heinemann, 1987). It is possible that higher concentrations of these drugs have to be achieved in the medium bathing hippocampal slices to impair deeply the electrical synaptic responses.

A non specific influence of these high diltiazem and verapamil concentrations on other systems in addition to, or instead of inhibition of calcium channels could contribute to the epileptiform activity of the drugs. For example, verapamil and diltiazem at concentrations five to ten fold higher than those inhibiting calcium channels have been found (Erdreich *et al.*, 1983) to inhibit 'fast' sodium channels and sodium-calcium exchange. In addition, it has also been argued that the effects of verapamil and diltiazem at high concentrations are due to a common non-specific action such as pertubation of membrane fluidity which might lead to impairment of channel function (Thayer *et al.*, 1985). However, the similarity of the synaptic effects (biphasic excitatory/depressant effects) of low-calcium solutions and high concentrations of diltiazem, as well as the antagonism exerted by high calcium solutions and cromakalim on the excitatory activity of verapamil and diltiazem seem to indicate a main involvement of calcium and potassium channels in the epileptiform effects of these drugs.

In conclusion, diltiazem and verapamil present dose-related anticonvulsant/convulsant effects. The excitatory effects of high doses of these drugs may restrict the spectrum of activity of the anticonvulsant effects exerted by lower doses of diltiazem and verapamil.

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Effect of respiratory tract viral infection on murine airway β-adrenoceptor function, distribution and density

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1 The effects of a respiratory tract viral infection on β-adrenoceptor density, distribution and function were investigated in murine airways.

2 Following intranasal inoculation of CBA/CaH mice with influenza A/PR-8/34 virus, the virus proliferated rapidly in trachea (peak titres 2 days post-inoculation) and lung (peak titres 4–6 days post-inoculation). Respiratory tract viral infection was associated with a significant increase in lung weight (88% higher than control mice at day 6 post-inoculation) that was related temporally to the development of peripheral lung inflammation and consolidation.

3 Analysis of specific binding of [^{125}I]-cyanopindolol to β-adrenoceptors revealed that on days 2, 4 and 8 post-inoculation with virus, mouse isolated tracheal sections contained, on average, 40% more β-adrenoceptors than tracheal sections from time matched control mice. Subsequent quantitative autoradiographic studies demonstrated that this increase in total tracheal β-adrenoceptors was due primarily to a 90% increase in the density of β-adrenoceptors in the tracheal epithelium in virus-infected mice.

4 In contrast, virus-infection had no significant effect on the density of β-adrenoceptors in tracheal airway smooth muscle, although within 2 days of inoculation with virus, mouse tracheal smooth muscle segments were approximately 2 fold less sensitive to the β-adrenoceptor agonist, noradrenaline (mean $pD_2 = 6.57 \pm 0.04$, $n = 24$) and to the adenylyl cyclase-activator forskolin (mean $pD_2 = 6.78 \pm 0.04$, $n = 12$) compared to segments from control mice (mean $pD_2 = 6.84 \pm 0.06$ for noradrenaline; mean $pD_2 = 7.03 \pm 0.07$ for forskolin). Similar values were obtained 8 days post-inoculation. At day 2, but not day 8 post-inoculation with virus, relaxation responses to theophylline were also marginally attenuated compared with controls.

5 Mouse isolated tracheal segments obtained 2 days after virus inoculation and segments from time-matched control mice were equisensitive to the spasmogenic actions of the muscarinic cholinoceptor agonist, carbachol. However, tracheal segments from mice inoculated with virus were less responsive to carbachol on day 4 (mean $pD_2 = 6.45 \pm 0.04$, $n = 8$) and day 8 (mean $pD_2 = 6.45 \pm 0.02$, $n = 12$) compared to control preparations (day 4, mean $pD_2 = 6.73 \pm 0.06$, $n = 8$; day 8, mean $pD_2 = 6.65 \pm 0.04$, $n = 12$, $P < 0.05$). In contrast, endothelin-1-induced contractions of tracheal smooth muscle were not affected by virus-infection.

6 These data demonstrate that respiratory tract viral infection can produce significant tissue-selective changes in airway β-adrenoceptor density as well as small reductions in airway smooth muscle muscarinic cholinoceptor and β-adrenoceptor function.

Keywords: β-Adrenoceptors; influenza A virus; mouse trachea

Introduction

Respiratory tract viral infections in both asthmatic and otherwise healthy individuals are frequently associated with increased airway reactivity (Empey *et al.*, 1976; Little *et al.*, 1978). Virus-induced damage to the airway epithelium may contribute to this hyperreactivity by eliminating epithelium-derived relaxant factors, by removing enzymes such as enkephalinase which metabolize airway smooth muscle spasmogenic peptides including substance P, or by exposing afferent nerve endings (Empey *et al.*, 1976; Jacoby *et al.*, 1988; Jacoby & Fryer, 1990). In addition, increased vagal efferent activity, enhanced mast cell histamine release and impaired β-adrenoceptor function may also be contributing factors (Buckner *et al.*, 1981; 1985; Busse *et al.*, 1983).

Both bacterial and viral respiratory pathogens have been reported to modulate variably the function of airway β-adrenoceptors. Bacterial infections have been shown to attenuate the airway smooth muscle relaxant effects of β-adrenoceptor agonists, possibly by reducing receptor concentration (Schreurs *et al.*, 1980; Norris & Eyre, 1981; Engels *et al.*, 1987). Similarly, exposure of various individual inflammatory cell types including T-lymphocytes (Lee, 1980), neutrophils (Lee, 1980; Busse *et al.*, 1979) and pulmonary alveolar macrophages (Ogunbiyi *et al.*, 1988) to respiratory tract viruses is associated with a reduction in cell responsiveness to β-adrenoceptor agonists. Furthermore, whole lung membrane fractions from mice infected intranasally with a respiratory tract virus have also been shown to be hyporesponsive to β-adrenoceptor stimulation (Scarpace & Bender, 1989). The mechanisms responsible for the virus-induced reduction in β-adrenoceptor function is unclear, although altered β-adrenoceptor coupling to adenylyl cyclase and/or reduced adenylyl cyclase activity may be involved (Scarpace & Bender, 1989). In addition, recent evidence suggests that the β_1-adrenoceptor subtype may be more susceptible to virus-induced impairment than β_2-adrenoceptors (Ogunbiyi *et al.*, 1988).

Although respiratory tract viruses produce β-adrenoceptor hyporesponsiveness in many lung cell types, airway smooth muscle responses to β-adrenoceptor agonists have been reported to be either enhanced (Eyre *et al.*, 1982) or unchanged (Buckner *et al.*, 1981; Lemanske *et al.*, 1989) following respiratory tract virus infection. In the present investigation, we have used functional and autoradiographic techniques to assess further the effects of a respiratory tract viral infection on the density, distribution and function of β-adrenoceptors in murine airways.

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Methods

Virus stock and animal inoculation

The A/PR-8/34 strain (H1N1) of influenza virus was grown in the allantoic fluid of 10-day-old embryonated eggs at 37°C for 3 days as described previously (Williams & Mackenzie, 1977). The crude allantoic fluid was harvested and contained 4×10^7 egg infectious doses (EID_{50}) of virus ml^{-1} as determined by the method of allantois-on-shell titration for infectivity (Fazekas de St. Groth & White, 1958). Virus-containing allantoic fluid was stored in 0.5 ml aliquots at −70°C.

Four-week-old male CBA/CaH mice, specified pathogen-free, were anaesthetized (pentobarbitone sodium, 80 mg kg^{-1}, i.p.) and inoculated intranasally with 2000 EID_{50} of virus or 25 μl phosphate-buffered saline (PBS) (control mice). Otherwise, all mice were housed in the same environment and treated similarly. Mice received food and water *ad libitum*.

Tracheal and lung virus titres

On specified days post-inoculation, mice were killed (pentobarbitone sodium, 200 mg kg^{-1}, i.p.) and exsanguinated by section of the renal artery. The trachea and lungs were excised, separated and placed in sterile PBS on ice. Lungs from each animal were blotted dry, weighed and then immersed in 2 ml PBS. Tracheal segments from 3 animals were dissected free of surrounding tissue and immersed in 1 ml PBS. Tissues were homogenized with motorized glass tissue grinders and the suspension clarified by centrifuging at 2000 *g* for 5 min at 4°C. Infectious virus was assayed by allantois-on-shell titration for infectivity. Briefly, 6 mm × 6 mm pieces of allantois-on-shell from 11-day-old embryonated chicken eggs were incubated in sterile round bottom tubes containing 0.35 ml of Standard Medium (SM) and 25 μl aliquots of serial 10 fold dilutions of virus (10^{-1} to 10^{-9}) in SM. Five replicates were used at each dilution. Tubes were sealed, mounted in holders and placed in a horizontal shaker working in a 35°C room for 48 h. The fluid from each tube was transferred to a haemagglutination tray and one drop of 10% washed, goose red blood cells was added to each cell. The trays were shaken briefly and left to stand for 40 min. Positive haemagglutination indicated infection and infectious virus titre (EID_{50}) was calculated by the method of Thomson (1947). The composition of SM (pH 7.0) was (mM): NaCl 137, KCl 8, $CaCl_2$ 7.2, $MgCl_2$ 0.52, glucose 1.7, and acid-free gelatin 2.0 g l^{-1}, phenol red 2.5 mg l^{-1}, gentamycin 10 mg l^{-1} and penicillin 100 mg l^{-1}.

At the same time that mouse lungs and trachea were removed for infectious virus assay, replicate mice were killed and their lungs and trachea fixed in 10% buffered formal saline. After fixation, tissues were embedded in paraffin, cut to a thickness of 5 μm, stained with haematoxylin and eosin, and viewed by light microscopy.

Radioligand binding and quantitative autoradiography

On days 2, 4 and 8 post-inoculation, frozen tissue blocks of mouse trachea were prepared as described previously (Henry *et al.*, 1990). Serial frozen transverse sections (10 μm) were cut at −20°C and thaw-mounted onto gelatin/chrom alum-coated glass slides. Each slide contained 18 tracheal sections from different mice. For radioligand binding experiments, slides were incubated with the β-adrenoceptor radioligand (−)-[^{125}I]-cyanopindolol (I-CYP, 10-240 pM) in Tris-HCl buffer (170 mM, pH 7.6, 22°C) containing the protease inhibitor phenylmethylsulphonyl fluoride (10 μM). Non-specific binding was estimated in the presence of 1 μM (−)-propranolol. After a 150 min incubation period, slides were washed for 2 × 15 min in Tris-HCl buffer to remove unbound I-CYP. Tissue sections were wiped onto GF/A glass fibre filter papers (Whatman) and counted in a gamma counter (Packard). Specific binding for trachea was expressed as fmol/tracheal section.

For autoradiographic studies, slides were incubated with I-CYP and washed in Tris-buffer as described above for the radioligand binding studies. Slides were then briefly rinsed in distilled water and rapidly dried by a stream of cold, dry air. Emulsion-coated coverslips (Kodak NTB-2) were cemented to the non-tissue end of the slide and preparations exposed for 41 h at 4°C in X-ray cassettes. The emulsion was developed (Kodak Dektol; 1:1, 3 min), rinsed briefly in dilute acetic acid (2%) containing 2.5% hardener (Ilford Hypam Hardener) and fixed (Ilford Hypam; 1:4, 2.5 min). Tissue sections were stained with haematoxylin, dehydrated in ethanol, cleared in xylene and mounted for light microscopy. The relative densities of β-adrenoceptors over tracheal epithelium and tracheal smooth muscle from virus-inoculated and control mice were determined by a quantitative autoradiographic technique as described previously (Henry *et al.*, 1990). In these experiments, 8 fields were viewed from each tracheal section; 4 over the epithelial layer, 3 over the smooth muscle band and 1 over non-tissue (background). Thus approximately 4000 fields were analyzed (8 fields from each of 6 tracheal rings on 84 slides). Grain densities were expressed as grains 1000 μm^{-2}.

Functional studies

Isolated tracheal smooth muscle segments from virus-inoculated and control mice were prepared for isometric tension recordings as described previously (Henry & Goldie, 1990). The epithelium was not removed intentionally from any tracheal segment. In each experiment, a total of 4 segments were prepared; 2 tracheal segments from a virus-inoculated mouse and 2 tracheal segments from a control mouse. All preparations were suspended under 500 mg resting tension in a 3 ml organ bath containing Krebs-Henseleit solution at 37°C bubbled with 5% CO_2 in O_2. Following a 45 min equilibration period, the tissues were primed by cumulative addition of 0.2 and 10 μM carbachol. Tissues were washed and rested for 40 min and a concentration-effect curve subsequently obtained to carbachol (0.1–30 μM).

Mouse isolated tracheal preparations do not exhibit intrinsic tone (Henry *et al.*, 1990) and therefore all concentration-effect curves to relaxant agonists were constructed in preparations pre-contracted with carbachol. In preliminary experiments, the influence of the initial level of carbachol-induced tone on the subsequent relaxant potency of noradrenaline was assessed in tracheal segments from virus-inoculated and control mice. In the functional antagonism studies, preparations were precontracted with carbachol to produce specified levels of induced tone and cumulative concentration-effect curves obtained to noradrenaline (0.01–10 μM). In all other studies, preparations were contracted with carbachol (EC_{50} concentration estimated for that particular preparation) and cumulative concentration-effect curves obtained to one of the relaxant agonists, noradrenaline, forskolin or theophylline. Another 2 concentration-effect curves to the relaxants were subsequently constructed in each preparation. Cocaine (10 μM) and 10 μM phentolamine were added to the bath 30 min before the start of all concentration-effect curves to inhibit α-adrenoceptors and neuronal and extraneuronal uptake systems (Goldie & Paterson, 1982). Relaxation responses were expressed as % reversal of the carbachol-induced tone and the relaxation response induced by the highest concentration of agonist was taken as the maximal response (E_{max}). Agonist potency, defined as the concentration of agonist that produced 50% E_{max} (EC_{50} value), was estimated by fitting the concentration-effect data to a logistic function by computer-assisted nonlinear least squares regression analysis.

Drugs

Drugs used were: (−)-noradrenaline bitartrate, carbamylcholine chloride (carbachol), cocaine hydrochloride, forskolin, theophylline (Sigma Chemical Company, St. Louis, U.S.A.); endothelin-1 (Auspep, Melbourne, Australia); phen-

tolamine mesylate (Ciba-Geigy, Sydney, Australia). The composition of the Krebs Henseleit solution was (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4 \cdot 7H_2O$ 0.57, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.1.

Statistical analyses

All data are presented as arithmetic mean ± s.e.mean. Estimates of the dissociation constant (K_d) and maximum binding capacity (B_{max}) of specific I-CYP binding were obtained by non-linear least squares regression analyses of data fitted to a single site model. Estimates were also obtained by Scatchard analysis. The nature of the binding was determined by Hill plot analysis. Differences between means were established by Student's two-tailed, non-paired t test and were considered significant if $P < 0.05$.

Results

General effects of influenza A/PR-8/34 virus infection

Following intranasal inoculation, the virus proliferated rapidly in the trachea during the initial 2 days, then declined progressively to undetectable levels by day 8 (Figure 1a). In contrast, lung virus titres peaked later (between days 4 and 6) and then fell rapidly to levels that could not be detected by day 10 (Figure 1a). As shown in Figure 1b, virus infection was associated with marked increases in lung weight which peaked at day 6 post-inoculation (88% higher than control mice) and was still elevated by 46% on day 21. The significant increase in lung weight from day 4 onwards was apparently due to the development of peripheral lung inflammation and consolidation which was observed macroscopically and microscopically. The percentage survival rates for mice were 100% up to day 7, 86.7% at day 8, 66.1% at day 9 and 57.8% at day 10. No further deaths were observed between day 10 and 21.

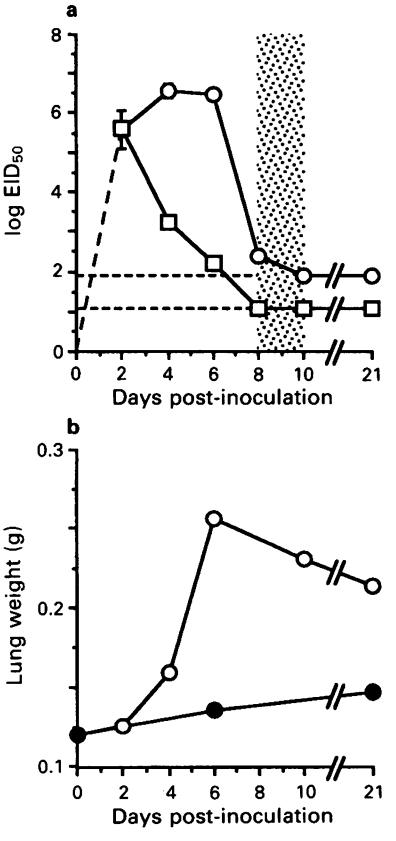


Figure 1 (a) Influenza A/PR-8/34 virus titres in mouse trachea (□) and lungs (○) at various time points post-inoculation. Data are presented as log EID_{50}/lung or /trachea (mean; $n = 3$ animals per time point, vertical bars show s.e.mean). Detection limits of virus titres were 1.1 log EID_{50}/trachea and 1.9 log EID_{50}/lung. No virus was detected in the trachea or lungs of control mice. The shaded area indicates the period over which some animals died. (b) Lung weights of mice inoculated with virus (○) or sterile PBS (●) at various time points up to 21 days post-inoculation. Data are presented as mean ($n = 3$ mice per time point).

Radioligand binding studies

Specific I-CYP binding to mouse tracheal sections obtained from virus-inoculated and control mice was saturable, involving a single population of non-interacting sites (nH = 0.96 ± 0.01, $n = 6$ experiments). The mean density of specific binding sites for I-CYP in tracheal sections obtained from control mice was 23 ± 6 amols/tracheal section, which is similar to the density previously reported in control mice from this laboratory (22 ± 3 amol/tracheal section, Henry *et al.*, 1990). As shown in Figure 2 and Table 1, the density of specific binding sites for I-CYP (B_{max}) was higher in tracheal sections obtained from virus-inoculated mice than from control mice. On average, tracheal sections from virus-inoculated mice had 41 ± 12% more specific binding sites for I-CYP than control mice (mean increase calculated over the 3 days tested, i.e. days 2, 4 and 8). There was no significant difference between the estimates obtained for the dissociation constant

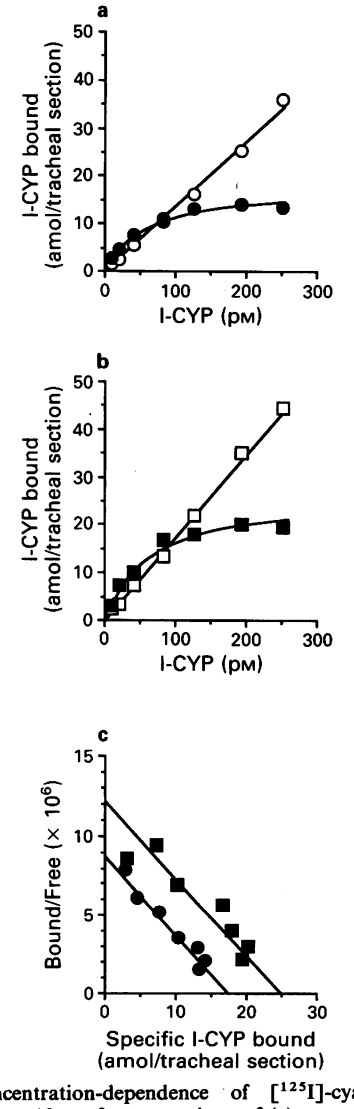


Figure 2 Concentration-dependence of [^{125}I]-cyanopindolol (I-CYP) binding to 10 μm frozen sections of (a) control and (b) virus-inoculated mouse trachea (8 days post-inoculation) showing specific (closed symbols) and non-specific (open symbols) binding. Each point represents the mean data from a single experiment conducted using triplicate slides. Also shown is Scatchard analysis (c) of specific I-CYP binding for control (●) and virus-inoculated (■) mouse trachea. Estimates of B_{max} and K_d for days 2, 4 and 8 are presented in Table 1.

Table 1 Binding parameter estimates for B_{max} and K_d obtained from analysis of [^{125}I]-cyanopindolol (I-CYP) saturation binding curves in mouse tracheal sections

Days post-inoculation	*Treatment*	K_d (pM)	B_{max} (amol/tracheal section)
Day 2	Virus	117.2	51
	Control	112.3	33
	#Ratio	1.04	1.57
Day 4	Virus	45.9	22
	Control	52.6	18
	#Ratio	0.87	1.23
Day 8	Virus	53.8	25
	Control	53.2	17
	#Ratio	1.01	1.44

#Ratio = Virus: Control, for respective K_d and B_{max} values.

(K_d) of specific I-CYP binding in tracheal sections from virus-inoculated and control mice (Table 1).

Quantitative autoradiography

As shown in Figures 3 and 4, significant densities of autoradiographic grains were located over the tracheal smooth muscle band and epithelial layer in tracheal sections from virus-inoculated and control mice. Quantitative analysis of saturation binding curves constructed from autoradiographic grain counting data demonstrated that there was no significant difference between tracheal sections from virus-inoculated and control mice with respect to the density of specific I-CYP binding sites associated with the smooth muscle band (Figure 3 and Table 2). In sharp contrast, the density of specific grains over the epithelial layer was, on average, 95 ± 13% higher in tracheal sections from virus-inoculated mice than from control mice (Figures 3 and 4). Similar results were obtained at each time point examined (days 2, 4 and 8 post-inoculation; Table 2). Further analysis of saturation binding curves indicated that the apparent dissociation constant for I-CYP binding to the epithelial layer was consistently lower in tracheal sections from virus-inoculated mice than in control mice (Table 2).

Functional studies

The effects of influenza A/PR-8/34 virus inoculation on mouse isolated tracheal smooth muscle responses to carbachol are shown in Figure 5. Carbachol-induced contractions of tracheal smooth muscle preparations from control mice (mean pD_2 value = 6.62 ± 0.03; mean maximum contraction (C_{max}), 1.29 ± 0.06 g; n = 32 preparations) and from mice 2-days post-inoculation (mean pD_2 value = 6.64 ± 0.05; C_{max} = 1.35 ± 0.10 g; n = 12) were not significantly different. However, smooth muscle responsiveness to carbachol was significantly lower in trachea from virus-inoculated mice at both 4 days (virus-inoculated, mean pD_2 value = 6.45 ± 0.04; control, mean pD_2 value = 6.73 ± 0.06, n = 8, $P < 0.05$) and 8-days post-inoculation (virus-inoculated, mean pD_2 value = 6.45 ± 0.02; control, mean pD_2 value = 6.65 ± 0.04, n = 12, $P < 0.05$). In addition, the mean maximum contraction produced by carbachol was marginally, but statistically significantly lower on day-8 post-inoculation (C_{max} = 1.11 ± 0.06 g; $P < 0.05$) than in preparations from day-matched control mice. In contrast, the contractile responses of mouse isolated

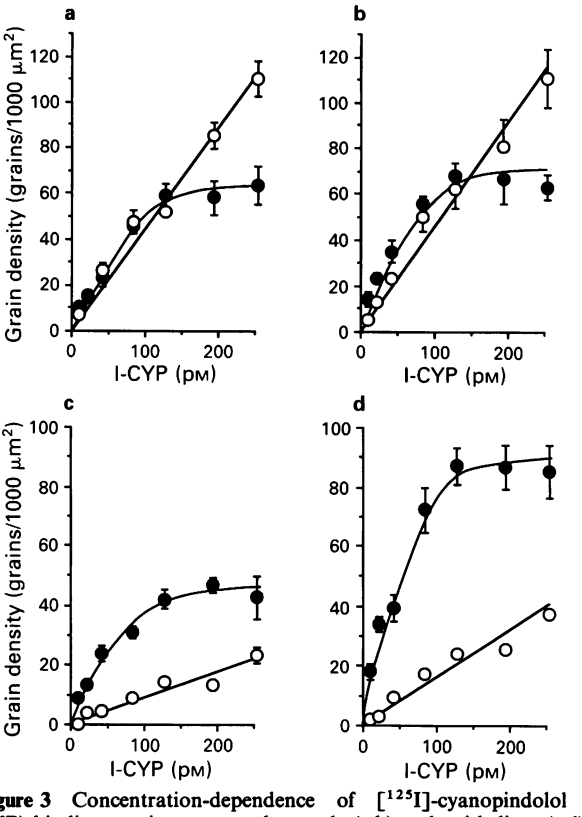


Figure 3 Concentration-dependence of [^{125}I]-cyanopindolol (I-CYP) binding to airway smooth muscle (a,b) and epithelium (c,d) of control (a,c) and virus-inoculated (b,d) mouse trachea showing specific (●) and non-specific (○) binding. I-CYP binding is presented in terms of grain densities estimated from quantitative autoradiography (see Methods). Each curve represents the mean responses of preparations from 6 mice; s.e.mean shown by vertical bars. Trachea were obtained 8 days post-inoculation. Estimates of B_{max} and K_d for days 2, 4 and 8 are presented in Table 2.

Table 2 Binding parameter estimates of B_{max} and K_d obtained from analysis of [^{125}I]-cyanopindolol (I-CYP) saturation binding curves constructed from autoradiographic grain (G) counting data

		Smooth muscle		*Epithelium*	
Days post-inoculation	*Treatment*	K_d (pM)	B_{max}[a] (G 1000 μm^{-2})	K_d (pM)	B_{max} (G 1000 μm^{-2})
Day 2	Virus	113.5	159.5	84.2	186.9
	Control	146.4	159.3	130.8	104.7
	#Ratio	0.78	1.00	0.64	1.79
Day 4	Virus	56.3	119.8	34.4	194.8
	Control	53.9	120.0	61.0	90.4
	#Ratio	1.04	1.00	0.56	2.15
Day 8	Virus	49.9	84.1	53.1	110.8
	Control	89.2	90.8	62.4	57.9
	#Ratio	0.56	0.93	0.85	1.91

#Ratio = Virus: Control, for respective K_d and B_{max} values.
[a] B_{max} values are presented as G 1000 μm^{-2}. Note that differences in B_{max} values for control tissues between days 2, 4 and 8 do not represent real differences in maximum β-adrenoceptor densities but simply reflect differences in the specific activity of I-CYP on each of these days.

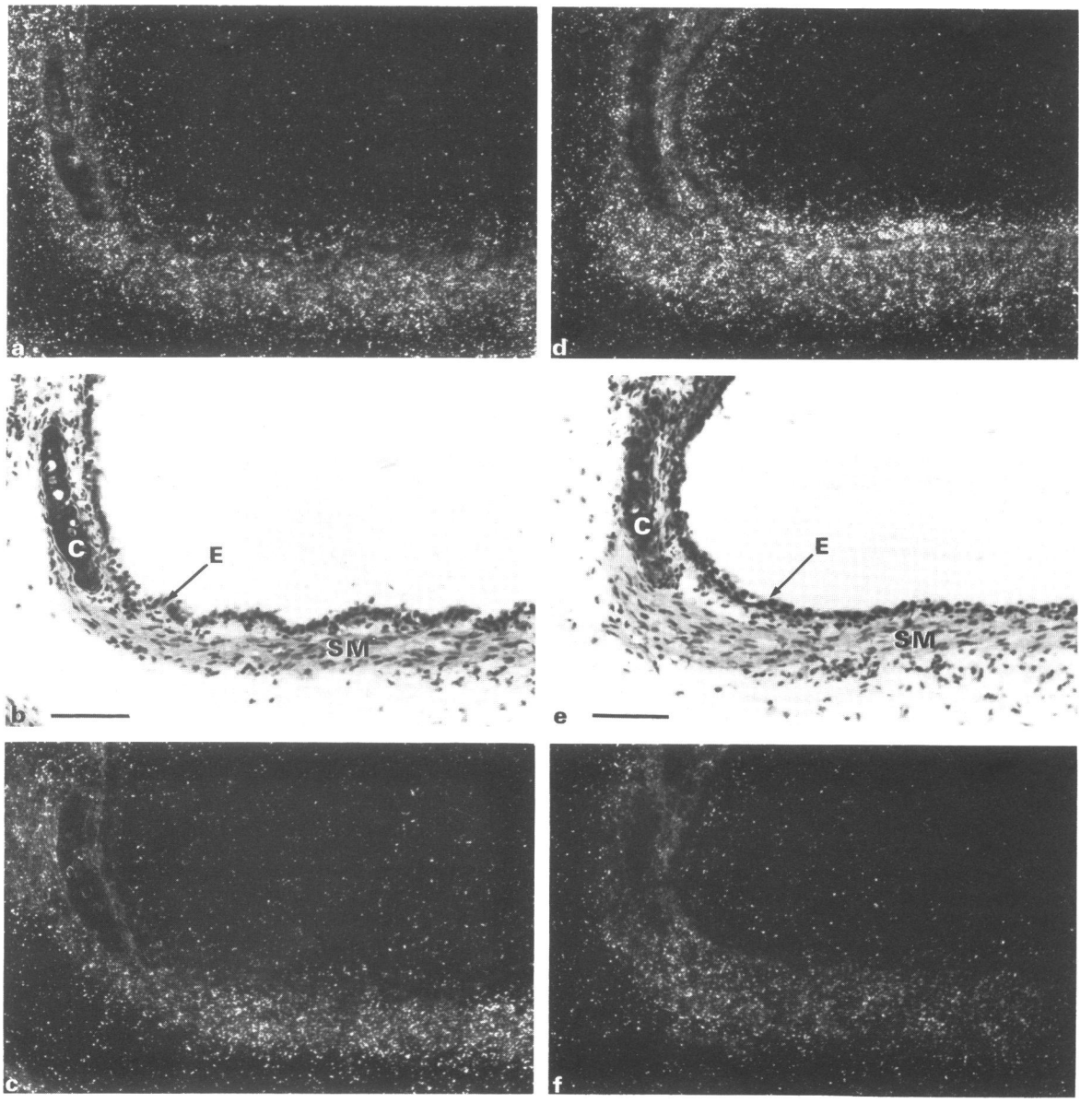


Figure 4 (a) Dark-field photomicrograph of a tracheal section from a control mouse showing the distribution of autoradiographic grains derived from [^{125}I]-cyanopindolol (I-CYP) binding (20 pM). (b) Bright-field photomicrograph of the above section. SM = smooth muscle, C = cartilage, E = epithelium. Bar = 100 μm. (c) Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in a neighbouring section incubated with 20 pM I-CYP and 1 μM (−)-propranolol. (d), (e) and (f) are the corresponding micrographs obtained from a virus-inoculated mouse (4 days post-inoculation).

tracheal smooth muscle preparations to endothelin-1 were not significantly attenuated by virus inoculation (Day 2 post-inoculation, mean control $pD_2 = 7.99 \pm 0.05$, mean virus-inoculated $pD_2 = 8.07 \pm 0.15$, $n = 6$; Day 4 and 8 post-inoculation, mean control $pD_2 = 7.99 \pm 0.17$, mean virus-inoculated $pD_2 = 8.00 \pm 0.17$, $n = 6$).

In view of (a) the variable changes in the potency of carbachol observed during the time-course of virus-infection (Figure 5) and (b) the likelihood that β-adrenoceptor agonist relaxant potency will vary depending on the level of carbachol-induced tone (Buckner & Saini, 1975), a series of preliminary experiments was completed to investigate the respective influences of virus-infection and functional antagonism in determining noradrenaline relaxant potency (Figure 6). As early as 2 days post-inoculation with virus, mouse tracheal segments pre-contracted with 0.5 μM carbachol were hyporesponsive to noradrenaline (mean pD_2; control, 6.84 ± 0.06 versus virus-inoculated 6.57 ± 0.04, $n = 24$, $P < 0.05$, Figure 6a). On the other hand, at 8 days post-inoculation, mouse tracheal segments from virus-inoculated and control mice precontracted with 0.5 μM carbachol were equiresponsive to noradrenaline (Figure 6b). However, at this later time point, the level of tone induced by 0.5 μM carbachol was significantly lower in preparations from virus-inoculated mice ($43 \pm 2\%$ C_{max}) compared with controls ($53 \pm 2\%$ C_{max}). Subsequent functional antagonism experiments in virus-inoculated preparations (and control preparations, data not shown) revealed that noradrenaline was significantly less potent in relaxing preparations precontracted to 53% C_{max} than to 43% C_{max} (mean pD_2 values, 6.36 ± 0.05 and 6.61 ± 0.06 respectively, $n = 12$, $P < 0.05$; Figure 6c). Indeed, when the influence of functional antagonism was reduced (by precontracting day 8 virus-infected and control preparations to similar levels of carbachol-induced tone), noradrenaline was less potent in relaxing tracheal preparations from virus-infected mice (Figure 6d), as previously observed on day 2 post-inoculation (Figure 6a). In all subsequent experiments investigating the influence of virus-inoculation on the relaxant potencies of noradrenaline, forskolin and theophylline, tracheal preparations were precontracted with an EC_{50} concentration of carbachol, rather than a preselected concentration of carbachol (Figure 7).

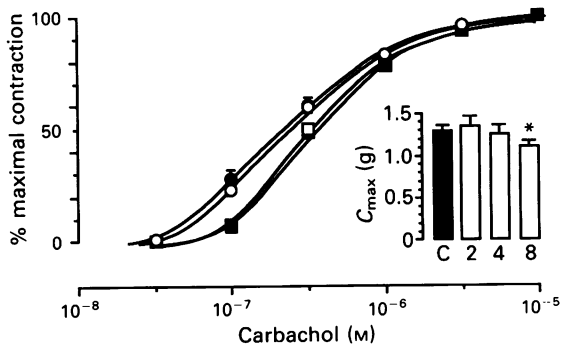


Figure 5 Mean concentration-effect curves to carbachol for contraction of tracheal segments obtained from control mice (○) and virus-inoculated mice at 2 (●), 4 (□) and 8 (■) days post-inoculation. As expected, there was no significant difference between the mean concentration-effect curves to carbachol in tracheal segments from control mice at days 2, 4 and 8 post-inoculation. Thus, for clarity, the control concentration-effect curve shown represents the combined control data from days 2, 4 and 8 ($n = 32$). Also shown are the maximal levels of carbachol-induced contraction (C_{max}) produced in control preparations (C, $n = 32$) and at 2, 4 and 8 days post-inoculation with virus ($n = 8$–12). Data are presented as mean ± s.e.mean (* $P < 0.05$ compared to controls).

At both 2 and 8 days post-viral inoculation, mouse tracheal segments were significantly less responsive to noradrenaline and forskolin than were trachea from control animals ($P < 0.05$; Figure 7 and Table 3). At 2 days, but not 8 days post-inoculation, tracheal smooth muscle sensitivity to theophylline was also marginally attenuated compared to control preparations. The maximum relaxation responses (E_{max}) produced by noradrenaline, forskolin and theophylline in tracheal preparations from virus-inoculated mice were not significantly different from the respective E_{max} values obtained in control preparations at 2, 4 or 8 days post-inoculation.

Discussion

In the current study, quantitative autoradiographic analyses of I-CYP binding to mouse tracheal β-adrenoceptors revealed that infection with the respiratory pathogen, influenza A/PR-8/34, was not associated with significant changes in either the density or binding characteristics of tracheal smooth muscle β-adrenoceptors. However, functional studies showed that relaxation responses to the β-adrenoceptor agonist noradrenaline were depressed in tracheal segments from virus-infected mice. These data suggest a functional lesion distal to the β-adrenoceptor moiety. This conclusion is supported by the fact that relaxation responses to the adenylyl cyclase-activator forskolin, were also impaired following virus infection. Although direct measures of adenylyl cyclase activity in tracheal smooth muscle have not been attempted in the current study, it appears that a component of the virus-induced lesion involves processes initiated following β-adrenoceptor activation. There is other evidence which also suggests that adenylyl cyclase activity may be reduced by respiratory tract viral infection (Halbach & Koschel, 1979). In addition, Scarpace & Bender (1989) found that isoprenaline- and NaF-stimulated adenylyl cyclase activity of lung membranes obtained from mice infected with influenza A/Port Chalmers/73 virus was significantly lower than that observed in membranes from control mice. These findings are consistent with the earlier observation of Busse and coworkers (1979, 1980) showing that the inhibition of β-glucuronidase release from granulocytes exerted by agonists acting via adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation (isoprenaline, histamine and prostaglandin E_2 (PGE_2)) was impaired following infection with Rhinovirus or bivalent (A + B) influenza vaccine. In none of these studies was impaired adenylyl cyclase activity associated with any significant alteration in the number of β-adrenoceptors (Busse *et al.*, 1980; Scarpace & Bender, 1989). At present the mechanism of virus-induced attenuation of relaxation responses to noradrenaline and forskolin is not fully resolved, although several general mechanisms have previously been proposed to explain virus-induced attenuation of adenylyl cyclase activity (e.g. localized physical insult, locally released and/or systemic mediators, direct effect of the virus) (Scarpace & Bender, 1989). The focus of the lesion may be at the level of adenylyl cyclase activity. However, other more distal processes may also be impaired following virus infection, since relaxation responses to theophylline were marginally, but significantly, attenuated at day 2, although no change was seen at day 8 post-inoculation.

Radioligand binding experiments revealed that tracheal sections obtained from mice inoculated with influenza A/PR-8/34 virus, contained 40% more β-adrenoceptors than tracheal sections from control mice. Subsequent quantitative autoradiographic analyses revealed that this increase in total tracheal β-adrenoceptors was due to a 90% increase in the density of β-adrenoceptors in the epithelial layer. The reason for this large increase in tracheal epithelial β-adrenoceptor density following virus inoculation is not clear, although we are currently investigating the possibility that the presence of damaged or regenerating epithelial cells, the influx of inflammatory cells or virus-activated β-adrenoceptor synthesis contribute to this effect. The observation that the density of β-adrenoceptors within different tracheal structures may be variably modified following respiratory tract virus infection is in accord with the recent report of Engels and coworkers (1989) showing that another respiratory pathogen, *Haemophilus influenzae*, significantly changed the density of β-adrenoceptors over many lung structures (alveolar septa, bronchial epithelia etc.) but not airway smooth muscle. In the current study, epithelial function was not assessed. Thus, it is not clear whether the virus-induced increase in epithelial β-adrenoceptor density is linked to changes in epithelial function, e.g. β-adrenoceptor-mediated increases in ciliary beat frequency (Lopez-Vidriero *et al.*, 1985), ion/water secretion (Davis *et al.*, 1979). It has been well established that the epithelium can alter airway smooth muscle reactivity to some bronchodilator and bronchoconstrictor agents. For example, studies of guinea-pig isolated

Table 3 Mean pD_2 values of noradrenaline, forskolin and theophylline for relaxation of tracheal smooth muscle preparations obtained from control and virus-inoculated mice 2 and 8-days post-inoculation

Days post-inoculation	*Treatment*	*Noradrenaline*	*Forskolin*	*Theophylline*
Day 2	Control	6.84 ± 0.06	7.03 ± 0.07	3.78 ± 0.04
	Virus	6.57 ± 0.04*	6.78 ± 0.04*	3.63 ± 0.04
	#*n*	24 (12)	12 (6)	12 (6)
Day 8	Control	6.81 ± 0.04	6.91 ± 0.04	3.69 ± 0.04
	Virus	6.54 ± 0.04*	6.70 ± 0.04*	3.63 ± 0.03
	n	12 (6)	12 (6)	12 (6)

#*n* = number of preparations (number of animals).
*$P < 0.05$ compared to Control value.

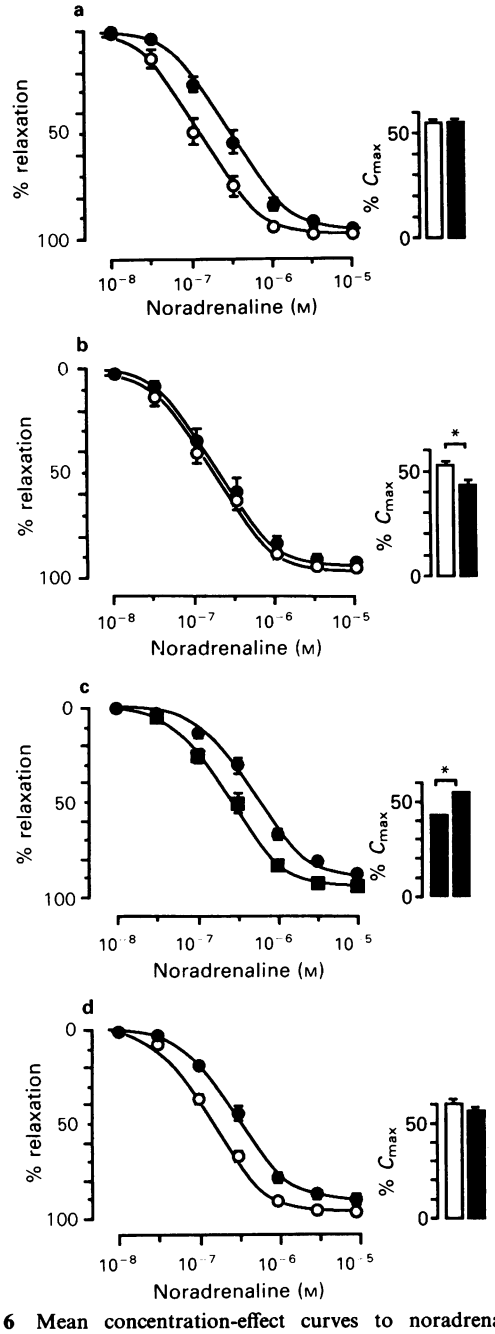


Figure 6 Mean concentration-effect curves to noradrenaline for relaxation of mouse isolated tracheal preparations. Each curve shows mean response of 12 preparations from 6 mice; vertical bars show s.e.mean. (a) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 2 days post-inoculation. All preparations were precontracted with 0.5 μM carbachol which produced the same level of tone (% C_{max}) in virus-inoculated (filled column) and control (open column) preparations. (b) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 8 days post-inoculation. All preparations were precontracted with 0.5 μM carbachol. (c) Tracheal segments obtained from virus-inoculated mice 8 days post-inoculation. Preparations were precontracted with carbachol to produce an initial tonus level of 55% (●) or 43% (■) C_{max}. (d) Tracheal segments obtained from virus-inoculated (●) and control (○) mice 8 days post-inoculation. All preparations were precontracted with carbachol to produce a similar level of induced tone (56–59% C_{max}).

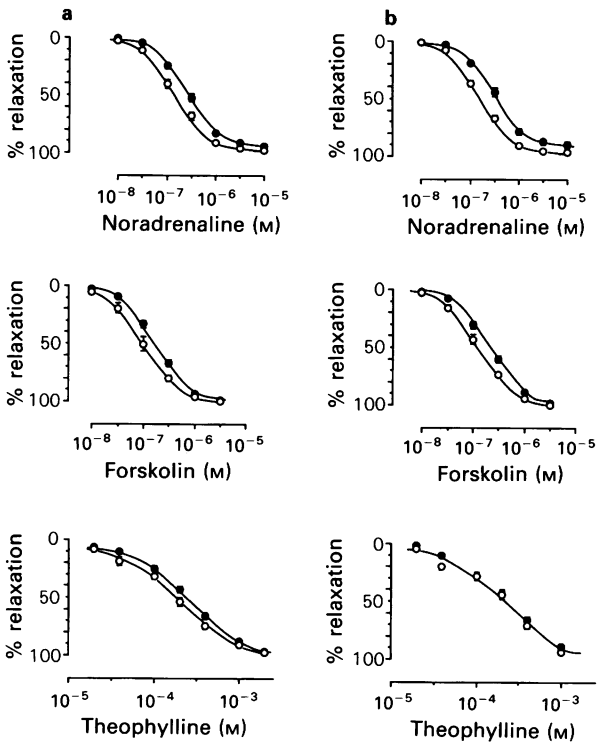


Figure 7 Mean concentration-effect curves to noradrenaline, forskolin and theophylline for relaxation of carbachol-contracted tracheal segments obtained from virus-inoculated (●) and control (○) mice. Tracheal preparations were used at either (a) 2 days or (b) 8 days post-inoculation. Response data are expressed in terms of mean ± s.e.mean (n = 12 preparations).

trachea indicate that removal of the epithelium is associated with either small increases in smooth muscle sensitivity to β-adrenoceptor agonists (Hay *et al.*, 1986; Holroyde, 1986) or no change in sensitivity (Goldie *et al.*, 1986). However, at present, the influence of the epithelium on β-adrenoceptor-mediated relaxation of isolated tracheal smooth muscle from either virus-infected or control mice is not known. Thus, the extent to which virus-induced changes in the tracheal epithelium contributed to the observed reduction in β-adrenoceptor agonist relaxant potency is not clear, but warrants further investigation.

Although β-adrenoceptor-mediated responses in many preparations are impaired following infection with respiratory tract viruses (Busse *et al.*, 1979; 1980; Lee, 1980; Ogunbiyi *et al.*, 1988; Scarpace & Bender, 1989), attenuation of β-adrenoceptor-mediated relaxation of airway smooth muscle has not previously been reported. Indeed, Eyre and coworkers (1979) found that guinea-pig isolated tracheal strips from animals inoculated with equine influenza virus were 10 fold more sensitive to the relaxant actions of isoprenaline than were non-infected controls. Furthermore, tracheal strips obtained from control guinea-pigs or from animals infected with parainfluenza-3 virus were equiresponsive to the relaxant actions of the β-adrenoceptor agonist sulfonterol (Buckner *et al.*, 1981). The reason for these differences is unclear, but may reflect differences between animal species, virus strains or the severity of infection. Whether the small reductions in β-adrenoceptor-mediated relaxations of isolated tracheal smooth muscle observed in the current study would be associated with a significant reduction *in vivo* in airway responsiveness to β-adrenoceptor bronchodilators is not known.

In the present murine model, virus infection was associated with attenuated tracheal smooth muscle responsiveness to the muscarinic agonist carbachol. However, no direct temporal relationship was observed between periods of maximal virus growth (day 2) and attenuated tracheal responsiveness to carbachol (days 4 and 8), suggesting the involvement of subsequent inflammatory processes in this effect, rather than the presence of the virus *per se*. Furthermore, these results are consistent with recent reports that, compared to muscarinic M_2-cholinoceptors, the M_3-cholinoceptors of airway smooth muscle are resistant to the neuraminidase, sialic acid-cleaving actions of respiratory tract viruses such as influenza and parainfluenza (Fryer *et al.*, 1990; Jacoby & Fryer, 1990).

Although a small reduction (17%) was observed in the magnitude of the maximum carbachol-induced contraction in trachea taken from mice at day 8 post-inoculation, the mechanism responsible is not clear. It cannot be discounted that differences in either smooth muscle mass or in the length-tension relationship may have contributed to this reduction. However, the attenuated responses to carbachol did not appear to be the result of a generalized effect on the airway smooth muscle since contractile responses to endothelin-1 were not affected by virus-infection.

The present study has clearly demonstrated that influenza A virus infection in mouse trachea resulted in significant attenuation of both airway smooth muscle β-adrenoceptor and muscarinic cholinoceptor function. Additionally, in airway epithelial cells, which act as the airway site of virus replication, major changes in the density of β-adrenoceptors were apparent. The possibility that these effects were primarily related to inflammatory processes associated with viral infection is under investigation.

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Aggregation of pigment granules in single cultured *Xenopus laevis* melanophores by melatonin analogues

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1 Isolated melanophores were differentiated from aggregates of neural crest obtained from neurula stage *Xenopus laevis* embryos after 2 days in culture.

2 Condensation of pigment granules in these cells by melatonin (5-methoxy N-acetyltryptamine, aMT) and various novel analogues was monitored with an image analysis system to quantitate the area occupied by pigment in individual cells.

3 Melanophores exposed to vehicle (a maximum of 0.1% MeOH) showed little (<5%) change in pigment area. aMT produced a dramatic condensation of pigment granules (EC_{50} = the concentration producing a half maximal condensation, 9 pM). The response was rapid, reached a maximum (~80% decrease in pigmented area) by 10 min, and was reversible after removal of aMT from the culture medium.

4 Aggregation to aMT was blocked by treating melanophores with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) indicating a role for a guanosine 5' triphosphate (GTP)-binding protein in transducing the aMT receptor signal.

5 Structure-activity studies indicated that analogues of aMT lacking a side-chain N-acyl substituent (5-methoxytryptamine, MT) or a group at the 5-position of the indole ring (N-acetyltryptamine, aT) were unable to induce pigment aggregation ($EC_{50} > 10\,\mu M$).

6 Lengthening the side-chain N-acyl group (N-propionyl, N-butanoyl) was tolerated to some degree but eventually (N-valeroyl and larger) activity diminished. Of the 5-position analogues tested 5-methoxy (aMT) was by far the most potent.

7 Halogen substitution in the 6-position of the indole ring led to some loss of activity as did a 6-OH substitution. The 6-OCH_3 compound was inactive.

8 These studies demonstrate the utility of this model in investigations of structure-activity relationships at the aMT receptor and suggest that it may be a valuable system for determining the transduction mechanisms coupled to the aMT receptor.

Keywords: Melatonin; melatonin analogues; *Xenopus* melanophores; pigment aggregation; structure-activity relationships

Introduction

Melatonin (5-methoxy N-acetyltryptamine, aMT), synthesized by the vertebrate pineal gland and secreted into the general circulation, plays a fundamental role in regulating seasonal changes in the reproductive axis in photoperiodic species (Tamarkin *et al.*, 1985) and is involved in regulating circadian rhythms in birds, reptiles (Underwood, 1989) and, possibly, mammals (Armstrong, 1989). aMT, probably synthesized locally, also modulates various aspects of retinal physiology such as photoreceptor outer segment disc shedding and phagocytosis, melanosome aggregation in pigment epithelium, cone photoreceptor retinomotor movements and intraocular pressure (Besharse *et al.*, 1988). Recent evidence indicates that aMT exerts its biological effects through specific receptors in the brain and eye. The development of 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT) initially for use in aMT radioimmunoassay (Vakkuri *et al.*, 1984), has enabled several groups to begin to identify and characterize high affinity aMT-binding sites in brains of various species including man (for review, see Morgan & Williams, 1989). Recent studies have also begun to focus on the transduction mechanisms utilized by the aMT receptor (Morgan *et al.*, 1989a; Vanecek & Vollrath, 1989). One of the few cellular models which exploits a known physiological response to aMT is the amphibian melanophore (for review see Rollag, 1988). In dermal melanophores aMT induces a condensation of pigment granules around the nucleus, producing a lightening of skin colour, a response which is part of the process adapting skin colour to external illumination. Pigment aggregation can also be produced in cultured melanophores *in vitro*. In the present study, melanophores obtained from the neural crest of *Xenopus laevis* embryos have been isolated and used to quantitate the pigment aggregation response to aMT and a number of novel analogues. These studies begin to allow an understanding of the structure-activity relationships at the amphibian aMT receptor and the transduction mechanisms triggered by aMT receptor activation.

Methods

Tissue culture

Xenopus laevis embryos were produced by adult frogs induced to lay by injection of human chorionic gonadotrophin (Chorulon, Intervet Laboratories Ltd., 400 iu/male, 600 iu/female). The embryos were reared in tap water until they reached stage 20, assessed from the normal table of *Xenopus* development (Nieuwkoop & Faber, 1956). The neural plate from about 20 embryos was dissected out and dispersed into small aggregates as described previously (Messenger & Warner, 1977). Tissue aggregates attached to the petri dish and after 2 to 3 days large pigmented cells, often at the edge of the aggregate, were readily visible among many nerve, muscle and undifferentiated cells. Cells were cultured in medium containing (mM): NaCl 100, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 5 plus 10% foetal calf serum and penicillin 100 u ml^{-1} and streptomycin 100 $\mu g\,ml^{-1}$. All experiments were done between 7 and 12 days of culture.

Aggregation responses

On the day of the experiment, cells were washed twice with fresh culture medium. The number of melanophores in each dish was variable (between 10 and 100). Typically the response of 5 or 6 individual pigment cells per dish, chosen at random,

was assessed. Drugs were dissolved in MeOH at 10 mM and stored at −30°C. Drugs were diluted just before use with deionized water and added from 100× concentrated stock solutions. The maximal concentration of MeOH added (0.1%) did not induce pigment granule aggregation. For each individual cell, the area occupied by pigment was measured (generally 2000 to 10000 μm^2) and designated 100%. At rest, pigment occupied the full area of the cell. The area covered by pigment after drug addition was related to the initial pigment area. Cells were observed through a Leitz compound microscope (×100 magnification) attached to a See-Scan image analysis system (See-Scan, Cambridge, U.K.). Routinely drugs were added for 10 min, then the area of the cell covered by pigment determined. As it required approximately 0.5 min to 1 min to measure each cell and about 5 cells per dish were assayed, measurements were generally done between 10 and 15 min after drug addition. In this way cumulative concentration-response curves were constructed. When a cell was fully aggregated the area occupied by pigment was usually between 15 and 25% of the initial area. All cells responded equally well to melatonin, irrespective of their size. An EC_{50} value, calculated from each concentration-response curve, was defined as the concentration of agonist causing the area occupied by pigment granules to decrease by 50% relative to the maximum aggregation responses.

Drugs

The compounds used in these studies were obtained from the following sources: melatonin (aMT), 6-hydroxymelatonin (6-OHaMT), N-acetyl 5-hydroxytryptamine (OHaT), 5-methoxytryptamine (MT), 5-methyltryptamine (MeT), tryptamine (T) (Sigma Chemical Co., Poole, Dorset); 5-benzyloxytryptamine (BzOT), 4-methoxy-3-(2-aminoethyl) benzo[b]thiophene (4-MBTh), 6-methoxy-3-(2-aminoethyl) benzo[b]thiophene (6-MBTh) (Aldrich Chemical Co., Gillingham, Dorset); 2-iodomelatonin (2IaMT) (Research Biochemicals Inc., Mass., U.S.A.); 6-chloromelatonin (6-ClaMT), 6-fluoromelatonin (6-FaMT) and 6,7-dichloro 2-methylmelatonin (6,7-Cl2MeaMT) (Lilly Research Laboratories, Indianapolis, U.S.A.); 2-chloromelatonin (2-ClaMT) (Dr C.E. Smithen, Roche Products Ltd., Herts.); 5-methoxy N-hexanoyltryptamine (hMT) (Dr D.J. Skene, University of Surrey); 5-methoxy N-acetyl 3-(2-aminoethyl) benzo[b]thiophene (MaBTh) (Dr T.R. Bosin, Indiana University, U.S.A.); 6-methoxymelatonin (6-MaMT) (Dr D.C. Klein, NIH, Bethesda, U.S.A.). Several N-acylated compounds were synthesized: 5-methyl N-acetyltryptamine (MeaT); 5-benzyloxy N-acetyltryptamine (BzOaT); 5-methoxy N-formyltryptamine (fMT); 5-methoxy N-propionyltryptamine (pMT); 5-methoxy N-butanoyltryptamine (bMT); 5-methoxy N-isobutanoyltryptamine (ibMT); 5-methoxy N-valeroyltryptamine (vMT); 6-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (6-MaBTh); 4-methoxy N-acetyl 3-(2-aminoethyl)benzo[b] thiophene (4-MaBTh). The procedure used to synthesize all of these compounds was similar to that described by Ho *et al.* (1968) except that one equivalent of the appropriate anhydride was used. fMT was prepared by addition of acetic anhydride to 5-methoxytryptamine dissolved in formic acid. All products were characterized after recrystallization as single spots on two thin layer chromatography (t.l.c.) systems: (I) chloroform/methanol/acetic acid/water, 60:30:3:1; (II) ethyl acetate/butan-1-ol, 90:10 and structures were confirmed by nuclear magnetic resonance. The abbreviations used for melatonin analogues follow, as far as is possible, the code outlined previously (Smith, 1981).

Pertussis toxin was obtained from Porton Products (Porton Down, Wilts).

Results

Large numbers of nerve and muscle cells differentiated from the fragments of neural crest placed in each culture dish as was seen previously (Jackson *et al.*, 1974; Messenger & Warner, 1977). Among these were smaller numbers of melanophores (10–100) easily recognised by their large size (50–100 μm diameter), distinctive shape and dark appearance due to the presence of many melanin-containing pigment granules (Figure 1a). Addition of aMT to these cultures resulted in a dramatic condensation of pigment granules in the melanophores (Figure 1b). The response was readily quantitated by determining the area of the cell occupied by pigment (Figure 1c, d) and could be detected within 1–2 min and had reached a maximum by 10 min (Figure 2). The continued presence of aMT in the culture medium maintained the pigment granules of melanophores in an aggregated state. Removal of aMT by washing the cells with fresh aMT-free medium resulted in a dispersal of pigment and a return, after about 20 min, of pigmented cell area to values very similar to those seen initially. Treatment of melanophores with vehicle (0.1% v/v MeOH) did not alter the mean pigmented cell area (Figure 2). In some cells there were movements of pigment granules into and out of projections as the shape of the cell slowly changed during the course of the experiment. Such changes produced only minor alterations in the area occupied by pigment.

Treatment of cultures with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) completely prevented the aggregating effect of aMT (10 nM) (Figure 3). Removal of Ca^{2+} from the culture medium by chelation with EGTA (2.3 mM, calculated free Ca^{2+} 0.2 μM) prevented the aggregation response to aMT (10 nM) but the response returned when Ca^{2+} was restored to the culture medium (Figure 4).

aMT produced a concentration-related aggregation of pigment granules in melanophores (EC_{50} 9 pM). N-acetyl-5-hydroxytryptamine (OHaT), the immediate precursor of aMT in the pineal gland, did not aggregate pigment granules in melanophores even at a high concentration (10 μM). The major metabolite of aMT, 6-OHaMT was a weak agonist and induced pigment aggregation with an EC_{50} of 63 nM. Other endogenous indoles related to aMT were inactive (no aggregation at 10 μM with 5-hydroxytryptamine, tryptamine and MT).

All of the aMT analogues tested, with the exception of fMT, gave concentration-response curves parallel to that for aMT (Figure 5). The fMT concentration-response curve was shallow. Several compounds were tested in which the N-acetyl group on the side chain of aMT was changed. A smaller group (N-formyl, fMT) led to a marked loss of activity (EC_{50} 296 nM) but compounds with larger substitutions (N-propionyl, pMT; N-butanoyl, bMT) were recognised by the aMT receptor (EC_{50} 11 pM and 60 pM respectively). Substitution of a larger group in the indole side-chain led to a loss of activity (N-isobutanoyl, ibMT EC_{50} 2.4 nM; N-valeroyl, vMT EC_{50} 14.1 nM; N-hexanoyl, hMT $EC_{50} > 10\,\mu M$).

Analogues with substitutions other than methoxy on the 5-position of the indole ring were inactive ($EC_{50} > 1\,\mu M$ for 5-methyl, 5-benzyloxy, 5-H and 5-hydroxy analogues; Figure 6).

Analogues of aMT with a substituent in the 6-position of the indole ring retained agonist activity but were less potent than aMT itself (6-fluoro, EC_{50} 2.5 nM; 6-chloro, EC_{50} 5.3 nM; 6,7-dichloro 2-methyl, EC_{50} 52 nM; 6-methoxy, $EC_{50} > 1\,\mu M$; Figure 7).

A series of analogues in which the indole nucleus of aMT was replaced by a benzo[b]thiophene ring were studied (Figure 8). The direct benzo[b]thiophene analogue of aMT (MaBTh) was an agonist at the aMT receptor in melanophores (EC_{50} 1.5 nM) although much less potent than aMT itself. The effect of the position of the 5-methoxy group on the ring was examined in this series. The 4-methoxy derivative retained some potency (EC_{50} 41 nM) but the 6-methoxy derivative was completely inactive ($EC_{50} > 1\,\mu M$).

Discussion

The ability of aMT to induce aggregation of pigment granules in amphibian melanophores has long been recognized (Rollag,

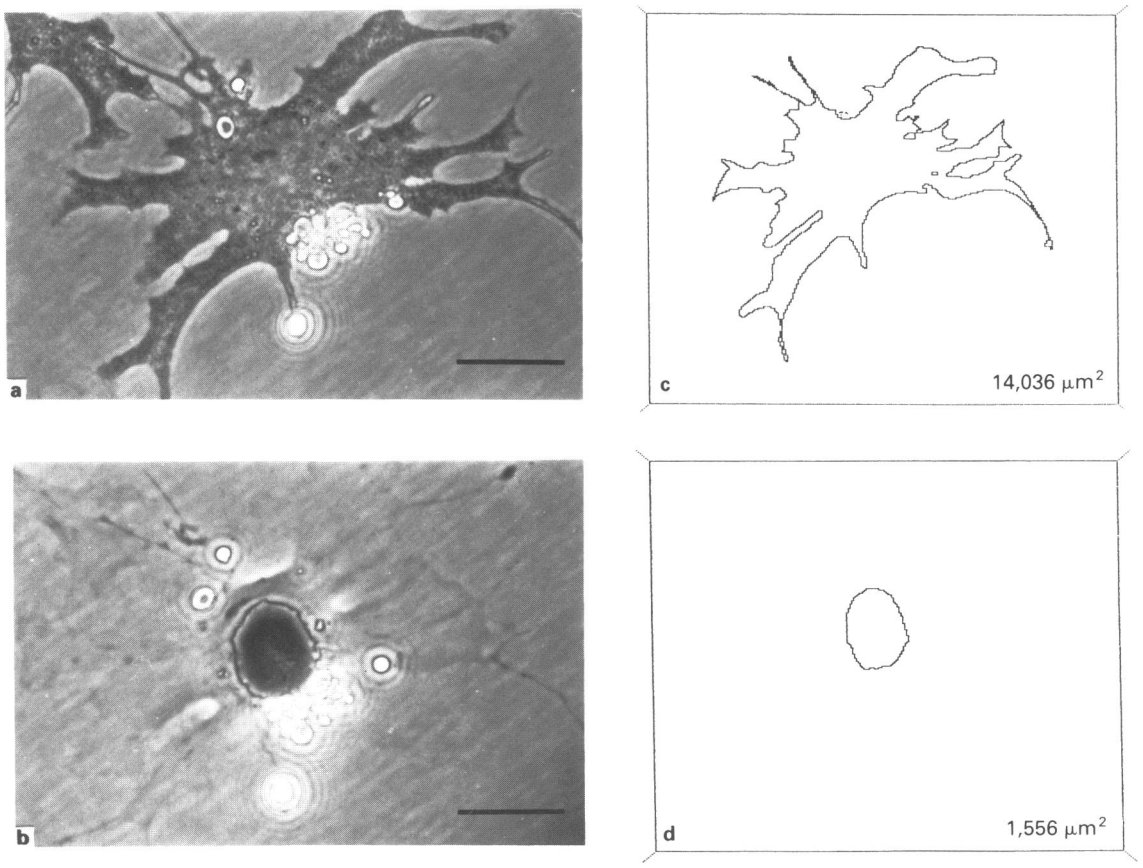


Figure 1 Photographs (a, b) and digitized processed images (c, d) of a typical melanophore before (a, c) and after (b, d) addition of melatonin (1 nM, 10 min). The horizontal bar represents 50 μm. The area occupied by pigment in (c) is 14036 μm^2 and in (d) 1556 μm^2.

1988). Indeed, this sensitive and specific response played an essential part in the original isolation and chemical characterization of aMT over 30 years ago as the skin-lightening factor present in pineal gland extracts (Lerner *et al.*, 1958). Since that time it has been recognised that aMT participates in regulating physiological colour changes in amphibians such as *Xenopus* (Bagnara, 1960). The response to aMT which occurs in melanophores *in vivo* can also be readily demonstrated in isolated melanophores prepared either from *Xenopus* embryos (Messenger & Warner, 1977) or tadpole skin (Fukuzawa & Ide, 1983; Rollag, 1988). In the present study, *Xenopus* melanophores have been used as a model system to study the

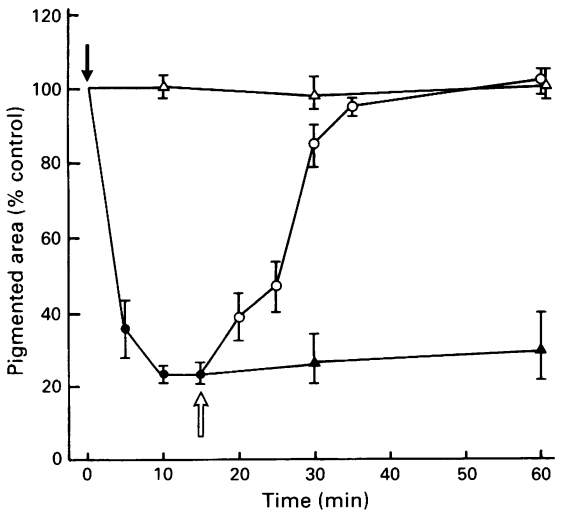


Figure 2 Time-course of pigment aggregation. Melanophores were treated with vehicle (△, 0.1% MeOH, $n = 10$) or 5-methoxy N-acetyltryptamine, (aMT, ●, 1 nM) at the arrow (↓). aMT-treated cells were washed with fresh medium (○, $n = 5$) or aMT-containing medium (▲, $n = 5$) at the second arrow (⇧). The area of each cell occupied by pigment granules was determined as described in Methods. All area measurements were related to the initial pigmented area of each cell. Each point represents the mean with s.e.mean shown by vertical bars.

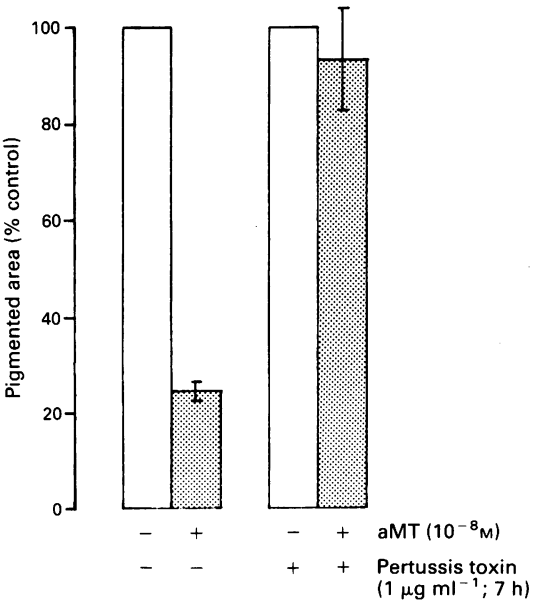


Figure 3 Effect of pertussis toxin on the pigment aggregation response to 5-methoxy N-acetyltryptamine (aMT). Cultures were treated with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) or vehicle (0.25% glycerol/0.25 mM sodium phosphate/2.5 mM sodium chloride) then the response to aMT (10 nM) tested. Each column represents the mean of 6 cells; vertical bars show s.e.mean.

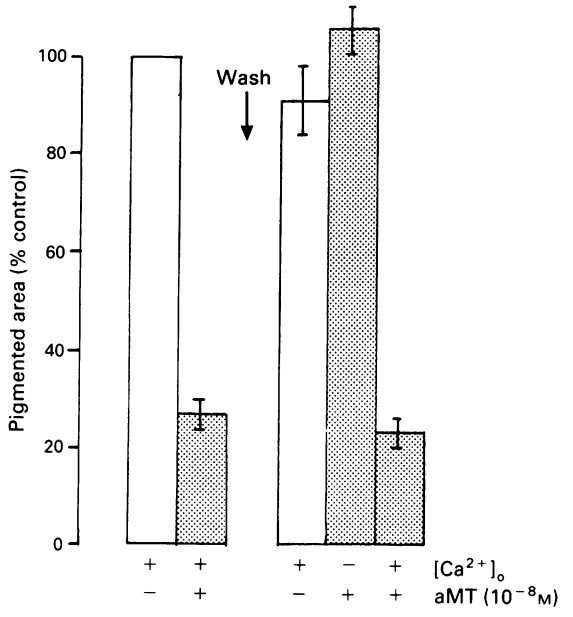


Figure 4 Effect of low external Ca^{2+} $[Ca^{2+}]_0$ on the pigment aggregation response to 5-methoxy N-acetyltryptamine (aMT). Six melanophores in a single culture were exposed to the following treatments: aMT (10 nM), which aggregated pigment granules as expected; fresh aMT-free medium, which reversed the aggregation produced by aMT; aMT (10 nM) plus EGTA (2.3 mM) to chelate extracellular Ca^{2+} (calculated free $Ca^{2+} = 0.2\,\mu M$), which prevented the expected response to aMT; restoration of extracellular Ca^{2+} (2 mM), by adding fresh medium, in the presence of aMT (10 nM) which resulted in pigment aggregation.

structure-activity relationships at the aMT receptor and to begin to examine the molecular mechanisms involved in aMT action.

Cultures pretreated for several hours with pertussis toxin failed to show aggregation in response to a high concentration (10 nM) of aMT (Figure 2). This finding suggests that the action of aMT is mediated by a guanine nucleotide-binding protein which is a pertussis toxin substrate, such as G_i. This idea is consistent with the finding that aMT inhibits the MSH-induced increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in melanophore-rich skin of *Xenopus* (Van de Veerdonk & Konijin, 1970) and *Rana pipiens* (Abe *et*

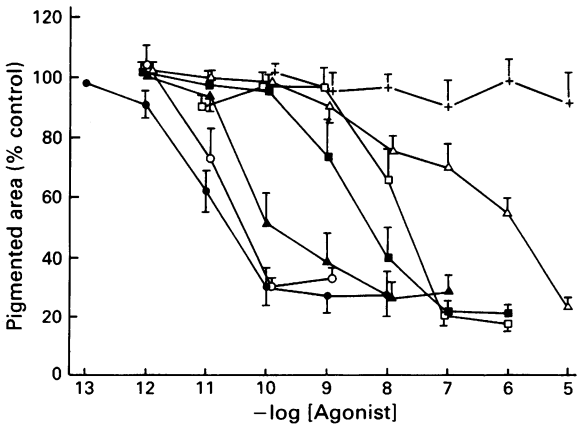


Figure 5 Pigment aggregation in response to N-acyl 5-methoxytryptamine derivatives. Concentration-response curves were obtained as described in Methods. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: N-formyl 5-methoxytryptamine (△, $n = 7$); aMT (●, $n = 18$); N-propionyl 5-methoxytryptamine (○, $n = 6$); N-butanoyl 5-methoxytryptamine (▲, $n = 6$); N-isobutanoyl 5-methoxytryptamine (■, $n = 6$); N-valeroyl 5-methoxytryptamine (□, $n = 8$); N-hexanoyl 5-methoxytryptamine (+, $n = 5$).

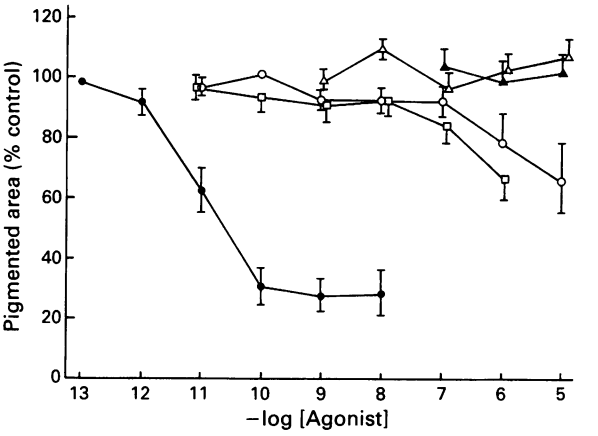


Figure 6 Pigment aggregation responses to 5-substituted N-acetyltryptamines. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: 5-methyl N-acetyltryptamine (○, $n = 7$); N-acetyltryptamine (▲, $n = 5$); 5-hydroxy N-acetyltryptamine (△, $n = 6$); 5-benzyloxy N-acetyltryptamine (□, $n = 9$).

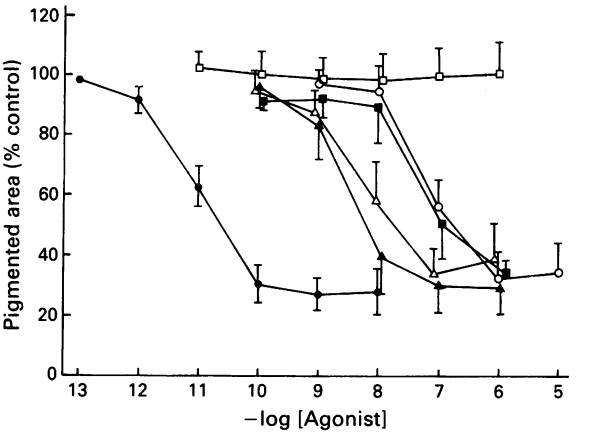


Figure 7 Pigment aggregation responses to 6-substituted melatonin (aMT) analogues. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: aMT (●, $n = 18$); 6-hydroxymelatonin (○, $n = 8$); 6-fluoromelatonin (▲, $n = 6$); 6-chloromelatonin (△, $n = 8$); 6-methoxymelatonin (□, $n = 6$); 6,7-dichloro 2-methylmelatonin (■, $n = 6$).

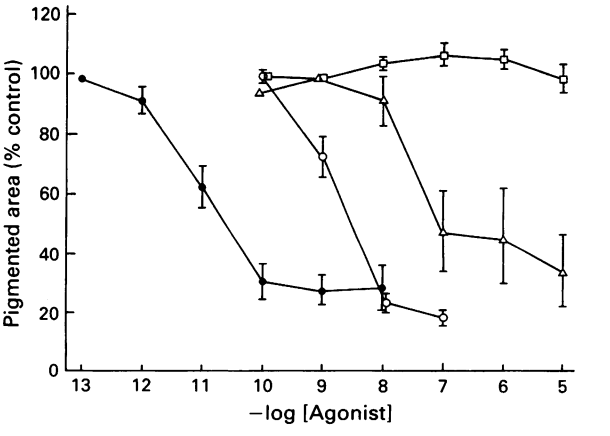


Figure 8 Pigment aggregation in response to methoxy substituted N-acetylaminoethyl benzo[b]thiophenes. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: 5-methoxy N-acetyltryptamine (●, $n = 18$); 5-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (○, $n = 7$); 4-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (△, $n = 5$); 6-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (□, $n = 5$).

al., 1969). Furthermore, agents elevating intracellular cyclic AMP such as forskolin, MSH and cell-permeable analogues of cyclic AMP induce an opposite response to aMT, namely pigment granule dispersal (Seldenrijk *et al.*, 1979). Pertussis toxin treatment of meningeal explants blocks the ability of aMT to reverse pigment granule dispersal induced by forskolin in melanophores in this tissue (White *et al.*, 1987), again suggesting that aMT may initiate aggregation by inhibiting adenylate cyclase activity via G_i.

The aggregation response to aMT was also absent when Ca^{2+} was removed from the incubation medium by addition of EGTA but was rapidly restored when extracellular Ca^{2+} was replenished (Figure 3). The inhibition of the aMT response in the presence of low (0.2 μM) extracellular Ca^{2+} may reflect a requirement for Ca^{2+} of the cellular machinery controlling the physical movement of pigment granules. Alternatively, extracellular Ca^{2+} may be involved in the aMT receptor transduction mechanism. However, an increase in extracellular K^+ did not induce pigment aggregation (data not shown), and an earlier study on pigment granule movement in melanophores of *Rana* skin (Wright & Lerner, 1960) showed that extracellular Ca^{2+} was not required for the aggregating action of aMT.

In agreement with earlier studies using either an indirect photometric method (Heward & Hadley, 1975) or the semi-quantitative 'melanophore index' (Quay, 1968), the present studies indicate that the 5-methoxy group on the indole nucleus and the N-acetyl side chain of aMT are important determinants of agonist activity at the melanophore aMT receptor. However, examination of several novel aMT analogues on the melanophore response has allowed a finer characterization of structure-activity relationships at the aMT receptor (Table 1). In particular, it is apparent that larger N-acyl groups on the indole side-chain are recognized by the receptor almost as well as the N-acetyl group of aMT. The EC_{50} value for the N-propionyl substituted compound is similar to aMT (11 pM) while that for the N-butanoyl analogue is reduced only slightly (60 pM); larger side-chain substitutions lead to a marked, progressive loss of activity as the length of the side-chain increases. An N-formyl substitution is too small to be recognised by the receptor. Broadly this agrees with recent data from radioligand binding experiments in which 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT) was used to identify aMT binding sites; in membranes prepared from both whole chick brain and sheep pars tuberalis, N-propionyl and N-butanoyl analogues displace 2-[^{125}I]-aMT binding with affinity constants (K_i) actually slightly higher ($\sim$10 fold) than aMT itself (Sugden & Chong, 1991). Radioligand binding experiments on *Xenopus* tissue with the use of a wide range of aMT analogues will be needed to determine if the *Xenopus* aMT receptor is identical to the high affinity site characterized in chick brain and retina, sheep pars tuberalis and some specific nuclei of rat brain (Dubocovich, 1989; Morgan *et al.*, 1989b; Laitinen *et al.*, 1990; Sugden & Chong, 1991).

Table 1 Potency of 5-methoxy N-acetyltryptamine (aMT) and some analogues in producing pigment aggregation in isolated *Xenupus laevis* melanophores

Compound	EC_{50} (pM)
N-acyl substitution	
aMT	9
pMT	11
bMT	60
ibMT	2424
vMT	14125
fMT	296000
hMT	>10000000
6-substitution	
6FaMT	2540
6ClaMT	5280
6,7diCl2MeaMT	52000
6OHaMT	63400
6MaMT	>1000000
5-substitution	
BzOaT	1000000
MeaT	10000000
aT	>10000000
OHaT	>10000000
Benzo[b]thiophenes	
5MaBTh	1560
4MaBTh	41300
6MaBTh	>1000000

EC_{50} values were determined from Figures 5 to 8 using a weighted log-logit equation.
For abbreviations, see *Drugs* section of text.

None of the substitutions at the 5-position of the indole nucleus which were tested (-H, -OH, -CH_3, -benzyloxy), other than the -OCH_3 of aMT, gave compounds which aggregated melanophore pigment granules ($EC_{50} > 1\,\mu$M) suggesting that the size and/or electronic properties of the substituent at this 5-position is critical for agonist activity. Previous work on pigment aggregation responses (Quay, 1968; Heward & Hadley, 1975) supported the idea that the substituent at the 5-position of the indole ring was essential for agonist activity and that the side-chain N-acetyl substituent determined the affinity of binding to the receptor. Clearly the 5-OCH_3 substituent is vital for agonist activity but this is also influenced by the N-acyl group. Furthermore, radioligand binding studies have shown that substitutions at both positions can markedly affect binding affinity (Sugden & Chong, 1991).

The indole nucleus is not essential for aMT activity in that a benzo[b]thiophene analogue with a -OCH_3 substitution at the appropriate position aggregated melanophore pigment granules although this compound was considerably less potent (EC_{50} 1560 pM) than aMT itself. Interestingly, a comparison of -OCH_3 substitution at different positions on the benzo[b]thiophene ring showed that the 6-OCH_3 compound was inactive ($EC_{50} > 1\,\mu$M) yet the 4-OCH_3 derivative retained some activity (EC_{50} 41 nM). A similar order of potency has been seen with these analogues in radioligand binding experiments on chick brain and sheep pars tuberalis membranes (Sugden & Chong, unpublished) again suggesting a close similarity of the amphibian, avian and mammalian aMT receptors.

Some analogues of aMT with 6-position substituents retained some activity at the melanophore receptor (e.g. -F, -Cl, -OH). However, a 6-OCH_3 substitution leads to a loss of activity (EC_{50} >1000 nM). This finding clearly distinguishes the amphibian aMT receptor from a low affinity (K_d 1.5–3 nM) 2-[^{125}I]-aMT binding site reported to be widely distributed in the brain of hamsters (Duncan *et al.*, 1988; 1989) which recognizes 6-MaMT as readily as aMT itself.

It appears that the aMT receptor in *Xenopus* melanophores is similar to the high affinity aMT receptor, now well-characterized pharmacologically in 2-[^{125}I]-aMT radioligand binding assays in a mammal (sheep pars tuberalis), bird (chick brain and retina) and a marsupial (brain of the Bennett's wallaby) (Sugden & Chong, 1991; Dubocovich, 1989; Sugden *et al.*, unpublished). The structural requirements for pigment aggregating activity seem to be more strict than for binding to the high affinity 2-[^{125}I]-aMT binding site identified in membrane assays. For example, while 5-substitutions other than 5-OCH_3 certainly reduce binding affinity they lead to a complete loss of agonist activity on melanophores. Similarly 6-substitutions are better tolerated by the site detected by 2-[^{125}I]-aMT than by the melanophore receptor. Conceivably this may represent a 'relaxation' of the aMT receptor, in terms of the structures it will accommodate, during evolution. The validity of such a speculation awaits binding data from *Xenopus* which can be directly compared with that available in higher vertebrates and the development of simple cellular models in these vertebrates which can compare the biological activity of aMT and its analogues. It must be remembered that of the 2-[^{125}I]-aMT binding sites detected in vertebrates, only that found in the neuronal retina has clearly been shown

to mediate a biological response, namely aMT-induced inhibition of Ca^{2+}-dependent [^{3}H]-dopamine release (Dubocovich, 1988). Indeed, few *in vitro* models exist in which a response to aMT can be quantitated in order to study either structure-activity relationships or aMT receptor transduction mechanisms (Morgan *et al.*, 1989a; Vanecek & Vollrath, 1989). The melanophore model described here will continue to be useful for such studies.

I would like to thank Sally Rowe and Professor Anne Warner, University College, London for their help with the *Xenopus* cultures, and Dr C.E. Smithen, Dr D.C. Klein, Dr D.J. Skene and Dr T.R. Bosin for providing analogues of melatonin.

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Attenuated responses to endothelin-1, KCl and $CaCl_2$, but not noradrenaline, of aortae from rats with streptozotocin-induced diabetes mellitus

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1 This study investigated the responsiveness to vasoconstrictor agents (including endothelin-1, ET-1) of aortic rings from rats with two-week streptozotocin (STZ, 60 mg kg^{-1}, i.v.)-induced diabetes and vehicle-treated control rats. The basal tension was 10 g, which was estimated to be more physiological than the tension of 1–2 g that has been previously used for most studies of aortic rings from diabetic rats.

2 Maximum responses to ET-1 (0.13–18 nM), KCl (2–20 mM) or $CaCl_2$ (10 μM–10 mM) were reduced in aortae from STZ-treated rats compared to those from control rats. Such reductions were still evident after removal of the endothelium.

3 Responses to noradrenaline (NA, 0.1 nM–26 μM) of aortae from STZ-treated rats were not significantly different from responses of aortae of control rats.

4 Removal of endothelium resulted in a significant reduction in the EC_{50} values for NA of rings from both STZ-treated rats (6.90 $\pm$ 0.13 and 8.17 $\pm$ 0.35 (−log M) with and without endothelium, respectively, $n = 5$) and control rats (6.90 $\pm$ 0.15 and 8.37 $\pm$ 0.44 (−log M) with and without endothelium, respectively, $n = 5$).

5 In calcium-free medium (with 1 mM EGTA), responses to NA and ET-1 were reduced compared with those in normal Krebs solution and maximum responses were less in rings from STZ-treated compared with control rats.

6 Indomethacin (5 μM) did not prevent the reduced maximum responsiveness to ET-1 in rings from STZ-treated rats compared with those from controls.

7 This study indicates that changes in vascular responsiveness to ET-1, KCl and $CaCl_2$ (but not NA) occur in aortae of two-week STZ-treated rats. The endothelium does not appear to play a major role in mediating changes in responsiveness to ET-1.

Keywords: Diabetes mellitus; rat aortae; endothelial cells; endothelin; noradrenaline; calcium; indomethacin

Introduction

The release of the potent vasoconstrictor peptide endothelin (Yanagisawa *et al.*, 1988), from endothelial cells, can be triggered by elevated glucose levels (Yamauchi *et al.*, 1990). Changes in endothelial cell function have been linked to various pathological states such as hypertension, atherosclerosis and vascular occlusion (Vanhoutte, 1986; Lüscher *et al.*, 1989; Vane *et al.*, 1990), all of which occur more frequently in diabetes mellitus (Colwell *et al.*, 1979). This study examined the role of the endothelial cell in modulating responsiveness to various vasoconstrictors in aortic rings from rats with streptozotocin (STZ)-induced diabetes mellitus of two weeks duration. Most previous studies of vascular responsiveness of aortic rings from diabetic rats have used basal tensions of 1 g (e.g. Mulhern & Docherty, 1989) or 2 g (e.g. Owen & Carrier, 1979; Scarborough & Carrier, 1983; Head *et al.*, 1987; Harris & MacLeod, 1988). Such tensions probably do not approximate to those found *in vivo* (see below, and Mulvany & Halpern, 1977; Angus *et al.*, 1986), and therefore the present study was designed to examine the responsiveness of aortic rings under a more physiological tension.

Methods

Male Wistar rats (300–425 g) were lightly anaesthetized (4% halothane, O_2/N_2O 2 : 1) and made diabetic by a single injection into the tail vein of STZ (60 mg kg^{-1}) in citrate buffer (50 mM, pH 4.5), as previously described (Law & King, 1990). Control rats received an equivalent volume of citrate buffer. The animals were then housed in treatment pairs (i.e. one STZ-treated and one control). Rats were allowed free access to food and water at all times. Animals were weighed and their blood glucose levels measured (Ames Glucometer II), from a tail vein sample, immediately prior to injection, and 1 and 2 weeks after injection. Only rats displaying elevated blood glucose levels (> 18 mM) after 2 weeks were considered to be diabetic. Control rats had normal blood glucose levels over the two week period (4–8 mM).

After 2 weeks, a STZ-treated and a control animal were decapitated and a section of each descending thoracic aorta removed, flushed through with Krebs solution, and placed in a Petri dish containing oxygenated Krebs solution. Fat and connective tissue were removed under magnification. Two rings (5 mm in length) were cut from each aorta with a twin-bladed scalpel. Endothelial cells were removed from 2 of the 4 aortic rings (i.e. 1 from each treatment group but randomly caudal or cephalad) by gentle rubbing with stainless steel wire within the lumen. Each ring was then placed with 2 hooks through its lumen in a 15 ml organ bath containing Krebs solution. To avoid absorption of peptides onto glass, baths were siliconized. One hook from each aorta was connected to a tissue holder, the other to a force displacement transducer (Grass FTO3). Contractions were recorded on a Grass polygraph (Model 7D). Tissues were equilibrated for 60 min under a resting tension of 1 g. After this period tissues were placed under a resting tension of 10 g for the duration of the experiment. This tension of 10 g results in a more close approximation to the wall tension of the aorta *in vivo*, than the more commonly used tension of 1–2 g.

The effective transmural pressure 'p' is an estimate of the pressure which would have been required to maintain the *in situ* vessel at the same internal wall circumference and wall tension. 'p' can be estimated, assuming the vessel wall is sufficiently thin for Laplace's equation to apply (Mulvany & Halpern, 1977).

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$p = 2\pi$ T/L, where 'T' is the wall tension, and 'L' is the internal circumference (even if non circular). At a tension of 10 g between hooks, T = 10 g/2 × 5 mm (i.e. 10 g ÷ twice the length of the ring); thus T = 1 g/mm. L = πd + 2x, where 'x' is the distance between the two hooks, and 'd' is the diameter of the hooks (0.6 mm). 'x' was found to be relatively constant between aortae (2 mm). Thus, at a tension of 10 g between hooks, L = 5.88 mm, and $p = 2\pi$ T/L = 1.07 g mm^{-2} = 0.107 kg cm^{-2} = 10.5 kPa ≈ 80 mmHg. i.e. the effective transmural pressure approximates to that *in vivo*.

Krebs solution was of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 . 7H_2O$ 1.2 and glucose 11.1. Where indicated, calcium-free Krebs consisted of Krebs solution without Ca^{2+} but with 1 mM EGTA added. In high K^+ Krebs solution, the osmotic pressure was maintained by a corresponding reduction of Na^+. Physiological solutions were maintained at 37°C, and bubbled with carbogen (5% CO_2 in O_2). After equilibration, a submaximal concentration of phenylephrine (0.3 μM) was added to the bath. At the plateau of contraction, acetylcholine (10 μM) was added. The presence of functional endothelial cells was indicated by subsequent relaxation. Cumulative concentration-response curves to agonists were then obtained, with only 1 agonist being examined in each experiment. Noradrenaline, KCl and $CaCl_2$ were added at intervals of between 1–5 min depending on the time taken for the response to reach a plateau. ET-1 was added at intervals of between 5–15 min due to the longer time taken for the responses to reach a plateau. Where stated, indomethacin was added to the Krebs solution reservoir for the duration of the experiment. After each experiment, aortic rings were dried and then weighed. The order of killing STZ-treated and control animals was alternated between days and the arrangement of tissues in organ baths randomised.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), ethyleneglycol-*bis*-(β-amino-ethyl ether)*N,N'*-tetra-acetic acid (EGTA; Sigma), endothelin-1 (Auspep), indomethacin (Sigma), noradrenaline bitartrate (Sigma), phenylephrine HCl (ICN Pharmaceutical), streptozotocin (Sigma). Noradrenaline and phenylephrine were made up in catecholamine diluent (0.312 g NaH_2PO_4 and 0.08 g ascorbic acid per litre of 0.9% (w/v) saline). Acetylcholine was dissolved in distilled water. Indomethacin was dissolved in 1% Na_2CO_3. Endothelin was initially dissolved in distilled water and frozen. On the day of use it was thawed and diluted in 0.9% saline. All other drugs were prepared daily.

Statistics

Single comparisons between responses in aortae from STZ-treated and control rats were made by Student's unpaired *t* test. Values shown are means ± s.e.mean. Parallel shifts and EC_{50}s were calculated by regression analysis of the linear portions of the log concentration-response curves. Multiple comparisons between aortae from STZ-treated and control rats were analyzed for interactions using two way analysis of variance (ANOVA) on the CLR ANOVA package (Apple Macintosh), and a series of 4 one-way ANOVAs for individual comparisons. In all cases, statistical significance is indicated by $P < 0.05$.

Results

Table 1 shows rat body weights and dry weights of aortic rings. There was no significant difference in pre-injection weights of STZ-treated and control rats. However, 1 and 2 weeks post-injection, STZ-treated rats showed a significant decrease in body weight relative to control rats, and also relative to their pre-injection weight (ANOVA, $P < 0.05$). There was no significant difference between aortic ring weights of STZ-treated and control rats with or without endothelial cells.

Vasoconstrictor responses to endothelin-1 (ET-1)

Cumulative concentration-response curves were obtained to ET-1 (0.13 nM–18.00 nM) in aortic rings from STZ-treated and control rats, with and without endothelial cells (Figure 1a, b). Maximum responses of aortae from STZ-treated rats, with or without endothelial cells, were reduced relative to those of aortae from controls (ANOVA, $P < 0.05$, Table 2). There was

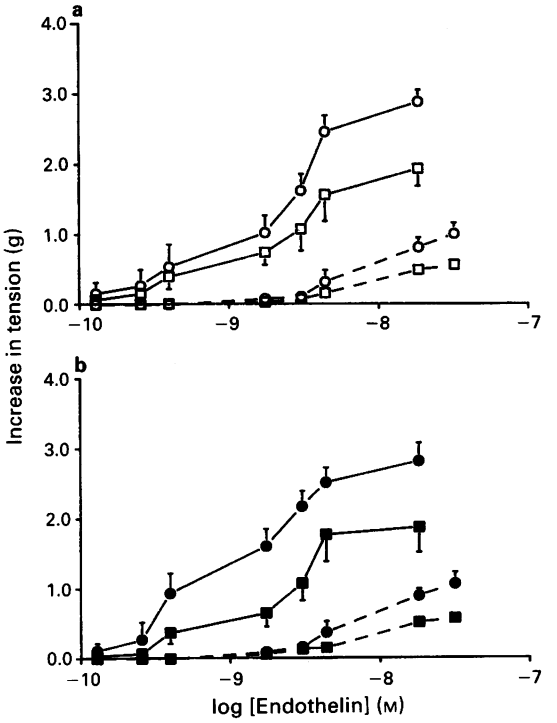


Figure 1 Responses of rat aortic rings to endothelin-1 ($n = 7$). (a) Streptozotocin (STZ)-treated (□—□ normal calcium; □---□ calcium-free) and control (○—○ normal calcium; ○---○ calcium-free) with endothelium. (b) STZ-treated (■—■ normal calcium; ■---■ calcium-free) and control (●—● normal calcium; ●---● calcium-free) without endothelium.

Table 1 Body weights and aortic ring dry weights of control and 14-day streptozotocin (STZ)-treated rats

	Animal weights (g)			*Aortic ring dry weights* (mg)	
	Initial	1 week	2 weeks	With EC	Without EC
Control ($n = 43$)	338 ± 3	369 ± 4#	378 ± 3#	1.82 ± 0.03	1.84 ± 0.03
STZ-treated ($n = 43$)	343 ± 4	315 ± 3*#	307 ± 4*#	1.83 ± 0.03	1.83 ± 0.02

EC = endothelial cells.
Significantly different from control at corresponding time, *$P < 0.05$.
Significantly different from initial weight in same treatment group, #$P < 0.05$.

Table 2 Maximum responses and EC_{50} values for control (C) and 14-day streptozotocin (STZ)-treated rats, with (+) and without (−) endothelial cells (EC)

	n		*C + EC*	*STZ + EC*	*C − EC*	*STZ − EC*
ET-1		Max response (g)	2.88 ± 0.17	1.93 ± 0.25*	2.81 ± 0.26	1.87 ± 0.36*
	7	EC_{50} (−log M)	8.80 ± 0.20	8.71 ± 0.22	8.95 ± 0.19	8.58 ± 0.06
ET-1 + Indo		Max response (g)	2.74 ± 0.34	1.90 ± 0.16*	3.66 ± 0.31	2.26 ± 0.12*
	5–7	EC_{50} (−log M)	8.61 ± 0.22	8.40 ± 0.07	8.71 ± 0.33	8.43 ± 0.12
ET-1 Ca^{2+}-free		Max response (g)	1.00 ± 0.16	0.56 ± 0.04*	1.06 ± 0.17	0.57 ± 0.07*
	5	EC_{50} (−log M)	8.10 ± 0.10	8.15 ± 0.10	8.14 ± 0.09	8.10 ± 0.03
NA		Max response (g)	3.12 ± 0.33	2.70 ± 0.36	2.96 ± 0.40	2.86 ± 0.34
	5	EC_{50} (−log M)	6.90 ± 0.15	6.90 ± 0.13	8.37 ± 0.44#	8.17 ± 0.35#
NA Ca^{2+}-free		Max response (g)	0.99 ± 0.15	0.57 ± 0.11*	0.88 ± 0.06	0.51 ± 0.14*
	5	EC_{50} (−log M)	7.20 ± 0.25	7.03 ± 0.24	6.65 ± 0.16	6.86 ± 0.21
KCl		Max response (g)	2.03 ± 0.16	1.34 ± 0.12*	2.18 ± 0.17	1.73 ± 0.12*#
	9	EC_{50} (−log M)	2.21 ± 0.04	2.27 ± 0.05	2.26 ± 0.06	2.35 ± 0.07
$CaCl_2$		Max response (g)	3.22 ± 0.15	2.02 ± 0.25*	3.54 ± 0.14	2.32 ± 0.28*
	5	EC_{50} (−log M)	3.30 ± 0.19	2.98 ± 0.14	3.53 ± 0.16	3.17 ± 0.29

Indo = indomethacin; NA = noradrenaline.
Significantly different from corresponding control value, $*P < 0.05$.
Significantly different from same treatment group with endothelial cells, $\#P < 0.05$.

no significant difference between EC_{50} values for STZ-treated versus controls, with or without endothelial cells (Table 2). As shown in Figure 2a and b, in the presence of the cyclo-oxygenase inhibitor indomethacin (5 μM), maximum responses to ET-1 of aortic rings from STZ-treated rats, with or without endothelial cells, were significantly reduced compared to control (ANOVA, $P < 0.05$). There were no significant differences in EC_{50} values between aortae from STZ-treated versus control rats, either with or without endothelial cells (Table 2, with indomethacin). Removal of endothelial cells did not significantly affect maxima or EC_{50} values of rings from either STZ-treated or control rats.

In calcium-free Krebs solution with 1 mM EGTA, there were significant reductions in maximum responses to ET-1 of aortae from STZ-treated rats, with or without endothelial cells, compared to those of controls (Figure 1a and b, Table 2, ANOVA, $P < 0.05$). Also, in calcium-free Krebs solution with EGTA, responses to ET-1 in all groups were markedly reduced compared with those obtained in normal Krebs solution.

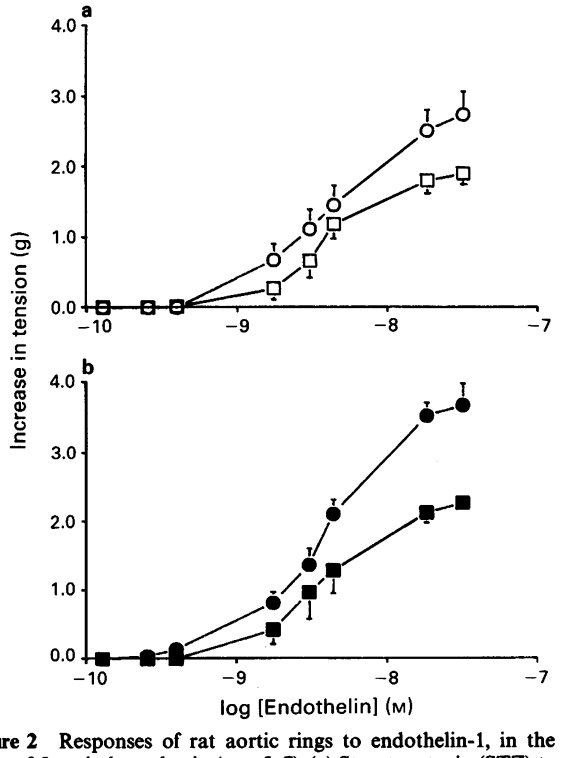


Figure 2 Responses of rat aortic rings to endothelin-1, in the presence of 5 μM indomethacin ($n = 5$–7). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium.

Vasoconstrictor responses to noradrenaline

Cumulative concentration-response curves were obtained to noradrenaline (NA, 0.1 nM–26.0 μM) in aortic rings, with or without endothelial cells. As shown in Figure 3a and b and Table 2, there was no significant difference between maximum responses of rings from STZ-treated and control rats, with or without endothelium. Removal of endothelium resulted in a parallel shift to the left in concentration-response curves of aortae from STZ-treated and control rats with a corresponding significant change in EC_{50} values (ANOVA, $P < 0.05$;

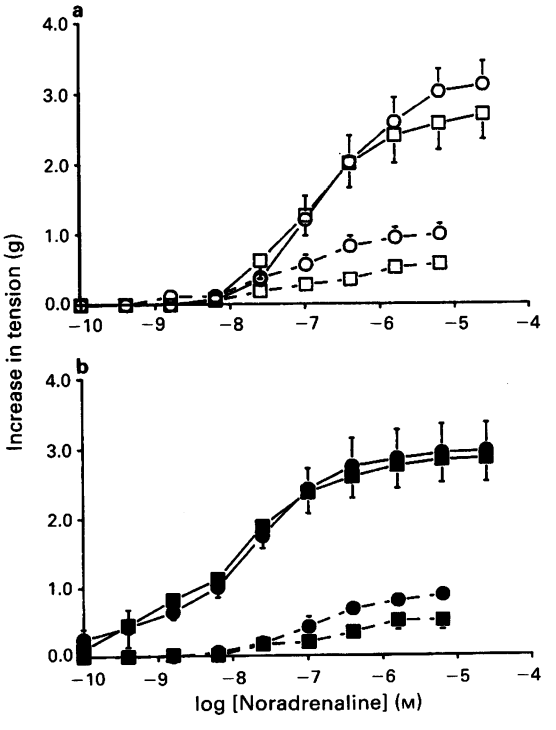


Figure 3 Responses of rat aortic rings to noradrenaline ($n = 5$). (a) Streptozotocin (STZ)-treated (□—□ normal calcium; □---□ calcium-free) and control (○—○ normal calcium; ○---○ calcium-free) with endothelium. (b) STZ-treated (■—■ normal calcium; ■---■ calcium-free) and control (●—● normal calcium; ●---● calcium-free) without endothelium.

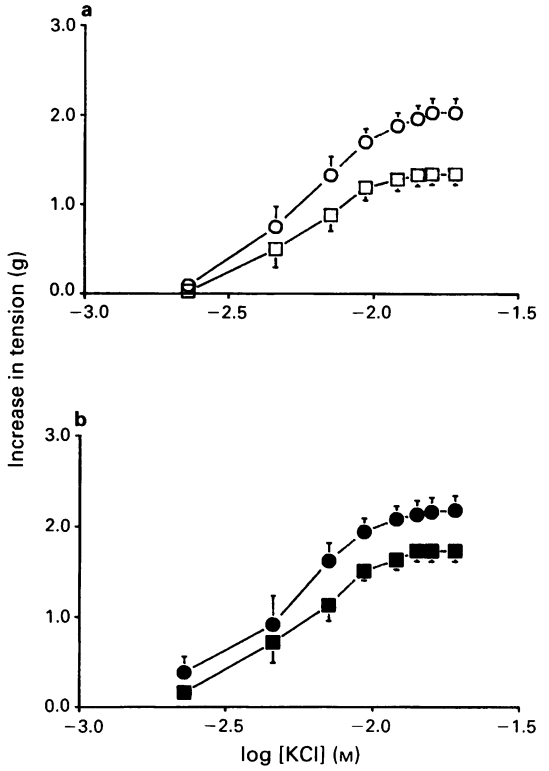


Figure 4 Responses of rat aortic rings to KCl ($n = 9$). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium.

Table 2). Maximum responses to NA in calcium-free Krebs solution with EGTA were significantly reduced in aortic rings from STZ-treated rats compared to those from controls, both with or without endothelial cells (ANOVA, $P < 0.05$; Figure 3a and b, Table 2). Also, in calcium-free Krebs solution with EGTA, responses to NA in all groups were markedly reduced compared to those obtained in normal Krebs solution.

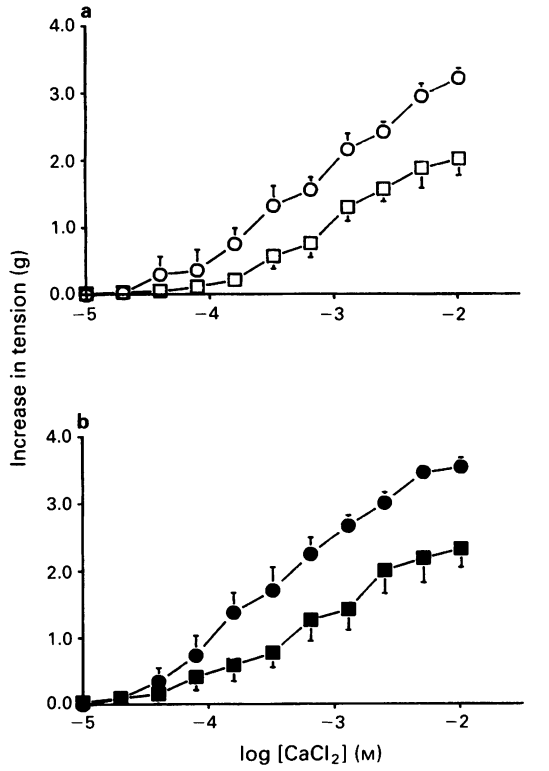


Figure 5 Responses of rat aortic rings to $CaCl_2$ ($n = 5$). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium.

Vasoconstrictor responses to KCl

Cumulative concentration-response curves to KCl (2 mM–20 mM) were obtained. As shown in Figure 4 and Table 2, maximum responses to KCl in aortic rings from STZ-treated animals, with or without endothelial cells, were significantly reduced relative to control (ANOVA, $P < 0.05$). There was no significant difference in EC_{50} values between aortae from STZ-treated and control rats, with or without endothelium (Table 2).

Vasoconstrictor responses to $CaCl_2$

Cumulative concentration-response curves to $CaCl_2$ (10 μM–10 mM), in high K^+ (60 mM) Krebs solution, were obtained in aortic rings from STZ-treated and control rats, with or without endothelial cells. Maximum responses of rings from STZ-treated rats were significantly reduced compared to those from control rats, either with or without endothelium (Figure 5 and Table 2, ANOVA, $P < 0.05$). However, there were no significant differences in EC_{50} values between responses of rings from STZ-treated and control rats, with or without endothelial cells (Table 2).

Discussion

In the present study, maximum responses to ET-1 were reduced in aortic rings from STZ-treated rats when compared to those from control rats. This reduction did not appear to be due solely to alterations in the endothelium, or to altered output of cyclo-oxygenase products and still occurred in the absence of extracellular calcium. Elevated plasma glucose levels in diabetes may cause an increase in secretion of ET. Takahashi *et al.* (1990) found elevated plasma ET levels in patients with diabetes, and elevated glucose levels can release ET (Yamauchi *et al.*, 1990). Such elevation of ET levels may result in homologous down-regulation of receptors (Miasiro & Paiva, 1990). This may account for at least part of the decreased responsiveness to ET-1 found in the present study but further experiments are required to substantiate this hypothesis. Furthermore, it is not known what the effect would be on ET-1 responses of bathing solutions appropriately adjusted for the differences in the composition of the extracellular fluid (including osmolality) of control and STZ-treated rats. Indeed, no such comparative *in vitro* studies with adjusted bathing solutions have been performed (Kiff *et al.*, 1991a). Also, changes in aortic endothelial cell function may not reflect changes in other vascular beds. Indeed Kiff *et al.* (1991b) found regionally selective and agonist-specific vasodilator abnormalities in diabetes, and emphasised the problems associated with *in vitro* studies of aortic ring preparations.

In the present study, the dry weights of aortic rings were not significantly affected by 2 weeks of diabetes. Although other workers have found a small but significant, reduction of aortic weights in diabetes, the diabetes has been of longer duration, and wet rather than dry weights were quoted (Scarborough & Carrier, 1983; 1984; Head *et al.*, 1987).

Results of the present study indicate that the reduced maximum responsiveness to ET-1 of aortic rings from STZ-treated rats was not totally non-specific. Although significantly reduced maximum responsiveness was observed to KCl and $CaCl_2$, it did not occur to NA, either in the presence or absence of endothelium. Also, reduced maximum responsiveness to ET-1 in STZ-treated rats was not totally dependent on extracellular calcium. Indeed, in the absence of extracellular calcium, maximum responsiveness to NA was reduced in STZ-treated rats compared to controls, suggesting that in diabetes there may be (at least in some circumstances) an impairment of mobilisation of intracellular calcium stores.

It has been shown that ET-1 stimulates the release of the vasodilator eicosanoid, prostacyclin, from rat mesenteric arteries (Rakugi *et al.*, 1989). However, in the present study indomethacin had no significant effect on EC_{50}s or maximum responses, suggesting that cyclo-oxygenase products do not normally play a major role in modifying constrictor effects of ET-1 in this preparation. The concentration of indomethacin used was greater than that previously shown to inhibit prostaglandin production in aortae from rats (Jeremy *et al.*, 1990).

Increased maximum responsiveness of aortae from STZ-treated rats to NA has been reported previously (Owen & Carrier, 1979 (4–5 week diabetic rats); Scarborough & Carrier, 1983 (8–10 week diabetic rats); Harris & MacLeod, 1988 (3 month diabetic rats)). In the latter study, the altered sensitivity to NA was no longer apparent after removal of endothelial cells. Abebe *et al.* (1990) have recently reported that enhanced responses of arteries from 3 month STZ-treated rats to α-adrenoceptor agonists are mediated by α_1-adrenoceptors and are largely dependent on the presence of extracellular calcium. It is possible that changes in responsiveness to NA of rat aortae are dependent on the duration of diabetes, since Mulhern & Docherty (1989), using aortae from 2–3 week STZ-treated rats found no change in responsiveness to NA. However, many previous studies have examined the responsiveness of isolated aortae to NA in the presence of a β-adrenoceptor antagonist (Harris & MacLeod, 1988; Mulhern & Docherty, 1989; Abebe *et al.*, 1990), since it is known that β-adrenoceptors mediate relaxation in rat aortae and that diabetes may change the β-adrenoceptor-mediated response (Kamata *et al.*, 1989). Nevertheless, the present study supports the findings of Mulhern & Docherty (1989), since after 2 weeks of diabetes, aortic responses to NA were not significantly different between STZ-treated and control rats. Results of the present study also support previous reports of decreased maximum responsiveness to KCl of aortae from diabetic rats (Pfaffman *et al.*, 1980; Head *et al.*, 1987) which is not altered by endothelial removal (Wakabayashi *et al.*, 1986).

In the present study, removal of endothelium reduced the EC_{50} value to NA by factors of 29.5 (2.5, 347, 95% confidence limits, controls) and 18.6 (2.6, 135, 95% confidence limits, diabetics). These shifts in potency are somewhat greater but not significantly so, than those reported by Martin *et al.* (1986) (6 fold, control) and Harris & MacLeod (1988) (4.6 fold, control; 5.6 fold, STZ-treated). The latter study was performed in the presence of a β-adrenoceptor antagonist.

In summary, a decreased responsiveness to endothelin, KCl and $CaCl_2$ (but not NA) of aortic rings from STZ-treated rats, was observed in the present study. The decreased responsiveness to ET-1 does not appear to be dependent on the presence of endothelial cells, or due to altered output of cyclo-oxygenase products or availability of extracellular calcium.

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Interaction of vasoactive substances released by platelet-activating factor in the rat perfused heart

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1 The coronary vascular effects of platelet-activating factor (PAF) have been intensively studied and it has been proposed that they are mediated by the release of vasoactive substances. In this study, a cascade perfusion model using two rat perfused hearts was developed to investigate the properties of PAF-released vasoactive substances and the interplay of these substances. The properties of the vasoactive substances after an injection of PAF (100 pmol) in the rat perfused heart were examined by collecting the effluent from the first heart for the perfusion of a second (recipient) heart. The presence of vasoconstrictor substances in the effluent was characterized by an increase in the perfusion pressure of the recipient heart.

2 Previous exposure of the recipient heart of PAF (100 pmol) abolished the response of the heart to subsequent administration of PAF, but did not affect the response of the recipient heart to the effluent. This suggested that the coronary vasoconstrictor response of the recipient heart was not due to the presence of PAF in the effluent but to other vasoactive substances.

3 Pretreatment of the recipient heart with the leukotriene receptor antagonist, L-649,923 (5 μM), partially reduced the vasoconstrictor effect of the effluent. Pretreatment of the first heart with indomethacin (2.8 μM) also partially reduced the vasoconstrictor effect of the effluent. The combination of indomethacin pretreatment of the first heart and L-649,923 pretreatment of the recipient heart completely abolished the vasoconstrictor effect of the effluent suggesting that both prostaglandins and leukotrienes are involved in the vasoconstrictor effect of the effluent.

4 Pretreatment of both hearts with L-649,923 or the first heart with the leukotriene synthesis inhibitor (MK-886, 10 μM) completely abolished the vasoconstrictor effect of the effluent. This suggested that the indomethacin sensitive vasoconstrictor component of the effluent might be regulated by leukotrienes in the first heart. However, infusion of leukotrienes (LTB_4, LTC_4 and LTD_4) to the first heart did not reproduce this vasoconstrictor component of the effluent in the recipient heart.

5 In conclusion, our study demonstrated through the use of a leukotriene receptor antagonist, a leukotriene synthesis inhibitor and a cyclo-oxygenase inhibitor that the vasoconstrictor effect of the effluent of the rat perfused heart after an injection of PAF is mediated by leukotrienes and prostaglandins. The ability of leukotriene receptor blockade and inhibition of leukotriene synthesis to mimic the effect of indomethacin indicates that the production and/or release of cyclo-oxygenase products in the effluent by PAF can be modulated by leukotrienes. The inability of exogenously applied leukotrienes to modulate the production and/or the release of cyclo-oxygenase products in the effluent suggests that the PAF-induced production of prostaglandins may be mediated by intracellular leukotrienes or at sites not accessible to exogenously applied leukotrienes.

Keywords: Platelet-activating factor; coronary vascular effects; leukotrienes; prostaglandins; rat perfused heart; cascade perfusion model

Introduction

Platelet-activating factor (PAF), a new class of phospholipid mediator, has been identified as a very potent vasoactive compound (Braquet *et al.*, 1987). PAF elicits a variety of striking cardiovascular changes including hypotension, selective vasodilatation, vasoconstriction, and cardiac depression (Braquet *et al.*, 1987). PAF has been detected in the effluent blood from the coronary sinus in patients with coronary artery disease undergoing atrial pacing (Montrucchio *et al.*, 1986) and release of PAF from ischaemic-reperfused rabbit heart has been demonstrated (Montrucchio *et al.*, 1989). The effects of PAF in the coronary circulation have been shown in a number of *in vivo* (Levi *et al.*, 1984; Piper & Stewart, 1986; 1987; Man *et al.*, 1990) and *in vitro* studies (Feuerstein *et al.*, 1984; Jackson *et al.*, 1986; Mehta *et al.*, 1986; Ezra *et al.*, 1987; Fiedler *et al.*, 1987).

Regarding the mechanism of the coronary vascular actions of PAF, it has been suggested that the effects of PAF are largely mediated by the release of other vasoactive substances. The importance of lipoxygenase products, leukotriene B_4 (LTB_4), LTC_4, and LTD_4 and cyclo-oxygenase products, prostaglandins and thromboxane A_2 in the coronary vascular effects of PAF has been demonstrated (Piper & Stewart, 1986; 1987). More recently, we showed that LTC_4 and LTD_4 may be responsible for the vasoconstrictor effect while LTB_4 may be responsible for the vasodilator effect of PAF in the rat perfused heart (Hu *et al.*, 1991). Inasmuch as the precise mechanisms of actions of PAF on circulatory function have not been fully elucidated, the complex interactions between PAF and archidonic acid metabolities in the coronary vascular effects of PAF are also not well defined. In the present study, a cascade perfusion model of rat isolated heart was developed to investigate the properties of vasoactive substances released by PAF. The objectives of this study were (a) to clarify the role of arachidonic acid metabolites in the coronary vascular effects of PAF and (b) to gain additional insight into the characteristics of the vasoactive mediators released by PAF.

Methods

Rat heart perfusion

Following cervical dislocation, hearts from Sprague-Dawley rats (250–350 g) were rapidly excised and placed in cool

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Krebs-Henseleit solution oxygenated with 95% O_2 : 5% CO_2. The solution had the following composition (mM): NaCl 120, NaH_2PO_4 1.18, $MgSO_4$ 1.18, KCl 4.76, $CaCl_2$ 1.25, $NaHCO_3$ 25.0 and glucose 11. The aorta was cannulated for coronary perfusion. The heart was allowed to beat spontaneously. The temperature of the perfusate was maintained at 37 ± 0.5°C and the coronary flow was controlled by a roller pump. The perfusion pressure was measured by a pressure transducer attached to a side arm of the aortic cannula. The perfusion pressure was recorded on a Gould chart recorder and monitored with a digital display. The detailed methodology of the isolated heart perfusion system had been reported previously (Hu *et al.*, 1991).

The cascade perfusion model

In the cascade perfusion model, two hearts were first separately perfused with oxygenated Krebs-Henseleit solution. Each heart was equilibrated with Krebs-Henseleit solution for 20 min. During this period, the flow rate was adjusted to obtain a control perfusion pressure of 65–75 mmHg and the flow rate was maintained constant for the rest of the experiment. Coronary vascular effects were measured as changes in the perfusion pressure as described previously (Man *et al.*, 1990). The perfusate for each heart could be controlled by a multiple way selector to enable drug pretreatments. During cascade perfusion, the effluent from the first heart was collected by a funnel and immediately pumped to a second (recipient) heart. The effluent was oxygenated with 95% O_2 : 5% CO_2 to maintain the Po_2, and Pco_2 at the same level as the perfusate for the first heart. The pH, Po_2 and Pco_2 of the solutions were measured by a pH/blood gas analyzer. Na^+ and K^+ contents were measured by flame photometry. The coronary flow rates of both hearts were controlled by the same roller pump motor so that the flow rate was kept identical. Solution accumulation in the funnel was kept to a minimum in order to reduce the dilution of vasoactive substances released from the first heart after challenge with PAF. One min before the injection of PAF to the first heart, the perfusion of the recipient heart was switched to the effluent from the first heart and continued to the end of experiment. Heart rates were monitored from the electrocardiographic recordings.

Drug preparations and administration

In the experiments which required a drug pretreatment, 10 min of equilibration with Krebs-Henseleit solution was followed by 10 min of pretreatment with a Krebs-Henseleit solution containing a leukotriene antagonist (L-649,923, Jones *et al.*, 1986) or the leukotriene synthesis inhibitor (MK-886, Gillard *et al.*, 1989), and continued throughout the administration of PAF. The cyclo-oxygenase inhibitor, indomethacin, was added to the perfusion solution at the start of the stabilization period and the effect of PAF injection was tested after 20 min of perfusion in the presence of indomethacin. All stock solutions were made fresh daily and kept at 4°C between experiments. The leukotriene antagonist, L-649,923 (sodium (βS^*, R^*)-4-(3-4(-acetyl-4-hydroxy-2-propylphenoxy)- propylthio)-γ-hydroxy-β-methylbenzenebutanoate, Merck Frosst Canada Inc.) and the leukotriene synthesis inhibitor, MK-886 (3-[1-(4-chrofobenzyl)-3-*t*-butyl-thio-5-iospropylindol - 2 - yl] - 2,2-dimethylpropanoic acid, Merck Frosst Canada Inc.) were dissolved in distilled water and then diluted in Krebs-Henseleit solution to a concentration of 5 μM and 10 μM respectively. Indomethacin (Sigma Chemical Co.) was dissolved in 0.1 M Na_2CO_3 solution and diluted in Krebs-Henseleit solution to a final concentration of 2.8 μM. PAF, (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, prepared from bovine heart, Sigma Chemical Co.) was prepared fresh daily in saline (0.9% NaCl) containing 0.25% bovine serum albumin (Sigma Chemical Co.). Bolus injections of PAF were given in a volume of 0.1 ml and over a 1 s period, into the perfusion line, 5–6 cm proximal to the aortic cannula. LTB_4, LTC_4 and LTD_4 (Merck Frosst Canada Inc.) were prepared fresh daily in saline (0.9% NaCl). The appropriate amount of LTB_4, LTC_4 or LTD_4 was continuously infused to the perfusion line 5–6 cm proximal to the aortic cannula for 5 min. Changes in perfusion pressure and the time at which maximum changes occurred were recorded.

Statistical analyses

Data were analyzed by Students *t* test and analysis of variance (ANOVA) followed by Duncan's test where appropriate. Values are expressed as means ± standard deviations (s.d.) and $P < 0.05$ was considered statistically significant.

Results

The effect of the effluent from the first heart on the recipient heart in the cascade perfusion model

After perfusion through the first heart, the Po_2 of the effluent was reduced. In order to maintain the pH, Po_2, and Pco_2 constant, the effluent from the first heart was oxygenated with 95% O_2 : 5% CO_2 before being perfused into the recipient heart. The pH, Po_2, and Pco_2 of the effluent were in the same range as normal oxygenated Krebs-Henseleit solution (Table 1). The Na^+ and K^+ concentrations of the effluent and normal Krebs-Henseleit solution were identical. Heart rate, contractility and the electrocardiogram were not altered by perfusion for up to 60 min with the effluent from the first heart (data not shown). There was also no significant alteration in the perfusion pressure of the recipient heart by perfusion with effluent (66.5 ± 1.3 and 68.8 ± 1.0 mmHg at time 0 and after 30 min of perfusion with the effluent respectively, $n = 4$).

Table 1 Po_2, Pco_2, pH, Na^+ and K^+ concentrations of the solutions used in the cascade perfusion model: (A) solution before perfusion into the first heart, (B) effluent collected from the first heart, (C) solution before perfusion into the second heart, and (D) effluent collected from the second heart

	A	*B*	*C*	*D*	n
Po_2 (mmHg)	462.4 ± 34.0	199.3 ± 32.3	434.0 ± 14.0	196.2 ± 20.9	7
Pco_2 (mmHg)	43.6 ± 9.3	40.4 ± 5.7	42.6 ± 8.7	40.7 ± 4.9	7
pH	7.37 ± 0.07	7.37 ± 0.05	7.36 ± 0.07	7.36 ± 0.04	6
Na^+ (mEq l^{-1})	149 ± 2	150 ± 2	150 ± 2	151 ± 2	6
K^+ (mEq l^{-1})	5.5 ± 0.5	5.5 ± 0.5	5.5 ± 0.5	5.5 ± 0.5	6

Values represent mean ± s.d., n = number of experiments. Na^+ concentration was determined to 1 mEq l^{-1} accuracy and K^+ concentration to 0.1 mEq l^{-1} accuracy.

Assessment of PAF-released vasoactive substances in the effluent using the recipient heart of the cascade perfusion model

When 100 pmol PAF was administered to the first heart, a brief vasodilator response followed by a vasoconstrictor response were observed as described previously. The effluent from this heart caused an increase in the perfusion pressure of the recipient heart (30.8 ± 8.7 mmHg, $n = 6$) at about 1.5 min after the injection of PAF to the first heart. However, no vasodilator response was observed in the recipient heart. The results of the effects of PAF on the perfusion pressure of the first and recipient hearts are summarized in Figure 1. To determine whether the coronary vascular effect of the effluent was due to the presence of PAF *per se* or of released vasoactive substances, the recipient heart was pretreated with PAF (100 pmol) in order to eliminate the vasoconstrictor response to subsequent exposure to PAF (Piper & Stewart, 1986; Man *et al.*, 1990). The vasoconstrictor response of the recipient heart to the effluent was not affected by such a pretreatment (Figure 1). When both hearts were pretreated with PAF, no vasoconstrictor response to the effluent was observed in the recipient heart (Figure 1). The above results demonstrated that the vasoconstrictor response of the recipient heart to the effluent from the first heart was not caused by PAF itself, but by the presence of other PAF-released vasoactive substances. To eliminate the possible interference of the presence of PAF in the effluent in the determination of the properties of the PAF-released vasoactive substances in the effluent, 100 pmol PAF was administered to the recipient hearts in all subsequent experiments.

The effects of L-649,923, indomethacin and MK-886 on the response to PAF in the cascade perfusion model

To gain further insight into the characteristics of PAF-released vasoactive substances, pretreatments with a leukotriene receptor antagonist (L-649,923, 5 μM), a cyclo-oxygenase inhibitor (indomethacin, 2.8 μM) and a leukotriene synthesis inhibitor (MK-886, 10 μM) were used. The effects of pretreatment of the first heart with L-649,923, indomethacin and MK-886 on the coronary responses of these hearts to a bolus injection of 100 pmol PAF are shown in Figure 2. Pretreatment with indomethacin did not significantly affect the vasodilator or the vasoconstrictor responses to PAF while hearts

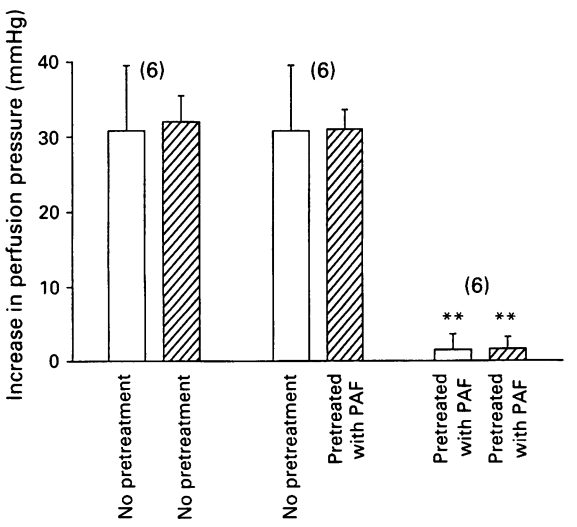


Figure 1 The effects of 100 pmol PAF on the perfusion pressure of the first (open column) and recipient (hatched column) hearts with and without pretreatment with PAF. Columns represent mean with s.d. shown by vertical bars. Numbers in parentheses indicate the number of experiments. $^{**}P < 0.01$ when compared to the response of the corresponding group with no pretreatment.

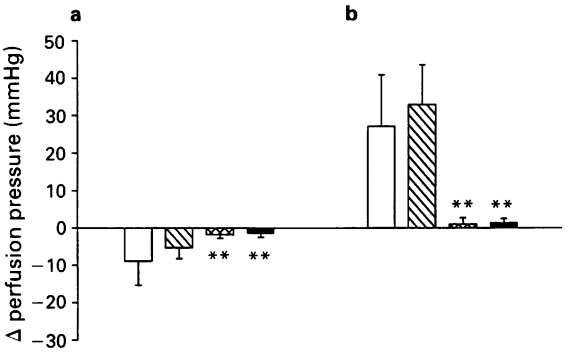


Figure 2 The effects of 100 pmol PAF on the perfusion pressure of the first heart after various pretreatments: (a) shows the initial vasodilator response to PAF and (b) shows the subsequent vasoconstrictor response to PAF. The concentrations of L-649,923, indomethacin and MK-886 were 5.0, 2.8 and 10 μM, respectively. Pretreatments: none (9), open columns; indomethacin (7) hatched columns; L-649,923 (6) cross-hatched columns; MK-886 (6) solid columns. Numbers in parentheses indicate the number of experiments. Columns represent mean with s.d. shown by vertical bars. $^{**}P < 0.01$ when compared to the response of hearts with no pretreatment.

pretreated with L-649,923 and MK-886 abolished both the vasodilator and vasoconstrictor responses to PAF.

The responses of the recipient hearts following the various pretreatments are summarized in Figure 3. Pretreating only the recipient heart with L-649,923 significantly attenuated ($P < 0.01$) but did not completely abolish the vasoconstrictor response of the recipient heart to the effluent from the first heart after an administration of 100 pmol PAF. The remaining vasoconstrictor component was abolished by pretreating the first heart with indomethacin and the recipient heart with L-649,923 ($P < 0.05$ when compared to the group pretreated with L-649,923 alone). Pretreatment of both hearts with L-649,923 or the first heart with MK-886 also abolished the vasoconstrictor effect of the effluent from the first heart on the recipient heart (Figure 3).

The coronary vascular effects of the effluent from hearts given leukotriene infusions

The effect of a 5 min infusion of LTC_4 or LTD_4 (300 pmol min^{-1}) on the perfusion pressure of the first and

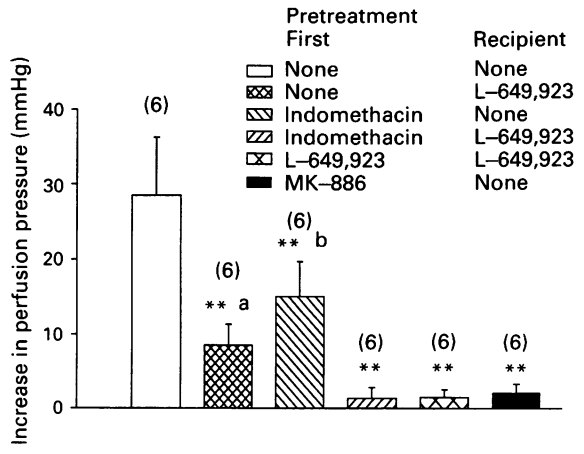


Figure 3 The response of the recipient heart to the effluent from the first heart: the effect of various pretreatments to the first and recipient hearts. The concentrations of L-649,923, indomethacin and MK-886 were 5.0, 2.8 and 10 μM. Columns represent mean with s.d. shown by vertical bars. Numbers in parentheses indicate the number of experiments. $^{**}P < 0.01$ when compared to the response of recipient hearts with no pretreatment. $^{a}P < 0.05$ when compared to the responses of the last four groups. $^{b}P < 0.01$ when compared to the responses of the last three groups.

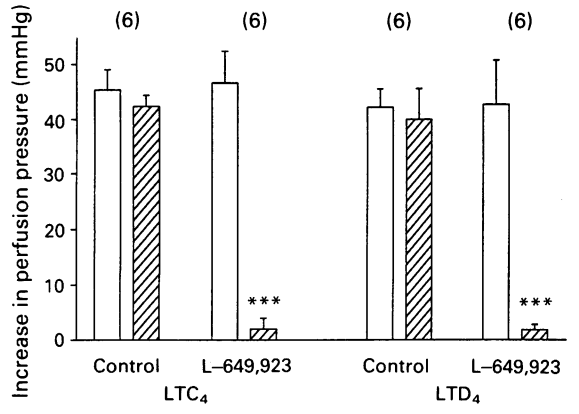


Figure 4 The effects of leukotriene C_4 (LTC_4) and LTD_4 infusion on the perfusion pressure of the first (open columns) and recipient (hatched columns) hearts. LTC_4 and LTD_4 infusion rates were 300 pmol min^{-1} and the amount of L-649,923 used for pretreatment of the recipient heart was 5.0 μM. Columns represent mean with s.d. shown by vertical bars. Numbers in parentheses indicate the number of experiments. ***$P < 0.001$ when compared to the response of the corresponding values with no L-649,923 pretreatment.

recipient hearts is summarized in Figure 4. With no pretreatment, LTC_4 and LTD_4 infusion resulted in a prominent increase in perfusion pressure in the first and recipient hearts and the increases were similar (Figure 4). With pretreatment of only the recipient heart with L-649,923 (5 μM), the effect of the effluent from hearts given LTC_4 or LTD_4 infusion on the perfusion pressure of the recipient heart was completely blocked ($P < 0.001$). Infusion of LTB_4 (300 or 600 pmol min^{-1}) resulted in vasodilatation in the first heart (-5.7 ± 1.2, $n = 12$ and -10.7 ± 1.9 mmHg, $n = 12$ respectively). However, only a small vasodilatation was detected in the recipient heart when perfused with the effluent from hearts with these LTB_4 infusions (-2.2 ± 0.9, $n = 6$ and -4.3 ± 2.4 mmHg, $n = 6$ for 300 and 600 pmol min^{-1} LTB_4 infusions, respectively). Pretreatment of the recipient heart with L-649,923 also did not result in any significant change in the response to effluent from hearts given LTB_4 in infusions (-1.5 ± 0.8, $n = 6$ and -1.2 ± 1.5 mmHg, $n = 6$ for 300 and 600 pmol LTB_4 infusions, respectively).

Discussion

In the cascade perfusion model, the recipient heart was pretreated with a bolus injection of PAF (100 pmol). After this treatment, the recipient heart loses the ability to respond to the direct effects of a further exposure to PAF (Piper & Stewart, 1986; 1987; Man *et al.*, 1990). This enabled us to determine the properties of PAF-released vasoactive substances in the effluent of the first heart without the possible interference of the effect of PAF being presence in the effluent.

Our results confirmed the existence of vasoactive substances in the effluent of the rat perfused heart after an administration of PAF. This is consistent with the concept that PAF itself does not directly initiate the coronary vascular effects but mediates its effects through the release of other vasoactive substances (Piper & Stewart, 1986; 1987; Hu *et al.*, 1991). Our results showed that one PAF-induced vasoconstrictor component in the effluent was mediated by leukotrienes (blocked by the leukotriene receptor antagonist in the recipient heart or the leukotriene synthesis inhibitor in the first heart) while another component was mediated by cyclo-oxygenase products (blocked by the cyclo-oxygenase inhibitor pretreatment in the first heart). Use of the cascade model enabled us to study the interplay of the various vasoactive substances in mediating the coronary vascular effects of PAF.

There was an interaction between the two major arachidonic acid products, prostaglandins and leukotrienes in the perfused heart. This conclusion is based on the observation that the indomethacin-sensitive vasoactive component could also be blocked by pretreatment of the first heart with a leukotriene receptor antagonist or leukotriene synthesis inhibitor. These results demonstrate for the first time that cyclo-oxygenase products may be regulated by leukotrienes in the rat perfused heart and that this effect is receptor-mediated. Although it is more common to observe regulation of 5-lipoxygenase products by cyclo-oxygenase products, there have been a number of studies that demonstrate leukotrienes directly regulating the synthesis and/or release of cyclo-oxygenase products in other systems. Busija & Leffler (1986) found that leukotrienes were able to increase the levels of prostaglandins ($PGF_{2\alpha}$ and PGE_2) in the cerebral cortex of new born pigs. A dose-dependent increased production of PGI_2 from human lymphatics by LTC_4 and LTD_4 has also been described and each type of leukotriene tested (LTC_4, LTD_4 and LTE_4) has a similar effect on prostaglandin synthesis (Sinzinger *et al.*, 1986). In addition it was suggested that the synthesis and release of 6-keto-$PGF_{1\alpha}$ and thromboxane A_2 in guinea-pig lung by LTD_4 and LTE_4 was receptor-mediated (Mong *et al.*, 1986).

It has been reported in several studies that MK-886 is a potent inhibitor of leukotriene biosynthesis *in vivo* (Gillard *et al.*, 1989; Miller *et al.*, 1990; Rouzer *et al.*, 1990) and MK-886 has no direct effect on 5-lipoxygenase activity (Rouzer *et al.*, 1990) or cyclo-oxygenase activity (Gillard *et al.*, 1989). The ability of MK-886 to inhibit leukotriene biosynthesis is mediated via binding to the 5-lipoxygenase activating protein resulting in the prevention of translocation of 5-lipoxygenase from the cytosol to the active membrane location (Ford-Hutchinson, 1991). Our results have shown that pretreatment of the first heart with MK-886 completely abolished the PAF-induced vasoconstrictor effect of the effluent in the recipient heart. In contrast, L-649,923 pretreatment of the first heart would still require pretreatment of the recipient heart with L-649,923 since PAF-induced leukotriene production would not be affected. Although a direct inhibition of cyclo-oxygenase by L-649,923 cannot be ruled out in the present study, the evidence from using both L-649,923 and MK-886 strongly supports the concept that the synthesis and/or release of cyclo-oxygenase products are leukotriene receptor-mediated in the coronary circulation of the rat perfused heart as concluded in the previous section.

Piper & Stewart (1986, 1987) showed that indomethacin attenuated the increase in perfusion pressure in rat and guinea-pig isolated hearts following bolus injections of PAF. It had also been reported that indomethacin blocked the coronary vasoconstriction produced by intracoronary injection of PAF in the pig heart *in vivo* (Feuerstein *et al.*, 1984). However, our previous study (Hu *et al.*, 1991) and current data showed that pretreatment of the heart with indomethacin did not affect the coronary vascular effect of PAF in the same heart. Thus cyclo-oxygenase products are unlikely to participate in the coronary vascular response under our experimental conditions, although results obtained from the cascade perfusion model indicate the presence of cyclo-oxygenase products based on the response of recipient hearts to effluents from hearts with or without pretreatment with indomethacin. One possible explanation is that the cyclo-oxygenase products are released from the venous side of the coronary circulation and therefore cause vasoconstriction only in the recipient heart.

Leukotrienes and prostaglandins are extensively metabolized. However, it is clear that a sufficient amount of these vasoactive arachidonic acid products was present in the coronary effluent after an administration of PAF to initiate a vasoconstrictor response in the recipient heart comparable to that in the first heart. In contrast, in spite of the presence of a vasodilator response in the first heart, no noticeable vasodilatation was observed in the recipient heart. This suggests that LTB_4, proposed as the mediator of the vasodilator response in the first heart (Hu *et al.*, 1991), is unstable in coronary effluent and was therefore present in insufficient quantity to produce vasodilatation in the recipient heart.

In summary, the present study used a cascade perfusion model to investigate the characteristics and the interaction of PAF-released vasoactive substances. Our results suggest the following. Upon the administration of PAF in the rat perfused heart, leukotrienes (LTB_4, LTC_4 and LTD_4) are released. While LTB_4 may be responsible for the observed vasodilator effect of PAF, LTC_4 and LTD_4 are responsible for the vasoconstrictor effect of PAF (Hu *et al.*, 1991). In addition to directly mediating part of the coronary vascular effects of PAF, leukotrienes also modulate the synthesis and release of cyclo-oxygenase products via a receptor-mediated mechanism. Both lipoxygenase and cyclo-oxygenase products are found in the effluent after the administration of PAF in the rat perfused heart and contribute to the coronary vascular effects of the effluent observed in the recipient heart. However, exogenously applied leukotrienes cannot mimic the endogenously produced leukotrienes released by PAF in regulating the synthesis of cyclo-oxygenase products. This suggests that the PAF-induced production of prostaglandins may be mediated by intracellular leukotrienes or at sites not accessible to exogenous leukotrienes. Indeed it has been demonstrated that leukotrienes, in particular LTC_4, may serve as intracellular mediator of somatostatin-induced increase of neuronal M-current (Schweitzer *et al.*, 1990) and regulation of prostaglandin synthesis by leukotrienes. Hence, further work regarding the cellular mechanism(s) for the interaction between these PAF-released vasoactive substances and the elucidation of the site where this interaction occurs in the coronary vasculature seems warranted.

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Effects of vasoactive intestinal polypeptide antagonists on cholinergic neurotransmission in dog and cat trachea

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1 The effects of vasoactive intestinal polypeptide (VIP) antagonists [AC-Tyr[1], D-Phe[2]]-GRF(1-29)-NH_2 and [4-Cl-D-Phe[6], Leu[17]]-VIP on excitatory neuroeffector transmission in the dog and cat trachea were investigated by use of microelectrode, double sucrose-gap and tension recording methods.

2 In the dog trachea, repetitive stimuli at high frequency (20 Hz) markedly enhanced the amplitude of contraction, the amplitude of contractions evoked by 50 stimuli at 20 Hz relative to that evoked by 5 stimuli being 14.2 ± 3.8 times ($n = 7$, ± s.d.). In the cat, the summation was much less marked, the amplitude of contractions evoked by 50 stimuli relative to that evoked by 5 stimuli being only 2.1 ± 0.6 times ($n = 5$, ± s.d.). Neither VIP antagonist had any effect on the relationship between the number of stimuli at 20 Hz and the relative amplitude of contraction in the dog trachea, but did enhance the amplitude of contractions to 1.1–1.5 times control in the cat trachea.

3 VIP antagonists dose-dependently enhanced the amplitude of excitatory junction potentials (e.j.ps) evoked by a single stimulus in the cat trachea, without changing the resting membrane potential or input membrane resistance of the smooth muscle cells. However, neither antagonist had any effect on the amplitude of the e.j.p. in the dog trachea.

4 Neither VIP antagonist had any effect on the post-junctional response of smooth muscle cells to exogenously applied acetylcholine (ACh; 10^{-9}–10^{-5} M) in the dog or cat trachea.

5 In the cat trachea, VIP (10^{-11} M) suppressed the e.j.p. amplitude to 0.74 ± 0.09 times the control value ($n = 6$). However, after pretreatment of the tissue with the VIP antagonists [Ac-Tyr[1], D-Phe[2]]-GRF(1-29)-NH_2 (10^{-8} M) and [4-Cl-D-Phe[6], Leu[17]]-VIP (10^{-8} M), VIP (10^{-11} M) did not suppress the e.j.p. amplitude, indicating that VIP antagonists block the presynaptic inhibitory action of exogenous VIP.

6 In parallel with the enhancement of contraction, e.j.ps showed marked summation when repetitive field stimulations were applied at high frequency (20 Hz) in the dog trachea. The relationship between the relative amplitude of the e.j.p. and number of stimuli at 20 Hz was linear and the slope was 2.2 ± 0.3 mV/stimulation. VIP antagonists did not affect this relationship. However, in the cat trachea, summation of e.j.ps was not at all marked and a linear relationship was not observed with the double sucrose-gap method. Incubation of the cat tracheal tissue with either of the VIP antagonists (10^{-8} or 10^{-7} M) markedly enhanced the summation of e.j.ps evoked by repetitive field stimulation at 20 Hz, and after the treatment a linear relationship between the number of stimuli and the amplitude of e.j.ps was observed, the slopes being 0.6 ± 0.1 ($n = 8$) and 0.55 ± 0.1 mV/stimulation ($n = 5$), respectively.

7 These results indicate that both VIP antagonists, [Ac-Tyr[1], D-Phe[2]]-GRF(1-29)-NH_2 and [4-Cl-D-Phe[6], Leu[17]]-VIP, have a prejunctional action accelerating the excitatory neuroeffector transmission, presumably by enhancing transmitter release from the vagus nerves in the cat, but not in the dog trachea.

Keywords: VIP; VIP-antagonists; trachea; cholinergic neurotransmission; modulation; excitatory junction potentials (e.j.ps)

Introduction

Recent investigations have revealed that the parasympathetic nervous supply to the airways contains non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitters such as vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) or peptide histidine methionine (PHM) in addition to acetylcholine (see, for example, Barnes, 1986). The presence of NANC inhibitory neurotransmitters in the nervous system was first demonstrated by relaxation of the airway smooth muscle produced in response to electrical field stimulation in the presence of cholinergic and adrenergic blocking agents in the guinea-pig (Coburn & Tomita, 1973), baboon (Middendorf & Russell, 1978), cat (Ito & Takeda, 1982) and human (Richardson & Be'land, 1976). Subsequently, immunofluorescence techniques demonstrated the presence of VIP-immunoreactive nerve fibres in human and animal airway smooth muscle layers including those of the dog and cat (Dey *et al.*, 1981; Polak & Bloom, 1982; Hakanson *et al.*, 1983). Further, VIP-immunoreactivity was localized to cholinergic nerves (Lundberg, 1981; Laitinen *et al.*, 1985a), and ultrastructural studies have demonstrated the presence of peptide containing granules within cholinergic nerves in the airway smooth muscle (Laitinen *et al.*, 1985b).

Hakoda & Ito (1990) reported that VIP, exogenously applied in low concentrations, has a prejunctional action which inhibits excitatory neuroeffector transmission in the dog and cat trachealis, and that commercially available VIP antiserum or the VIP antagonists [Ac-Tyr[1], D-Phe[2]]-GRF(1-29)-NH_2 and [4-Cl-D-Phe[6], Leu[17]]-VIP enhance the amplitude and summation of excitatory junction potentials (e.j.ps) in the trachea of the cat, but not of the dog. Further, overnight incubation of the cat tracheal tissue with VIP antiserum markedly reduced the amplitude of the muscle relaxation evoked by electrical field stimulation during contraction induced by 5-hydroxytryptamine in the presence of atropine (10^{-5} M) and guanethidine (10^{-6} M). These observations indicate that in the cat trachea, VIP may contribute to NANC relaxation and that endogenous VIP also inhibits the release of ACh from the vagus nerve when a single stimulus or repetitive stimuli are applied (Hakoda & Ito, 1990).

In the dog, on the other hand, although the occurrence of VIP-immunoreactive nerves in the walls of bronchi, bronchioles and pulmonary vessels has been demonstrated (Dey *et al.*, 1981), it has been reported that NANC relaxations are not demonstrable in tracheal or bronchiolar tissues (Ito & Tajima,

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1981; Inoue & Ito, 1986). In previous work, the actions of VIP-antagonists on dog tracheal tissue were not investigated systematically. To study the possible role of endogenous VIP on excitatory neuro-effector transmission in the airways, we have performed comparative studies on the actions of the VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP on excitatory neuro-effector transmission in dog and cat trachea.

Methods

Adult mongrel dogs and cats of either sex, weighing 10–13 kg and 2–4 kg respectively were anaesthetized with i.v. administration of pentobarbitone (30 mg kg^{-1}). Segments of cervical trachea were excised, and a dorsal strip of transversely running smooth muscle was separated from the cartilage. The mucosa and adventitial areolar tissue were carefully removed. The tracheal smooth muscle was cut into a strip 2.0–2.5 mm wide and about 15 mm long for the double sucrose gap method. The preparation was bathed in a modified Krebs solution with the following ionic composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was bubbled with 97% O_2 and 3% CO_2 and the pH was 7.3–7.4. The double-sucrose gap method was used to record the membrane potential and tension development in the tissue. The chamber used has been described elsewhere (Ito & Tajima, 1981). To produce neurogenic responses, electrical field stimulation was applied via a ring electrode placed in the centre pool of the apparatus using an electronic stimulator (Nihon Kohden SEN-7103). Single and repetitive stimulation was applied with current pulses of 50 μs duration and about 10–20 V intensity. The voltage of the current pulse was adjusted so that an e.j.p. of a certain fixed amplitude was evoked by a single pulse. Drugs were dissolved in Krebs solution and applied to the tissue through the centre pool of the double sucrose-gap apparatus with a multi-way tap (dead-time approximately 30 s).

For intracellular recording of the membrane potential from single cells, thin strips of tissue 10–15 mm long, 4–5 mm wide and 0.3–0.4 mm thick were used. A conventional microelectrode filled with 3 M KCl (30–50 MΩ) was inserted from the outer surface of the preparation. Field stimulation was applied to the nerves through a pair of Ag-AgCl wires 3–5 mm apart, placed so that a current pulse would pass transversely across the tissue. Single and repetitive stimuli at 20 Hz were applied, with a pulse of 30–100 μs duration and 30–50 V intensity, using an electronic stimulator (Nihon Kohden SEN-7103). The chamber in which the strips were mounted had a volume of 2 ml, and was superfused at a rate of 3 ml min^{-1} at a temperature of 35–36°C. To avoid recording artifacts due to twitch-like contractions of the muscle tissue, the preparation was firmly fixed onto the rubber plate in the chamber with insect pins of 100 μm diameter.

For the measurement of the mechanical changes, the tissue was mounted in a 1 ml organ bath through which the test solution flowed continuously at a rate of 3 ml min^{-1} at a temperature of 35–36°C. The preparation was placed vertically and one end of each strip was tied to a mechanotransducer (Nihon-Kohden Ltd., RCA-5734) and the other end to a hook at the bottom of the bath with fine silk thread. The strips were set up with an initial tension of 0.1–0.2 g and mechanical activity was recorded with a pen recorder.

The following drugs were used: indomethacin and acetylcholine hydrochloride (Sigma), guanethidine (Tokyo kasei), vasoactive intestinal polypeptide (VIP) (Peptide Institute, Osaka, Japan), VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP (Cosmo Bio. Co. Ltd., Tokyo) and atropine sulphate (Daiichi).

Results (amplitude of contractions or e.j.ps) are expressed as mean ± s.d. and were analyzed for statistical significance with Student's t test.

Results

Effects of VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP on contraction of dog and cat trachea evoked by electrical field stimulation

The effects of VIP antagonists on the contractions evoked by electrical field stimulation of cholinergic nerve fibres were studied in dog and cat tracheal tissues. The dog and cat airway smooth muscle cells are innervated by both cholinergic and adrenergic nerves (Russell, 1980), and noradrenaline released from sympathetic nerves can activate prejunctional β-adrenoceptors to inhibit cholinergic neurotransmission. In the dog trachea, the contraction evoked by nerve stimulation decreased progressively in amplitude, because endogenous prostaglandin E compounds inhibit cholinergic transmitter release from the vagus nerves (Ito, 1990). Therefore, the experiments on the dog trachea were carried out in the combined presence of guanethidine (10^{-6} M) and indomethacin (10^{-5} M), but those on the cat trachea in the presence of guanethidine (10^{-6} M) alone.

Figure 1 shows the effects of the VIP antagonist [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 on the amplitude of contractions evoked by repetitive field stimulation (5, 10, 20, 30 and 50 stimuli at 20 Hz) in trachea of the dog (a and a') and cat (b and b'). One of most striking differences between the dog and cat trachea was that the contraction was monophasic in the dog (Ito & Tajima, 1981) but biphasic in the cat trachea (Ito & Takeda, 1982). There was also a pronounced difference in the summation of the amplitude of contraction. Namely, in the dog trachea, repetitive stimuli at high frequency (20 Hz) markedly enhanced the amplitude of the contraction, the amplitude of contraction evoked by 50 stimuli at 20 Hz being 14.2 ± 3.8 times (n = 7, range 10–16 times) that evoked by 5 stimuli. On the other hand, in the cat trachea, repetitive stimuli at 20 Hz enhanced the amplitude of the twitch contraction only slightly, the amplitude of contraction evoked by 50 stimuli at 20 Hz being 2.1 ± 0.5 times (n = 5, range 1.7–2.5 times) that evoked by 5 stimuli.

A VIP antagonist, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (10^{-7} M), changed neither the resting tone of the muscle preparation nor the amplitude of twitch contraction evoked by repetitive stimuli in the dog trachea (Figure 1a and a'). On the other hand, in the cat trachea, the same antagonist (> 10^{-8} M) significantly increased the amplitude of contractions evoked by repetitive stimuli at 20 Hz (Figure 1b and b'). Similar results were obtained with the other VIP antagonist [4-Cl-D-Phe^6,Leu^{17}]-VIP (> 10^{-7} M): this too significantly increased the amplitude of contraction in the trachea of the cat (Figure 1e and f), but not of the dog (Figure 1c and d).

Effects of VIP antagonists on the amplitude of excitatory junction potentials (e.j.ps)

The mechanisms involved in the enhancement by VIP antagonists of twitch contractions in the cat trachea were studied and the effects were compared with those in the dog trachea by use of microelectrode and double sucrose gap-methods. A single field stimulation evoked an e.j.p. and the VIP antagonist, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (10^{-9}–10^{-7} M) produced no effects on either the e.j.ps or the resting membrane potential of the smooth muscle cells measured by the microelectrode in the dog trachea. The other VIP antagonist [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-9} M) also produced no effects on the amplitude of e.j.ps. However, at increased concentration (10^{-7} M), this antagonist suppressed the e.j.p. amplitude to 0.81 ± 0.05 times control (n = 5, P < 0.01) without changing the resting membrane potential in the dog trachea (Figure 2a and b).

In the cat trachea, both VIP antagonists enhanced the amplitude of e.j.ps. For example, 10^{-10} M [Ac-Tyr^1, D-Phe^2]-

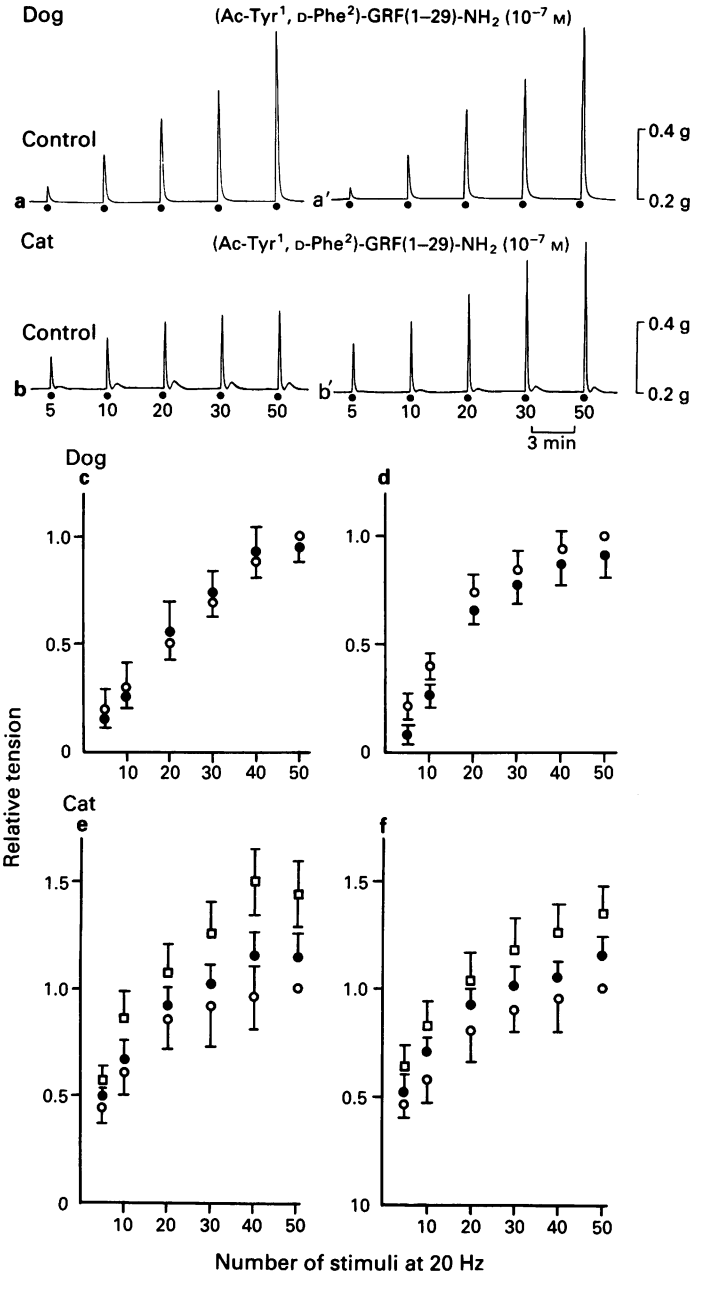


Figure 1 Effects of the vasoactive intestinal polypeptide (VIP) antagonist [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 on twitch contractions of dog (a and a') and cat (b and b') trachea evoked by field stimulation (5, 10, 20, 30 and 50 stimuli at 20 Hz). Effects of VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (c and e) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (d and f) on the relationship between the number of stimuli (5–50 at 20 Hz) and relative amplitude of twitch contractions in the dog and cat trachea. In (c–f): (○) control; [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 10^{-7} M (●) in (c), 10^{-9} M (●) and 10^{-8} M (□) in (e); [4-Cl-D-Phe^6, Leu^{17}]-VIP 10^{-7} M (●) in (d), 10^{-8} M (●) and 10^{-7} M (□) in (f). The amplitude of twitch contractions evoked by 50 stimuli at 20 Hz in normal Krebs solution was given the relative value 1.0.

GRF(1-29)-NH_2 increased the e.j.p. amplitude to 1.21 ± 0.06 times control ($n = 6$, $P < 0.01$), and 10^{-8} M [4-Cl-D-Phe^6, Leu^{17}]-VIP enhanced the e.j.p. amplitude to 1.35 ± 0.15 times control ($n = 7$, $P < 0.01$) without affecting the resting membrane potential or the input membrane resistance. At higher concentrations (10^{-8} M and 10^{-7} M respectively), both agents reduced input membrane resistance in both tissues (Figure 2a, b and c, d). Figure 2c and d summarizes the effects of both VIP antagonists on the relative amplitude of the e.j.ps and input membrane resistance and on the resting membrane potential of smooth muscle cells in the cat trachea.

Effects of VIP antagonists on acetylcholine-induced contractions

As low concentrations of VIP antagonists increased the amplitude of both the e.j.ps and contractions evoked by field stimulation with no change in the membrane potential or input membrane resistance in the cat trachea, the effects of VIP-antagonists were observed on the sensitivity of smooth muscle cells of the cat tracheal tissue to acetylcholine (ACh). For this purpose, the tension development induced by various concentrations of ACh (10^{-9}–10^{-5} M) was examined in the presence of one or the other VIP antagonist. The relationship between tension development and concentration of ACh produced a sigmoidal curve for both the dog and the cat trachea, neither of which was affected by [Ac-Tyr^1, D-Phe^2]-GRF(1-29)- NH_2 or by [4-Cl-D-Phe^6, Leu^{17}]-VIP at 10^{-8} M. Figure 3a and b shows the effects of the VIP antagonists on the relationship between tension development and concentration of ACh in the cat trachea. Similar experiments were performed with 10^{-8} M [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 in the dog trachea, and again the relationship between tension development and the concentration of ACh was not affected (data not shown).

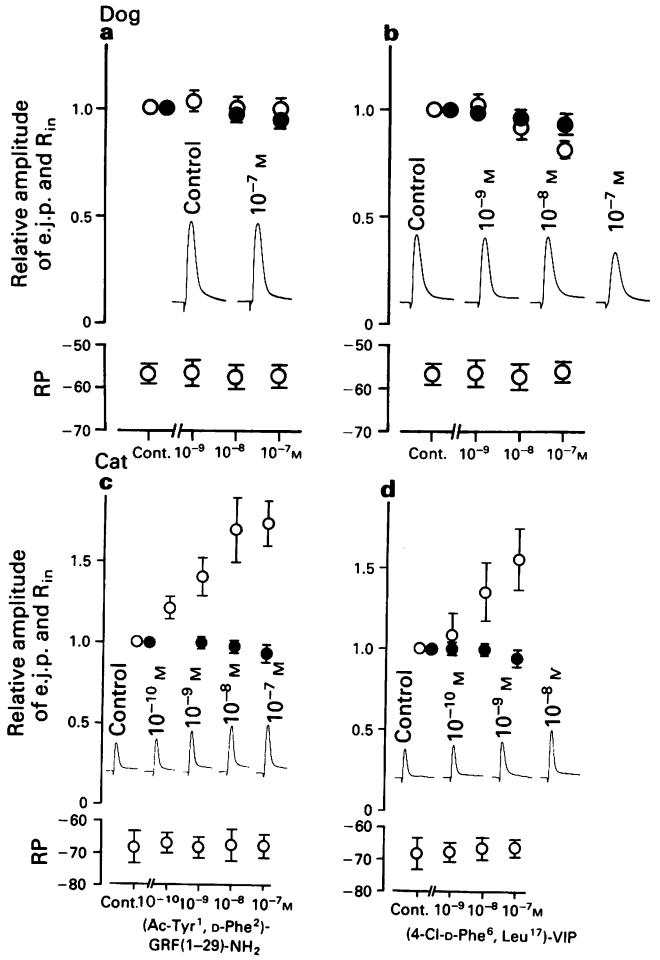


Figure 2 Relationships between the concentration of vasoactive intestinal polypeptide (VIP) antagonist and relative amplitude of e.j.p. (○), relative value of input membrane resistance (R_{in}, ●) and resting membrane potential (RP, ○) of the smooth muscle cells of a dog (a and b) and cat trachea (c and d). The amplitude of e.j.p. evoked by single field stimulation in normal Krebs solution, and the amplitude of electrotonic potentials produced by rectangular pulses in normal Krebs solution were given the relative value 1.0. Each point is the mean value derived from 5–30 experiments, and vertical bars indicate 2 × s.d. Microelectrode recording of resting membrane potential was used, and e.j.p. and electrotonic potentials were recorded by the double sucrose-gap method.

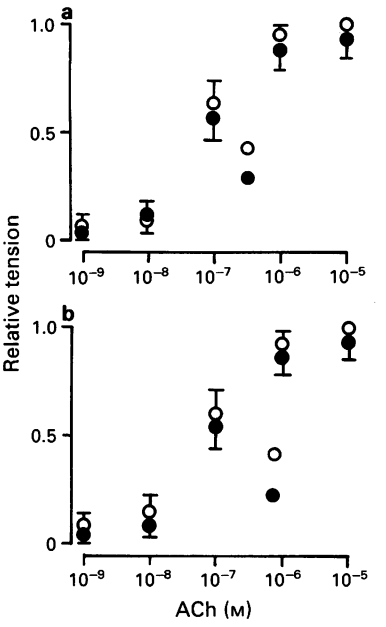


Figure 3 Effects of [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (a) and [4-Cl- D-Phe^6, Leu^{17}]-VIP (b) on dose-response curves (acetylcholine (ACh) against tension development) of the cat trachea. Ordinate scale: relative tension development where the amplitude of contracture evoked by ACh 10^{-5} M was given the relative value 1.0. Abscissa scale: dose of ACh. Points indicate mean values with bars showing 2 × s.d. of 4–6 preparations. Data are control (○) and after treatment of the tissue with VIP-antagonists (10^{-8} M for both) (●). Absolute values of tension development induced by various concentration of ACh in the presence or absence of VIP antagonists were between 0.1 and 1.5 g.

Effects of VIP antagonists on the action of exogenous VIP on the e.j.p. amplitude

Low concentrations of VIP antagonists increased the e.j.p. amplitude with no change in the membrane potential and post-junctional response of smooth muscle cells to exogenous ACh. VIP, exogenously applied in low concentrations, has a prejunctional action, inhibiting excitatory neuroeffector transmission in the cat trachea (Hakoda & Ito, 1990). Therefore we observed the effects of VIP antagonists on the action of exogenous VIP on the e.j.p. amplitude. In the cat trachea, VIP (10^{-11} M) suppressed the e.j.p. amplitude to 0.74 ± 0.09 times the control when a single stimulus was applied with no change in the membrane potential and the input membrane resistance (data not shown). However, after the pretreatment of the tissue with the VIP antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (10^{-8} M) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (10^{-8} M), VIP (10^{-11} M) did not change the e.j.p. amplitude (to 1.02 ± 0.04 (n = 4) or to 1.01 ± 0.03 (n = 4) times control, respectively).

Effects of VIP antagonists on the e.j.ps evoked by repetitive stimulation

In parallel with the summation observed in the contractions of the dog and cat trachea, e.j.ps also showed summation when repetitive stimulation at high frequency (>10 Hz) was applied to either tissue. The summation of e.j.ps was more prominent in the dog than in the cat. Figure 4a and b shows an example of e.j.ps and contractions evoked by repetitive stimuli at 20 Hz and recorded from dog and cat tracheal tissues. In the dog trachea, the summation of e.j.ps was striking, the relative amplitude of e.j.ps evoked by 10 stimuli at 20 Hz being 21.5 ± 5.0 times the control evoked by a single stimulus (n = 4), and the relationship between the relative amplitude of e.j.ps and the number of stimulations was linear. Accordingly, the amplitude of contraction recorded simultaneously also showed marked summation (Figure 4a). In the cat trachea, on the other hand, repetitive stimuli at 20 Hz enhanced the amplitude of e.j.ps only slightly so that the e.j.p. amplitude evoked by 10 stimuli at 20 Hz was only 2.1 ± 3.0 times (n = 4) that evoked by a single stimulus, and a linear relationship between the number of stimuli at 20 Hz and the amplitude of e.j.ps was not observed.

The effects of VIP antagonists were therefore investigated on the relationship between the number of stimuli at 20 Hz and e.j.p. amplitude in dog and cat trachea. It has been reported that overnight incubation of cat tracheal tissue with [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 markedly enhances the amplitude of e.j.ps evoked by repetitive field stimulation (Hakoda & Ito, 1990). Therefore, dog tracheal tissues were treated with one or the other antagonist overnight and tracheal tissue treated with Krebs solution overnight was used for the control experiments. In the dog trachea, a linear relationship between the number of stimuli and the amplitude of the e.j.p. could be seen when several stimuli at 20 Hz were applied under control conditions (Ito & Tajima, 1981; Ito & Yoshitomi, 1988; Hakoda & Ito, 1990). Overnight treatment with either of the VIP antagonists (10^{-7} M), produced no effect on the relationship between the e.j.ps and number of stimuli at 20 Hz (Figure 4g and h). Thus, the slopes of the linear relationship between the number of stimuli and the amplitude of the e.j.ps were 2.1 ± 0.5 mV/stimulation (n = 4) and 2.2 ± 0.4 mV/stimulation respectively, after treatment with [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (10^{-7} M) or with [4-Cl-D- Phe^6, Leu^{17}]-VIP (10^{-7} M),

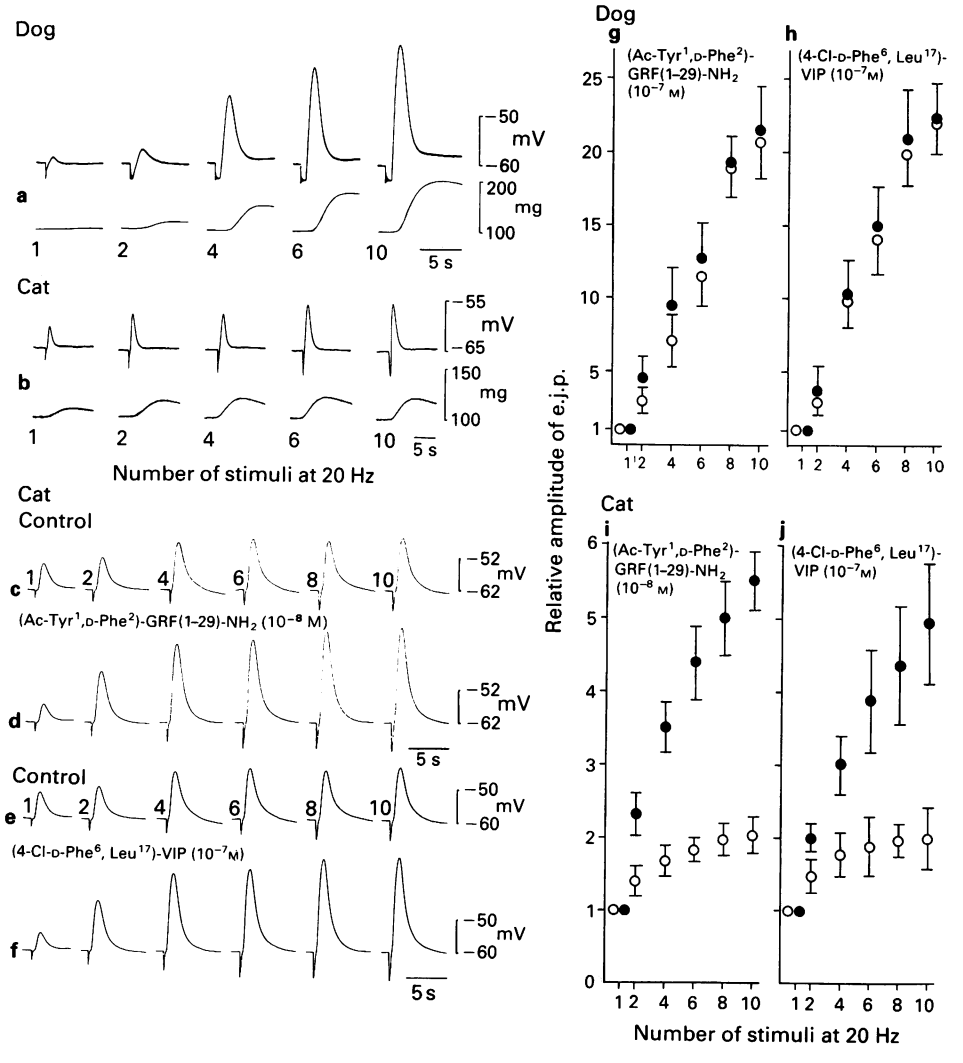


Figure 4 Effects of repetitive stimulation at high frequency (20 Hz) on the amplitude of e.j.ps and contractions of dog (a) and cat (b) tracheal tissues. (c–f) Effects of repetitive stimulation at high frequency (20 Hz) on the amplitude of e.j.ps of cat trachea in the absence (c and e) and presence of [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (d) or [4-Cl-D-Phe^6, Leu^{17}]-VIP (f). The double sucrose-gap method was used to record e.j.ps. (g–j) Relationships between the amplitude of the e.j.p. and the number of stimuli at 20 Hz recorded from dog (g and h) and cat (i and j) in the presence (●) or absence (○) of the VIP antagonists. Each point is the mean value of 5–8 experiments, and the vertical bars indicate 2 × s.d. Absolute values of e.j.p. amplitude were between 2 and 40 mV.

the control value being 2.2 ± 0.3 (n = 5). In the cat trachea, however, treatment of the tissues with the VIP antagonists for more than 2–3 h markedly enhanced the amplitude of e.j.p. evoked by repetitive field stimulation (Figure 4c–f). After treatment of the tissue with [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (10^{-8} M) or [4-Cl-D- Phe^6, Leu^{17}]-VIP (10^{-7} M), a linear relationship was observed, the slopes being respectively 0.6 ± 0.1 mV/stimulation (n = 8) and 0.55 ± 0.1 mV/stimulation (n = 5), indicating that the summation was enhanced by the VIP antagonists in the cat trachea (Figure 4i and j).

Discussion

The present experiments show that the VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, act prejunctionally to enhance excitatory neuro-effector transmission presumably by enhancing ACh release from the vagus nerve in the cat trachea, since a low concentration of either antagonist significantly enhanced the amplitude of the e.j.p. and the contractions evoked by nerve stimulation without affecting the resting membrane potential, input membrane resistance or ACh-sensitivity of the smooth muscle cells. These results confirmed previous observations (Hakoda & Ito, 1990). However, in the dog trachea neither antagonist produced any effect on the amplitude of the e.j.p. or the contractions evoked by field stimulation at concentrations ranging from 10^{-9} to 10^{-8} M. It has been reported that low concentrations of exogenous VIP (10^{-11}–10^{-9} M) act prejunctionally to inhibit ACh release from the vagus nerves in the dog, cat and ferret trachea (Sekizawa *et al.*, 1988). Furthermore in the present experiments, VIP antagonists blocked the prejunctional action of exogenous VIP to suppress the e.j.p. amplitude. Therefore, it seems reasonable to assume that VIP antagonists act on the VIP receptor located at prejunctional sites, and block the inhibitory action of endogenous VIP on transmitter release in the cat trachea. The inhibitory action of endogenously released VIP on ACh release from the vagus nerve terminal would explain the less prominent summation of e.j.ps and contractions in the cat trachea than in the dog trachea. In the dog trachea, the prominent summation of e.j.ps and contraction in the presence of indomethacin, the lack of effect of VIP antagonists or of VIP antiserum (Hakoda & Ito, 1990) on excitatory neuroeffector transmission even though the cholinergic nerve fibres are also immunoreactive to VIP (Dey *et al.*, 1981) suggests that endogenous VIP may not be released in response to nerve stimulation. This view was supported by the observations that, while dog tracheo-bronchial tissues do not show NANC-induced relaxation, exogenously applied VIP

relaxed airway smooth muscle when the muscle tone was elevated with 5-hydroxytryptamine (the same authors unpublished observations). It might be that the electrical field stimulation used in the experiments was not sufficient to release the peptide from the vagal nerve terminals. It is known that classical neurotransmitters such as ACh or noradrenaline (NA) are released at low stimulus frequencies (< 10 Hz), and peptides at higher stimulus frequencies (> 20 Hz) (Bartfai *et al.*, 1988). However, VIP antagonists enhanced the e.j.p. amplitude when a single field stimulation was used to evoke e.j.p. and a single field stimulation induced NANC relaxation in the cat trachea (the same authors unpublished observations). These observations may indicate that peptides are also released at low stimulus frequencies in the cat trachea. The reason for the lack of NANC relaxation in the dog airway needs to be clarified by further experiments.

The VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, which had been found to have a competitive action against VIP in intestinal and pancreatic cells (Waelbroeck *et al.*, 1985; Pandol *et al.*, 1986), showed no effect on the NANC-induced relaxation in the guinea-pig and cat trachea (Ellis & Farmer, 1989; Hakoda & Ito, 1990). Furthermore, Ellis & Farmer (1989) reported that these agents were without effect on the responsiveness of the guinea-pig trachea to exogenous VIP or PHI. Therefore, the VIP receptors located on the cholinergic nerve terminals may differ from those on airway smooth muscles, on the assumption that a part of NANC relaxation of the airway smooth muscles, at least, is mediated by VIP (Barnes, 1986).

At present, it is generally accepted that adrenergic neuroeffector transmission has two fundamental features, i.e., the coexistence and co-release of several transmitter substances along with NA and the negative feedback control on the NA release generally referred to as α_2-adrenoceptor autoinhibition (see, for example, Stjärne, 1989). However, it seems that these are also essential features of cholinergic neuroeffector transmission in the autonomic nervous system, since in the vagus nerve terminal, for example, peptides, such as VIP, PHI or PHM depending on species, are co-released and ACh release is modulated in many ways. For example, prejunctional inhibition of the release of ACh from the vagal nerve terminals has been noted with ACh, NA, prostaglandins and VIP, whereas mediators such as histamine, 5-hydroxytryptamine and substance P enhance the release of ACh by prejunctional mechanisms (Ito, 1990). Airway smooth muscle tissues receive a rich cholinergic innervation (Richardson, 1979), and ACh is one of the most potent agents causing bronchoconstriction. This being the case, investigations on modulatory mechanisms involved in the release of ACh from the vagus nerve terminal would seem to be particularly important.

In the cat trachea, VIP and ACh co-exist in the same nerves (Lundberg, 1981; Dey *et al.*, 1981), and VIP may be released as a co-transmitter (Barnes, 1986). VIP, when released, directly relaxes the airway smooth muscle without changing the electrical membrane properties of the smooth muscle cells (Ito & Takeda, 1982), and probably inhibits the release of ACh from the nerve, thus playing a 'double-braking' role on bronchoconstriction. On the other hand, in the dog airway smooth muscle, it has been reported that e.j.ps and twitch contractions elicited by nerve stimulation show a gradual and continuous reduction in amplitude during perfusion with normal physiological solution (Ito & Tajima, 1981; Walters *et al.*, 1984; Inoue & Ito, 1986). The prostaglandin synthesis inhibitor, indomethacin, and the prostaglandin antagonist, SC-19220, suppress the decline of the e.j.ps and twitch contractions evoked by nerve stimulation. During the action of indomethacin or SC-19220, no change in the membrane potential, membrane input resistance or sensitivity to ACh of the smooth muscle was observed, indicating that these chemicals are acting on the cholinergic nerve terminals in the muscle tissue. When the amplitude of e.j.ps or twitch contractions evoked by field stimulation were stabilized in the presence of indomethacin, prostaglandin E series in very low concentrations (10^{-12}–10^{-11} M) reduced the amplitude of twitch contractions and e.j.ps. Furthermore, the amount of prostaglandin E released into the perfusate was increased by field stimulation to 3–4 times that observed in the absence of stimulation. This increase would explain the decremental responses of the e.j.ps and twitch contractions (Inoue *et al.*, 1984; Ito, 1991). Furthermore, subcutaneous injection of indomethacin has been shown to induce wheezing or coughing in the dog (Ito & Tajima, 1981). These observations strongly indicate that inhibitory mechanisms mediated by endogenous prostaglandins on ACh release from the nerve terminals of the vagus play important roles in the regulation of the motility of tracheal and bronchial muscle in the dog. However, it appears that the mechanisms involved in the modulation of ACh release from the vagus nerve terminals are different in different species.

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Differential effects of phosphoramidon on neurokinin A- and substance P-induced airflow obstruction and airway microvascular leakage in guinea-pig

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1 The effects of the inhaled neuropeptides, neurokinin A (NKA) and substance P (SP) on lung resistance (R_L) and airway microvascular permeability were studied in anaesthetized guinea-pigs.

2 Single doses of inhaled NKA (3×10^{-5}, 1×10^{-4}, 3×10^{-4} M; 45 breaths) and SP (1×10^{-4}, 3×10^{-4}, 1×10^{-3}; 45 breaths) caused a dose-dependent increase in both R_L and airway microvascular leakage, assessed as extravasation of the albumin marker, Evans blue dye.

3 NKA at 1×10^{-4} and 3×10^{-4} M resulted in a significantly higher increase in R_L than SP at the same doses.

4 Inhaled SP (3×10^{-4} M; 45 breaths) caused significantly higher Evans blue dye extravasation in main bronchi and proximal intrapulmonary airways compared to the same dose of NKA.

5 Pretreatment with the specific inhibitor of neural endopeptidase (NEP24.11), phosphoramidon, caused an approximately 100 fold leftward shift of the R_L responses to inhaled NKA and SP.

6 Phosphoramidon significantly potentiated both NKA- and SP-induced airway microvascular leakage at proximal intrapulmonary airways, but not at any other airway level.

7 Inhibition of NEP24.11 potentiate both the SP- or NKA-induced airflow obstruction to a larger extent than the induced airway microvascular leakage, suggesting that NEP24.11 is more important in the modulation of the airflow obstruction observed after these mediators.

Keywords: Neurokinin A; substance P; neutral endopeptidase 24.11; airflow obstruction; airway microvascular leakage in guinea-pig

Introduction

Neurokinin A (NKA) and substance P (SP) are potent neuropeptides present in the airways of guinea-pig, and are found in close relation to the airway smooth muscle, beneath and within the epithelium, and around blood vessels in the airway submucosa (Nilsson *et al.*, 1977; Wharton *et al.*, 1979; Lundberg *et al.*, 1984; Hua *et al.*, 1985; Barnes, 1986; Saria *et al.*, 1988). These tachykinins can cause several pathological effects in the airways, including airway smooth muscle contraction, mucus secretion and increased microvascular permeability (Nilsson *et al.*, 1977; Andersson & Persson, 1977; Saria *et al.*, 1983; Hua *et al.*, 1984; Coles *et al.*, 1984; Rogers *et al.*, 1988; Lötvall *et al.*, 1990a). It has also been shown that inhaled NKA, but not SP, causes airway narrowing in asthmatic patients, but not in non-asthmatics (Joos *et al.*, 1986; Fuller *et al.*, 1987).

Neutral endopeptidase (NEP24.11), which may be an important enzyme in degrading tachykinins, is present in high concentrations in the airway epithelium, and in close relation to nerve fibres and smooth muscle (Johnson *et al.*, 1985). Recent work has shown that NEP24.11 is important in regulating *in vivo* responses to both exogenous and endogenous tachykinins (Thompson *et al.*, 1988; Shore *et al.*, 1988; Dusser *et al.*, 1988; Umeno *et al.*, 1989; Lötvall *et al.*, 1990b; Martins *et al.*, 1990). Thus, the bronchoconstrictor response to inhaled SP is shifted close to two log units to the left when NEP24.11 is inhibited (Lötvall *et al.*, 1990b), and the bronchoconstrictor response to endogenous tachykinins, released by electrical vagal stimulation, is potentiated in the presence of an NEP24.11-inhibitor (Dusser *et al.*, 1988). It has also been shown that airway microvascular leakage induced by endogenous tachykinins, is potentiated in rats pretreated with inhibitors of NEP24.11 (Umeno *et al.*, 1989). This study was performed to evaluate the importance of NEP24.11 in the regulation of airway narrowing and airway microvascular leakage induced by inhaled NKA, because both substance P and NKA can be released by airway sensory nerves (Saria *et al.*, 1988). NKA, given by the i.v. route, is a more potent airway smooth muscle constrictor than substance P, but has smaller effects on airway microvascular leakage (Hua *et al.*, 1984; Rogers *et al.*, 1988). We examined the responses in R_L and extravasation of plasma albumin induced by inhaled NKA in guinea-pigs treated with inhaled phosphoramidon, a specific inhibitor of NEP24.11. In addition, we compared these responses to those induced by inhaled SP.

Methods

Preparation

We studied specific pathogen-free Dunkin-Hartley guinea-pigs with a body weight of 400–550 g. On the day of study, the animals were anaesthetized with an initial dose of 6–8 ml kg^{-1} of urethane diluted to 25% w/v in 0.9% saline, injected intraperitoneally. Additional urethane was given as required to maintain an appropriate level of anaesthesia. A tracheal cannula (10 mm long and with 2.7 mm inner diameter) was inserted into the lumen of the upper cervical trachea through a tracheostomy, and tied snugly with suture material. A polyethylene catheter was inserted into the left carotid artery to monitor blood pressure and heart rate with a pressure transducer. The right external jugular vein was cannulated for the administration of drugs or fluids.

The guinea-pigs were placed in a supine position with the intratracheal cannula connected to a constant volume mechanical ventilator (Model 50-1718; Harvard Apparatus Ltd,

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Edenbridge, Kent, U.K.). A tidal volume of 10 ml kg^{-1} and a frequency of 56 breaths per min was used. Transpulmonary pressure was measured with a pressure transducer (Model FCO 40; $\pm$1000 mmH_2O, Furness Controls Ltd, Bexhill, E. Sussex), with one side attached to a catheter connected to a side port of the intratracheal cannula, and the other side attached to a catheter inserted into the right pleural cavity. The pleural catheter was carefully inserted during mechanical ventilation, thus avoiding effects of spontaneous breathing. Furthermore, the pleural catheter was air filled, and an additional volume of 0.5 ml of air was injected into the pleural cavity, to acquire a pleural signal. The ventilatory circuit had a total volume of 20 ml. Airflow was measured with a pneumotachograph (Model F1L; Mercury Electronics Ltd, Glasgow), connected to a transducer (Model FCO 40; $\pm$20 mmH_2O: Furness Controls Ltd). The signals from the transducers were digitalised with a 12-bit NB-MIO-16 analog-digital board connected to a Macintosh II computer (Apple Computer Inc., Cupertino, CA, U.S.A.) and analysed with a software (LabVIEW, National Instruments™, Austin, TX, U.S.A.), which was programmed to calculate expiratory lung resistance (R_L) instantaneously by the method of von Neergaard & Wirz (1927). Transpulmonary pressure and mean blood pressure were also monitored throughout the experiments. The aerosols were generated with an ultrasonic nebuliser (Model 2511; PulmoSonic, DeVilbiss Co, PA, U.S.A.), and were administered to the airways through a separate ventilatory system, that bypassed the pneumotachograph. The volume of this circuit was 50 ml. The output from the nebuliser at the port of the tracheal cannula, measured with an air flow of 0.3 l min^{-1} was 70 $\pm$ 5 μl min^{-1}. The particle size produced by the nebuliser was 3.8 μm, with a geometric standard deviation of 1.3, measured with a laser droplet and particle analyser (Model 2600C, Malvern Instruments, U.K.).

Protocols

The animals were divided into seven groups in order to study the effects of three different single concentrations of inhaled NKA (3×10^{-5}, 10^{-4}, 3×10^{-4} M), SP (10^{-4}, 3×10^{-4}, 10^{-3} M), or vehicle (saline; 0.9%), all given for 45 breaths, on lung resistance (R_L) and airway microvascular leakage in animals pretreated with inhaled saline (0.9%, 60 breaths). In another six groups we investigated the effects of inhaled phosphoramidon (0.1 mM, 60 breaths; 7.5 nmol) on three different doses of NKA (3×10^{-7}, 3×10^{-6}, 3×10^{-5} M), and SP (1×10^{-6}, 1×10^{-5}, 1×10^{-4} M). These different doses were chosen because preliminary studies showed marked potentiation of the R_L effects by phosphoramidon. Inhaled phosphoramidon at the same dose (7.5 nmol, inhaled) does not influence baseline R_L or baseline Evans blue dye measurements (Lötvall *et al.*, 1991).

All animals were pretreated with propranolol (1 mg kg^{-1}, i.v.) to inhibit adrenergic effects of the anaesthesia (Spriggs, 1965). Five minutes later, phosphoramidon or saline was administered. Ten minutes later, suxamethonium (0.5 mg, i.v.) was given to induce neuromuscular blockade, and after a further 5 min, baseline R_L was measured, and Evans blue dye injected slowly i.v. (20 mg kg^{-1} over 1 min). One minute later, the nebulsing circuit was opened for administration of inhaled SP, NKA or vehicle. R_L transpulmonary pressure and mean blood pressure were recorded every 15 s. Five minutes later, the animals were hyperinflated with twice the tidal volume by manually blocking the outflow of the ventilator. No hyperinflation was performed if the transpulmonary pressure was higher than 40 cmH_2O. One minute later, the chest was opened, and a cannula was inserted into the aorta through the left ventricle. The animals were perfused through this cannula with saline at a pressure of 100 mmHg, to eliminate intravascular dye. After 2 min of perfusion through this route, a polyethylene cannula was inserted into the pulmonary artery, and the pulmonary circulation was perfused with 15 ml of saline over another minute. The lungs were then removed, and the parenchyma carefully scraped off. The trachea, main bronchi, and intrapulmonary airways were separated from each other, and the intrapulmonary airways divided lengthwise into two equally long portions, arbitrarily named proximal and distal. All tissues were weighed wet, and Evans blue dye was extracted in 2 ml of formamide in a 40°C water bath for 24 h. Absorption at 620 nm was measured in a spectrophotometer (Model 8480, Philips, Cambridge). The extracted Evans blue was quantified by interpolation on a standard curve of dye concentrations in the range of 0.5–10 μg ml^{-1}, and expressed as ng dye mg^{-1} wet tissue. The Evans blue dye technique has previously been shown to correlate closely with the extravasation of radiolabelled albumin in guinea-pig airways (Rogers *et al.*, 1989).

Drugs

We used the following drugs: propranolol (Inderal, ICI, U.K.); urethane, phosphoramidon substance P and neurokinin A (Sigma Chemical Company, Poole, Dorset), suxamethonium (Duncan, Flockhart & Co. Ltd, Greenford).

Data analysis

Data are given as mean $\pm$ s.e.mean. Kruskal-Wallis test was used to determine significant differences among groups. If a significant difference was found, the Mann-Whitney U-test was performed to determine significance between individual groups. A P value less than 0.05 was considered significant. Data were analysed with a Macintosh computer (Apple Computer Inc., Cupertino, CA, U.S.A.) using standard statistical packages.

Results

Effects on lung resistance

There were no significant differences in baseline R_L between the different treatment groups (saline; 0.23 $\pm$ 0.02 cmH_2O/ml $\times$ s^{-1}: phosphoramidon; 0.25 $\pm$ 0.04 cmH_2O/ml $\times$ s^{-1}).

Peak R_L induced by different single doses of inhaled NKA or SP are shown in Figure 1. NKA caused a significantly higher increase in R_L compared to SP at 1×10^{-4} and 3×10^{-4} M ($P < 0.05$). Inhaled NKA was approximately 7–10 times more potent than inhaled SP, when approximate doses to require different levels of bronchoconstriction were compared (log-linear interpolation at 1–10 cmH_2O/ml $\times$ s^{-1}; Figure 1). Figure 1 also shows the effect of pretreatment with the NEP24.11-inhibitor phosphoramidon (0.1 mM, 60 breaths; 7.5 nmol). Phosphoramidon pretreatment shifted the bronchoconstrictor effects of both inhaled NKA and SP approximately 2–2.5 log units to the left. After phosphoramidon, inhaled NKA was still approximately 4–20 times more potent than SP, depending on which level of airflow obstruction was compared (log-linear interpolation at 1–10 cmH_2O/ml $\times$ s^{-1}; Figure 1).

Effects of plasma exudation

NKA caused a small but significant and dose-dependent increase in Evans blue dye extravasation in trachea and main bronchi in saline pretreated animals ($P < 0.05$), and at all airway levels after pretreatment with phosphoramidon (Figure 2; 0.1 mM, 60 breaths; 7.5 nmol, $P < 0.05$ for trachea and main bronchi, and 0.01 for proximal and distal intrapulmonary airways). SP caused a significant, dose-dependent, Evans blue dye extravasation at all airway levels, and significantly more than NKA at main bronchi and proximal intrapulmonary airways at 3×10^{-4} M (59.3 $\pm$ 4.5 and 21.4 $\pm$ 2.7 ng Evans blue dye mg^{-1} tissue at main bronchi for SP and NKA respectively, $P < 0.01$, and 21.1 $\pm$ 4.1 and 11.1 $\pm$ 2.0 ng Evans blue dye mg^{-1} tissue at proximal intrapulmonary airways for SP and NKA respectively, $P < 0.05$). Phosphoramidon caused

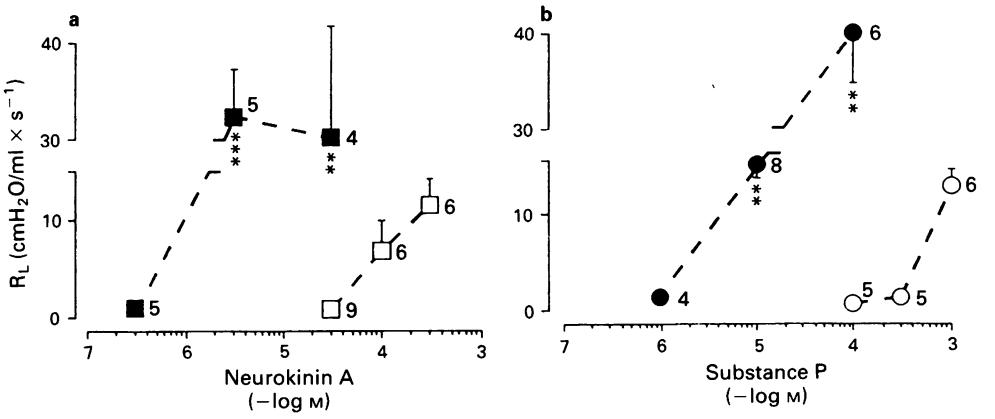


Figure 1 Peak R_L induced by single doses of inhaled neurokinin A (NKA, a; squares) or substance P (SP, b; circles), both given for 45 breaths. Pretreatment with inhaled phosphoramidon (Ph: 0.1 mM, 60 breaths; 7.5 nmol; filled symbols) caused a marked leftward shift of the response to both inhaled NKA and SP, amounting to approximately 2–2.5 log-units. Data are shown as mean for 4–9 animals as indicated in the figure; s.e.mean shown by vertical bars; $^{**}P < 0.01$ or $^{***}P < 0.005$ compared to the lowest dose of either mediator in animals pretreated with inhaled saline (0.9%; 60 breaths).

a small but significant potentiation of both NKA- and SP-induced Evans blue dye extravasation at both proximal and distal intrapulmonary airways (IPA) in the case of NKA, and at proximal IPA in the case of SP (Figure 2). After phosphoramidon-pretreatment and exposure to NKA (3×10^{-5} M), three out of four animals died before the end of the 6 min post challenge period, and these were perfused approximately 3 min earlier than the control animals. However, the Evans blue dye measurement at proximal IPA was still significantly higher in this group, compared to animals pretreated with saline (Figure 2, $P < 0.05$).

Discussion

In the present study we gave the tachykinins NKA and SP to guinea-pigs by the inhaled route, in order to evaluate the importance of enzymatic degradation by airway neutral endopeptidase (NEP24.11). Pretreatment with the NEP24.11-inhibitor phosphoramidon, also given by the inhaled route, caused a pronounced potentiation of the increase in lung resistance induced by inhaled NKA, as well as a small but significant potentiation of airway microvascular leakage in intrapulmonary airways. We found similar effects with inhaled SP, confirming a previous study showing potentiation of the bronchoconstrictor response to SP by another NEP24.11-inhibitor, acetorphan (Lötvall *et al.*, 1990b). In addition, we found that inhaled NKA was more potent than SP in increasing R_L whereas inhaled SP was more potent than NKA for induction of airway microvascular leakage, which is consistent with previous studies in which these tachykinins were administered by the i.v. route (Rogers *et al.*, 1988).

NKA and SP are generated from a large precursor preprotachykinin (Hua *et al.*, 1985). Exogenous NKA and SP have been shown to cause both airway smooth muscle contraction as well as increased airway microvascular permeability in guinea-pig (Saria *et al.*, 1983; Rogers *et al.*, 1988). Furthermore, these two tachykinins are released in approximately equimolar concentrations in the lungs when airway sensory nerves are stimulated either electrically or with capsaicin (Saria *et al.*, 1988).

It has been suggested that NKA and SP cause their effects by activating two distinct receptors, called neurokinin$_1$ (NK_1) and neurokinin$_2$ (NK_2) receptors (Iversen, 1985). SP is believed to be more potent in stimulating NK_1 receptors, and NKA more potent in stimulating NK_2 receptors (Iversen, 1985). A third receptor subtype, neurokinin$_3$ receptor (NK_3), is more specific for neurokinin B (NKB), but this peptide has not been found to be present in the airways of guinea pig (Hua *et al.*, 1985). The NK_1 receptor is believed to be predominant in the vasculature, causing increased microvascular permeability and vasodilatation when activated, whereas the

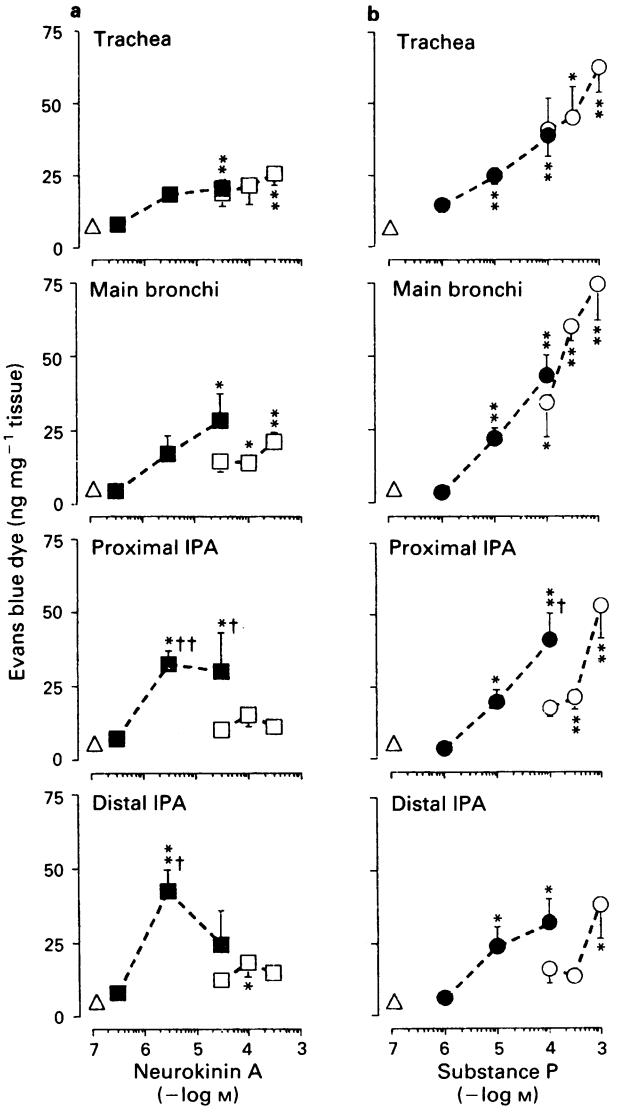


Figure 2 Extravasation of Evans blue dye induced by inhaled neurokinin A (NKA, a, left panels; squares) or substance P (SP, b, right panels; circles) in saline pretreated animals or after pretreatment with inhaled phosphoramidon (0.1 mM, 60 breaths: 7.5 nmol; filled symbols). All responses are compared to sham stimulation (open triangle, $n = 7$). Data are shown as mean for 4–9 animals, vertical bars show s.e.mean (see Figure 1). $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to sham-stimulated animals. $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P < 0.01$ compared to the lowest dose of either mediator in animals pretreated with inhaled saline (0.9%; 60 breaths).

NK_2 receptor is present in higher concentration in airway smooth muscle, causing contraction when activated (Iversen, 1985). Our data, showing that NKA is relatively more potent in causing an increase in R_L than airway microvascular leakage, compared to inhaled SP, provides further evidence that such receptors may be important in the *in vivo* situation in guinea-pig.

Neutral endopeptidase (NEP24.11) is present in high concentrations in airway epithelium (Johnson *et al.*, 1985; Sekizawa *et al.*, 1987). In several studies the bronchoconstrictor response induced by inhaled SP has been shown to be potentiated by inhibition of this enzyme (Dusser *et al.*, 1988; Lötvall *et al.*, 1990b), and a leftward shift of a dose-response curve of up to 2 log units has been observed (Lötvall *et al.*, 1990a). In the present study we have shown that inhibition of NEP24.11 also leads to a potentiation of the bronchoconstrictor response to inhaled NKA to a similar degree. NKA- and SP-induced airway microvascular leakage was slightly potentiated in intrapulmonary airways in phosphoramidon pretreated animals, but not to the same degree as the R_L responses were. Therefore, the potentiation of tachykinin-induced airflow obstruction following NEP24.11-inhibition is likely to be due to an effect on airway smooth muscle contraction rather than airway microvascular leakage. Possibly, other enzymes in the vicinity of airway microvessels may be more important than epithelial NEP24.11 in modulating tachykinin-induced airway microvascular leakage.

We are confident that the increase in airway microvascular leakage seen with tachykinins is independent of severe bronchoconstriction, since inhaled methacholine causes large increases in lung resistance through airway smooth muscle contraction, without an effect on airway microvascular permeability (Lötvall *et al.*, 1990a).

It seems likely that NEP24.11 is also important in the modulation of tachykinin-effects in human airways. Recently, Joos and coworkers (1986) showed that inhaled NKA will produce bronchoconstriction in asthmatic subjects, but not in non-asthmatics. Since one of the major features of asthma is epithelial damage, it may be suggested that lack of airway epithelial NEP24.11 in the asthmatic group may have contributed to the hyperresponsiveness to NKA. This hypothesis has been supported by a recent study (Cheung *et al.*, 1991), showing that inhalation of an inhibitor of NEP24.11 (thiorphan) potentiated the bronchoconstriction induced by inhaled neurokinin A in non-asthmatics. However, it is not known whether NEP24.11 modulates tachykinin-induced airway microvascular leakage in man.

This study shows that inhaled NKA is more potent than SP in causing airflow obstruction in guinea-pig, supporting previous studies using i.v. administration of these agonists. Importantly, inhibition of NEP24.11 potentiates both the SP- or NKA-induced airflow obstruction to a larger extent than the induced airway microvascular leakage. Thus, NEP24.11, presumably present in airway epithelium, is important in the modulation of the airflow obstruction observed after both SP and NKA.

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Effects of porcine relaxin on contraction, membrane response and cyclic AMP content in rat myometrium in comparison with the effects of isoprenaline and forskolin

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1 The longitudinal muscle from the uterus of oestrogen-treated rats was quiescent in Mg-free Krebs solution. Electrical stimulation generated phasic contraction, which was depressed to 35% and 18% by 50 mu and 150 mu porcine relaxin, respectively.

2 The phasic contractions were more strongly depressed to 26% by 50 mu relaxin in solution containing 0.6 mM Mg, and the depression lasted for more than 4 h after the removal of relaxin. During the persisting depression, raising the external Ca to 7.5 mM did not restore the contraction, but the contraction was restored by removal of Mg.

3 The depression of the phasic contraction by relaxin, examined in Mg-free solution, was enhanced and reduced by pretreatment of the tissue with 0.6 mM Mg and 0.6 mM Mn, respectively, for about 15 min. In contrast, the depression of contraction by isoprenaline or forskolin was enhanced by pretreatment with either Mg or Mn.

4 The cellular content of cyclic AMP was measured in Krebs solution containing 0.6 mM Mg. The values were 1.24 (pmol mg^{-1} protein) in control solution, and 2.31 and 1.56 when the tissues were treated with 150 mu relaxin and 10^{-9} M isoprenaline, respectively.

5 The cyclic AMP production in response to 10^{-7} M forskolin measured in Mg-free solution was enhanced when the tissue was pretreated with either 0.6 mM Mg or Mn for 15 min. The cyclic AMP production in response to 100 mu relaxin was increased when the tissue was pretreated with 0.6 mM Mg, and was unchanged by pretreatment with Mn. The cyclic AMP production in response to 10^{-9} M isoprenaline was unchanged by pretreatment with the divalent cations.

6 The membrane potential of the muscle was −60.8 mV in Krebs solution containing 0.3 mM Mg, and electrical stimulation induced an action potential which consisted of spike and plateau components. Application of 150 mu relaxin reduced the duration of the plateau; the contractions were progressively depressed. The resting membrane potential and membrane resistance were unchanged by application of 150 mu relaxin. The membrane was hyperpolarized by 2.8 mV, accompanied by a decrease in membrane resistance, when 10^{-9} M isoprenaline was applied.

7 Although there were several differences between the effects of relaxin and isoprenaline, it is probable that some process, which is cyclic AMP-dependent, accelerated by Mg and depressed by Mn, is involved in the depressant action of relaxin on contraction.

Keywords: Relaxin; isoprenaline; forskolin; Mg; Mn; cyclic AMP; myometrium

Introduction

Relaxin, a peptide hormone produced mainly in the ovary, has several target tissues, including the myometrium on which it exerts a relaxant effect (rat: Sawyer *et al.*, 1953; Bradshaw *et al.*, 1981; mouse: Wiqvist, 1959; man: Szlachter *et al.*, 1980; guinea-pig: Porter, 1972; mini-pig: Porter & Watts, 1986). It was recently reported that porcine relaxin specifically depressed the plateau component of the action potential in the myometrium of the pregnant and oestrogen-treated rat, thereby inhibiting the contraction (Chamley & Parkington, 1984). It was further shown that the cellular adenosine 3′,5′-cyclic monophosphate (cyclic AMP) was increased by relaxin in dispersed myometrial cells in culture, as with isoprenaline (Hsu *et al.*, 1985).

The relaxant effect presumably depends on the population of relaxin receptors in the surface membrane. Relaxin receptor population in rat myometrium was shown to change during pregnancy, its peak being attained on day 17 of pregnancy (Mercardo-Simmen *et al.*, 1980; 1982). In addition, the binding of relaxin to the membrane is accelerated when Mg, Ca or Mn is present in the incubation media (Mercardo-Simmen *et al.*, 1980).

The aim of the present experiments was to obtain further insight into relaxin action in comparison with β-adrenoceptor effects. For this purpose, the effects of porcine relaxin were examined mainly on isolated longitudinal muscle strips in oestrogen-primed rat uterus. The influence of Mg and Mn on the relaxant effects of relaxin was investigated, since these ions are known to modify the adenylate cyclase activity in cell-free myometrium (Krall *et al.*, 1988; Arkinstall & Jones, 1990). Secondly, the effects of relaxin, isoprenaline and forskolin on cellular cyclic AMP production were compared. The results indicated several differences between the effects of relaxin and β-adrenoceptor agonists, leading to the conclusion that some cyclic AMP-dependent process, which is accelerated by Mg and decelerated by Mn, is involved in the depressive action of relaxin on rat myometrial contraction.

Methods

Preparations

Wistar-strain female rats (200–250 g) were ovariectomized under pentobarbitone anaesthesia and about 2 weeks later were given a single subcutaneous injection of 50 μg oestradiol-17 β-benzoate dissolved in sesame oil. Ovariectomized rats are called 0E, and those injected with oestradiol and left for 24, 48 and 72 h are called 1E, 2E and 3E, respectively. Animals were mainly used 72 h after oestrogen treatment (3E). In other

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experiments, in which 17 day-pregnant rats were used, pro-oestrus rats were housed with males overnight and day 1 of pregnancy was taken as the day on which copulatory plugs were found in the vagina.

Animals were stunned and bled, and uterine horns removed. The uterus was cut open along the mesometrium, and the endometrium was removed carefully with jeweler's forceps. Longitudinal and circular muscle strips (0.5 mm wide, 5 mm long) were dissected from the ovarian portion of the uterus. Muscle strips were mounted vertically in an organ bath. One end was fixed and the other end was connected to a force transducer. The bathing solution (36.0 ± 0.5°C) flowed continuously at a rate of 8 ml min^{-1}, and the capacity of the organ bath was 0.2 ml. Platinum electrodes were placed near the ends of the muscle strip, and electric pulses, 1 s duration and supramaximal intensity, were applied. The preparation was equilibrated with warm control solution for 1–2 h before the experiment began.

For measurement of electrical activity, the muscle strip was mounted in a partition chamber (1 ml in volume) as described by Abe & Tomita (1968), and superfused at a rate of 5 ml min^{-1}. Electrical activity of the muscle cell was recorded intracellularly with glass microelectrodes filled with 3M KCl (30–50 MΩ in resistance). For the recording of electrotonic potentials, the microelectrode was placed 0.2 mm from the stimulating electrode. For simultaneous records of electrical and mechanical activity, one end of the muscle strip was tied with a thread and connected to a force transducer. Signals were amplified and recorded by an ink-writing pen recorder. Membrane responses were in some cases stored on a data recorder and reproduced at appropriate sweep speed and magnitude.

Measurements of contraction and membrane response are expressed as mean ± s.d.

Solutions and drugs

The control solution (Mg-free Krebs solution) had the following composition (mM): NaCl 123.1, $NaHCO_3$ 15.5, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, and glucose 11.5. It was equilibrated with a gas mixture of 95% O_2 + 5% CO_2 (pH of 7.4). A solution of $MgCl_2$ or $MnCl_2$ (both 1 M) was added to give the desired concentration. To prepare high Ca solution, KH_2PO_4 was omitted to prevent the precipitation of Ca, and $NaHCO_3$ was decreased to 12 mM, where 4.7 mM NaCl was added to balance the osmolarity.

Porcine relaxation (NIH-RXN-P1,3000 guinea-pig units 1 mg^{-1}) was a gift from National Hormone and Pituitary Program, U.S.A. It was dissolved in distilled water and stored at −40°C. Acryl and silicone wares were used at all times when relaxin was used. The concentration of relaxin is expressed in guinea-pig units. Isoprenaline-HCl and forskolin were from Nikken and Sigma, respectively. Stock solution of forskolin was prepared by dissolving it in dimethylsulphoxide (DMSO), so that the final concentration of DMSO in the Krebs solution was at most 0.1%. Molar concentrations of these agents are given. Drugs were added to the perfusion solution and took 30 s and 3 min to reach the tissue in the contraction and microelectrode organ baths, respectively.

Cyclic AMP measurement

The blood attached to the surface of the uterus was removed with a cotton bud prior to preparing muscle strips. In one series of experiments (Table 1), longitudinal muscle strips (1–3 mg wet weight) were incubated in 0.6 mM Mg containing solution for 3 h and were treated with 10^{-9} M isoprenaline or 150 mu relaxin.

In the divalent cation pretreatment experiments (Figure 11), longitudinal muscle strips were incubated in Mg-free Krebs solution for 2 h, and were exposed to 0.6 mM Mg or 0.6 mM Mn for 15 min. Then muscle strips were washed with Mg-free Krebs solution for 45 min, and muscles were exposed to 10^{-9} M isoprenaline, 10^{-7} M forskolin and 100 mu relaxin for 5, 10 and 10 min, respectively, when cyclic AMP in the cells attained maximal value. At appropriate points in the above sequential procedures, muscle strips were harvested, and were immediately frozen with liquid nitrogen, then lyophilized for 2–3 h. The lyophilized muscles were homogenized with an agate mortar and pestle, after an aqueous solution containing 6% trichloroacetic acid (1.5 ml, 3°C) had been added to terminate the reactions in muscle cells. The homogenate was centrifuged at 2500 *g* for 15 min at 4°C, and the precipitate was used to determine protein content by the method of Lowry *et al.* (1951). The supernatant was washed 3 times with 5 volumes of water-saturated diethyl ether. The upper ether layer was discarded at each wash. Cyclic AMP content retained in the water layer was measured by a radioimmunoassay system (Amersham, UK), which allows high sensitivity without interference from ATP (Volker *et al.*, 1985).

Measurements are expressed as mean ± s.e.mean in Figure 11 and Table 1, and statistical significance of differences between means was tested by Student's *t* test.

Results

Hormonal influence on relaxin action

Effects of 150 mu relaxin on the contractile activity of rat myometria were first examined in Mg-free solution (Figure 1). Longitudinal muscle strips excised from ovariectomized rat (0E) exhibited frequent spontaneous contractions. On application of relaxin the baseline tension declined slightly, and the frequency and amplitude of spontaneous contraction decreased slightly. A similar observation was made on the 1E preparations. Spontaneous contraction was absent in the 3E preparations, but phasic contractions could be evoked by electrical stimuli. Relaxin strongly depressed the contraction and recovery from the depression was very slow.

Circular muscle strips of the 0E rat were spontaneously active, and relaxin caused slight depression of the phasic contraction. The frequency of spontaneous contractions was less in 1E preparations, and both the frequency and amplitude were slightly diminished when relaxin was applied. Spontaneous activity was absent in 3E preparations, and phasic contraction evoked by electrical stimulation was strongly depressed by relaxin. As with the longitudinal muscle, the recovery from the depressant effect was slow.

Results are summarized in Figure 1b, where the effects of relaxin are indicated by % response of phasic contraction during 8–10 min application of relaxin relative to the mean control amplitude of 5 successive phasic contractions which occurred prior to the application of relaxin. These results indicate that the inhibitory effect of relaxin increased as exposure time to oestrogen increased in both the longitudinal and circular muscle strips.

The effects of 150 mu relaxin on the longitudinal and circular muscle strips excised on day 17 of pregnancy were also examined (Figure 1a, bottom). The longitudinal muscle strips on day 17 of pregnancy were spontaneously active only for about 30 min after being placed in the organ bath, but phasic contractions could be elicited by electrical stimuli. Relaxin (150 mu) did not cause a significant effect on the amplitude of evoked contractions, though the contraction was partly depressed temporarily after relaxin was removed. Relaxin (150 mu) depressed the contraction to 97 ± 4% ($n = 10$) of the control value in Mg-free solution, and to 91 ± 13% ($n = 10$) in the solution containing 0.6 mM Mg. Phasic contractions were spontaneously generated in the circular muscle strips excised on day 17 of pregnancy, and relaxin caused slight depression. In all cases, the spontaneous contractions were strongly depressed for about 3 min after relaxin was removed. Relaxin (150 mu) depressed the contraction to 89 ± 9%

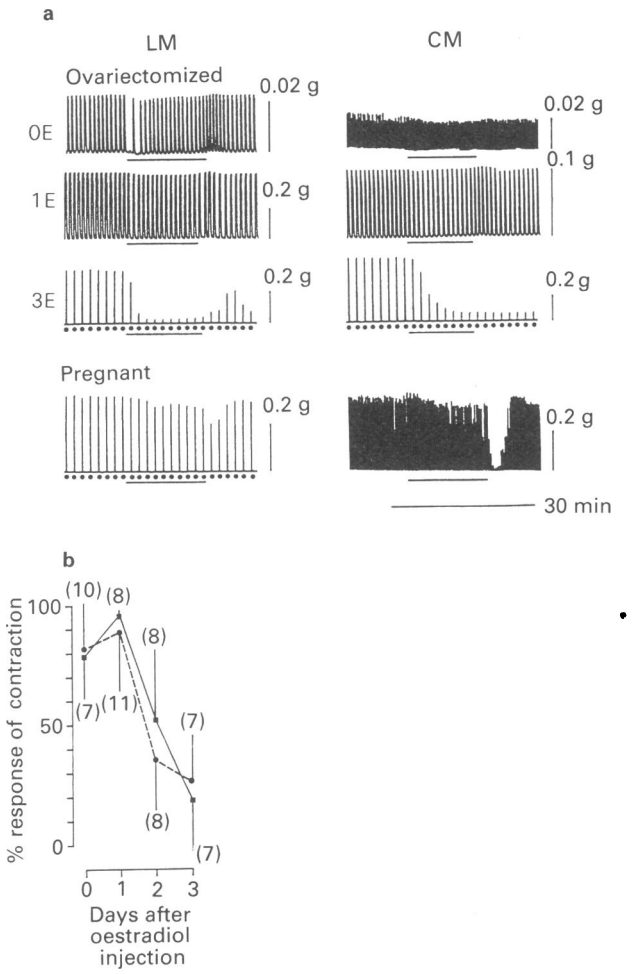


Figure 1 (a) Effects of porcine relaxin on the phasic contractions of uterine longitudinal (LM) and circular (CM) muscle strips, examined in Mg-free Krebs solution. Muscle strips were taken from ovariectomized rats at 0 (0E), 1 (1E) and 3 (3E) days after 50 μg oestradiol-17β injection, and on day 17 of pregnancy. Application of relaxin (150 mu) is indicated by bars. Dots indicate electrical stimulation of the muscle. (b) Shows the average inhibition of phasic contractions by relaxin (ordinate; mean $\pm$ s.d.). Figures in parentheses are the number of muscle strips used for measurements; (■——■) longitudinal muscle; (●---●) circular muscle.

($n = 18$) of the control value in Mg-free solution, and to 49 $\pm$ 24% ($n = 9$) in the solution containing 0.6 mM Mg, respectively.

For the sake of simplicity in analyzing the relaxin action, longitudinal muscle strips from rats treated with oestrogen for 3 days (3E) were used in the following experiments.

Effects of Mg and Ca on relaxin action

It has been reported that depressive effects of relaxin on myometrial contraction *in vitro* varied depending on the incubation period with artificial saline media (Wiqvist & Paul, 1955). The influence of incubation and Mg ion contained in the bathing solution were therefore examined. The muscle strips were freshly dissected, and 50 mu relaxin was applied intermittently during 10 h incubation. In the tissue shown in Figure 2, relaxin initially depressed the contractions in Mg-free solution, and the degree of contractile depression became weaker when relaxin was applied about every 2 h. Exchange of the bulk solution in the organ bath took 30 s. However, it took about 6 min to reach the maximum depression. Contractions recovered completely in 10 min on washing with normal solution. In the presence of 0.6 mM Mg (b), the amplitude of the phasic contractions was also depressed by the first application of 50 mu relaxin, and the recovery in the control solution was incomplete even after by 4 h washing. During the recovery, phasic contractions were again depressed by relaxin, and the minimum amplitude was nearly the same with repeated application of relaxin. The amplitude of phasic contractions was increased when the external Mg was removed, and again depressed when Mg was readmitted (bottom trace). Such a 'persistent' depression of contraction in the presence of Mg was observed in 10 other preparations. In the absence of relaxin, the phasic contraction remained nearly identical in size for 10 h in the solution containing 0.6 mM Mg.

Experiments similar to those shown in Figure 2 were carried out on different muscle strips taken from different animals, and representative results are illustrated graphically in Figure 3. From results from 12 preparations studied in Mg-free solution, responses can be classified into 3 types. In 6 preparations out of 12, designated type i, the depressive effect of relaxin was progressively reduced; in type ii, the degree of depression by relaxin was nearly identical throughout 9 h incubation; in type iii which was observed only once, the depression was initially weak, and became stronger as the incubation time increased. In solution containing 0.6 mM Mg (Figure 3b), the mean of 5 successive phase contractions before the first application of relaxin was taken as 100%. The 3 different patterns of response in terms of incubation time were also observed. Only in one preparation out of 11 muscle strips examined, was the depressive effect reduced after 9 h incubation (b(i)). In 10 preparations, the degree of contractile depression by relaxin was nearly steady after 3 h incubation (b(ii); b(iii)). The % contraction estimated at the second application of relaxin (3 h incubation) was 35 $\pm$ 16% ($n = 12$) in Mg-free solution, and 26 $\pm$ 18% ($n = 11$) in the solution containing 0.6 mM Mg. Comparison of Figures 3a and b suggests that external Mg is needed to keep the relaxation action stable *in vitro* for several hours.

An explanation of the persistent effect of relaxin is that a stabilizing action of Mg was enhanced by relaxin. The effects of raising the concentration of Ca on relaxin depression were examined in the absence and presence of Mg (Figure 4). The amplitude of evoked phasic contractions was enhanced when the external Ca concentration was raised to 7.5 mM (Figure 4a). About 7 min was needed to reach the maximal amplitude. The effect was the same when Ca was applied 30 min after application of 50 mu relaxin in the Mg-free solution. Mg, 0.6 mM, depressed the phasic contractions, but their amplitudes were restored by application of Ca, 7.5 mM (b). The contractions were strongly depressed in the presence of 0.6 mM Mg by applying 50 mu relaxin, and the persisting depression lasted for about 5 h. The amplitude of the depressed contractions was increased only slightly by 7.5 mM Ca during the initial 2 h after removal of relaxin. Thereafter contractions of large amplitude occurred on exposure to 7.5 mM Ca. It took about 10 min after each admission of 7.5 mM Ca to produce full-size phasic contractions. Removal of external Mg also restored the phasic contraction (bottom trace).

A question arising from the foregoing results is why the recovery was incomplete after the removal of relaxin when Mg was in the perfusing solution. Furthermore, is the responsiveness of muscles to relaxin changed during preparation (30 min) in Mg-free solution at room temperature (20–27°C)? Figure 5 demonstrates the effect of brief treatment with Mg and of cooling on the depression produced by 50 mu relaxin. The amplitude of contraction was depressed by the first application of relaxin (a). The muscle was treated with 0.6 mM Mg for 15 min, then the inhibition by relaxin was stronger. When the muscle was cooled down to 24°C for 40 min, the baseline tension rose and the phasic contraction became smaller (b). When the muscle was rewarmed to 36°C, the baseline tension declined, phasic contractions were restored, and the depressive effect by relaxin became very small. The muscle was again treated with Mg, the depressive effect of relaxin returned (c). From these results, it is suggested that pretreatment with Mg produced a residual effect on the muscle to cause stronger

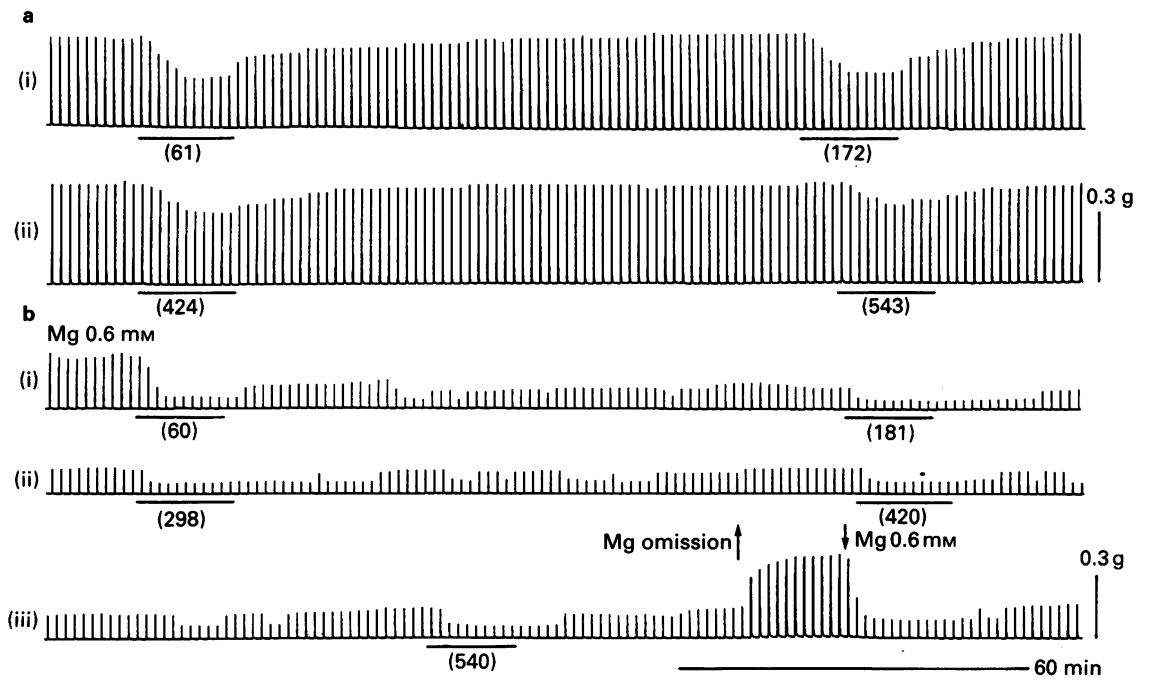


Figure 2 Effects of relaxin on the phasic contractions of uterine longitudinal muscle of oestrogen-treated rats (3E). The 2 muscle strips were excised from different animals; (a) responses to application of 50 mu relaxin (bars) examined in Mg-free solution. (b) Sequential responses examined in solution containing 0.6 mM Mg. Phasic contractions were evoked by supramaximal electrical stimulation at 90 s intervals. Figures in parentheses show the time in min after mounting the muscle strip in the warm organ bath.

contractile depression by relaxin, and the effect of Mg could be removed by low temperature.

Relaxin action on membrane responses

In order to correlate electrical and mechanical events, the membrane activity and phasic contraction were recorded simultaneously, and effects of relaxin were examined in Krebs solution containing 0.3 mM Mg. The preparations were quiescent, and the resting membrane potential was -60.8 ± 4.1 mV (mean $\pm$ s.d., $n = 41$). Activity was generated by electrical pulses which produced a large action potential with a plateau upon which spikes were superimposed (Figure 6). The duration of the action potential decreased gradually when 50 mu

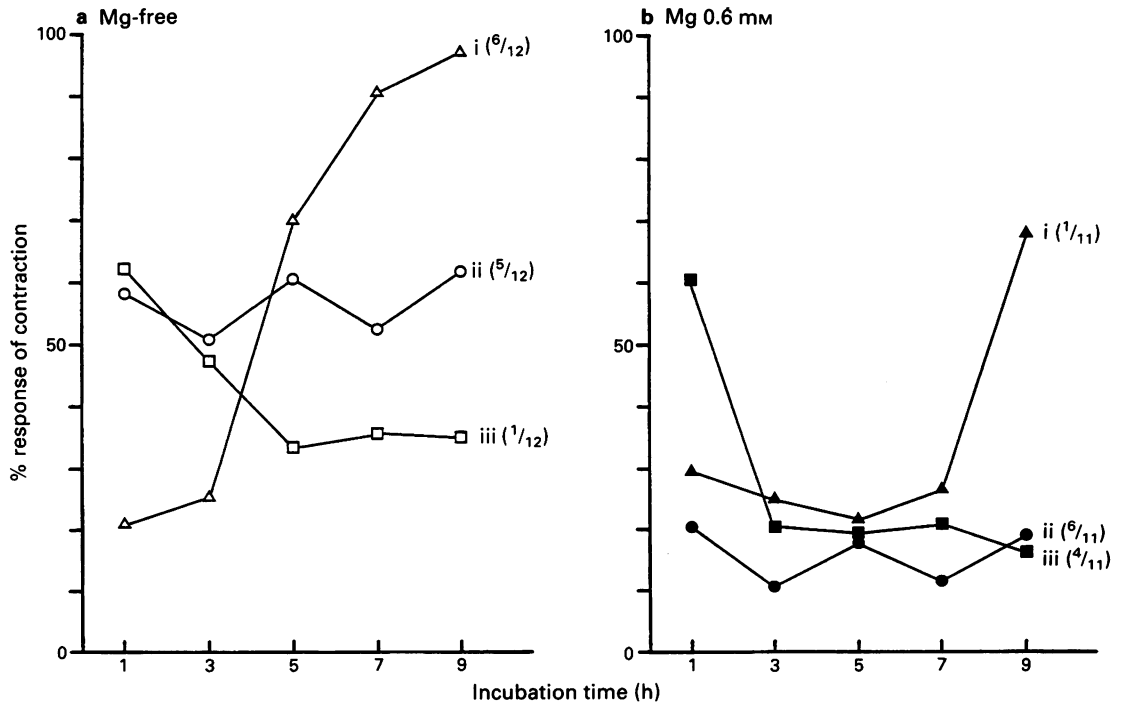


Figure 3 Changes with time of the inhibitory effects of relaxin on the phasic contractions in the absence (a) and presence (b) of 0.6 mM Mg. Longitudinal muscle strips freshly excised were incubated in warm solution for 1 h, and 50 mu relaxin was applied about every 2 h thereafter (abscissae). In (a) the minimum amplitude during relaxin application is expressed as a % of the phasic contraction immediately prior to the application; whereas in (b) all inhibitions are expressed relative to the control response after the initial 1 h incubation. In both (a) and (b), the responses are categorized into 3 types (i, ii, iii), and the occurrence in the total preparations examined are indicated in parentheses.

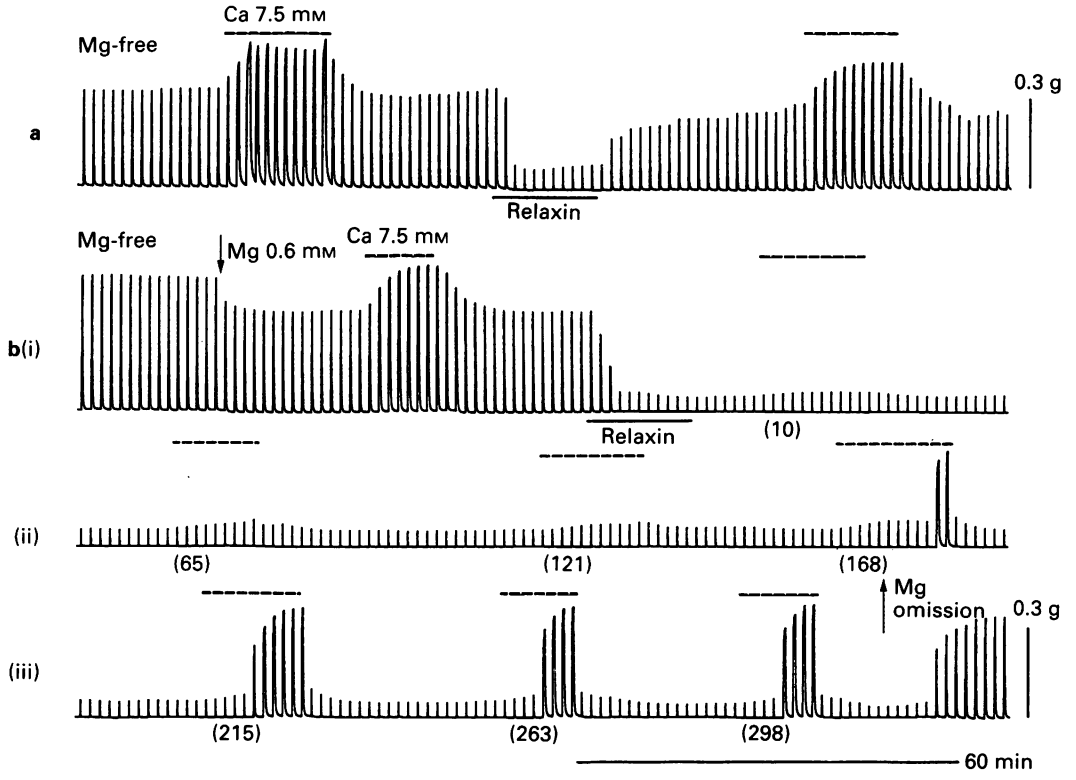


Figure 4 Effects of high Ca (7.5 mM) on the depression of contractions by relaxin, examined in the absence (a) and presence (b) of 0.6 mM Mg. Application of 7.5 mM Ca, 50 mu relaxin and 0.6 mM Mg are indicated by broken lines above, bars below and arrows, respectively. Successive records are shown in (b). Figures in parentheses indicate the time in min after removal of relaxin.

relaxin was applied, and accordingly the duration of the phasic contraction became shorter, then the amplitude became reduced. The depression of both membrane and contractile activities was still apparent 20 min after the removal of relaxin (Figure 6b). When Mg was omitted from the bathing solution, the action potential became prolonged, the contraction became larger, and spontaneous activity occurred. On re-exposure of the muscle to 0.3 mM Mg and 150 mu relaxin the action potentials were not much depressed, whereas the contractions gradually decreased. When relaxin was removed, the amplitude of contraction gradually increased, while the action potentials were not much altered (Figure 6d). The resting membrane potential remained unchanged throughout.

The effects of relaxin on membrane response were compared in the presence of 0.6 mM Mg with those of isoprenaline which also caused depression. The concentrations of the agents were chosen so that they depressed the phasic contraction to a similar degree (Table 1). In the cell illustrated in Figure 7, the membrane potential was -59 mV, and application of 10^{-9} M isoprenaline hyperpolarised the membrane by 3.5 mV (a), and reduced the amplitude of the electrotonic potential by 5% (ai, aii). From different muscle strips, the mean membrane hyperpolarization and the reduction of electrotonic potential by 10^{-9} M isoprenaline were 2.8 ± 0.3 mV ($n = 9$) and 4.2 ± 1.2%, respectively. The membrane potential and the electrotonic potential were not changed by 150 mu

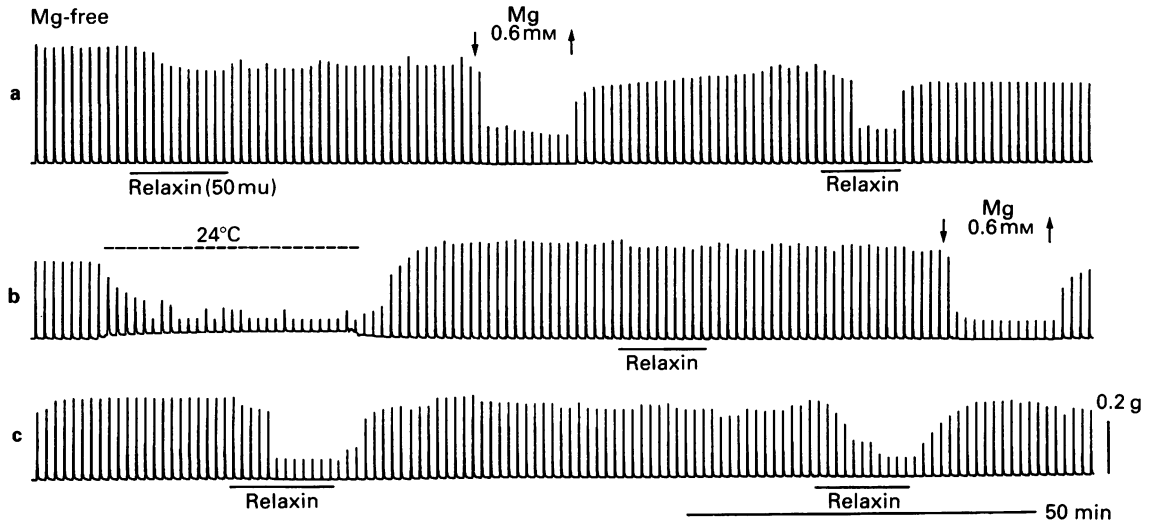


Figure 5 Effects of low temperature on the interaction between Mg and relaxin actions. The muscle was perfused with Mg-free Krebs solution (36°C), and 50 mu relaxin and 0.6 mM Mg were applied during the periods indicated by bars and arrows, respectively. The muscle was cooled to 24°C during the period indicated by the broken line. Successive records are shown.

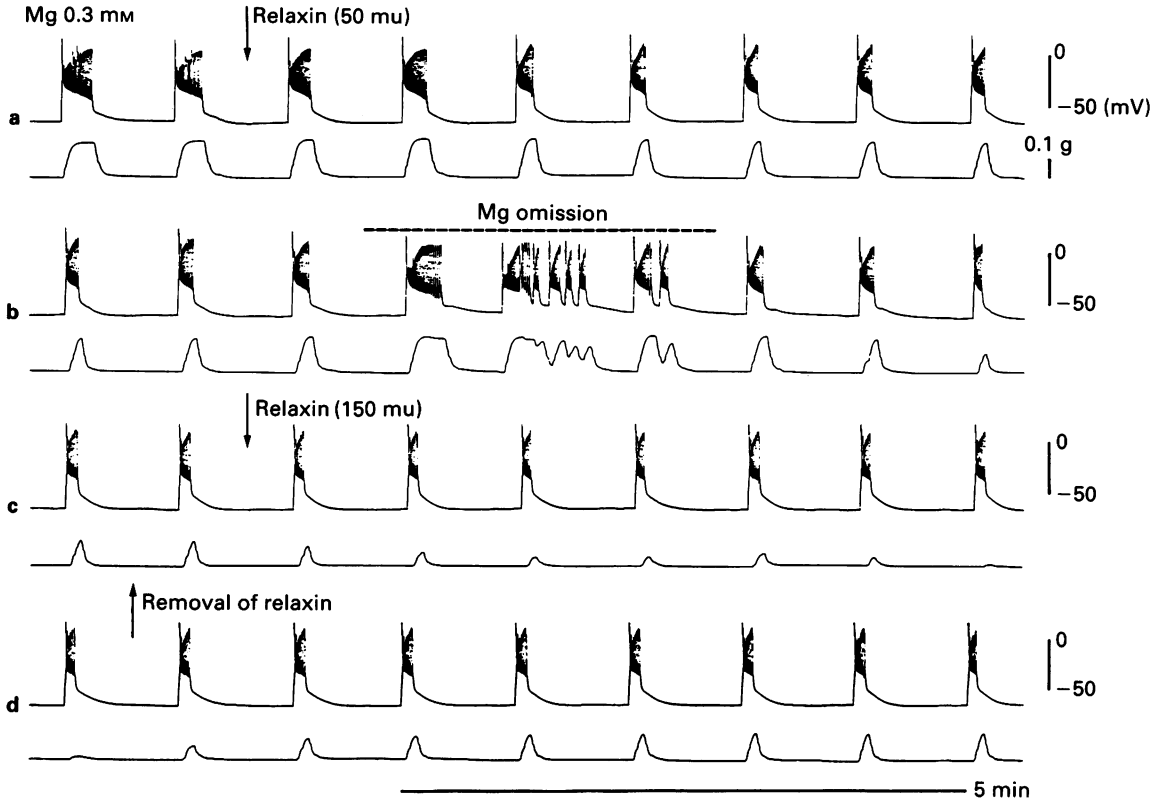


Figure 6 Effects of relaxin on the intracellularly recorded membrane activity (upper traces) and simultaneously recorded contraction (lower traces), examined in the presence of 0.3 mM Mg. The muscle was stimulated by electrical pulses at intervals of 1 min. All electrical records were obtained from the same cell. Record (b) shows the activities 20 min after relaxin was removed, and Mg was omitted during the period indicated by the broken line. Relaxin and Mg were applied or removed 3 min before the time indicated by signals, because of slower exchange of the solutions in the organ bath.

relaxin ($n = 7$). indicating that isoprenaline but not relaxin alters passive resting membrane properties.

Effects of Mn on relaxin action in comparison with β-adrenoceptor stimulation

It has been reported that in cell-free systems the increase in adenylate cyclase activity linked to β-adrenoceptor activation is facilitated by adding Mn (myometrium, Krall *et al.*, 1988; ocular ciliary process membrane, Mittag *et al.*, 1988). It has also been shown that relaxin increased the cellular content of cyclic AMP in myometrial cells in culture (Hsu *et al.*, 1985). The question arises as to whether or not the treatment of intact muscle with Mn affects the cyclic AMP-mediated response. In the experiment shown in Figure 8, the influence of Mn on the effects of isoprenaline and relaxin was studied. Application of 3×10^{-10} M isoprenaline and 50 mu relaxin in Mg-free solution depressed the phasic contractions to 39% and 47% of the control value, respectively. Contractions were strongly depressed by 0.6 mM Mn and recovered on removal of Mn. After this procedure, contractions were depressed by 3×10^{-10} M isoprenaline more strongly than before, whereas the depression by 50 mu relaxin became markedly reduced. The muscle was then exposed to 2 mM Mg for 1 h, and the evoked contractions were strongly depressed. Phasic contractions gradually developed when Mg was removed until the amplitude recovered almost to the previous control level. Contractions were then strongly depressed by 50 mu relaxin. Thus, as with Mg, Mn pretreatment caused a residual influence in the muscle so that the inhibitory effects of isoprenaline and relaxin were affected in opposite directions.

Forskolin is an agent which activates the catalytic unit of adenylate cyclase (Seamon *et al.*, 1981), and increases intracellular cyclic AMP concentration in uterine smooth muscle (Marshall & Fain, 1984). The inhibitory effect of forskolin on myometrium was shown to be enhanced when the muscle was pretreated by Mg (Maruta & Osa, 1989). The effects of Mn on the action of 10^{-7} M forskolin were therefore examined in

Table 1 Effects of relaxin (150 mu) and isoprenaline (10^{-9} M) on phasic contractions and tissue cyclic AMP content in the longitudinal muscle from oestrogen-primed rat uterus, examined in Krebs solution containing 0.6 mM Mg

	Control	*Relaxin*	*Isoprenaline*
Time s to reach the minimum contraction		368 ± 55* ($n = 8$)	172 ± 45 ($n = 8$)
Amplitude of contraction (% of control)		16 ± 8.3 ($n = 8$)	21 ± 8.7 ($n = 8$)
Cyclic AMP (pmol mg^{-1} protein)	1.24 ± 0.24 ($n = 9$)	2.31 ± 0.25* ($n = 9$)	1.56 ± 0.28* ($n = 8$)

The phasic contractions were generated every 60 s by electric pulses, and the time in s to reach the minimum value of phasic contraction was determined by the time after the exchange of bath solution. They were significantly different from each other. The minimum amplitude of contraction during exposure is expressed as a percentage of the mean of amplitude of 5 successive contractions immediately before the drug application. Cyclic AMP content was determined after 3 min and 6 min exposure to isoprenaline and relaxin, respectively and were significantly different from the control. Values are means ± s.d. n = number of preparations. Statistical analysis for the significance of differences between means was by Student's t test: * $P < 0.02$.

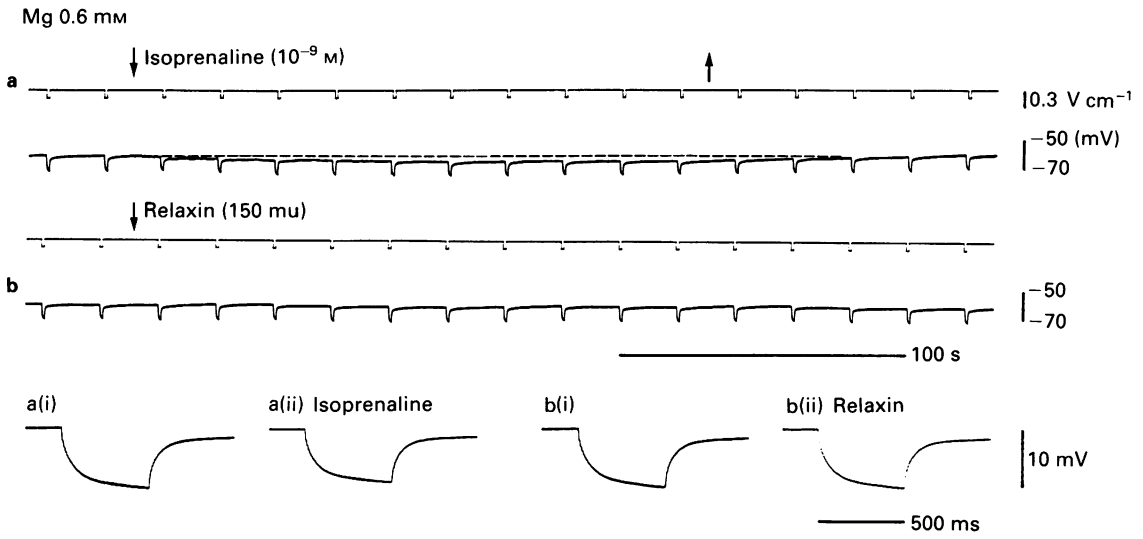


Figure 7 Effects of isoprenaline (a) and relaxin (b) on the membrane potential and membrane resistance examined in the presence of 0.6 mM Mg. Upper and lower traces show current monitor and membrane potential, respectively. Inward current pulses (500 ms) at constant intensity were applied at intervals of 20 s. Magnified electrotonic potentials with a faster sweep are reproduced in a(i) and a(ii) (10 s before and 35 s after the application of isoprenaline), and b(i) and b(ii) (15 s before and 120 s after the application of relaxin). The muscle was washed with Krebs solution for 20 min, after application of isoprenaline. All records were obtained from the same cell.

Mg-free Krebs solution. The phasic contractions were depressed only slightly by 0.1% DMSO, the concentration present in a 10^{-7} M solution of forskolin. Forskolin gradually depressed the phasic contractions, up to 8 min, when the maximal inhibition was reached (Figure 9). Following application of Mn (0.4 mM) for 15 min, and 40 min wash in control solution, forskolin caused a stronger depression of evoked contraction. The enhanced depression lasted more than 3 h after the removal of Mn, though the degree was slightly reduced.

The fact that the inhibitory action of isoprenaline and forskolin was enhanced, whereas that of relaxin was reduced by pretreatment of the muscle strip with Mn, was further investigated. Pairs of muscle strips freshly excised were incubated in the same organ bath with Mg-free Krebs solution, and the effects of agents on the phasic contractions were examined 2 h later. The contractions of agents were adjusted so that the contractile inhibition was not too strong for isoprenaline (3×10^{-10} M) and forskolin (10^{-7} M), and moderate inhibition (100 mu) was caused by relaxin. After 45 min in Mg-free Krebs solution, 0.6 mM was applied for 15 min, and the depressive effect of Mn was examined. The effects of agents were again examined 40 min after the removal of Mn. Figure 10a shows the depression of phasic contractions by isoprenaline examined in 7 muscle strips. The amplitude of the evoked contraction was measured 5 min after the administration of

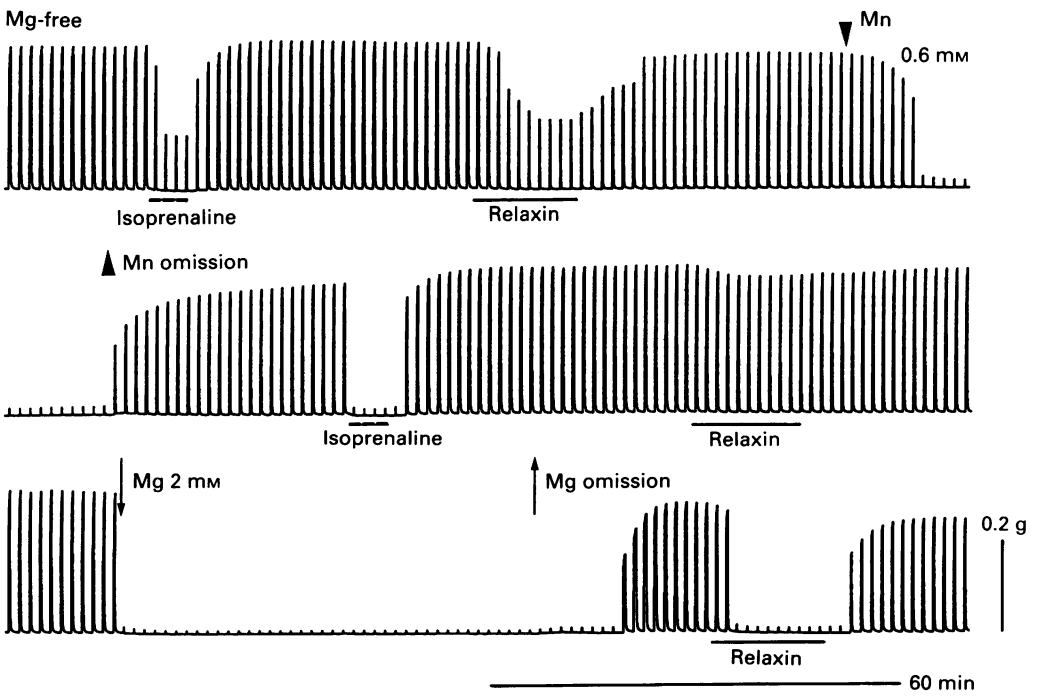


Figure 8 Effects of Mn and Mg on depression of contraction produced by isoprenaline and relaxin. The muscle strip was perfused with Mg-free Krebs solution, and 3×10^{-10} M isoprenaline and 50 mu relaxin were applied during the periods indicated by broken lines and bars, respectively. Application and removal of 0.6 mM Mn and 2 mM Mg are indicated by arrow heads and arrows, respectively. Sequential records from top to bottom.

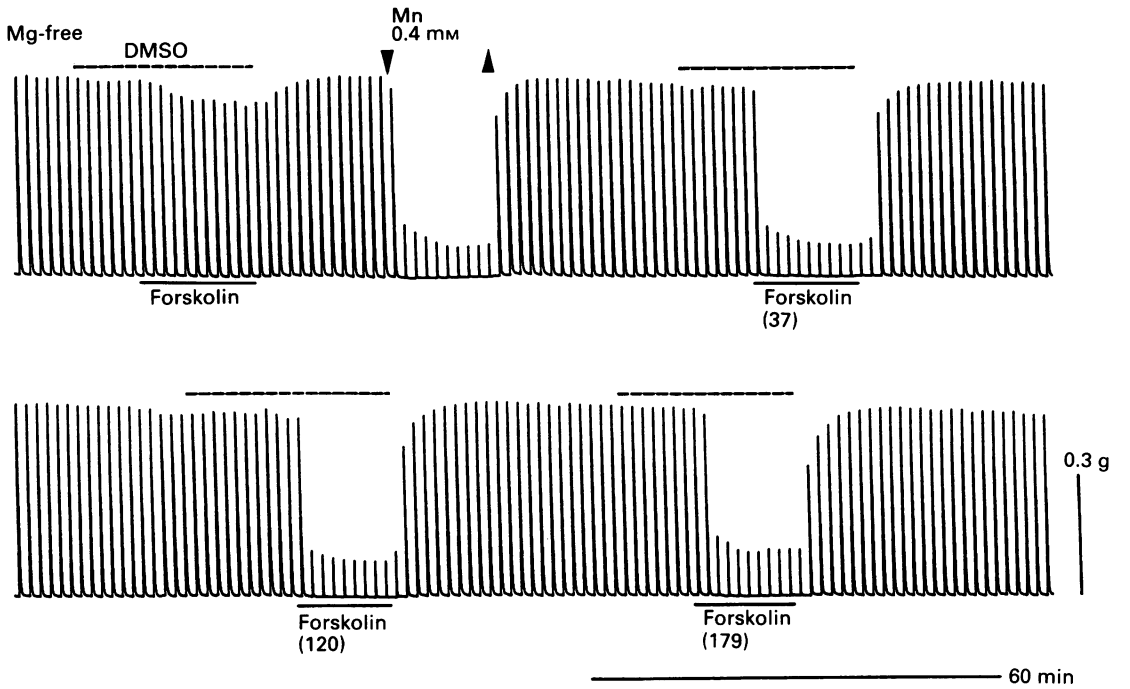


Figure 9 Effects of Mn on the inhibitory action of forskolin. The muscle strip was perfused with Mg-free Krebs solution, and 10^{-7} M forskolin was applied during the periods indicated by bars below. Dimethylsulphoxide (DMSO, 0.1%), the vehicle, was applied during the period indicated by broken lines above. Times in min after removal of Mn are indicated in parentheses.

isoprenaline, and the contractile inhibition was expressed as a % of the control and ranged between 100% and 17%. The depression by isoprenaline was enhanced by Mn in all muscle strips examined. In the case of forskolin, maximum inhibition was measured 10 min after addition of the agent. The degree of the contractile depression caused by forskolin was less variable for 7 muscle strips (Figure 10b). Again, the depression by forskolin became enhanced after pretreatment with Mn. In the case of 100 mu relaxin, the degree of contractile depression was measured for the evoked contraction generated 10 min after the administration of the agent (Figure 10c). The degree of the contractile inhibition was always smaller after pretreatment with Mn. The averages of inhibition expressed as a % response of the amplitude of control were 58 ± 38% (n = 7) and 8 ± 6% for isoprenaline, 88 ± 8% (n = 7) and 19 ± 6% for forskolin, 43 ± 22% (n = 8) and 72 ± 32% for relaxin, before and after the Mn pretreatment, respectively. These results therefore suggest that the intracellular signal transduction system could be different in the case of relaxin, although intracellular cyclic AMP is increased by this agent (Hsu *et al.*, 1985).

Influence of Mg and Mn on cyclic AMP content

Since isoprenaline and relaxin both depress the contractions, their effects on the cellular content of cyclic AMP were com-

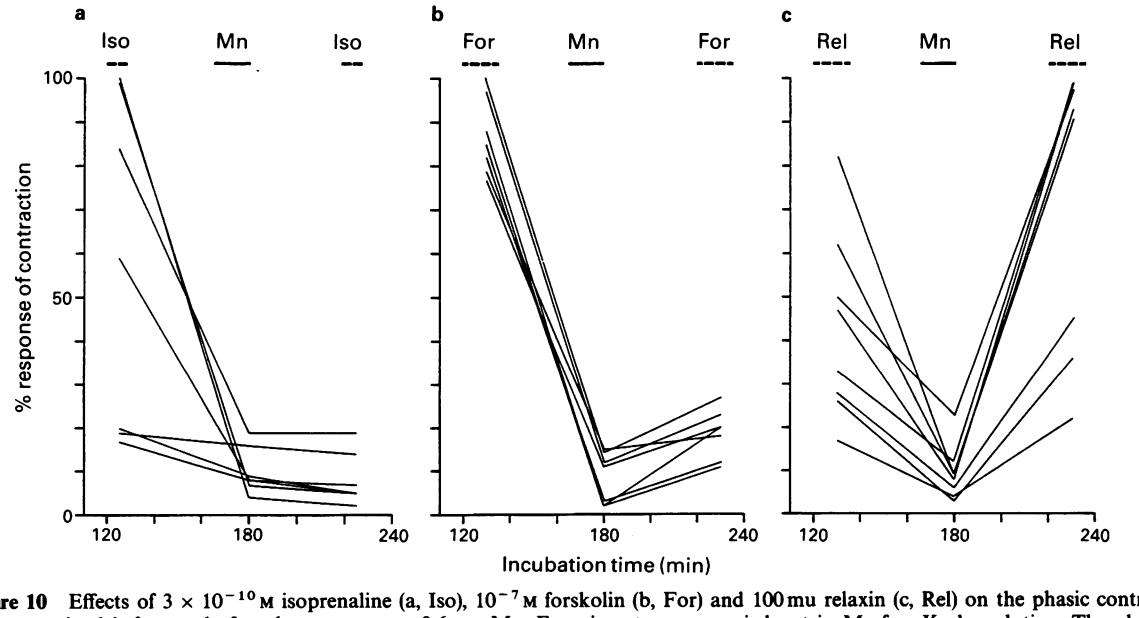


Figure 10 Effects of 3 × 10^{-10} M isoprenaline (a, Iso), 10^{-7} M forskolin (b, For) and 100 mu relaxin (c, Rel) on the phasic contractions, examined before and after the exposure to 0.6 mM Mn. Experiments were carried out in Mg-free Krebs solution. The phasic contractions were generated by electric pulses, and the minimum amplitude of phasic contraction during exposure to agents is expressed as a percentage of the mean of 5 successive contractions immediately before the drug application. The application of agents and Mn is indicated by broken lines and bars. Abscissae indicate the time (in min) after freshly excised muscle strips were exposed to warm Krebs solution (Mg-free). Responses in the same muscle strip are connected by lines.

pared in the presence of 0.6 mM Mg. A higher concentration of isoprenaline (10^{-9} M) was used in the present case, because the change in cyclic AMP content due to 3×10^{-10} M isoprenaline application was hard to detect. It was found that the time for relaxin and isoprenaline to produce maximum depression after the agents were perfused in the bulk solution was different (Table 1). In order to minimize the time resolution, the phasic contractions were generated every 60 s. The mean times in seconds to reach the maximum depression were 368 s ($n = 8$) and 172 s ($n = 8$) for relaxin (150 mu) and isoprenaline (10^{-9} M), respectively. Contractions were depressed to almost the same extent by these doses of the agents. The slower time course of relaxation by relaxin does not appear to be due to diffusion in the extracellular space, because a similar relaxation was reported for a single rat myometrial cell in culture (Hsu & Sanborn, 1986).

Because of the different times to reach the maximum depression, the muscle strips (about 1–3 mg) were treated with relaxin for 10 min and with isoprenaline for 5 min, and the cellular content of cyclic AMP was then measured. Values were 2.31 ± 0.25 pmol mg^{-1} protein ($n = 9$) for relaxin and 1.56 ± 0.28 pmol mg^{-1} protein ($n = 8$) for isoprenaline; cyclic AMP content was 1.24 ± 0.24 pmol mg^{-1} protein ($n = 9$) in non-treated muscle, and the difference from the control value was statistically significant for relaxin ($P < 0.02$).

In relation to the contractile investigation shown in Figure 10, effects of the pretreatment with 0.6 mM Mg or 0.6 mM Mn on cellular cyclic AMP content were examined (Figure 11). Several muscle strips, each weighing about 1–3 mg wet weight, were taken from a uterine horn, and subjected to sequential procedures. In order to minimize possible differences due to individual animals (cf. Figure 10), the value in a muscle strip exposed to the control solution was taken as 100% for all other muscle strips taken from the same animal. The average control value of cyclic AMP measured in Mg-free solution for 52 muscle strips excised from 37 rats was 0.98 ± 0.02 pmol mg^{-1} protein (mean ± s.e.mean). The cyclic AMP content was increased (1.17 ± 0.06 pmol mg^{-1} protein, $n = 18$) by exposure of the tissues to 0.6 mM Mg, and the level remained elevated during a 45 min rinse with the control solution. The muscle strip was rapidly frozen by liquid nitrogen 5 min after exposure to isoprenaline, and 10 min after exposure to forskolin or relaxin. The cyclic AMP content in response to either forskolin or relaxin was increased more following pretreatment with 0.6 mM Mg than in tissues kept in Mg-free solution throughout (Figure 11a). In the case of Mn, the cyclic AMP content was significantly increased (1.11 ± 0.06 pmol mg^{-1} protein, $n = 16$) by exposing the tissue to 0.6 mM Mn, but had returned to the control level by 45 min rinse with control solution. The increase in cyclic AMP content in response to forskolin was greater following pretreatment with 0.6 mM Mn, whereas the increase to relaxin was unchanged by pretreatment with Mn (Figure 11b).

The cyclic AMP contents in response to 10^{-8} M isoprenaline, by which evoked contractions were almost abolished, were 1.44 ± 0.07 (pmol mg^{-1} protein, $n = 4$) and 1.66 ± 0.02 ($n = 3$) before and after the treatment with 0.6 mM Mg and 1.42 ± 0.04 ($n = 3$) and 1.54 ± 0.06 ($n = 3$) before and after the treatment with 0.6 mM Mn, respectively.

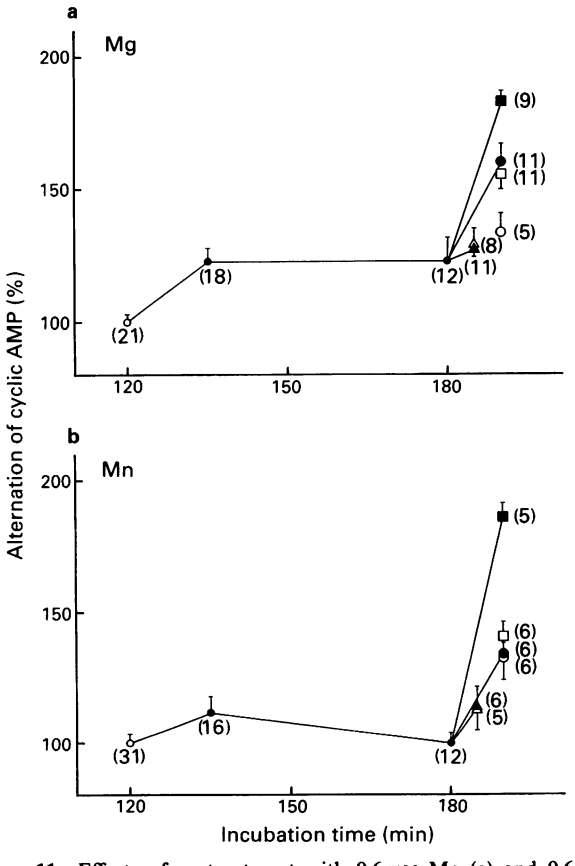


Figure 11 Effects of pretreatment with 0.6 mM Mg (a) and 0.6 mM Mn (b) on the cyclic AMP content increased by 10^{-9} M isoprenaline (△, ▲), 10^{-7} M forskolin (□, ■) and 100 mu relaxin (○, ●). Open and filled symbols show cyclic AMP content measured without and with pretreatment with Mg or Mn. The cyclic AMP content is expressed as a % of the content measured 120 min after incubation with Mg-free solution for other muscle strips taken from the same animal. The application of Mg or Mn, and agents is indicated by bars and broken lines. Figures in parentheses indicate the number of measurements, and vertical bars indicate s.e.mean. See text for further explanation.

Discussion

It has been shown that oestrogen treatment is needed to reveal the effect of relaxin by bioassay for either the lysis of the public symphysis or the relaxation of the myometrium (for review, see Schwabe *et al.*, 1978; Weiss, 1984). The present results are in agreement with the above finding on the myometrium, and further show that extracellular Mg was not necessary for relaxin to produce strong inhibition in both longitudinal and circular muscles in oestrogen-treated rats. In contrast, in spite of the abundance of relaxin receptors in rat myometrium on day 17 of pregnancy (Mercado-Simmen *et al.*, 1980; 1982), 150 mu porcine relaxin was almost without effect when Mg was absent in the bathing solution. Thus, a different requirement of external Mg for the relaxin-receptor binding is suggested between pregnant and oestrogen-primed rat myometrium. The following discussion will be confined to the action of relaxin on the longitudinal myometrium of the oestrogen-treated rat.

It was demonstrated by Marshall & Kroeger (1973) that the cellular content of cyclic AMP was increased when isoprenaline was administered to rat myometrium. Since then, it has been further proposed that cyclic AMP is most probably the second messenger for β-adrenoceptor action on myometrial tissues, because dibutyryl-cyclic AMP and forskolin, which permeates into the cell interior and activates the catalytic unit of adenylate cyclase, are both capable of causing an inhibitory effect similar to isoprenaline (for review see Bülbring & Tomita, 1987; Marshall & Fain, 1984; Maruta & Osa, 1989). Furthermore, it was recently shown that relaxin increased the cellular content of cyclic AMP in the rat myometrical cells in culture (Hsu *et al.*, 1985). In the present experiments, the cyclic AMP content of the longitudinal myometrium of the oestrogen-primed rat was increased to 186% of the control by the application of 150 mu relaxin in Krebs solution containing 0.6 mM Mg, and the dose of the agent depressed the phasic contraction to 16% of the control response (Table 1). Therefore, cyclic AMP may in some way participate in the inhibitory effect of relaxin.

It has been reported that the external presence of Mg and other divalent cations enhances the physiological effects of several agents such as vasopressin and oxytocin (vascular

muscle, Somlyo *et al.*, 1966; myometrium, Schild, 1969). One action was shown to be due to the enhancement of binding between the agonist and receptor (Soloff & Grzonka, 1986). On the other hand, the enhancement of β-adrenoceptor inhibition by Mg application via an intracellular process has been proposed in some smooth muscle tissues (myometrium, Maruta & Osa, 1989; taenia, Maruta *et al.*, 1989). In the present experiments, the intracellular cyclic AMP content was more elevated by administration of β-agonists and relaxin measured in the Mg-free solution, after brief application of 0.6 mM Mg, where the experimental design eliminated the facilitatory effect of Mg on the binding of relaxin to the surface membrane receptors (Mercado-Simmen *et al.*, 1980).

A question that arises is what happens during pretreatment with divalent cations. Free $[Mg]_i$ in immature rat uterus was estimated to be between 200 and 400 μM, and increased by 25% at 24 and 48 h after intraperitoneal injection of 17 β-oestradiol (Degani *et al.*, 1984). A recent report (Nakayama & Tomita, 1990) has shown that intracellular free Mg concentration ($[Mg]_i$) of guinea-pig taenia caeci is estimated to be 310 μM, and can be changed by altering the extracellular ionic environment. The $[Mg]_i$ was not altered by removal of external Mg, but decreased to 8.3 μM in 100 min when both Mg and Ca were omitted. The $[Mg]_i$ recovers completely in about 25 min on readmission of 1.2 mM Mg. On the other hand, Mn ion has long been known as an inorganic Ca antagonist (for reviews see Bolton, 1979; Weiss, 1981). Yet its action on contraction is complicated, and is not fully understood; $MnCl_2$ modifies the Ca influx and penetrates the cell, thus causing either a suppression (Keene *et al.*, 1972) or an acceleration (Shibata, 1969; Osa, 1974; Sakai & Uchida, 1981; Itoh *et al.*, 1982; Lategan & Brading, 1988) of the mechanical responses in various visceral muscles. Intracellular accumulation of Mn was experimentally proved to occur in rat ventricular muscle, in which the baseline tension was gradually elevated when exposed to 0.2 mM Mn (Chapman & Ellis, 1977). Thus, if a similar situation is applicable to myometrium, it can be assumed that the cells are loaded with Mg or Mn by pretreatment with the respective ions, and these are not removed.

In intact cells and cell-free systems, it has been reported that the adenylate cyclase activity linked to β-adrenoceptor activation is facilitated by adding Mg or Mn (Krall *et al.*, 1988; Mittag *et al.*, 1988) or can be directly activated by these ions (Braun *et al.*, 1972; Neer, 1979; Cech *et al.*, 1980; Cech & Maguire, 1982; Mittag *et al.*, 1988; Arkinstall & Jones, 1990). Effects of Mg and Mn could be in the same or the opposite direction in regulating adenylate cyclase activity depending on the agonists and tissues (Limbird *et al.*, 1979; Cech *et al.*, 1980; Cech & Maguire, 1982). As far as the present results are concerned, 'intracellular' Mg and Mn share in common the enhancing effect by themselves on the cyclic AMP production (Figure 11). The relationship between cyclic AMP production and contractile depression is linear in the case of forskolin application such that cyclic AMP was more increased and contraction was more strongly depressed when the muscle was pretreated with either Mg or Mn. Therefore, the action site of divalent cations is probably the catalytic unit of adenylate cyclase. The same idea could also be applied to the case of relaxin action after the pretreatment with Mg. On the other hand, cyclic AMP production in response to 10^{-9} M isoprenaline was not significantly changed by pretreatment with either Mg or Mn, while the contractile depression was strongly enhanced (Figure 10; Maruta & Osa, 1989). Furthermore, the cyclic AMP production by relaxin was not significantly altered with or without Mn pretreatment, whereas the contractile depression became much smaller after Mn pretreatment. These are the experimental findings suggesting that the cellular content of cyclic AMP does not parallel the contractile depression. In general, there are several sequential interactions from the activation of a receptor by a hormone to the final effect, and the interactions take place in the membrane as well as the intracellular phase involving myosin kinase (for review see Adelstein, 1980). H-8, a potent protein kinase A inhibitor, effectively reversed the attenuation by both isoprenaline and relaxin of the oxytocin-induced increase in $[Ca]_i$ and phosphoinositide turnover of myometrial cells in culture (Anwer *et al.*, 1990). At present, therefore, it seems possible that Mn blocks or potentiates some step(s), e.g. protein kinase(s), so that contractile depression was changed in different ways in response to relaxin or isoprenaline, respectively.

Investigations of the contractile response revealed some unexpected features of the action of relaxin especially concerning the effects of divalent cations; the depression of phasic contractions persisted after the removal of relaxin, when Mg was present in the recovery solution, and raising the external Ca to 7.5 mM did not restore the amplitude of phasic contraction. During the persistent depression, the removal of external Mg was effective in restoring the phasic contraction. Therefore, the enhancement of stabilizing action of Mg at the membrane to prevent Ca-influx is suggested. Assuming that the increase in $[Mg]_i$ enhances the relaxin action and *vice versa*, it may be deduced that $[Mg]_i$ was reduced when the tissue was cooled in Mg free solution and hence the depressive action of relaxin on contraction became reduced.

As frequently reported by previous investigators, hyperpolarization of the membrane and reduction of membrane resistance are the electrophysiological manifestation of stimulation of β-adrenoceptors in the myometrial membrane, suggesting an increase in K-conductance (for review see Bülbring & Tomita, 1987). On the other hand, Chamley & Parkington (1984) showed that the plateau potential in the myometrium of the pregnant and oestrogen-treated rat is specifically depressed by relaxin added to the Krebs solution, and the resting membrane was not hyperpolarized by relaxin up to 300 mu. This observation was confirmed on the longitudinal myometrium (3E) in the present experiments. It is furthermore observed that the depression of contraction by 50 mu relaxin is probably caused secondarily to the shortening of the action potential, whereas the contractile depression was caused by 150 mu relaxin in spite of no change in action potential. For the latter finding, inhibitory effects of relaxin on the contractile machinery bypassing the membrane response should be taken into account. It has been proposed that a cyclic AMP-mediated phosphorylation of myosin light chain kinase decreases its affinity for calmodulin (Conti & Adelstein, 1981; Higashi *et al.*, 1983), whereby relaxin relaxes the myometrium (Nishikori *et al.*, 1983; Hsu & Sanborn, 1986).

In conclusion, the present physiological results are compatible with the view that cyclic AMP acts as a second messenger of relaxin in depressing the myometrial contraction. However, it is possible that intracellular signal transduction systems are different between β-adrenoceptor-mediated and relaxin actions.

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Three distinct binding sites for [^{3}H]-prazosin in the rat cerebral cortex

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1 The putative α_1-adrenoceptor subtypes of rat cerebral cortex membranes were characterized in binding experiments with [^{3}H]-prazosin.

2 Specific binding of [^{3}H]-prazosin was saturable between 20–5000 pM. Scatchard plots of the binding data were non-linear, indicating the presence of two distinct affinity sites for prazosin ($pK_{D,high} = 10.18$, $R_{high} = 308$ fmol mg^{-1} protein; $pK_{D,low} = 8.96$, $R_{low} = 221$ fmol mg^{-1} protein).

3 In the membranes pretreated with chlorethylclonidine (CEC) two affinity sites for prazosin were also observed: the affinities were similar to those without CEC pretreatment, but the maximum numbers of binding sites were reduced by CEC pretreatment to 23 and 62% for prazosin-high (R_{high}) and low affinity sites (R_{low}), respectively.

4 The prazosin-high affinity sites were further subdivided into two subclasses by WB4101(2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) and phentolamine; the low affinity sites for WB4101 and phentolamine were more potently inactivated by CEC as compared with the high affinity sites. On the other hand, prazosin, HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile fumarate) and yohimbine inhibited [^{3}H]-prazosin binding to prazosin-high affinity sites monophasically.

5 In addition to the high affinity sites, the prazosin-low affinity sites were labelled at high concentrations of [^{3}H]-prazosin. Thus, prazosin and WB4101 showed shallow displacement curves. On the other hand, HV723 and yohimbine did not discriminate between prazosin-high and low affinity sites.

6 Two distinct α_1-adrenoceptor subclassifications have been recently proposed (α_{1A}, α_{1B} subtypes and α_{1H}, α_{1L}, α_{1N} subtypes). According to the criteria defined with competitive antagonists in both the subclassifications, the present results indicate that the α_1-adrenoceptors of rat cerebral cortex consist of three different subtypes, presumably α_{1A}, α_{1B} and α_{1L}, and suggest that the α_{1A} and α_{1B} subtypes are identified as a single affinity site for prazosin (α_{1H}). The results also indicate that care must be taken in the use of CEC for α_1-adrenoceptor subclassification because of its low selectivity.

Keywords: $\alpha_1$adrenoceptor subtype; α_1-adrenoceptor subclassification; rat cerebral cortex; α_{1A} subtype; α_{1B} subtype; α_{1L} subtype

Introduction

There is increasing evidence for the existence of α_1-adrenoceptor subtypes. Radioligand binding studies with [^{3}H]-prazosin and [^{125}I]-BE2254 (2[β-(4-hydroxyphenyl)-ethyl-aminomethyl]tetralone) have demonstrated that α_1-adrenoceptors of rat cerebral cortex and other tissues can be divided into two subclasses: putative α_{1A} and α_{1B}. The α_{1A}-subtype has a high affinity for the competitive antagonists WB4101(2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane), benoxathian, phentolamine and 5-methylurapidil and is not inactivated by the alkylating agent, chlorethylclonidine (CEC). The α_{1B}-subtype shows a lower affinity for the competitive antagonists mentioned and is potently inactivated by CEC (Morrow & Creese, 1986; Han *et al.*, 1987a,b; Minneman, 1988; Hanft & Gross, 1989). However, both α_{1A}- and α_{1B}-subtypes cannot be discriminated by prazosin and yohimbine (Hanft & Gross, 1989). In contrast, functional studies with blood vessels have proposed another subclassification, where the α_1-adrenoceptors can be classified into putative two (α_{1H} and α_{1L}) or three (α_{1H}, α_{1L} and α_{1N}) subtypes (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990a,b). The α_{1H} subtype has a higher affinity for prazosin comparing with the α_{1L} and α_{1N} subtypes, while the latter two subtypes can be further distinguished by a novel α_1-adrenoceptor antagonist HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)propyl) benzeneacetonitrile fumarate) (Oshita *et al.*, 1988; Muramatsu *et al.*, 1990b). However, there is no evidence on the correlation between the two distinct subclassifications. The purpose of the present study was to determine the α_1-adrenoceptor subtypes of the rat cerebral cortex on the basis of the criteria proposed in the α_{1H}, α_{1L}, α_{1N} subclassification and to compare the approach with the α_{1A}, α_{1B} subclassification.

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Methods

Binding study

Male Wistar rats (300–450 g) were decapitated and the brain was rapidly removed. Cerebral cortex was homogenized in 100 vol. of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) using a polytron (setting 8, 15 s × 2). The homogenates were filtered through four layers of cheesecloth and centrifuged at 80,000 *g* for 20 min at 4°C. The pellets were resuspended in the same volume of assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated for 10 min at 37°C, and centrifuged again as described above. All procedures to prepare membranes were conducted at 4°C except preincubation of the membranes, and ice-cold buffers were used. The final pellet was resuspended in assay buffer and used for binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml (50–100 μg protein/tube), but displacement experiments with CEC-treated membranes were carried out in a final volume of 2 ml (approximately 200 μg protein/tube). Reactions were terminated by rapid filtration using a Brandel cell harvester with Whatman GF/C filters presoaked in 0.3% polyethylenimine for 5 min. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried; the filter-bound radioactivity was determined by liquid scintillation

counting. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine or 1 μM prazosin. Assays were conducted in duplicate.

CEC treatment: membrane preparations were incubated for 30 min at 37°C with 10 μM CEC and centrifuged at 80,000 g for 20 min. The pellet was washed once with assay buffer before the binding experiment.

Data were analysed by the weighted least-squares iterative curve fitting programme, LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by an F-test comparison, then the two-site model was accepted. P values less than 0.05 were considered significant.

Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Statistical analyses

Experimental values are given as the mean ± s.e.mean. Results were analysed by Student's t test and a probability of less than 0.05 was considered significant.

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 76.6 Ci mmol^{-1}, NEN, Boston, U.S.A.), prazosin hydrochloride (Sigma, St. Louis, U.S.A.), phentolamine mesylate (Ciba, Basel, Switzerland), yohimbine hydrochloride (Wako, Osaka, Japan), WB4101 hydrochloride, chloroethylclonidine dihydrochloride (CEC) (Research Biochemicals Inc., Natick, U.S.A.), noradrenaline bitartrate (Nacalai, Kyoto, Japan), HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)-ethyl)-amino)propyl)benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Japan).

Results

Saturation experiments

[^{3}H]-prazosin at concentrations ranging from 20 to 5000 pM was used to label α_1-adrenoceptors of rat cerebral cortex membranes. The specific binding was approximately 90% of the total binding at 200 pM [^{3}H]-prazosin and saturated at concentrations of 4000–5000 pM (Figure 1a). Scatchard plots of the binding data were curvilinear, suggesting more than a single class of binding site (Figure 1b). LIGAND analysis fitted the data to a two site model. The high affinity site showed a B_{max} (expressed as R_{high}) of 308 ± 19 fmol mg^{-1} protein and an equilibrium dissociation constant (expressed as $pK_{D,\,high}$) of 10.18 ± 0.07, while the low affinity site showed R_{low} of 221 ± 24 fmol mg^{-1} protein and $pK_{D,\,low}$ of 8.96 ± 0.17. There was an approximately 20 fold difference between the high and low affinity sites for prazosin. The maximum number of binding sites with a high affinity (R_{high}) was about 1.5 times greater than those with a low affinity (R_{low}). The specific binding of 200 pM [^{3}H]-prazosin was completely displaced by noradrenaline (0.1–1000 μM) with a pK_I of 5.7, and that of 3000 pM [^{3}H]-prazosin was inhibited to 6% by 10 mM noradrenaline (n = 2).

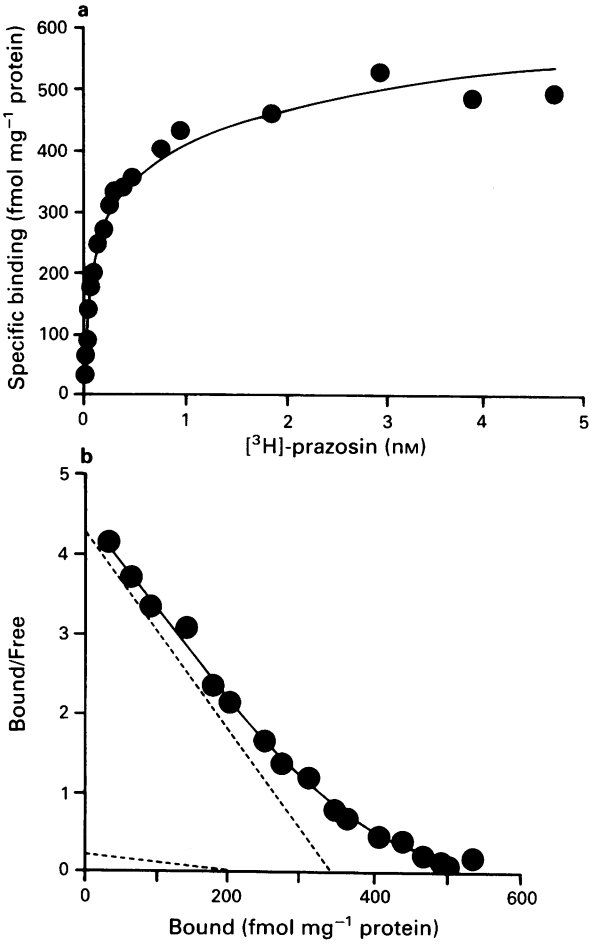


Figure 1 Saturation experiments of [^{3}H]-prazosin binding to rat cerebral cortex membranes. (a) Saturation curve of specific [^{3}H]- prazosin binding. (b) Scatchard analysis. The figures are obtained from a single experiment where each point is the mean of duplicate determinations. Scatchard transformed data were best fit to a two-binding site model (dashed lines) by LIGAND programme ($pK_{D,\,high}$ = 10.22, R_{high} = 336 fmol mg^{-1} protein; $pK_{D,\,low}$ = 9.12, R_{low} = 202 fmol mg^{-1} protein). Mean values derived from 4 independent experiments are presented in Table 1.

The membranes pretreated with 10 μM CEC also showed two sites of distinct affinity for [^{3}H]-prazosin; both affinities were not significantly different from the corresponding values estimated in the untreated membranes (Table 1). However, the maximum numbers of high and low affinity sites were reduced to 23 and 62%, respectively, by pretreatment with CEC.

Displacement experiments

The pharmacological profile of high and low affinity sites for prazosin was examined in displacement experiments. [^{3}H]-

Table 1 Binding of [^{3}H]-prazosin to α_1-adrenoceptors of rat cerebral cortex

Treatments	n	$pk_{D,\,high}$	$pK_{D,\,low}$	R_{high}	R_{low}
None	4	10.18 ± 0.07	8.96 ± 0.17	308 ± 19	221 ± 24
CEC	6	10.09 ± 0.06	8.60 ± 0.11	70 ± 9[a]	137 ± 13[a]

Scatchard curves were individually analysed by LIGAND programme and a two site fit was accepted as it was significantly better than a one site fit.
Data shown are mean ± s.e.mean, n = number of experiments.
$pK_{D,\,high}$ and $pK_{D,\,low}$: negative log of the equilibrium dissociation constants (−log M) at prazosin-high and low affinity sites.
R_{high} and R_{low}: maximal numbers of prazosin-high and low affinity sites (fmol mg^{-1} protein).
CEC: The cortex membranes were pretreated with 10 μM chlorethylclonidine (CEC) for 30 min and then washed repeatedly.
[a] Significantly different from control (no treatment) ($P < 0.05$).

prazosin, 200 pM, was used to label the high affinity sites for prazosin. Unlabelled prazosin displaced the binding in a monophasic manner with a high pK_I value (10.16 ± 0.03) (Figure 2 and Table 2). HV723 and yohimbine also antagonized the [^{3}H]-prazosin binding monophasically with a single pK_I value for each drug. On the other hand, displacement curves for WB4101 and phentolamine were shallow. Computerized analysis of the displacement curves revealed that WB4101 and phentolamine bound to two distinct sites, suggesting heterogeneity of the high affinity sites for prazosin. The proportion of the high affinity sites for WB4101 and phentolamine amounted to approximately 40% of total binding at prazosin-high affinity sites.

[^{3}H]-prazosin at 200 pM also bound to membranes pretreated with CEC, although the specific binding was reduced to approximately 34 ± 6% (a 66% loss, $n = 6$) compared with the binding of the membranes without CEC pretreatment. Computerized analysis of the displacement curves revealed that the displacement curves for prazosin, HV723 and yohimbine fitted better to a one-site model, whereas the curves for WB4101 and phentolamine were analysed in a two-site model (Table 3). The affinity constants obtained for all the displacers were not significantly different from the corresponding values of the membranes not pretreated with CEC. However, the proportion of high affinity sites for WB4101 and phentolamine increased to approximately 80% in the membranes pretreated with CEC, whereas that of low affinity sites decreased to 20%. Since 66% of the specific binding sites for 200 pM [^{3}H]-prazosin was lost by CEC pretreatment, from the ratio (80:20) obtained in CEC-pretreated membranes, it was estimated that 27 and 7% respectively of the number of high and low affinity sites for WB4101 or phentolamine recognized in CEC-untreated membranes remained unaffected by CEC. In other words, this indicates that 32 and 88% of high and low sites in CEC-untreated membranes were inactivated by CEC.

The low affinity sites for prazosin were characterized in the displacement experiments with a higher concentration of [^{3}H]-prazosin (3000 pM). Figure 3 shows the displacement curves for prazosin and WB4101 in [^{3}H]-prazosin binding over a wide range of concentrations. In contrast to HV723 and yohimbine, the slope factors for prazosin and WB4101 deviated from unity (Table 4). Conducting the LIGAND analysis of the displacement curve for prazosin utilizing the

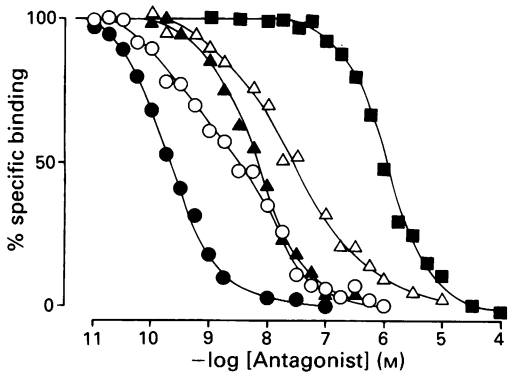


Figure 2 Displacement of [^{3}H]-prazosin binding from rat cerebral cortex membranes by prazosin (●), WB4101 (○), HV723 (▲), phentolamine (△) and yohimbine (■). [^{3}H]-prazosin (200 pM) was incubated with various concentrations of non-radiolabelled drugs under assay conditions described in Methods. The figure represents a single experiment for each drug, where each point is the mean of duplicate determinations. Displacement curves of WB4101 and phentolamine showed the best fit to a two-site model by LIGAND analysis.

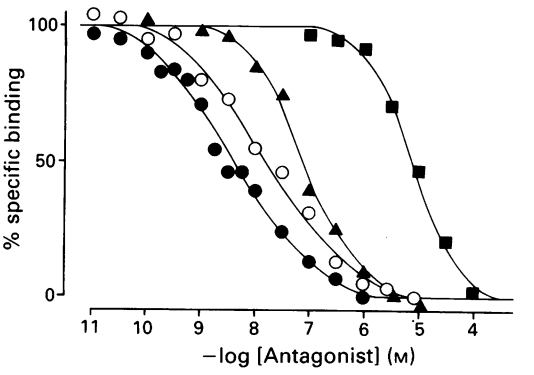


Figure 3 Displacement of [^{3}H]-prazosin binding from rat cerebral cortex membranes by prazosin (●), WB4101 (○), HV723 (▲) and yohimbine (■). The concentration of [^{3}H]-prazosin was 3000 pM. The other experimental conditions are the same as those in Figure 2.

Table 2 Inhibition of 200 pM [^{3}H]-prazosin binding to α_1-adrenoceptors of rat cerebral cortex by α-antagonists

Antagonist	n	$pK_{I,high}$	$pK_{I,low}$	*% high*	*Slope factor*
Prazosin	3	10.16 ± 0.03	—	100	0.95 ± 0.02
WB4101	3	10.29 ± 0.14	8.64 ± 0.02	39.0 ± 2.6	0.70 ± 0.01
HV723	3	8.76 ± 0.07	—	100	0.96 ± 0.01
Phentolamine	3	8.94 ± 0.21	7.55 ± 0.13	42.7 ± 10.5	0.75 ± 0.05
Yohimbine	4	6.47 ± 0.02	—	100	1.01 ± 0.04

Displacement curves were individually analysed by LIGAND programme. A two site fit was accepted only if it was significantly better than a one site fit.
Data shown are means ± s.e.mean, n = number of experiments.
$pK_{I,high}$ and $pK_{I,low}$: negative log of the equilibrium dissociation constants (−log M) at high or low affinity for antagonists tested.
% high: population binding at the high affinity site compared to the total specific binding sites.

Table 3 Inhibition by α-adrenoceptor antagonists of 200 pM [^{3}H]-prazosin binding to α_1-adrenoceptors of rat cerebral cortex pretreated with chlorethylclonidine (CEC)

Antagonist	n	$pK_{I,high}$	$pK_{I,low}$	*% high*	*Slope factor*
Prazosin	3	10.12 ± 0.02	—	100	0.88 ± 0.03
WB4101	3	10.32 ± 0.06	8.49 ± 0.13	80.0 ± 2.1	0.59 ± 0.01
HV723	4	9.10 ± 0.05	—	100	0.88 ± 0.03
Phentolamine	3	8.83 ± 0.02	6.93 ± 0.21	84.0 ± 1.2	0.67 ± 0.05
Yohimbine	3	6.52 ± 0.05	—	100	0.95 ± 0.02

The rat cortex membranes were pretreated with 10 μM CEC and then washed. The other experimental conditions are the same as those of Table 2.

Table 4 Inhibition of 3000 pM [^{3}H]-prazosin binding to α_1-adrenoceptors of rat cerebral cortex by α-antagonists

Antagonist	n	*pIC$_{50}$*	*Slope factor*
Prazosin	4	8.69 ± 0.23	0.69 ± 0.04
WB4101	3	7.54 ± 0.10	0.57 ± 0.05
HV723	3	7.32 ± 0.12	0.86 ± 0.04
Yohimbine	3	5.05 ± 0.02	1.09 ± 0.05

affinities of [^{3}H]-prazosin for its two binding sites as they were determined from saturation analysis (Table 1), two distinct affinity sites ($pK_I = 10.4 \pm 0.2$ and 8.2 ± 0.4) were detected. The proportion of high affinity site amounted to 65 ± 3.3% of the total [^{3}H]-prazosin binding (4 experiments).

Discussion

A single affinity site for prazosin or BE2254 has been demonstrated in the rat cerebral cortex (Minneman, 1983; Morrow & Creese, 1986; Hanft & Gross, 1989). However, the present study clearly demonstrates that prazosin binds to two distinct populations of binding sites in the cortex. This discrepancy is probably associated with the radioligand concentrations used, because the low affinity sites may be overlooked at low concentrations of radioligand. In fact, higher concentrations of [^{3}H]-prazosin (up to 5 nM) were used in the present study as compared with the previous studies (Morrow & Creese, 1986; Hanft & Gross, 1989). Like the present results, two distinct affinity sites for prazosin have also been demonstrated in other tissues such as rabbit thoracic aorta (Babich *et al.*, 1987; Piascik *et al.*, 1988; Mignot *et al.*, 1989; Muramatsu *et al.*, 1990a).

As mentioned in the Introduction, two different α_1-adrenoceptor subclassifications have been proposed. According to the criteria defined with competitive antagonists in the α_{1H}, α_{1L}, α_{1N} subclassification (Muramatsu *et al.*, 1990a, b), the prazosin-high and low affinity sites of rat cortex seem to correspond to α_{1H} and α_{1L} subtypes, respectively. An α_{1N}-selective drug HV723 did not discriminate both sites, resulting in a relatively low affinity.

On the other hand, the presence of prazosin-high and low affinity sites seems not to fit in with the α_{1A}, α_{1B} subclassification, as α_{1A} and α_{1B} subtypes show a similar affinity for prazosin (Hanft & Gross, 1989). Rather, the present study reveals that the prazosin-high affinity sites can be further subdivided into two classes by the α_{1A}, α_{1B}-discriminating drugs such as WB4101 and phentolamine (Morrow & Creese, 1986; Minneman *et al.*, 1988). Therefore, it would be reasonable to consider that the high and low affinity sites for WB4101 or phentolamine correspond respectively to α_{1A} and α_{1B} subtypes and that both the subtypes are identified as a single, high affinity site for prazosin. Similar subdivision has led to a single affinity site not only for [^{3}H]-prazosin but also for [^{125}I]-BE2254 in many tissues including rat cerebral cortex (Morrow & Creese, 1986; Han *et al.*, 1987a,b; Hanft & Gross, 1989).

In contrast to such a subclassification with the use of competitive antagonists, subclassification by CEC may be controversial. CEC was originally reported to cause a selective inactivation of the α_{1B} subtype (Han *et al.*, 1987b; Minneman *et al.*, 1988). However, the present results reveal that CEC can produce a loss (probably inactivation) of [^{3}H]-prazosin binding to all the three subtypes (α_{1A}, α_{1B} and α_{1L}) which are identified by different affinities for competitive antagonists as discussed above. The degree of inactivation by CEC varied among the subtypes, suggesting that α_{1B} subtype is more sensitive to CEC than the other subtypes. At a concentration of 10 μM CEC, the inactivation was approximately 90% of α_{1B} subtype and 30–40% of the α_{1A} and α_{1L} subtypes. Such differential inactivation by CEC can also be estimated from the results obtained by Hanft & Gross (1989): 33 and 92% inactivation respectively of α_{1A} (high affinity site for 5-methylurapidil) and α_{1B} (low affinity site) subtypes by 10 μM CEC.

Coexistence of distinct binding sites with different affinities implies that the proportion of each site recognized would vary according to the concentrations of radioligand used. At 3 nM [^{3}H]-prazosin, the proportion of the low affinity sites for prazosin was approximately 35% of total specific binding, which was close to the ratio of B_{max} value for R_{low} (42%) in the saturation experiments. When the concentration of [^{3}H]-prazosin was 200 pM, however, computer analysis of displacement curves for prazosin gave a better fit to a one-site model, detecting only high affinity sites. This suggests that the proportion of low affinity sites for prazosin becomes smaller at lower concentrations of [^{3}H]-prazosin and that the small proportion may be overlooked even though the slope factor of the displacement curve for prazosin deviated slightly from unity (0.88 in CEC-treated membranes). Relatively small differences (ratio: 17 fold) in the affinities for prazosin between two distinct sites would also make it difficult to separate them (Molinoff *et al.*, 1981).

Although α_1-adrenoceptors produce various responses, the diversity is thought to be in part related to distinct subtypes which may activate different mechanisms of signal transduction (Minneman, 1988). Responses to α_{1A} subtype reportedly require Ca^{2+} influx whereas responses to α_{1B} subtype seem to involve mobilization of intracellular Ca^{2+} stores by an inositol phosphate mechanism (Han *et al.*, 1987; Hanft & Gross, 1989; Tsujimoto *et al.*, 1989; Wilson & Minneman, 1990). However, the cellular mechanisms and functions for α_{1L} subtype remain to be clarified.

In conclusion, the present results show the existence of three distinct binding sites for prazosin in the rat cerebral cortex, presumably α_{1A}, α_{1B} and α_{1L} adrenoceptor subtypes which can be discriminated by different affinities for competitive antagonists. The results also suggest that great care must be taken when CEC is used for α_1-adrenoceptor subclassification because of its low selectivity for each subtype.

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Chronic treatment with the angiotensin I converting enzyme inhibitor, perindopril, protects *in vitro* carbachol-induced vasorelaxation in a rat model of vascular calcium overload

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1 Treatment of young rats with vitamin D_3 plus nicotine produced 31 and 4 fold increases in the calcium content of the aorta and the mesenteric arterial bed, respectively.

2 Aortic rings and perfused mesenteric arterial beds from vitamin D_3/nicotine-treated animals showed a diminished contractile response to noradrenaline *in vitro*.

3 In vascular preparations from vitamin D_3/nicotine-treated animals, precontracted with noradrenaline, relaxation by the endothelium-dependent vasodilator, carbachol, was attenuated but responses to sodium nitroprusside were not modified.

4 Prolonged treatment with the angiotensin I converting enzyme inhibitor, perindopril, at a dose (1 mg kg^{-1}) which did not significantly modify blood pressure, failed to prevent vascular calcium overload.

5 Perindopril treatment diminished noradrenaline-evoked vasoconstrictor responses of aortic rings in both groups, but restored responses in mesenteric arterial beds of vitamin D_3/nicotine-treated rats.

6 Perindopril treatment also restored the maximal responses to carbachol of both aortic rings and mesenteric arterial beds of vitamin D_3/nicotine-treated rats.

7 In conclusion, in the vitamin D_3 plus nicotine model of calcium overload, reduced endothelial-mediated relaxation can be prevented by perindopril treatment.

Keywords: Calcium overload; rat aorta; endothelium; angiotensin I converting enzyme; ACE inhibitor; perindopril

Introduction

Treatment of spontaneously hypertensive rats for 18–20 months with high doses of angiotensin I converting enzyme inhibitor, captopril (up to 350 mg kg^{-1} per day, orally) has been shown to normalize blood pressure and prevent calcium overload of the aorta, and the mesenteric and other arteries (Fleckenstein *et al.*, 1987). Drugs which modify the evolution, and/or the consequences, of vascular calcium overload may be more effective in the treatment of vascular diseases, since, in addition to their haemodynamic action, they may correct the pathogenetic abnormalities of vascular calcium handling and consequences such as atherosclerosis and hypertension (Fleckenstein *et al.*, 1987). As vascular calcium overload develops very slowly in the spontaneously hypertensive rat and treatment aimed at modifying this natural process has to be applied for several months, a shorter-term model was developed (Fleckenstein, 1985).

Administration to young rats of vitamin D_3 followed by daily injections of nicotine produces large increases in the calcium content of arteries, followed by death within 6 days; simultaneous administration of large doses of calcium entry blockers attenuates the vascular calcium overload and protects the rats against the lethal effect of such treatment (Fleckenstein, 1985). Death may be due to calcium overload in many organs not only arteries. Immediately following treatment, hypercalcaemia and increases in the plasma levels of urea and creatinine are observed (Thorin *et al.*, 1990). We reduced the toxicity of the original model by (i) decreasing the total amount of nicotine administered, and (ii) allowing the rats to recover for 16 days following the treatment.

Changes in endothelial function have been described in both atherosclerosis (Parmley, 1990) and hypertension (Hongo *et al.*, 1988). The end result we chose, therefore, was evaluation of endothelium-dependent relaxation *in vitro*. As angiotensin I converting enzyme inhibitors prevent vascular calcium overload in the spontaneously hypertensive rat (see above) we evaluated the effects of perindopril in this model. Perindopril is approximately 10 times more potent than captopril (Muller *et al.*, 1990).

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Methods

Animals

One hundred and six male, outbred Wistar rats (220–250 g; Iffa-Credo SA, L'Arbresle, France) were given a standard diet (A04, UAR, Villemoisson sur Orge, France; calcium 150 mmol kg^{-1}) and mineral water (calcium 0.26 mM; Société de Eaux de Volvic, Volvic, Puy-de-Dôme) *ad libitum*.

Vitamin D_3 and nicotine treatment

Fifty-five of the rats were injected with vitamin D_3 (300 000 iu kg^{-1}, i.m.) and nicotine (25 mg kg^{-1}, orally) at 08 h 30 min, on day 1. The nicotine administration was repeated at 18 h 00 min. Rats were allowed 16 days to recover. The remaining 51 rats (controls) were administered distilled water (5 ml kg^{-1}, orally) and 0.15 M NaCl (2 × 1 ml kg^{-1}, i.m.).

Perindopril treatment

All rats were injected at 09 h 00 min on days 2 to 16 with perindopril (0.3, 1 or 3 mg kg^{-1}, i.p.) or sodium chloride (0.15 M, 0.5 ml kg^{-1}, i.p.).

Blood pressure and heart rate measurements

On the 17th day, the right carotid artery was cannulated following anaesthesia with sodium pentobarbitone (50 mg kg^{-1}, i.p.), and the cannula connected to a pressure transducer

linked to a polygraph recorder (Narco Biosystems, Houston, Texas, U.S.A.) for the measurement of mean arterial pressure (mmHg). Heart rate (b.p.m.) was recorded with a ratemeter driven by the pulse pressure signal.

Blood sampling

Following recording of blood pressure, 5 ml of blood were collected with heparin (10 iu ml^{-1}) used as anticoagulant. Samples were centrifuged for 10 min at 1000 *g* and 4°C. Calcium, phosphate, magnesium, sodium, potassium, creatinine, urea, cholesterol, triglycerides, glucose, protein, aspartate aminotransferase and alkaline phosphatase activities were determined by standard clinical chemistry methods.

Aortic ring preparation

Following blood sampling, a ring (3 mm) was dissected from the thoracic aorta and mounted under a tension of 0.8 g in Krebs bicarbonate solution (mM: NaCl 118, KCl 4.7, $MgCl_2 \cdot 6H_2O$ 1.2, NaH_2PO_4 1.0, $CaCl_2 \cdot 2H_2O$ 2.6, $NaHCO_3$ 25, glucose 11.1), at 37°C, oxygenated with 95% oxygen plus 5% carbon dioxide (pH 7.4 ± 0.1). Isometric tension was recorded by a force transducer (Ugo Basile, Comerio, Italy). After equilibration (90 min), cumulative doses of noradrenaline were added to contract the rings to approximately the same relative tension compared to the maximal response of the tissue in all groups. When contraction reached a plateau, carbachol (10^{-10} M to 10^{-4} M) was added in a cumulative manner. After several washes with Krebs bicarbonate and a 30 min recovery period, a cumulative dose-response curve was constructed for noradrenaline (10^{-9} M to 3×10^{-6} M). Responses to noradrenaline were evaluated on the basis of: (i) maximal increase in tension (mg), (ii) threshold sensitivity estimated from $ED_{0.1g}$, and (iii) midrange sensitivity estimated from $ED_{50\%}$.

After several washes with Krebs bicarbonate and a 30 min recovery period, cumulative doses of noradrenaline were added to precontract the ring as described above and sodium nitroprusside (10^{-11} M to 10^{-5} M) was added in a cumulative manner. Relaxation of the precontracted aortic ring by either carbachol or sodium nitroprusside is expressed as (i) maximal inhibition (%) of the noradrenaline-induced tension observed at the highest dose of carbachol or sodium nitroprusside used, and (ii) $IC_{50\%}$.

All experiments described in this paper were done in the presence of endothelium, thus the number of rats in each group is equivalent to that given in Table 1. In other experiments (not reported here), in the absence of endothelium carbachol had no effect, and the relaxant effect of sodium nitroprusside was slightly potentiated in all groups.

Mesenteric arterial bed preparation

Before removal of the thoracic aorta (for the aortic ring preparations, see above), the superior mesenteric artery was cannulated, the ileo-colonic branches tied off, the gut removed (McGregor, 1965), and the mesenteric arterial bed removed and perfused at 4 ml min^{-1} with Krebs bicarbonate. A bubble trap system, with a flow rate of 0.2 ml min^{-1}, continuously removed a small volume of perfusate plus any air bubbles. Vasoconstriction was estimated from the increase in perfusion pressure (mmHg) by use of a strain gauge transducer (Beckman, Palo Alto, California, U.S.A.). After a 15 min stabilization period, noradrenaline was injected (3 nmol in 0.33 ml, during 20 s) into the perfusate at 5 min intervals for 25 min, by which time a reproducible vasoconstrictor response was obtained. The following protocol was then used in half the rats. Firstly, a noradrenaline dose-response curve was constructed by injection of noradrenaline (0.33 ml in 20 s, 0.3 to 300 nmol) every 5 min. Vasoconstrictor responses to noradrenaline were evaluated on the basis of: (i) maximal increases in perfusion pressure (mmHg), (ii) threshold sensitivity estimated from ED_{10mmHg}, and (iii) midrange sensitivity estimated from $ED_{50\%}$. Secondly, following construction of the dose-response curve to noradrenaline, the mesenteric bed was perfused for 5 min with carbachol (10^{-9} M) then noradrenaline was injected, at a dose chosen on the basis of the noradrenaline dose-response curves, to produce an approximately equal increase in perfusion pressure relative to the maximal response in each group. The protocol was repeated with stepwise increases in the concentration of carbachol up to 10^{-4} M. Thirdly, following perfusion with Krebs bicarbonate for 15 min, the protocol was repeated with sodium nitroprusside (10^{-10} to 10^{-5} M). Relaxation is expressed as maximal attenuation (%) of noradrenaline-induced contraction, and $IC_{50\%}$.

The protocol was repeated in half the animals after the endothelium had been removed by passing air bubbles through the perfusion system (Story & Ziogas, 1986) before the construction of the noradrenaline dose-response curve.

Tissue calcium and magnesium levels

Samples of the left ventricle, thoracic aorta, abdominal aorta, carotid artery, mesenteric arterial bed, tail artery, femur, small intestine, cerebral cortex, eyes, kidneys and liver were weighed and heated to constant dry weight. Dry tissue samples were dissolved in nitric acid (14 N, 72 h) then centrifuged (2000 *g*, 10 min). Lanthanum chloride was added to the supernatant, and calcium and magnesium (μmol g^{-1} dry weight, except for femur: mmol g^{-1} dry weight) measured by atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, Connecticut, U.S.A.).

Substances used

Vitamin D_3 (Vi-Dé) was a gift of Sandoz AG, Bern, Switzerland. Nicotine bitartrate, noradrenaline bitartrate, carbachol chloride, sodium nitroprusside and ascorbic acid were purchased from Sigma, St. Louis, Missouri, U.S.A. Noradrenaline bitartrate was dissolved in 0.1 mM ascorbic acid. Concentrations or doses are given as base. Sodium pentobarbitone was purchased from Sanofi SA, Paris, France. Other chemicals were purchased from Merck AG, Darmstadt, Germany or Biomérieux, Marcy l'Etoile, France. Perindopril was a gift of Servier Laboratories, Paris, France.

Statistics

Results are expressed as means ± s.e.mean. Significant differences between groups were determined by ANOVA and the Scheffé test. The probability level chosen was $P < 0.05$. Dose-response data were fitted to the sigmoid curve equation: response = c/(1 + exp ((*a* − $\log_{10}$ dose)/b)) + d, where response is expressed as 'mmHg' or 'mg', a = $\log_{10}$ ($ED_{50\%}$), b = slope, c = response at [dose] = ∞, d = response at [dose] = 0, using a computer programme written by M. Bordes, IMPC, Sophia-Antipolis, France, based on Bevington (1969). Dose-response data were also subjected to linear regression analysis following logit (response %) versus $\log_{10}$ [concentration] transformation. Threshold and midrange sensitivities were calculated from the regression lines obtained.

Results

Body weight and plasma profile

Control rats gained weight (4 to 6 g day^{-1}) throughout the experiment (Table 1). During the first 6 days following vitamin D_3 and nicotine, rats lost weight at a rate of 8 to 10 g day^{-1}. From day 7 onwards rats gained weight at a rate of 6 to 9 g day^{-1}. This growth rate was greater than that of control rats during the corresponding period. Perindopril had no

Table 1 Effect of chronic treatment with perindopril (Perind, mg kg^{-1} per day) on body weight in vitamin D/nicotine-treated and control rats

Group	n	Perind	Start (g)	Day 6 (g)	Day 17 (g)	Growth (g day^{-1})
Control	19	—	241 ± 8	269 ± 7	322 ± 6	5.6 & 5.3
Vitamin D/nicotine-treated rats	22	—	239 ± 9	189 ± 3	265 ± 9	−10.0 & 7.6
Control	8	0.3	231 ± 12	258 ± 13	315 ± 11	5.4 & 5.7
Vitamin D/nicotine-treated rats	8	0.3	219 ± 9	173 ± 5	264 ± 5	−9.2 & 9.1
Control	16	1	238 ± 9	269 ± 10	319 ± 10	6.2 & 5.0
Vitamin D/nicotine-treated rats	16	1	234 ± 8	193 ± 6	267 ± 5	−8.2 & 7.4
Control	8	3	221 ± 10	240 ± 9	296 ± 9	3.8 & 5.6
Vitamin D/nicotine-treated rats	9	3	220 ± 5	171 ± 5	233 ± 8	−9.8 & 6.2

Growth = growth rates (g day^{-1}) from day 1 to day 5, and from day 7 to day 17.

effect on growth rate of control or vitamin D_3/nicotine-treated rats. Neither vitamin D_3 plus nicotine nor perindopril had any effect on plasma ions, metabolites or enzymes (Table 2, results for perindopril: 1 mg kg^{-1} only).

Tissue calcium and magnesium contents

Treatment with vitamin D_3 plus nicotine increased the calcium content of blood vessels, heart and kidney. The calcium content of the femur decreased. Chronic treatment with perindopril had no effect on soft tissue calcium content in either vitamin D_3/nicotine treated or control rats, but prevented the fall in bone calcium following vitamin D_3 plus nicotine treatment (Table 3). Magnesium levels did not change (results not shown).

Blood pressure and heart rate

Mean arterial pressure (113 ± 6 and 113 ± 5 mmHg) and heart rate (409 ± 12 and 406 ± 11 b.p.m.) were similar in vitamin D_3/nicotine-treated and control rats. Lower doses (0.3 and 1 mg kg^{-1}, per day) of perindopril had no effect on either blood pressure or heart rate. Perindopril at a dose of 3 mg kg^{-1} per day had no effect on blood pressure (vitamin D_3/nicotine-treated rats 103 ± 4, and control rats 105 ± 3 mmHg) but lowered heart rate (vitamin D_3/nicotine-treated rats 342 ± 10, and control rats 360 ± 14 b.p.m., $P < 0.05$).

Aortic rings

Noradrenaline produced dose-related increases in tension in aortic rings; maximum and sensitivity were lower in aortic rings from vitamin D_3/nicotine-treated rats (Figure 1). In control rats, perindopril, at the lower dose of 0.3 mg kg^{-1} had no effect, but at the higher doses of 1 or 3 mg kg^{-1}, produced a decrease in maximum (see Figure 1 for results with 1 mg kg^{-1}). Perindopril treatment had no significant effect in vitamin D_3/nicotine-treated rats, although a similar tendency was observed.

Relaxation induced by carbachol was attenuated by treatment with vitamin D_3 and nicotine (Figure 2). The percentage relaxation induced by a concentration of carbachol of 1 μM was 73 ± 5 in controls and 35 ± 10% in vitamin D_3/nicotine-treated rats ($P < 0.5$). Treatment with higher doses of perindopril restored the maximal vasorelaxant response to carbachol in vitamin D_3/nicotine-treated rats (for 1 mg kg^{-1} see Figure 2; 3 mg kg^{-1}: 85 ± 3%). Relaxation at a concentration of carbachol of 1 μM was similar to that of controls. For example, following chronic treatment with 1 mg kg^{-1} perindopril, values were 62 ± 9 in controls and 59 ± 13% in vitamin D_3/nicotine-treated rats.

Although vitamin D_3 and nicotine treatment had no effect on the relaxation induced by sodium nitroprusside (Figure 3), chronic treatment with perindopril, albeit without any effect on the maximal response, produced dose-related increases ($P < 0.05$) in $IC_{50\%}$ values: 1.3 ± 0.3, 4.0 ± 0.4, 8.6 ± 0.7 and 15.0 ± 1.5 × 10^{-9} M in control rats, and 2.6 ± 0.6, 5.5 ± 0.7,

Table 2 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day) on plasma ions, metabolites and enzymes in vitamin D/nicotine treated and control rats

	Controls	Vitamin D/nicotine	Controls	Vitamin D/nicotine
Perindopril	—	—	1	1
n	19	22	16	16
Calcium (mM)	2.45 ± 0.05	2.52 ± 0.01	2.41 ± 0.1	2.39 ± 0.09
Magnesium (mM)	1.02 ± 0.03	1.10 ± 0.03	1.10 ± 0.06	1.06 ± 0.04
Sodium (mM)	143 ± 3	144 ± 6	145 ± 5	138 ± 5
Potassium (mM)	3.93 ± 0.08	3.84 ± 0.08	3.83 ± 0.07	3.71 ± 0.09
Phosphate (mM)	1.84 ± 0.11	1.50 ± 0.09	1.79 ± 0.13	1.65 ± 0.14
Creatinine (μM)	82 ± 15	65 ± 6	66 ± 6	61 ± 3
Urea (mM)	6.2 ± 0.4	6.4 ± 0.8	6.9 ± 0.6	6.9 ± 0.4
Cholesterol (mM)	1.25 ± 0.08	1.43 ± 0.12	1.11 ± 0.05	1.26 ± 0.10
Triglycerides (mM)	0.78 ± 0.12	0.73 ± 0.18	0.72 ± 0.10	0.62 ± 0.03
Glucose (mM)	10.3 ± 0.7	8.7 ± 0.5	8.9 ± 0.9	8.7 ± 0.8
Protein (g l^{-1})	58 ± 3	53 ± 3	57 ± 2	54 ± 1
Aspartate aminotransferase activity (iu l^{-1})	75 ± 5	92 ± 10	85 ± 9	98 ± 7
Alkaline phosphatase activity (iu l^{-1})	304 ± 18	280 ± 37	272 ± 27	355 ± 48

Table 3 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day) on tissue calcium content (μmol g^{-1} dry weight) in vitamin D/nicotine-treated and control rats

	Controls	*Vitamin D/ nicotine*	*Controls*	*Vitamin D/ nicotine*
Perindopril	—	—	1	1
n	19	22	16	16
Thoracic aorta	10.9 ± 0.9	307 ± 77*	12.5 ± 0.7	416 ± 143*
Abdominal aorta	14.8 ± 0.8	333 ± 44*	15.6 ± 1.8	232 ± 54*
Carotid artery	34 ± 5	543 ± 95*	33 ± 6	426 ± 99*
Tail artery	27 ± 3	67 ± 9*	27 ± 5	88 ± 7*
Mesenteric arterial bed	1.3 ± 0.2	5.1 ± 1.0*	1.1 ± 0.1	3.8 ± 0.7*
Myocardium	4.0 ± 0.1	38 ± 6*	4.5 ± 0.2	47 ± 5*
Kidney	6.2 ± 0.9	105 ± 33*	5.5 ± 0.5	103 ± 12*
Intestine	6.5 ± 0.4	9.0 ± 1.5	6.8 ± 0.4	10.9 ± 2.0
Liver	2.9 ± 0.2	2.6 ± 0.3	2.6 ± 0.3	2.3 ± 0.3
Cerebral cortex	5.5 ± 0.9	5.1 ± 0.7	5.3 ± 0.7	4.7 ± 0.6
Eye	9.3 ± 0.4	10.6 ± 0.4	8.8 ± 0.3	10.0 ± 0.3
Femur diaphysis (mmol g^{-1})	5.5 ± 0.2	4.7 ± 0.2*	5.2 ± 0.2	5.4 ± 0.3
Femur epiphysis (mmol g^{-1})	5.1 ± 0.1	4.6 ± 0.2*	5.3 ± 0.2	5.2 ± 0.3

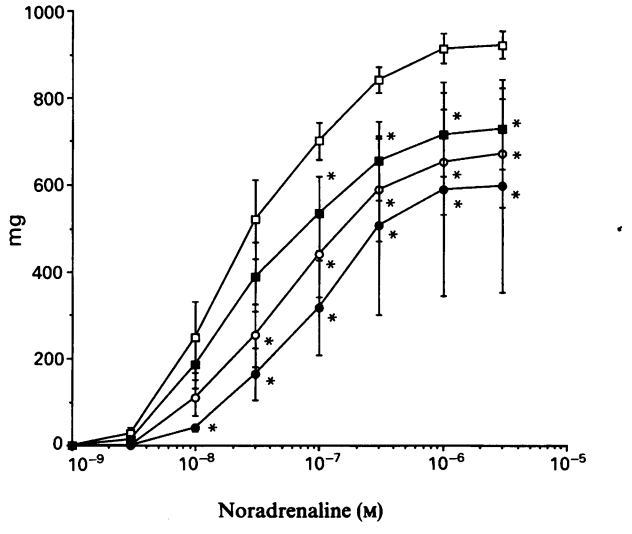
* $P < 0.05$ compared to means for control rats chronically treated with 0.5 M NaCl.

9.0 ± 1.5 and 12.5 ± 2.3 × 10^{-9} M in vitamin D_3/nicotine-treated rats, for doses of perindopril of 0, 0.3, 1 and 3 mg kg^{-1} per day, respectively.

Mesenteric arterial bed

Noradrenaline produced dose-related increases in perfusion pressure in mesenteric arterial beds perfused at a constant flow rate; maximum and sensitivity were lower in mesenteric arterial beds removed from rats previously treated with vitamin D_3 and nicotine (Figure 4). Perindopril had no effect in controls, but in mesenteric arterial beds from vitamin D_3/nicotine-treated rats the maximal response and sensitivity were increased at doses of 1 mg kg^{-1} (Figure 4) and 3 mg kg^{-1} per day. In all cases, removal of endothelium doubled mid-range sensitivity (for example in control rat preparations $ED_{50\%}$ decreased from 6.9 ± 0.8 to 2.9 ± 0.4 × 10^{-9} M, $n = 9$ per group, $P < 0.05$, following removal of endothelium), and slightly, but not significantly, increased the maximal response. The effects of chronic treatment with perindopril were proportionally similar to those observed in mesenteric arterial bed preparations with an intact endothelium.

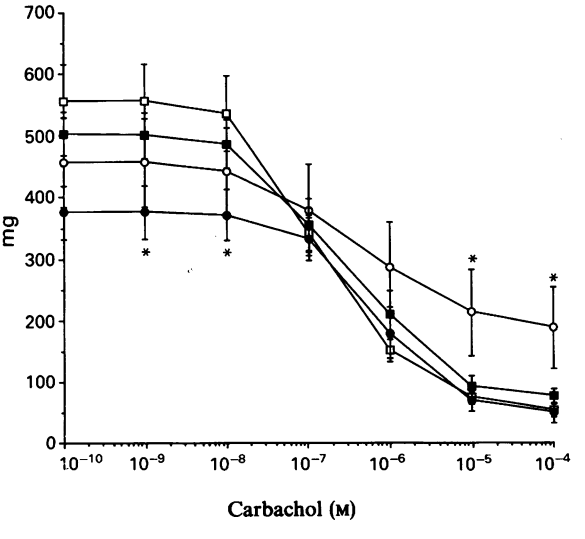
Relaxation induced by carbachol was decreased in vitamin D_3/nicotine-treated rats (Figure 5). Perindopril treatment had no effect in controls, but at doses of 1 (Figure 5) and 3 mg kg^{-1} per day, restored the maximal response to car-



Group	*Chronic treatment*	$ED_{50\%}$ (10^{-8} M)	*Maximum* (mg)
Control	0.15 M NaCl	1.6 ± 0.3	920 ± 30
Control	Perindopril	1.7 ± 0.3	730 ± 90*
Vitamin D/nicotine	0.15 M NaCl	4.3 ± 1.0*	670 ± 80*
Vitamin D/nicotine	Perindopril	8.4 ± 2.5*	600 ± 100*

* $P < 0.5$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

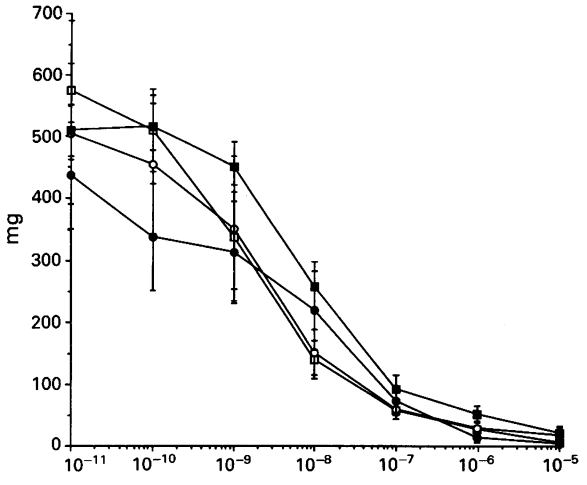
Figure 1 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on noradrenaline-induced contraction (mg) in aortic rings from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).



Group	*Chronic treatment*	$IC_{50\%}$ (10^{-7} M)	*Maximum* (%)
Control	0.15 M NaCl	1.5 ± 0.3	90 ± 2
Control	Perindopril	3.0 ± 0.7	87 ± 2
Vitamin D/nicotine	0.15 M NaCl	4.3 ± 1.0*	61 ± 9*
Vitamin D/nicotine	Perindopril	6.7 ± 2.0*	92 ± 5

* $P < 0.05$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

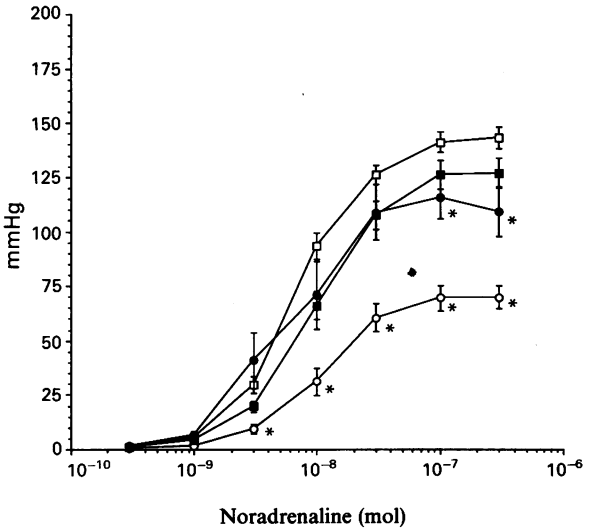
Figure 2 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on carbachol-induced relaxation of aortic rings precontracted with noradrenaline from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).



Group	Chronic treatment	$IC_{50\%}$ (10^{-9} M)	Maximum (%)
Control	0.15 M NaCl	1.4 ± 0.3	99 ± 2
Control	Perindopril	8.6 ± 0.7*	96 ± 2
Vitamin D/nicotine	0.15 M NaCl	1.6 ± 0.6	96 ± 3
Vitamin D/nicotine	Perindropril	9.0 ± 1.5*	98 ± 2

* $P < 0.05$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

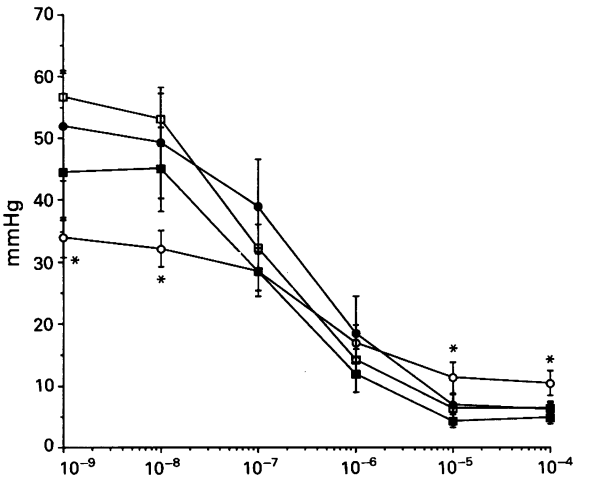
Figure 3 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on sodium nitroprusside-induced relaxation of aortic rings precontracted with noradrenaline from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).



Group	Chronic treatment	$ED_{50\%}$ (10^{-9} M)	Maximum (mmHg)
Control	0.15 M NaCl	6.9 ± 0.8	143 ± 5
Control	Perindopril	9.7 ± 1.0	127 ± 7
Vitamin D/nicotine	0.15 M NaCl	11.4 ± 2.2*	70 ± 5*
Vitamin D/nicotine	Perindopril	6.5 ± 0.6	116 ± 10*

* $P < 0.05$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

Figure 4 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on noradrenaline-induced vasoconstriction (mmHg) of mesenteric arterial beds removed from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).



Group	Chronic treatment	$IC_{50\%}$ (10^{-7} M)	Maximum (%)
Control	0.15 M NaCl	1.1 ± 0.3	89 ± 3
Control	Perindopril	3.0 ± 0.8	89 ± 4
Vitamin D/nicotine	0.15 M NaCl	4.5 ± 0.8*	69 ± 4*
Vitamin D/nicotine	Perindopril	6.7 ± 1.0*	88 ± 3

* $P < 0.05$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

Figure 5 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on carbachol-induced relaxation of mesenteric arterial bed preparations precontracted with noradrenaline from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).

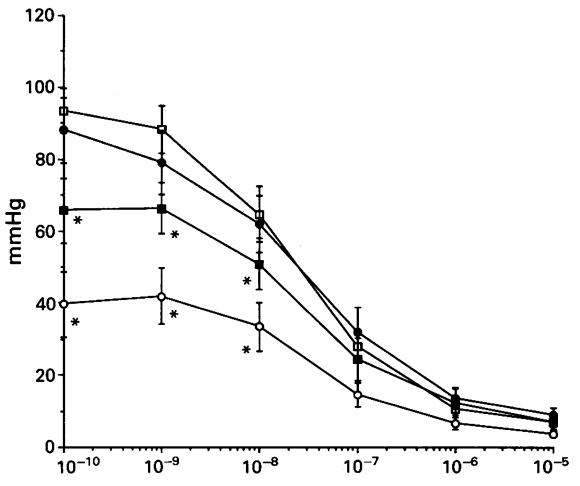
bachol in vitamin D_3/nicotine-treated rats. Following removal of endothelium, carbachol was without effect in mesenteric arterial bed preparations (results not shown).

Sodium nitroprusside-induced relaxation of mesenteric arterial bed preparations precontracted with noradrenaline was similar in controls and vitamin D_3/nicotine-treated rats (Figure 6). Perindopril treatment had no significant effect.

Discussion

Although body weight initially fell, vitamin D_3/nicotine-treated rats later showed a growth rate which was higher than that of controls. There were no signs of renal or other toxicity. Perindopril treatment had no effect on growth rate or measured plasma variables. Although the calcium content of the kidney increased 17 fold, plasma urea and creatinine levels in vitamin D_3/nicotine-treated rats were normal (Table 2; Waynforth, 1980). Endothelial function appeared to be modified in spite of normal plasma cholesterol levels, and this raises the possibility that vitamin D_3 plus nicotine treatment could provide one of the few models of altered endothelial function in the absence of any increase in the plasma level of cholesterol.

Calcium overloading of vessel walls presumably arises after the following sequence of events. Firstly, vitamin D, by increasing intestinal and renal calcium absorption (Guyton, 1991), and mobilizing bone calcium (present results) produces an initial hypercalcaemia (Thorin *et al.*, 1990). In the presence of increased extracellular calcium levels, nicotine causes intracellular accumulation of calcium, either directly (Nayler, 1963), or indirectly via release of catecholamines (Hass *et al.*, 1966). Intracellular accumulation of calcium is presumably lethal for the cell (Farber, 1981), and following cell death, extracellular deposition of calcium occurs (Seydewitz & Stau-



Group	Chronic treatment	$IC_{50\%}$ (10^{-8} M)	Maximum (%)
Control	0.15 M NaCl	2.4 ± 0.3	93 ± 3
Control	Perindopril	3.3 ± 0.3	89 ± 3
Vitamin D/nicotine	0.15 M NaCl	3.9 ± 0.5	90 ± 2
Vitamin D/nicotine	Perindopril	2.8 ± 0.4	90 ± 3

* $P < 0.05$ compared to means for control rats chronically treated with 0.15 M NaCl (□).

Figure 6 Effect of chronic treatment with perindopril (1 mg kg^{-1} per day, solid symbols) or 0.15 M NaCl (open symbols) on sodium nitroprusside-induced relaxation of mesenteric arterial bed preparations precontracted with noradrenaline from control rats (squares) or vitamin D_3/nicotine-treated rats (circles).

besand, 1988), especially in the media of the compliance vessels (Fleckenstein *et al.*, 1987; Henrion *et al.*, 1991). Calcium accumulation in the media may present a diffusion barrier for endothelium-derived relaxing factor (EDRF) and so explain the decrease in carbachol-induced relaxation. This hypothesis could be tested by superfusion experiments but appears unlikely as perindopril did not modify calcium accumulation and yet restored carbachol-induced relaxation.

The decrease in carbachol-induced relaxation could stem from a decrease of the affinity and/or number of the cholinoceptors on the endothelial cells following exposure to a high dose of nicotine. This appears unlikely as the relaxant properties of histamine are also diminished in aortic rings from vitamin D_3/nicotine-treated rats precontracted with noradrenaline ($IC_{50\%}$ 7.2 ± 0.3, controls 3.0 ± 0.2 μM, $n = 12$, $P < 0.05$, maximal relaxation 69 ± 5, controls 86 ± 1%, $P < 0.05$; Henrion *et al.*, unpublished results).

It is possible that calcium overloading of endothelial cells, produced by vitamin D_3 and nicotine, interferes with their intracellular calcium handling mechanisms and so diminishes release of EDRF, which is a calcium-dependent mechanism (Defeudis, 1985). Perindopril treatment could restore endothelial calcium handling mechanisms involved in carbachol-induced relaxation. Other changes in calcium handling are possible. Although vitamin D_3 plus nicotine produced calcium accumulation in small muscular arteries (tail artery) and in resistance vessels (mesenteric arterial bed) the rats did not become hypertensive. This raises the possibility that following vitamin D_3 plus nicotine treatment, calcium accumulates in smooth muscle cells at intracellular sites, such as the plasmalemma and the endoplasmic reticulum, and in some way interferes with the intracellular calcium handling. This may explain the decrease in the constrictor responses to noradrenaline in both the aortic rings and the mesenteric arterial bed preparations of vitamin D_3/nicotine-treated rats. Perindopril did not modify calcium accumulation in the mesenteric arterial bed of vitamin D_3/nicotine-treated rats but did restore noradrenaline-induced vasoconstriction (in both the presence and absence of endothelium). The effects of vitamin D_3 plus nicotine and/or perindopril treatment on calcium handling mechanisms could be investigated by following changes in intracellular free calcium levels with a fluorescent probe such as fura-2 (Thorin-Trescases *et al.*, 1990).

Converting enzyme inhibitors can modify prostanoid metabolism but the evidence as to whether or not such an effect is involved in cholinergic relaxation *in vitro* is contradictory. Indomethacin does not alter the potentiation by captopril of acetylcholine-induced relaxation of aortic rings (precontracted with noradrenaline) from normotensive rats (Schultz & Raij, 1989). Indomethacin did, however, decrease the ratio of 'relaxation divided by constriction' for 5-hydroxytryptamine in aortic rings from spontaneously hypertensive rats (Clozel *et al.*, 1990). The question as to whether or not chronic treatment with perindopril can prevent the changes in endothelial prostanoid metabolism induced by calcium overload could be investigated by using drugs such as indomethacin which interfere with prostanoid metabolism.

In conclusion, in both a compliance and a resistance vessel, treatment with vitamin D_3 and nicotine attenuated endothelial-dependent carbachol relaxation and perindopril treatment of these animals opposed this effect. This may be taken as evidence of a novel vascular action of angiotensin I converting enzyme inhibitors at the endothelial level.

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The effect of CCK_B/gastrin antagonists on stimulated gastric acid secretion in the anaesthetized rat

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1 The urethane-anaesthetized, vagotomised rat preparation was used to investigate the effects of the histamine H_2-antagonist ranitidine, the proton pump inhibitor omeprazole and the CCK_B/gastrin antagonists CI-988, PD 136450 and L-365,260 on pentagastrin-, histamine- and bethanechol-induced gastric acid secretion.

2 The novel CCK_B/gastrin antagonists CI-988 and PD 136450, and L-365,260 dose-dependently inhibited pentagastrin-induced secretion. The ED_{50} value for PD 136450 was 0.05 μmol kg^{-1}, the same following intravenous or subcutaneous administration.

3 CI-988 and PD 136450 administered subcutaneously at dose levels highly effective for antagonism of pentagastrin responses had no effect on basal acid secretion.

4 Ranitidine inhibited pentagastrin-, bethanechol-, and histamine-induced acid secretion, whereas the CCK_B/gastrin antagonists inhibited only the secretory response to pentagastrin.

5 The selective CCK_A antagonist, devazepide, was inactive at up to 300 μmol kg^{-1} i.p. against the three stimulants of acid secretion.

6 CI-988 and PD 136450 will be useful research tools with which to investigate the role of CCK_B/gastrin receptors in gastric acid secretion and the trophic activities of gastrin and cholecystokinin (CCK) on the gastrointestinal tract.

Keywords: CCK_A receptor; CCK_B receptor; gastrin receptor; vagotomised; gastric acid secretion; cholecystokinin

Introduction

The primary endogenous mediators of gastric acid secretion are acetylcholine, gastrin and histamine. Each of these secretagogues acts through its own receptor to trigger unique series of biochemical events within the parietal cell but the final step involves activation of the enzyme, H^+, K^+-ATPase, located in the membrane of intracellular tubulovesicles. As a result of this activation, potassium ions are reabsorbed from the lumen into the cytosol in exchange for the hydrogen ions that are excreted into the lumen of the canaliculi.

The interrelationship of the three physiological gastric secretagogues in the stimulation of gastric acid secretion remains unresolved. The physiological problem is in the relationship between gastrin and histamine, both being powerful stimulants of gastric acid secretion and both synthesized in the mucous membrane of the stomach. As early as 1938, MacIntosh had proposed that stimulation of the vagus resulted in the release of histamine, and Code (1965), and later Kahlson & Rosengren (1972) extended that idea to gastrin, making histamine the final common chemostimulant. However, many believe gastrin to be a direct hormone of secretion in its own right; thus, the question of the function and interrelationship of the three secretagogues in the stomach remains unsettled. The pharmacological problem at the basis of this has been the lack of antagonists for the gastrin receptor that have sufficient affinity and selectivity. Proglumide, a non-selective CCK receptor antagonist, blocks gastrin receptors (Magous & Bali, 1983), and has been used in the treatment of peptic ulcer disease in Europe and Japan (Rovati, 1976; Weiss, 1979). However, neither the specificity nor the affinity of proglumide has been sufficient to permit its use *in vivo* to define the role of gastrin in the regulation of gastric acid secretion. In recent years other CCK receptor antagonists from several chemical classes have been developed, and although there are now available such benzodiazepines as devazepide (MK-329, L-364,718 Chang & Lotti, 1986; Evans *et al.*, 1986) with high affinity and high selectivity for the CCK_A class of receptor found predominantly, though not exclusively in peripheral organs, and L-365,260 (Lotti & Chang, 1989; Bock *et al.*, 1989) which is relatively selective for the CCK_B ('brain') receptor and the gastrin receptor, no antagonist is known that discriminates between the CCK_B and gastrin receptor. More recently, non-peptide, non-benzodiazepine compounds, CI-988 (PD 134308) and PD 136450 have been described that have affinity at the CCK_B/gastrin receptor comparable to that seen with L-365, 260, but are more selective for the CCK_B receptor over the CCK_A receptor (Hughes *et al.*, 1990; Horwell *et al.*, 1991). While the antisecretory effects of L-365,260 have been described in several animal models of acid secretion (Lotti & Chang, 1989), the activity of the more selective compounds has not yet been reported. The importance of such studies with the CCK_B/gastrin antagonists is not only for an examination of their potential utility as anti-secretory agents, but also as possible adjuncts to therapies with currently available agents that carry the attendant risk of hypergastrineamia that is a consequence of the achlorhydria produced by long-term antisecretory therapy.

In the present study, an *in situ* perfused stomach preparation of the rat has been used for the quantitative investigation of the effect of the selective CCK/gastrin receptor antagonists devazepide, L-365,260, CI-988 and PD 136450 on gastric acid secretion evoked by pentagastrin, histamine or bethanechol. A preliminary account of these findings has been presented to the British Pharmacological Society (Hayward *et al.*, 1991).

Methods

Anaesthetized rat perfused stomach preparation

The perfused stomach preparation was that described by Parsons (1969) as a modification of the Ghosh & Schild (1958) method. Briefly, male Wistar rats (180–220 g) were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and the trachea and one or both jugular veins were cannulated. The stomach was perfused with 5.4% (w/v) glucose in water (37°C) at a rate of

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3 ml min^{-1} via perspex cannulae in the oesophagus and pyloric antrum; the perfusate issued through a perspex funnel in the non-secretory lumen and passed over a conventional pH electrode recording continuously onto a flatbed chart recorder. The cannula in the oesophagus was placed below the diaphragm and the oesophagus was cut.

In preliminary experiments to determine doses of the separate agonists that produce comparable, submaximal levels of acid output, the dose-effect relationship with pentagastrin, histamine or bethanechol was determined. Agonists were infused intravenously at a constant rate (1 ml h^{-1}) and the dose increased cumulatively when the effect of the preceding dose had reached a peak. The response of a given dose was measured as hydrogen ion concentration at the peak of the response minus basal hydrogen ion concentration. Hydrogen ion concentration at any time of interest was measured as the negative antilogarithm of the pH at that time.

For the work with antagonists only one dose of blocker was tested in each preparation. Once basal acid secretion was stable submaximal doses of either pentagastrin (1 $\mu g\,kg^{-1}\,min^{-1}$), histamine (30 $\mu g\,kg^{-1}\,min^{-1}$) or bethanechol (6 $\mu g\,kg^{-1}\,min^{-1}$) were administered intravenously via the right jugular vein. The stimulation of acid secretion produced by the secretagogue was allowed to reach a plateau for about 30 min before the dose of antagonist was administered by intravenous injection via the left jugular vein, or subcutaneously into the right flank. Acid secretion continued to be monitored for as long as possible after drug administration. Results are expressed as percentage inhibition of secretagogue-induced gastric acid secretion:

$$\% \text{ inhibition} = \left(\frac{\text{reversal-basal}}{\text{stimulated-basal}}\right) \times 100 - 100$$

where:

inhibition = hydrogen ion concentration ($[H^+]$) at the point of maximal inhibition of stimulated acid secretion by the compound under test

stimulated = $[H^+]$ at the stable level of stimulated acid secretion

basal = unstimulated $[H^+]$

In those experiments where the antagonists had little or no effect on stimulated acid secretion the preparation was validated at the end of the day by testing for complete block of stimulated acid secretion by administering the H_2-antagonist, ranitidine (1 $\mu mol\,kg^{-1}$ s.c./i.v.). No preparations were rejected on these grounds.

ED_{50} values with 95% confidence limits were calculated for inhibition of gastric acid secretion. At least four rats were used for each dose group.

Compounds

The following compounds were used: atropine sulphate (Sigma Chemical Co. Ltd.), carbamyl-β-methylcholine chloride (Sigma Chemical Co. Ltd.), histamine dihydrochloride (Sigma Chemical Co. Ltd.), pentagastrin (Bachem), ranitidine hydrochloride (Sigma Chemical Co. Ltd), omeprazole (Astra). CI-988 (PD 134308), [**R** − (**R***, **R***)]-4-[[2-[[3-(1H-Indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.$1^{3,7}$]dec-2-yloxy)carbonyl]-amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoic acid, and PD 136450, [**R** − (**R***, **R***)]-4-[[2-[[3-(1H-Indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.$1^{3,7}$]dec-2-oxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxo-2-butenoic acid, were synthesized in the Department of Medicinal Chemistry, Parke-Davis Neuroscience Research Centre, Cambridge, UK. Devazepide (MK-329, L-364,718 Evans *et al.*, 1986) and L-365,260 (3**R**(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N′-(3-methyl-phenyl)urea; Bock *et al.*, 1989) were synthesized in the Department of Medicinal Chemistry, Parke-Davis Ann Arbor, U.S.A. All compound doses are expressed in terms of free base.

Pentagastrin, histamine dihydrochloride and carbamyl-β-methylcholine chloride (bethanechol) were stored as frozen aliquots of 0.1 mg ml^{-1}, 10 mg ml^{-1}, and 1 mg ml^{-1} in 0.9% (w/v) NaCl respectively and diluted to the required concentration with 0.9% w/v NaCl. CI-988, PD 136450, ranitidine and atropine were dissolved in 0.9% w/v NaCl with brief sonication. L-365,260 and devazepide were made up fresh in 10% (v/v) ethanol in 90% carboxymethyl cellulose/Tween 80 (aqueous suspension, 1% w/v with respect to carboxymethyl cellulose, 0.1% w/v with respect to Tween 80). Omeprazole was made up fresh in distilled water with brief sonication. CI-988, PD 136450, ranitidine, atropine and omeprazole were administered intravenously or subcutaneously in a dose volume of 1 ml kg^{-1}, whilst L-365,260 and devazepide due to their poor solubility were administered via the intraperitoneal route in a dose volume of 4 ml kg^{-1}.

Results

Stimulation of gastric acid secretion

In preliminary experiments the dose-response relationship was established for the secretagogues pentagastrin, histamine and bethanechol. All three secretagogues caused a dose-dependent increase in gastric acid secretion. Doses of histamine (30 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) and bethanechol (6 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) that were on the linear portion of their log dose-response curves, and that produce increases in acid output comparable to that obtained with pentagastrin (1 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) were used to determine the specificity of CI-988 and PD 136450 as antagonists of pentagastrin *in vivo* (Figure 1). Each secretagogue stimulated a maintained plateau of acid output; pentagastrin and histamine 1 h following the start of infusion gave acid output levels of 1113.1 ± 413.5 and 1415.4 ± 159.0 $\mu mol\,l^{-1}\,H^+$, and at 3 h gave acid output levels of 866.4 ± 422.1 and 1254.2 ± 184.2 $\mu mol\,l^{-1}\,H^+$ respectively. In the case of bethanechol, stable levels of acid output were obtained only after 2 h following the start of infusion, the level being 1572 ± 486.6 $\mu mol\,l^{-1}\,H^+$; at 4 h the acid output was 1767.9 ± 290.6 $\mu mol\,l^{-1}\,H^+$.

Effects of CCK/gastrin antagonists, ranitidine, omeprazole and atropine on stimulated gastric acid secretion

In the anaesthetized rat preparation, CI-988 powerfully inhibited pentagastrin-induced gastric acid secretion following intravenous administration (Figure 2). CI-988, PD 136450, the H_2-antagonist ranitidine, the proton pump inhibitor omeprazole, administered subcutaneously, and the CCK_B antagonist L-365,260 following intraperitoneal administration, were all able to antagonize dose-dependently pentagastrin-induced

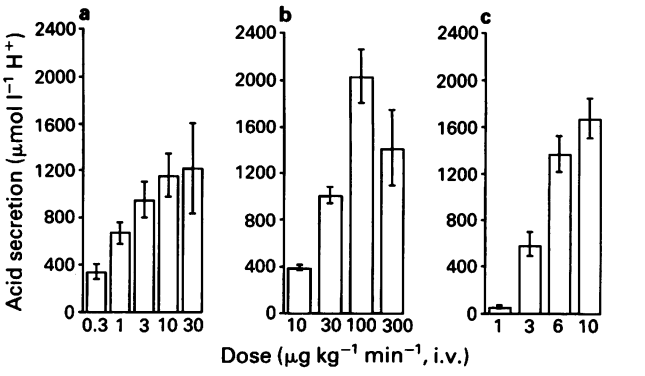


Figure 1 Histograms illustrating the dose-related stimulant effect of pentagastrin (a), histamine (b), and bethanechol (c) on gastric acid secretion. (Each column is the mean of 4–6 observations. Vertical bars represent s.e.mean).

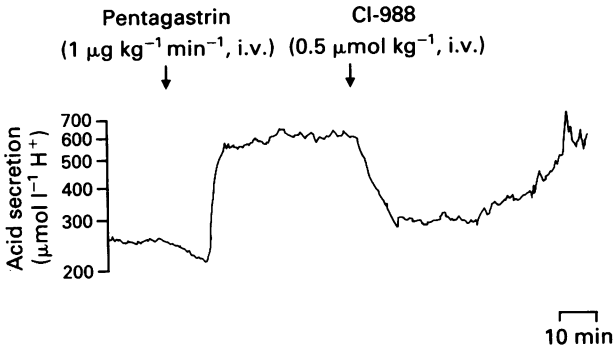


Figure 2 The effect of CI-988 (0.5 μmol kg^{-1}, i.v.) on pentagastrin (1 μg kg^{-1} min^{-1}, i.v.)-stimulated gastric acid secretion in the anaesthetized rat.

secretion (Figure 3). On a molar basis PD 136450 was found to be 3.8, 9.4, and 32.2 times more active than ranitidine, omeprazole and L-365,260 respectively (Table 1).

PD 136450 was as active at inhibiting pentagastrin-stimulated acid secretion following intravenous administration (ED_{50} 0.052 (0.01–0.26) μmol kg^{-1}), as after subcutaneous administration; thus indicating good absorption of the compound following subcutaneous administration. Ranitidine on the other hand was approximately 3 times more potent when administered intravenously compared to subcutaneously with an ED_{50} value of 0.067 (0.02–0.20) μmol kg^{-1}.

After subcutaneous administration, at dose levels highly effective for antagonism of the pentagastrin response (1–100 μmol kg^{-1}), neither CI-988 nor PD 136450 had any significant effect upon basal acid secretion in this model. All other antagonists tested had no effect upon basal acid secretion.

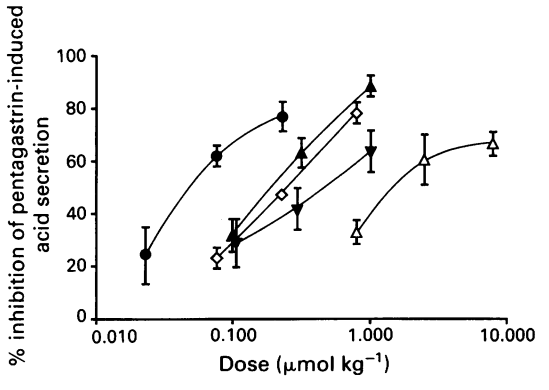


Figure 3 The effect of CI-988 s.c. (◇), PD 136450 s.c. (●), L-365,260 i.p. (△), ranitidine s.c. (▲) and omeprazole s.c. (▼) on pentagastrin-induced acid secretion in the anaesthetized rat. (Each point is the mean of 4–5 observations. Vertical lines represent s.e.mean).

As shown in Table 1, at doses 100 times their respective ED_{50} value against pentagastrin, the CCK_B/gastrin antagonists L-365,260, CI-988 and PD 136450 were without effect on gastric acid secretion by either histamine or bethanechol, thus highlighting the selectivity of the former compounds for the CCK_B/gastrin receptor. As expected, the proton pump inhibitor omeprazole, which inhibits the enzyme H^+, K^+-ATPase involved in the final step in the secretion of hydrogen ions in the stomach, and interestingly, the H_2-antagonist ranitidine, dose-dependently inhibited gastric acid secretion produced by either histamine or bethanechol with values for ED_{50} comparable to those against pentagastrin-induced acid secretion.

The selective CCK_A antagonist, devazepide, was found to be inactive at up to 300 μmol kg^{-1}, i.p. against the three stimulants of acid secretion (Table 1).

Discussion

Recently, non-peptide agents of the benzodiazepine structure with high affinity for peripheral CCK_A receptors and only low affinity for gastrin and CCK_B receptors have been described (Chang *et al.*, 1985; Chang & Lotti, 1986). The related compound with the reverse profile of affinity, L-365,260, was subsequently described (Bock *et al.*, 1989; Lotti & Chang, 1989). The present paper describes the effects of the new, non-benzodiazepine compounds, CI-988 and PD 136450, that exhibit high affinity for the stomach gastrin and brain CCK_B receptors, relative to the peripheral, pancreatic CCK_A receptor (Hughes *et al.*, 1990). CI-988 and PD 136450 are antagonists at CCK_B receptors in the brain, as highlighted by the observation that both compounds inhibit responses to agonists at this receptor in the ventromedial nucleus of the hypothalamus of the rat (Hughes *et al.*, 1990; Boden & Pinnock, personal communication).

CI-988 and PD 136450 inhibited pentagastrin-induced acid secretion, confirming the view that they are antagonists at the 'gastrin receptor'. The potency of CI-988 was comparable to that of the H_2-antagonist ranitidine, while PD 136450 was 4 times more potent, with an identical ED_{50} value when administered subcutaneously or intravenously. The intraperitoneal administration of the CCK_B/gastrin antagonist L-365,260 was active in producing a dose-dependent block of pentagastrin-induced acid secretion in the anaesthetized rat, confirming observations of Lotti & Chang (1989) in the anaesthetized mouse and conscious rat following oral or intraduodenal administration. The effect of this CCK_B/gastrin antagonist was relatively weak compared to PD 136450, whilst the selective CCK_A antagonist devazepide was inactive at up to 300 μmol kg^{-1} against the three stimulants. This latter observation is in keeping with the low affinity of devazepide for both the gastrin, and brain CCK_B receptors (Chang & Lotti, 1986; Evans *et al.*, 1986). However, our results with devazepide are in conflict with those in a similar study by Hirst *et al.* (1991). These authors observed that L-365,260 and devazepide are both active at a dose of 10 μmol kg^{-1}, administered intravenously, in blocking acid secretion by either pentagastrin or

Table 1 The effect of various compounds on pentagastrin, bethanechol, and histamine stimulated gastric acid secretion

	ED_{50} (95% confidence limits) μmol kg^{-1}		
Compound	*Pentagastrin*	*Histamine*	*Bethanechol*
CI-988	0.25 (0.06–1.05)	>25	>25
PD 136450	0.05 (0.01–0.26)	>5	>5
L-365,260	1.61 (0.61–4.21)	>160	>160
Devazepide	>300	>300	>300
Ranitidine	0.19 (0.11–0.36)	0.25 (0.03–2.30)	0.20 (0.08–0.49)
Omeprazole	0.47 (0.17–1.33)	0.23 (0.03–1.96)	0.19 (0.09–0.40)
Atropine	>0.2	>0.2	0.0021 (0.0003–0.0118)

All compounds were administered subcutaneously, except devazepide and L-365,260 which were administered via the intraperitoneal route. (Each value represents the mean with 95% confidence limits in parentheses).

histamine. The model used in the latter study is almost identical, to that in the present study, but in their case with the vagus nerve trunks kept intact. It may be that this fact alone is sufficient to reveal a role for the CCK_A receptor in affecting acid secretion induced by either pentagastrin or histamine. It is curious however, that in their hands, even with intravenous administration, L-365,260 is comparatively weak as a blocker of the response to pentagastrin (cf. ED_{50} value after i.p. administration in the present study, was only $1.6\,\mu mol\,kg^{-1}$).

Our studies confirm the findings by Bunce & Parsons (1978), in the conscious vagotomized rat, that H_2-antagonists inhibit gastric acid secretion in response to pentagastrin, histamine and bethanechol. The selectivity of CI-988 and PD 136450 was highlighted by the observation that they inhibited the response to pentagastrin, without altering the responses to histamine and bethanechol, in the vagotomised, anaesthetized rat at least.

At present there are two hypotheses for the regulation of gastric acid secretion; the 'permissive' and 'transmissive' theories. The permissive theory states that parietal cells express acetylcholine, gastrin and histamine receptors on their surface but that gastrin acting at its receptor is only fully effective if histamine has already occupied its own receptor, that is, gastrin acts by 'permission' of histamine (Grossman & Konturek, 1974; Soll, 1977). Our studies in the anaesthetized rat, confirm this view since ranitidine inhibited equally the acid secretion by either pentagastrin or histamine, whereas CI-988 and PD 136450 selectively inhibited the responses to pentagastrin alone. However, the corollary to this hypothesis is that pentagastrin will be inactive in the absence of histamine secretion. An interesting finding then, is that pentagastrin is fully active on the *in vitro* mouse-stomach preparation under standardized conditions where there is no pharmacological evidence that histamine secretion occurs (Black & Shankley, 1985). However, the ability of the supplementation by exogenous histamine to restore partially the response to pentagastrin in mast cell-deficient mice (Stechschulte *et al.*, 1990) suggests that gastrin requires the presence of histamine to act effectively as a secretagogue in the mouse. The ability of ranitidine to block pentagastrin-induced acid secretion as shown in this study also argues for the effect of gastrin on histamine-containing cells and against a significant direct effect of gastrin on parietal cells; the transmissive theory. This theory states that blood-borne gastrin and neurally-released acetylcholine acting on histamine-secreting cells located adjacent to the parietal cells release histamine, which then acts on parietal cells; thus histamine 'transmits' the actions of gastrin and the vagus (MacIntosh, 1938; Code, 1965; Kahlson & Rosengren, 1972). Earlier studies have demonstrated the ability of gastrin or pentagastrin to initiate histamine release from gastric mucosa (Ekblad, 1985) and mast cells (Tharp *et al.*, 1984), thus confirming the view that part of the gastric acid secretion effect of gastrin is through histamine. In the rat, histamine is stored in endocrine-like cells (Hakanson *et al.*, 1976) and gastrin stimulates the formation of histamine by inducing the activity of histidine decarboxylase (Kahlson & Rosengren, 1968; Hakanson & Leidberg, 1970) and diminishes gastric mucosal histamine (Haverback *et al.*, 1964; Bunce & Parsons, 1977; Main & Pearce, 1982).

Just as CCK_B/gastrin antagonists were seen to be selective for the one secretagogue, pentagastrin, atropine was selective in blocking acid secretion induced by the muscarinic agonist bethanechol, confirming earlier studies (Albinus & Sewing, 1969; Hedges & Parsons, 1977). Anticholinoceptor agents as well as H_2-antagonists have been shown to possess specific actions on the rabbit isolated parietal cell against cholinoceptor agonists and histamine, respectively (Soll & Berglindh, 1987). It has also been shown that ranitidine does not inhibit the action of bethanechol at muscarinic receptors in isolated guinea-pig ileum even when tested at concentrations greatly in excess of those required to block H_2-receptors (Daly *et al.*, 1981). These findings are thus at odds with those described in this study and with other *in vivo* observations (Brimblecombe *et al.*, 1975; Parsons, 1975) that H_2-antagonists block cholinergically stimulated secretion as well as that by histamine itself. Also, gastrin and acetylcholine have been shown to cause release of histamine from rabbit gastric glands (Bergqvist *et al.*, 1980). Thus, the powerful inhibition by ranitidine on bethanechol-induced acid secretion may imply that part of the stimulant effect of bethanechol on acid secretion is due to the release of endogenous histamine; thus suggesting the pivitol role of histamine in the control of acid secretion. The apparent contradiction may reflect the influences of the endogenous stimulants and their interactions (Soll & Berglindh, 1987) as observed in the intact animal.

Species differences are apparent among the different models that have been used to explore the regulation of acid secretion. In the dog, the cholinergic pathway seems to be of great importance since atropine is able to block not only the powerful effect of cholinomimetics to stimulate acid secretion, but also the response to histamine and pentagastrin. In the rabbit on the other hand, as in the vagotomized rat, highlighted in this study, the potent stimulant effects on acid secretion of pentagastrin and histamine are not inhibited by atropine (Albinus & Sewing, 1969; Hedges & Parsons, 1977; Muluihill *et al.*, 1985).

In conclusion, the present studies demonstrate that the selective CCK_B/gastrin antagonists CI-988 and PD 136450 potently inhibit pentagastrin-stimulated gastric acid secretion without having any efficacy by themselves on acid secretion. The inability of both compounds to antagonize either histamine- or bethanechol-induced acid secretion, compared to the H_2-antagonist ranitidine, and the proton pump inhibitor omeprazole, supports the view that, for the anaesthetized, vagotomized rat at least, pentagastrin stimulates acid secretion, indirectly via histamine. Additionally however, the data do not disprove a permissive role for otherwise subthreshold levels of histamine. Although this model is useful for demonstrating antisecretory activity, a conscious animal model would be more useful in elucidating the physiological control of acid secretion, since maximal acid output in the anaesthetized rat is considerably lower than in conscious rats.

CI-988 and PD 136450 have provided us with research tools to investigate the role of CCK_B receptors in the control of anxiety (Hughes *et al.*, 1990); in gastric acid secretion and the trophic activities of gastrin and CCK on the gastrointestinal tract, with their potential application in the prevention or treatment of gastrointestinal carcinoids. Moreover it may be possible to use these agents to block the rebound effect whereby gastrin levels rise when patients are on long term anti-secretory therapy and there are hazards in producing achlorhydria, such as gastrointestinal carcinoids.

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Potentiation by endothelin-1 of 5-hydroxytryptamine-induced contraction in coronary artery of the pig

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1 In order to elucidate the physiological and potential pathological roles of endothelin-1 (ET-1) in coronary artery contraction and relaxation, we undertook the present study to examine the action of ET-1 itself, and the combined effects of ET-1 with vasoconstrictor agonists such as acetylcholine (ACh), histamine, and 5-hydroxytryptamine (5-HT), all of which have been implicated in the genesis of coronary spasm.

2 Isometric tension and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a ring segment of porcine coronary artery loaded with fura-2 were measured simultaneously.

3 ET-1 contracted the artery in a concentration-dependent manner; and nisoldipine, a Ca^{2+} channel blocking drug of the 1,4-dihydropyridine type, antagonized the ET-1 action non-competitively. A radioreceptor binding assay also indicated the mutually exclusive binding of ET-1 and (+)-[^{3}H]-PN200-110, a Ca^{2+} channel ligand, to the membrane fraction of porcine coronary artery.

4 ET-1 (10–100 pM) increased tension and $[Ca^{2+}]_i$ in a parallel manner, while at higher concentrations (1–10 nM) it produced further contraction with a small increase in $[Ca^{2+}]_i$.

5 ET-1 (30–100 pM) selectively potentiated the 5-HT-induced contraction 1.5 to 2 times over the control without causing a significant increase in $[Ca^{2+}]_i$, which seems to be qualitatively similar to a tumour promoting phorbol ester, 12-deoxyphorbol 13-isobutylate (DPB). Bay K 8644 (10 nM), on the other hand, potentiated the contraction in response to practically all agonists used and affected a concomitant increase in $[Ca^{2+}]_i$.

6 A Ca^{2+} channel blocking drug such as diltiazem abolished the increase in $[Ca^{2+}]_i$ and partially attenuated the mechanical potentiation produced by a small amount of ET-1 in combination with 5-HT.

7 The results suggest that ET-1 and 5-HT interact functionally at the cellular or subcellular level and modulate the Ca^{2+} sensitivity of the contractile elements through the possible activation of protein kinase C.

Keywords: Endothelin; coronary artery; 5-hydroxytryptamine; contraction; cytosolic Ca^{2+} concentration; fura-2

Introduction

Endothelin-1 (ET-1), a 21-residue peptide originally isolated from vascular endothelial cells, exhibits potent vasoconstrictor activity (Yanagisawa *et al.*, 1988). In pig coronary artery, ET-1-induced contraction was shown to be greatly attenuated by Ca^{2+} antagonists of the 1,4-dihydropyridine type (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). Moreover, ET-1 increased the voltage-dependent Ca^{2+} channel current (Goto *et al.*, 1989) and phosphoinositide (PI) hydrolysis (Kasuya *et al.*, 1989a; Lee *et al.*, 1989; Danthuluri & Brock, 1990). It has also been reported that ET-1 produces endothelium-dependent vascular relaxation (De Nucci *et al.*, 1988; Sakata *et al.*, 1989).

Although ET-1 immunoreactivity is widely detected in various tissues including those of the central nervous system and kidney, the observations on vascular reactivity have led to the idea that ET-1 may act as an endogenous modulator of vascular tone. Most studies on the action of ET-1 in biological systems have employed relatively large concentrations of the peptide (nM to μM). If ET-1 is released locally and plays an important role in the cardiovascular system under physiological and pathological conditions, it is of particular significance to know how ET-1 at lower concentrations (pM to nM) can act *per se*, or if, at these lower levels, it modulates the actions of other vasoconstrictor stimuli on cardiovascular functions. It has been reported that a low dose of ET-1 sensitized vascular smooth muscle to various agents including biogenic amines such as noradrenaline, histamine and 5-hydroxytryptamine (5-HT) as well as adenosine 5'-triphosphate (ATP), vasopressin (La *et al.*, 1990; Aitkenhead *et al.*, 1990), neuropeptide Y (MacLean & McGrath, 1990), clonidine, and Bay K 8644, a Ca^{2+} agonist of the 1,4-dihydropyridine type responsible for the opening of Ca^{2+} channels (Godfraind *et al.*, 1989). Of various endogenous substances, 5-HT (Ashton *et al.*, 1986), histamine (Shimokawa *et al.*, 1983), and acetylcholine (ACh) (Yasue *et al.*, 1976) have been particularly implicated in the genesis of the coronary vasospasm of Prinzmetal's (variant) angina pectoris (Fleckenstein, 1983).

The present study was thus undertaken to improve our understanding of the effects of ET-1 on coronary artery contraction and relaxation. In this study, the mechanical activity and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured simultaneously (Nakayama & Tanaka, 1988; 1989). Since tumour promoting phorbol esters mimic 1,2-diacylglycerol (DAG)-like action and activate protein kinase C (PKC) directly (Castagna *et al.*, 1982), we compared the actions of ET-1 with those of 12-deoxyphorbol 13-isobutylate (DPB), a phorbol ester, and Bay K 8644, on the tension-$[Ca^{2+}]_i$ relationship in isolated porcine coronary artery.

Methods

Isolation of arteries

A main branch of the anterior descending coronary artery, (outer diameter of about 3 mm), was carefully isolated from healthy pig hearts dissected within 15 min after slaughter of the animals. The pigs were of Yorkshire strain, either sex, 6–8 months old and weighed about 100 kg. The coronary arteries were immersed in Tyrode solution at 4°C, oxygenated with

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95% O_2 and 5% CO_2, and transferred to the laboratory. The arteries were cleared of connective tissue and adventitia under a dissection microscope and cut into ring segments about 2 mm wide. In experiments designed to examine the effect of removal of endothelium, the intimal layer of the artery was rubbed with a moist cotton pledget. Repeated histological examination of the intimal surface of such arteries by scanning electron microscopy has indicated the absence of endothelial cells (Nakayama, 1988). The effectiveness of the endothelial removal was established by the absence of relaxation of the preconstricted artery to the endothelium-dependent relaxant, substance P.

Recording of isometric tension

Two L-shaped tungsten wires (150 μm diameter) were inserted through the lumen of the rings. The lower wire was attached to a supporting hook and the upper one was connected to the lever of a mechanoelectric transducer made in our laboratory. Each artery was mounted in an organ bath containing 10 ml of Tyrode solution (mM: NaCl 158.3, KCl 4.0, $NaHCO_3$ 10.0, NaH_2PO_4 0.42, $CaCl_2$ 2.0, $MgCl_2$ 1.05 and glucose 5.6). Isotonic high-K^+ Tyrode solution was prepared by replacing the NaCl by an equimolar amount of KCl. Ca^{2+}-free Tyrode solution was prepared by omitting $CaCl_2$ and adding 0.2 mM EGTA. The solution was bubbled with 95% O_2 and 5% CO_2 and maintained at a pH of 7.35 at 35°C. The coronary artery in the organ bath was allowed to equilibrate for 1.5 h under an optimal basal tension of 3.0 g before the actual experiments were started. Isometric tension measured with a force transducer was displayed on a penwriting recorder (R-52, Rikadenki, Tokyo, Japan).

Effects of antagonists on contractions produced by agonistic stimuli such as 5-HT, histamine, ACh, and Bay K 8644 were examined. The cumulative increase in concentrations of each agonist produced a concentration-dependent contraction. The pD_2 is the negative logarithm of the agonist concentration required to produce half the maximum response. The maximum contraction was taken as 100% in each preparation. After an incubation period of 30 min for each antagonist, a concentration-response curve for each agonistic stimulus was again obtained. The values of pA_2 and slope of Schild's plot were obtained according to the definition of Arunlakshana & Schild (1959).

The amplification phenomena described here comprises two elements; (1) augmentation, an increase in response amplitude at a given agonist concentration resulting in an increase in the agonist curve maximum with only a small effect of pD_2; (2) sensitization, an apparent increase in agonist potency which is manifest as an increase in pD_2, in this case accompanied by a change in curve maximum (Poech & Holzman, 1980; Leff & Morse, 1987).

Cytosolic Ca^{2+} level measured simultaneously with mechanical activity

Artery segments cleared from both adventitia and endothelium were loaded with 5 μM fura-2-AM for 4 to 5 h at room temperature in Tyrode solution containing 5 mg ml^{-1} albumin and a noncytotoxic detergent, Cremophor EL (0.5%). After the fura-2 loading, each segment was rinsed with normal Tyrode solution for 15 min, mounted in a temperature-controlled quartz glass chamber (7 ml), and perfused with normal Tyrode solution. $[Ca^{2+}]_i$ and isometric tension development were recorded simultaneously, the former with a fluorometer (CAF-100, Japan Spectrophotometric, Tokyo, Japan). U.v. excitation light obtained from a xenon high-pressure lamp (340 nm, F_{340}; and 380 nm, F_{380}; each within ± 5 nm) was focused on the artery and the corresponding emission signals (500 ± 10 nm) as well as the ratio signal (F_{340}/F_{380}), referred to as R, a measure of $[Ca^{2+}]_i$, were monitored. The emitted signals from the artery were collected into a photomultiplier through a 500 ± 10 nm filter. The time constant of the optical system was 0.25 ms. Two L-shaped tungsten wires (150 μm) were inserted through the lumen of the ring segment that was horizontally mounted in the organ bath. One end of each wire was anchored and the other end was connected to a force displacement transducer. All recordings were displayed on a multi-pen recorder.

Absolute $[Ca^{2+}]_i$ was calculated by the ratio method originally described by Grynkiewicz *et al.* (1985) and modified by Himpens *et al.* (1989). The following equation was used: Cytosolic Ca^{2+} concentration $= K_D \times B \times [(R - R_{min})/(R_{max} - R)]$, where K_D is the dissociation constant of fura-2 for Ca^{2+}, assumed to be 224 nM *in vivo* (Grynkiewicz *et al.*, 1985), and B is the ratio of F_{380} in Ca^{2+}-free solution to that in Ca^{2+}-containing solution. R is the fluorescence ratio at F_{340}/F_{380}. The minimum fluorescence (R_{min}) was obtained by superfusion of the artery with a 140 mM K^+, Ca^{2+}-free solution containing 2 mM EGTA. Twenty minutes after superfusion with this solution, 10 μM ionomycin was added, and R_{min} was determined. After determination of R_{min}, the artery was superfused with an excess of Ca^{2+} (10 mM), which gave the maximal signal ratio, R_{max}, $MnCl_2$ (20 mM) added thereafter totally quenched the fluorescence. The procedure for the calibration of $[Ca^{2+}]_i$ is depicted in Figure 1.

Binding assay

The coronary arteries (main branches of left anterior descending artery and left circumflex artery) of pigs were isolated. The arteries were cleared and minced with scissors and then homogenized by use of Potter and Polytron homogenizers in 10 volumes of 50 mM Tris-HCl buffer containing 0.25 M sucrose and 10 μM $MgCl_2$ at pH 7.5. The membrane fraction was prepared according to the method of De Pover *et al.* (1982) and modified by us (Yamada *et al.*, 1990). Briefly, the arterial homogenates were centrifuged at 500 *g* for 10 min and the supernatant fraction, after filtration through four layers of cheesecloth was centrifuged at 9,000 *g* for 10 min. The resulting supernatant fraction was further centrifuged at 120,000 *g* for 30 min and the pellet was resuspended in the buffer to a concentration of 0.5 to 1.5 mg ml^{-1} of protein and used in the binding assays. All steps were performed at 4°C. Freshly prepared membranes from pig coronary artery were incubated with 20 pmol–2 nmol (+)-[^{3}H]-PN200–110 in 50 mM Tris-HCl buffer (pH 7.5) for 60 min at 25°C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters by use of a cell harvester (Brandel, M-24R, Gaithersburg, U.S.A., M.D.), and the filters were immediately washed 3 times with 3 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid, and the radioactivity was determined by liquid scintillation counting. Specific binding of (+)-[^{3}H]-PN200-110 was defined as the difference in binding

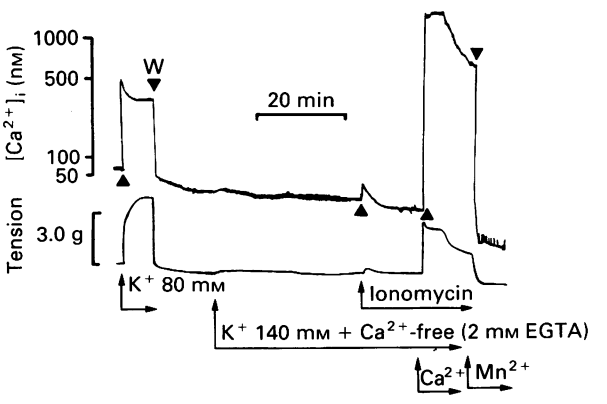


Figure 1 A tracing showing the tension-$[Ca^{2+}]_i$ relation in a coronary ring segment during 80 mM K^+-induced contracture and the actual procedure to obtain R_{min} and R_{max}. Ionomycin, 10 μM; Ca^{2+}, 10 mM; and $MnCl_2$, 20 mM. W: Washout. Muscle wet weight: 6.4 mg.

determined in the absence and presence of nifedipine (1 μM). The ability of drugs to inhibit specific (+)-[^{3}H]-PN200-110 binding was estimated by IC_{50} values which are the molar concentrations of unlabelled drugs necessary to displace 50% of the specific binding determined by log probit analysis. All experiments were carried out in duplicate.

Drugs

The drugs used in the present study were as follows: human endothelin-1 (Peptide Research Institute, Osaka, Japan), acetylcholine chloride (Daiichi, Tokyo, Japan), ionomycin calcium (Hoechst Japan, Tokyo, Japan), and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Bayer Yakuhin, Osaka, Japan). Fura-2/AM, ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Dojindo Laboratories (Kumamoto, Japan). 12-Deoxyphorbol 13-isobutylate (DPB) and 4α-phorbol didecanoate (4α-PDD) were obtained from Funakoshi (Tokyo, Japan), and dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1 mM and diluted to the desired concentrations with normal Tyrode solution. Ketanserin tartrate was supplied by Kyowahakko (Tokyo). Histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate and other drugs of reagent grade were obtained from Sigma (St. Louis, M.O., U.S.A.).

Statistical analysis

Results are expressed as means ± s.e. Statistical analysis was made by use of Student's paired or unpaired *t* test. *P* values less than 0.05 were considered significant.

Results

Recording of isometric tension in response to endothelin-1 and Bay K 8644

ET-1 and Bay K 8644 produced a concentration-dependent increase in tension of porcine coronary artery with and without endothelium. The sensitivities (pD_2) to ET-1 and Bay K 8644 of the coronary artery without endothelium were significantly increased in comparison with those of the arteries with endothelium (Table 1). The maximum tensions developed by ET-1 and Bay K 8644 in the endothelium-free artery were also increased, but not significantly in the case of ET-1, when compared with those of endothelium-intact artery. The amplitude of tonic contractions produced by 40 mM K^+ was about the same in the arteries with (6.4 ± 0.6 g, $n = 7$) and without (7.1 ± 0.5 g, $n = 7$) endothelium. Thus, the following experiments were carried out on arteries without endothelium in order to avoid the influence of endothelium on the contraction.

Table 1 Comparison of parameters of cumulative concentration-effect curves with and without endothelium elicited by ET-1 and Bay K 8644[a]

	pD_2	*Maximum response*[b]	n
ET-1			
E(+)	8.57 ± 0.06	105.6 ± 6.9	7
E(−)	9.02 ± 0.06**	122.6 ± 6.6	7
Bay K 8644			
E(+)	7.20 ± 0.08	37.0 ± 14.1	5
E(−)	7.40 ± 0.15**	69.6 ± 11.7*	5

[a] Rings of porcine coronary arteries with [E(+)] or without [E(−)] endothelium were studied in parallel by exposure to increasing concentration of ET-1 or Bay K 8644.
[b] The maximum contractile responses to ET-1 or Bay K 8644 were expressed as a percentage of the amplitude of tonic contraction produced by 40 mM K^+ (= 100%).
Each value represents means ± s.e. of number of preparations (*n*). The difference from the corresponding control response is statistically significant (* $P < 0.05$; ** $P < 0.01$).

Figure 2a shows that increasing concentrations of nisoldipine, a potent Ca^{2+} antagonist of the 1,4-dihydropyridine type, attenuated the slope as well as the maximum response of concentration-response curves for ET-1. Cumulative results are plotted in double reciprocal fasion in the inset of Figure 2a. Firstly, it can be seen that these two lines pass through different points on the Y axis, showing again the non-competitive nature of the interactions of nisoldipine and ET-1 with their binding site in the Ca^{2+} channel. Secondly, the K_D values can be calculated, and are (2.0 ± 0.4) × 10^{-9} M ($n = 4$) for the ET-1 control and (6.9 ± 4.2) × 10^{-9} M ($n = 4$) for nisoldipine (10 nM). These K_D values are not significantly different from each other. In contrast, nisoldipine caused a parallel shift in the concentration-response curves for Bay K 8644 (data not shown) and the pA_2 value was 9.0 ± 0.1 ($n = 6$), indicating an apparently competitive antagonism between Bay K 8644 and nisoldipine.

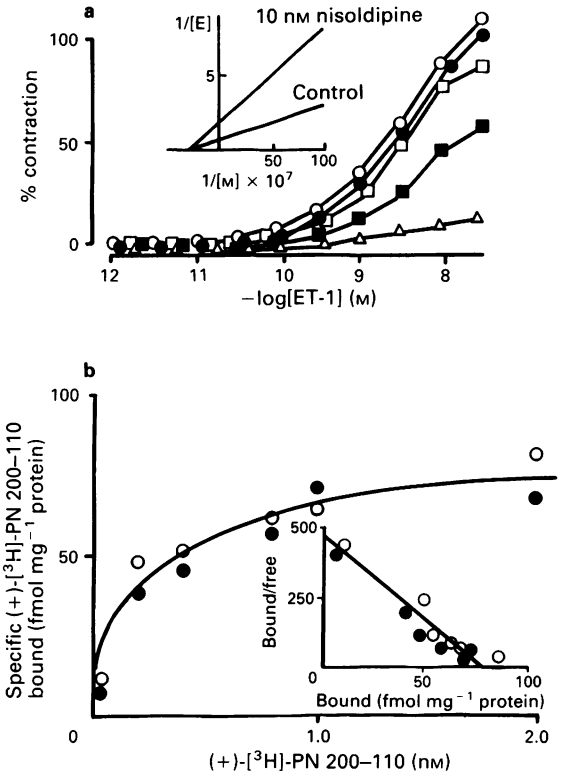


Figure 2 (a) Concentration-response curves for the effect of endothelin-1 (ET-1) on the basal tension of pig coronary artery. The increase in basal tension was normalized as % of amplitude of the tonic component of the 40 mM K^+-induced contracture (= 100%) in the corresponding coronary artery. Each curve shows mean value after the incubation with various concentrations of nisoldipine for 40 min: 0.1 nM (●), 1 nM (□), and 10 nM (■). Control concentration-response curves of the tonic contractions produced by ET-1 in the medium containing 2 mM Ca^{2+} (○) and in the Ca^{2+}-free medium containing 0.2 mM EGTA (△) are also given. Each point represents the mean of 6 to 8 experiments. Standard error at each point is smaller than symbols. Double reciprocal plots of either control response in the presence of 2 mM Ca^{2+} or the response after incubation of the coronary artery with nisoldipine (10 μM) are depicted in the inset. [E]: Effect, [M]: Molar concentration of ET-1. Calculated K_D value, i.e., dissociation constant, is (2.0–9.6) × 10^{-9} M. (b) Specific binding of (+)-[^{3}H]-PN200-110 in absence (○) and presence (●) of ET-1 (30 nM). Inset: Scatchard plot analysis in absence (○) and presence (●) of ET-1 (30 nM).

Binding studies

The effect of ET-1 on the specific binding of (+)-[^{3}H]-PN200-110, a Ca^{2+} antagonist of the 1,4-dihydropyridine type was studied. ET-1 (30 nM) had no apparent effect on the (+)-[^{3}H]-PN200-110-binding (Figure 2b). The Scatchard plot showed that the dissociation constant (K_D) and the maximum number of binding sites (B_{max}) of (+)-[^{3}H]-PN200-110 were 0.172 nM and 79.1 fmol mg^{-1} protein, respectively. In the presence of ET-1 (30 nM) the value was 0.17 nM for K_D, and 74.8 fmol mg^{-1} protein for B_{max}, values which were not significantly different from their controls.

Effects of endothelin-1, 12-deoxyphorbol 13-isobutylate and Bay K 8644 on the agonist-induced contractions

ET-1 (100 pM) potentiated 5-HT-induced contraction of the coronary segment (Figure 3a). Cumulative addition of 5-HT produced a concentration-dependent increase in tension. Low concentrations of ET-1 (10–100 pM) potentiated the contractile response to 5-HT in a concentration-dependent manner, whereas ET-1 in concentrations above 1 nM rather depressed or potentiated less reproducibly the 5-HT-induced contraction. In order to obtain a stable contractile potentiation for quantitative analysis the effects of low concentrations of ET-1 (30–100 pM) were examined. These amounts of ET-1 increased the basal tension to only about 10–15% of the contraction produced by 40 mM K^+ (see Figure 3a). The potentiation of the 5-HT-induced contraction by ET-1 was calculated from the following formula; (ii − i)/i × 100 (see Figure 3a), and normalized as % of amplitude of the tonic component of the 40 mM K^+-induced contraction in the corresponding coronary arteries. Pretreatment with ET-1 (100 pM) for 40 min significantly augmented the contraction amplitude in response to 5-HT (Figure 3b). The sensitivity to 5-HT was not significantly changed before ($pD_2 = 6.4 \pm 0.04$, $n = 6$) and after ($pD_2 = 6.5 \pm 0.04$, $n = 6$) application of ET-1. ET-1 (10 nM), on the other hand, depressed the 5-HT-induced contraction. The contractile response to 5-HT itself was not inhibited by prazosin, phentolamine (10 μM) or chlorpheniramine (1 μM), but was attenuated by ketanserin (0.1 μM). With a concentration range of ketanserin between 3 nM–0.1 μM, the concentration-response curves for 5-HT in the coronary artery showed a parallel shift to the right (data not shown). The pA_2 value for ketanserin was 8.77 ± 0.39 ($n = 5$), and the slope of Schild plot was 1.06. The value of pA_2 and slope of the Schild plot were not significantly affected by pretreatment with 1 μM prazosin; pA_2, 9.14 ± 0.25 ($n = 5$); the slope of the Schild plot, 0.86. Furthermore, the contractile response to 5-HT augmented by ET-1 was not inhibited by prazosin, phentolamine (10 μM) or chlorpheniramine (1 μM), but was attenuated by ketanserin (0.1 μM) (Figure 3a).

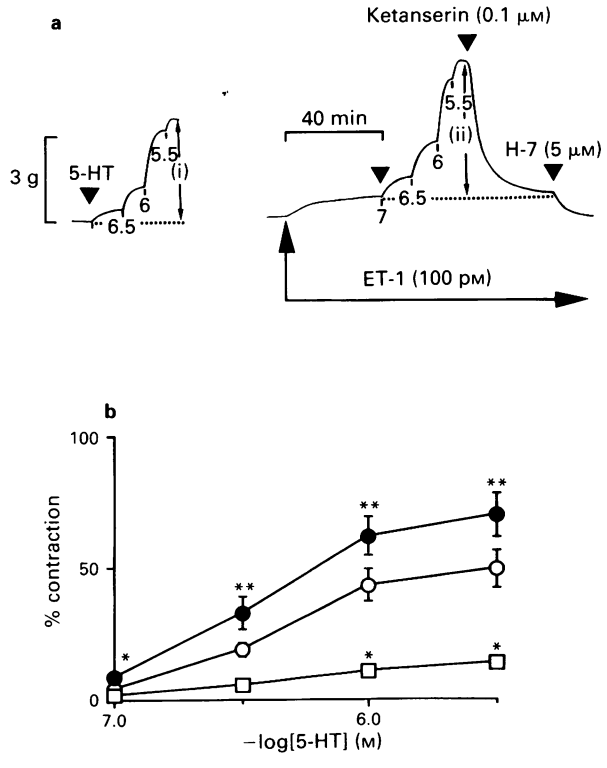


Figure 3 Effect of endothelin-1 (ET-1) on 5-hydroxytryptamine (5-HT)-induced contraction. (a) Representative tracings showing concentration-dependent increase in basal tension produced by 5-HT before (left panel) and after (right panel) administration of ET-1 (100 pM) to the same coronary ring. The numbers on the curves indicate the negative log of molar concentration of 5-HT. (i) and (ii) refer to the 5-HT-induced maximal contraction before and after application of ET-1 (100 pM), respectively. (b) Concentration-response curves for the effect of 5-HT on the basal tension of coronary arteries before (○) and after administration of ET-1 at 100 pM (●) or 10 nM (□). Points and bars indicate mean ± s.e. of 6 to 8 preparations. * $P < 0.05$; ** $P < 0.01$ vs corresponding control values of arteries.

When a single dose of DPB (0.1 nM–1 μM) was applied, the tension increased slowly in a concentration-dependent fashion (data not shown). The tension developed to DPB (3 nM) was less than 10% of that produced by the 40 mM K^+ medium, and the maximum tension developed in response to DPB (1 μM) was 147.9 ± 20.8% ($n = 6$) of the 40 mM K^+-induced contraction. The pD_2 value was 7.2 ± 0.3 ($n = 6$). In contrast, 4α-PDD (1 μM) showed no apparent effect on the tension.

Pretreatment with ET-1 (100 pM) or DPB (3 nM) (Table 2) potentiated the 5-HT-induced contraction, but did not show any apparent potentiating effect on the contractions produced by histamine and ACh. A small amount of Bay K 8644 (10 nM), (producing 10–15% of the increase in tension produced by 40 mM K^+) strongly potentiated the contraction in response not only to 5-HT but also histamine and ACh (Table 2). The contractile response to histamine, ACh, and 5-HT, and their amplification phenomena produced by ET-1 and DPB, or by Bay K 8644 were inhibited by chlorpheniramine and atropine (each 1 μM) (data not shown), or by ketanserin (0.1 μM), respectively. The results indicate that ET-1 selectively potentiated the 5-HT-induced contraction, while Bay K 8644 augmented the contraction amplitude and/or sensitized the coronary artery to the agonistic stimuli used in the present study.

Recording of tension and $[Ca^{2+}]_i$ in coronary artery

Effects of high-K^+, Bay K 8644, endothelin-1 and 12-deoxyphorbol 13-isobutyrate For further elucidation of the potentiating action of ET-1 on the 5-HT-induced contraction, $[Ca^{2+}]_i$ assessed by use of fura-2 and tension development were measured simultaneously. In the fura-2-loaded porcine coronary artery, high-K^+ (20–80 mM) increased $[Ca^{2+}]_i$ which was followed by an increase in basal tone (see Figure 1). The tension development due to high-K^+ was preceded by less than 1 s by the fluorescence changes and the rate of increase in the fluorescence was faster than that of tension development. The mean $[Ca^{2+}]_i$ at basal tone was 69.1 ± 2.3 nM ($n = 42$). The $[Ca^{2+}]_i$ increased during the tonic phase of 40 mM K^+-induced contraction was 228.4 ± 11.0 nM ($n = 42$). A single application of high-K^+ produced a concentration-dependent increase in both tension and $[Ca^{2+}]_i$, indicating the existence of a positive correlation between the two (Figure 4a).

Although Bay K 8644, a Ca^{2+} agonist of the 1,4-dihydropyridine type, was light-sensitive and the time for exposure to u.v. light was limited, a positive tension-$[Ca^{2+}]_i$ relationship could be obtained by single application of Bay K 8644. There is a positive correlation between tension and $[Ca^{2+}]_i$ measured after a 30 min exposure to Bay K 8644 (Figure 4b). Both mechanical activity and $[Ca^{2+}]_i$ augmented by 40 mM K^+ or Bay K 8644 (100 nM) were abolished by diltiazem (10 μM).

Table 2 Effects of endothelin-1 (ET-1, 100 pM), 12-deoxyphorbol 13-isobutylate (DPB, 3 nM), and Bay K 8644 (10 nM) on the contractions of porcine coronary artery produced by cumulative addition of 5-hydroxytryptamine (5-HT), histamine and acetylcholine (ACh)[a]

	Control + ET-1			*Control + DPB*			*Control + Bay K 8644*		
	n	pD_2	*Maximum response*[b]	n	pD_2	*Maximum response*	n	pD_2	*Maximum response*
5-HT	6	6.40 ± 0.03	49.6 ± 7.0	7	6.54 ± 0.09	55.2 ± 6.1	8	6.51 ± 0.07	52.7 ± 6.8
	6	6.46 ± 0.04	70.4 ± 8.2**	7	6.53 ± 0.11	66.3 ± 6.7*	8	6.72 ± 0.09**	84.7 ± 6.5**
Histamine	6	5.47 ± 0.10	135.6 ± 6.3	5	6.21 ± 0.13	121.6 ± 14.3	6	5.16 ± 0.08	141.1 ± 16.3
	6	5.51 ± 0.10	130.3 ± 7.9	5	6.14 ± 0.14	121.0 ± 12.6	6	5.55 ± 0.14**	156.1 ± 22.9
ACh	6	6.23 ± 0.11	61.2 ± 9.3	5	6.59 ± 0.11	101.6 ± 20.9	5	6.01 ± 0.06	24.5 ± 9.7
	6	6.17 ± 0.12	63.1 ± 9.4	5	6.43 ± 0.20	101.0 ± 17.3	5	6.37 ± 0.21	52.5 ± 7.5*

[a] Rings of porcine coronary arteries without endothelium were studied in parallel in the absence (control) or presence of ET-1 (100 pM), DPB (3 nM), or Bay K 8644 (10 nM) and were exposed to increasing concentration of 5-HT, histamine, or ACh.
[b] The maximum contractile response to each agonist was expressed as percentage of amplitude of tonic contraction produced by 40 mM K^+ (= 100%).
Each value represents mean ± s.e. of number of preparations (*n*). The difference from the corresponding control response is statistically significant (* $P < 0.05$; ** $P < 0.01$).

Figure 5 shows the tension-$[Ca^{2+}]_i$ relationship obtained by a single application of ET-1 (30 pM–10 nM). Figure 5a depicts an experiment showing that the $[Ca^{2+}]_i$ was increased from the control level (70.7 ± 2.9 nM, $n = 4$) to the initial peak (170.0 ± 17.1 nM, $n = 4$) within 3 min with a slight effect on the tension when ET-1 at a high concentration of 10 nM was applied. The muscle tension then increased gradually with time and reached a maximum within 20 to 30 min, while $[Ca^{2+}]_i$ slowly declined and reached a steady state level (120.1 ± 14.0 nM, $n = 4$). There was a positive correlation

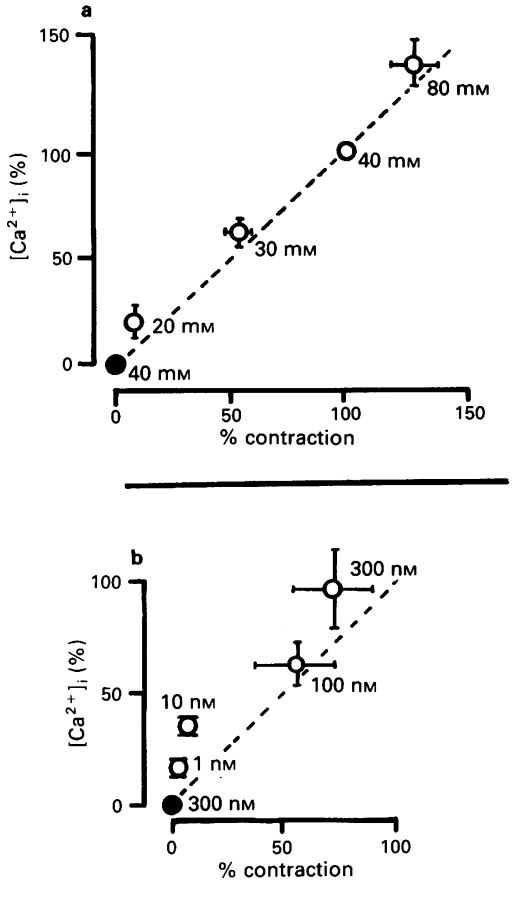


Figure 4 Relationship between tension development and cytosolic Ca^{2+} signal ($[Ca^{2+}]_i$) during contractions produced by high-K^+ (a) or Bay K 8644 (b). A positive correlation between tension and $[Ca^{2+}]_i$ in the amplitude of the tonic phase of high K^+- or Bay K 8644-induced contractions is evident. The responses to a single application of various concentrations of K^+ or Bay K 8644 were plotted, and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ augmented by 40 mM K^+ (a) or Bay K 8644 (b) were abolished by diltiazem (10 μM) (●). Points and bars indicate mean ± s.e. of at least 4 preparations for each concentration. (b) Relationship between tension development and $[Ca^{2+}]_i$ during Bay K 8644-induced contraction. The responses to a single application of various concentrations of Bay K 8644 were plotted, and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Points and bars indicate mean ± s.e. of at least 4 preparations for each concentration.

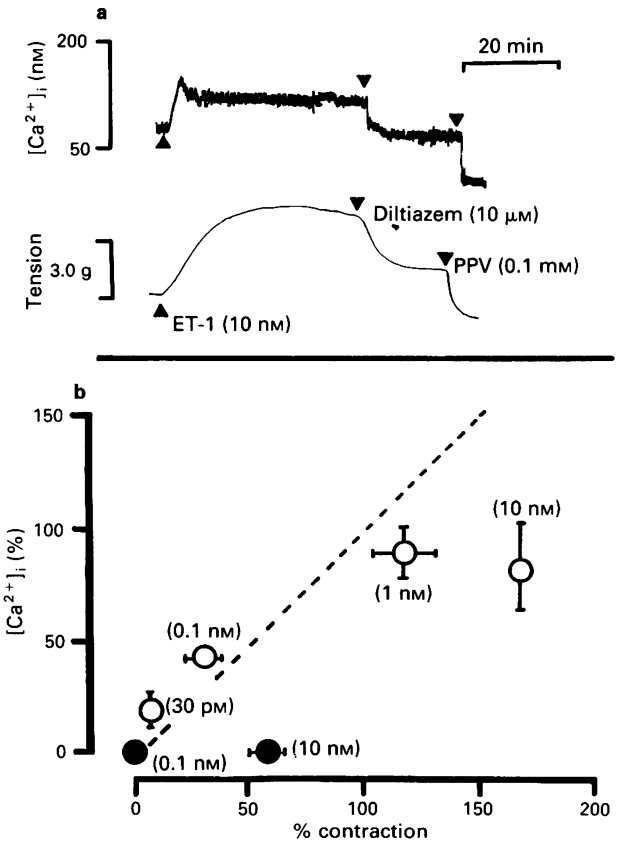


Figure 5 Relationship between tension development and $[Ca^{2+}]_i$ during endothelin-1 (ET-1)-induced contraction. (a) Typical tracings showing the tension-$[Ca^{2+}]_i$ relationship at 10 nM ET-1 in the coronary ring segment. PPV: Papaverine hydrochloride (0.1 mM). Muscle wet weight: 5.3 mg. (b) Relation between tension amplitude and $[Ca^{2+}]_i$ in the tonic phase of ET-1-induced contraction. The responses to a single application of various concentrations of ET-1 were plotted before (○) and after (●) administration of diltiazem (10 μM), and those to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ were augmented in parallel by ET-1 at relatively lower concentrations but that the tension was augmented without an increase in $[Ca^{2+}]_i$ at the higher concentrations of the peptide. Points and bars, indicate mean ± s.e. of 4–6 preparations.

between tension and $[Ca^{2+}]_i$ measured 40 min after application of ET-1 (30–100 pM) (Figure 5b). ET-1 at higher concentrations, over 1 nM, produced a larger contraction than that due to 40 mM K^+ with still only a small increase in $[Ca^{2+}]_i$. Diltiazem (10 μM) abolished the increase in tension and $[Ca^{2+}]_i$ produced by a low concentration of ET-1 (0.1 nM), while the antagonist only partially inhibited the augmented tension in response to ET-1 (10 nM) in spite of abolition of the increase in $[Ca^{2+}]_i$ (Figure 5a and b). Papaverine (0.1 mM) totally inhibited both tension and the increase in $[Ca^{2+}]_i$.

Figure 6 shows the tension-$[Ca^{2+}]_i$ relationship of DPB, which is qualitatively similar to that of ET-1: a single application of DPB (1 nM–30 nM) slowly increased the basal tension and $[Ca^{2+}]_i$ in a concentration-dependent manner, and there was a positive correlation between these. However, DPB at higher concentrations, over 0.1 μM, produced a larger contraction than that due to 40 mM K^+ with a slight increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ and the tension produced by low concentration of DPB (30 nM) was abolished by diltiazem (10 μM). However, diltiazem (10 μM) only partially inhibited the augmented tension in spite of abolition of the increase in $[Ca^{2+}]_i$ when a high concentration of DPB (1 μM) was given (Figure 6). 4α-PDD (1 μM) had no apparent effect on the tension and $[Ca^{2+}]_i$.

Effects of 5-hydroxytryptamine before and after application of endothelin-1, 12-deoxyphorbol 13-isobutylate and Bay K 8644 Figure 7 shows the tension-$[Ca^{2+}]_i$ relationship for a single application of 5-HT at various concentrations before and after application of ET-1 (a) or DPB (b). The $[Ca^{2+}]_i$-tension relationship of 5-HT alone was similar to that of high-K^+ (see open circles in Figure 4a). ET-1 (30 pM) or DPB (3 nM) strongly augmented the 5-HT-induced tension without a significant change in $[Ca^{2+}]_i$ such that the tension-$[Ca^{2+}]_i$ relation shifted almost parallel to the right (closed circles in Figure 7a and b). 4α-PDD (3 nM) showed no apparent effect on the tension-$[Ca^{2+}]_i$ relation.

When diltiazem (10 μM) was applied, the increase in tension and $[Ca^{2+}]_i$ induced by 5-HT (3 μM) itself was abolished (open squares in Figure 7a and b). The increased $[Ca^{2+}]_i$ produced by 5-HT (3 μM) plus ET-1 (30 pM) or DPB (3 nM) was completely inhibited by diltiazem (10 μM), while the 5-HT (3 μM)-induced contraction which was potentiated by ET-1 (30 pM) or DPB (3 nM) was partially attenuated (closed squares in Figure 7a and b).

Figure 8 shows the tension-$[Ca^{2+}]_i$ relation of 5-HT before and after application of Bay K 8644 (10 nM). Bay K 8644

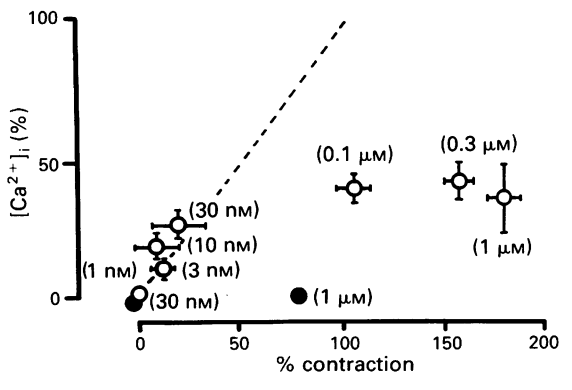


Figure 6 Relationship between tension development and $[Ca^{2+}]_i$ during 12-deoxyphorbol 13-isobutylate (DPB)-induced contraction. The tonic contractile responses to a single application of various concentrations of DPB were plotted (○); after administration of diltiazem (10 μM) (●). The responses to 40 mM K^+ were taken as 100% on the ordinate and abscissa scales. Note that tension and $[Ca^{2+}]_i$ were augmented in parallel by DPB at relatively lower concentrations but that the tension was augmented without an increase in $[Ca^{2+}]_i$ at the higher concentration of DPB. Points and bars, indicate mean ± s.e. of 4–6 preparations.

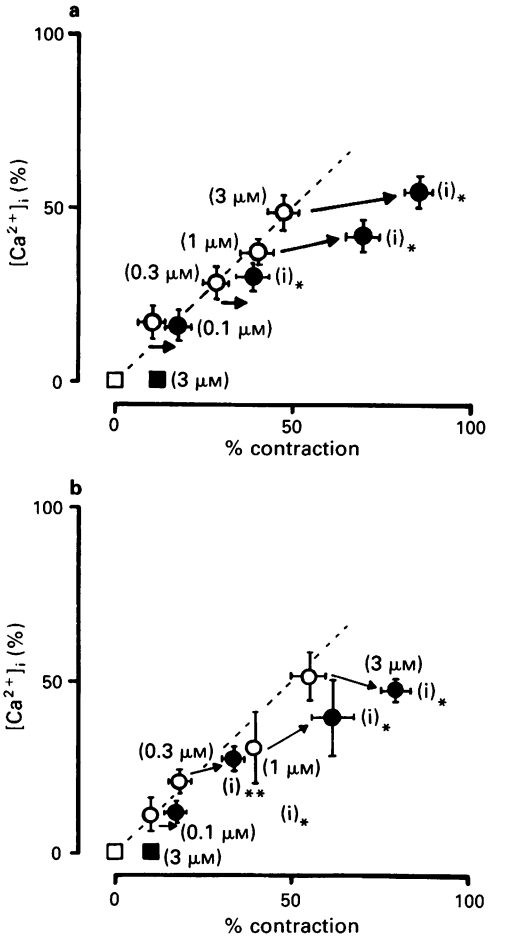


Figure 7 Changes in tension-$[Ca^{2+}]_i$ in 5-hydroxytryptamine (5-HT)-induced contractions before (○) and after (●) treatment with either endothelin-1 (ET-1, 30 pM) (a) or 12-deoxyphorbol 13-isobutylate (DPB) (3 nM) (b). Arrows show the direction of shift. Effects of diltiazem (10 μM) on the response to 5-HT alone (□), and that to 5-HT plus ET-1 or DPB (■). (i) indicates contraction. * $P < 0.05$, ** $P < 0.01$ vs. corresponding control values of arteries. Points and bars, indicate mean ± s.e. of 4–6 preparations.

(10 nM) significantly augmented not only the 5-HT-induced increase in $[Ca^{2+}]_i$ but also tension development (closed circles). Thus, the tension-$[Ca^{2+}]_i$ relationship shifted to the upper right (Figure 8). The 5-HT (3 μM)-induced tension and

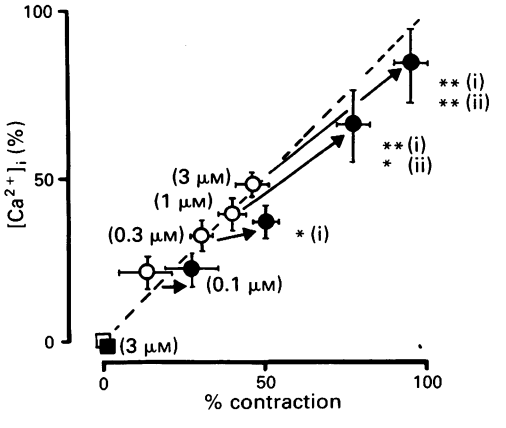


Figure 8 Changes in tension-$[Ca^{2+}]_i$ in 5-hydroxytryptamine (5-HT)-induced contractions before (○) and after (●) treatment with Bay K 8644 (10 nM). Effect of diltiazem (10 μM) on the response to 5-HT alone (□), and that to 5-HT plus Bay K 8644 (■). Arrows show the direction of shift. (i) and (ii) indicate contraction and $[Ca^{2+}]_i$, respectively. Further explanations are the same as those for Figure 7.

$[Ca^{2+}]_i$ potentiated by Bay K 8644 (10 nM) were abolished when diltiazem (10 μM) was applied (closed square in Figure 8).

Discussion

The present study showed that ET-1 *per se* (30–100 pM) produced a slight increase in basal tension, less than 10% of the 40 mM K^+-induced contraction, and strongly potentiated the 5-HT-induced contraction of the coronary artery without a significant increase in $[Ca^{2+}]_i$. The pharmacological properties of ET-1 seem to be qualitatively similar to those of a phorbol ester such as DPB. In contrast, the potentiation of the contractions produced by Bay K 8644 was considered to be attributable to Ca^{2+} agonistic augmentation of transmembrane Ca^{2+} influx (Schramm *et al.*, 1983). This is supported by the finding that the present study showed that Bay K 8644 potentiated the contraction with a concomitant increase in $[Ca^{2+}]_i$ in the coronary artery.

The present study confirmed previous reports by others (De Nucci *et al.*, 1988; Sakata *et al.*, 1989) that removal of endothelium sensitizes arterial smooth muscle and augments the contractile responses to ET-1. Although ET-1 has been reported to produce endothelium-dependent relaxation in the rat (De Nucci *et al.*, 1988), the porcine coronary artery with intact endothelium, which was preconstricted with U46619, a thromboxane A_2 analogue, did not relax in response to ET-1, while substance P relaxed the artery (our unpublished observations). Furthermore, the freshly isolated and dispersed endothelial cells of porcine coronary artery preloaded with fura-2 did not show any increase in $[Ca^{2+}]_i$ in response to 0.1 μM ET-1 ($[Ca^{2+}]_i$ at rest, 106 $\pm$ 8 nM, $n = 5$, and after 0.1 μM ET-1, 105 $\pm$ 15 nM, $n = 5$), while substance P (10 nM) increased the $[Ca^{2+}]_i$ (unpublished observations). The $[Ca^{2+}]_i$ before and at the peak after treatment with substance P was 105 $\pm$ 4 nM and 1,008 $\pm$ 8 nM ($n = 23$), respectively. The relaxation of the coronary artery produced by substance P was not inhibited by indomethacin but attenuated by methylene blue, indicating the release of EDRF concomitant with the increase in $[Ca^{2+}]_i$ (Uchida *et al.*, 1990). It has been reported that the release of vasorelaxant substances such as EDRF and PGI_2 inevitably produce a concomitant increase in $[Ca^{2+}]_i$ (Luckhoff *et al.*, 1988). Therefore, as for porcine coronary artery, it is unlikely that ET-1 can produce relaxation by stimulation of EDRF release. The removal of endothelium seems to inhibit the basal release of a relaxing substance such as EDRF; thus, this removal enhances the actions of practically all vasoconstrictor agonists used in the present study.

Earlier studies on ET-1-induced contraction of the coronary artery have suggested that nicardipine, a Ca^{2+} antagonist, shifted the concentration-response curves for ET-1 to the right in a parallel manner, indicating a competitive antagonism between ET-1 and the Ca^{2+} antagonist at the same binding site on the Ca^{2+} channel (Yanagisawa *et al.*, 1988). However, the results of radio-ligand binding assays of ET-1 binding to plasma membranes of the rat (Gu *et al.*, 1989) and chicken heart (Watanabe *et al.*, 1989) and of porcine coronary artery (Kasuya *et al.*, 1989b) have clearly shown that ET-1 does not bind to the Ca^{2+} channel receptor sensitive to Ca^{2+} antagonists of the 1,4-dihydropyridine type but to ET-1 receptor(s). Our radio-receptor binding assay on the Ca^{2+} antagonist receptor in the porcine coronary artery also showed a clear separation of the binding site between ET-1 and (+)-[^{3}H]-PN200-110. In accordance with the results of the radio-receptor binding assay, the present functional study showed that nisoldipine antagonized the ET-1-induced contraction in a non-competitive manner. Our previous radio-receptor binding study (Yamada *et al.*, 1990) and the present functional study indicate that Bay K 8644 and a Ca^{2+} antagonist of the 1,4-dihydropyridine type such as (+)-[^{3}H]-PN200-110 share the same binding site at the voltage-dependent Ca^{2+} channel in the porcine coronary artery. The functional and binding studies are thus well correlated, and both studies can aid in the elucidation of the pharmacological characteristics of ET-1 and Bay K 8644.

We can only speculate on the mechanism of the potentiation of the 5-HT-induced contraction produced by ET-1. It has been reported that in the rat aorta, 5-HT activates phospholipase C and thus promotes PI hydrolysis which leads to production of inositol 1,4,5-trisphosphate ($InsP_3$) and DAG (Nakaki *et al.*, 1985). Since $InsP_3$ releases Ca^{2+} from intracellular Ca^{2+} stores (Berridge, 1984), $InsP_3$-induced contractions are rather resistant to Ca^{2+} antagonists and withdrawal of extracellular Ca^{2+}. However, our present study showed that 5-HT- and high-K^+-induced contraction and the increase in $[Ca^{2+}]_i$ in particular at the tonic phase of contraction correlated well with contraction amplitude. The 5-HT-induced contraction was not affected by prazosin, phentolamine or chlorpheniramine, but competitively antagonized by ketanserin, indicating a pivotal role of 5-HT_2 receptors. Moreover, of various agonists including 5-HT, histamine, and ACh, the 5-HT-induced contraction of porcine coronary artery was the most susceptible to a Ca^{2+} antagonist such as nifedipine (Nakayama *et al.*, 1989) and to withdrawal of Ca^{2+}. Therefore, like high-K^+-induced contraction, the contraction produced by 5-HT in porcine coronary artery seems to be primarily dependent on the transmembrane supply of Ca^{2+}.

As to the mechanism of ET-1-induced contraction, the following two ideas have been proposed: (1) augmentation of Ca^{2+} influx (Yanagisawa *et al.*, 1989); (2) augmentation of turnover of PI and increase in intracellular messengers such as $InsP_3$ and DAG. These ideas seem to be qualitatively common to those for the mechanism of action of various agonistic stimuli including 5-HT, and tumour promoting phorbols on various biological systems such as vascular smooth muscles. In the present study, we observed that ET-1 and DPB at a low concentration (less than 1–3 nM) increased the tension and the $[Ca^{2+}]_i$ almost in a parallel manner, indicating that the first mechanism may be dominant, while the second mechanism of promotion of PI hydrolysis seems to be dominant when a large concentration of ET-1 or DPB over 10–100 nM is applied. Of the various biological roles of PKC promoted by DAG, the phosphorylation of various proteins (Nishizuka, 1984) is the first step, and as a consequence, the Ca^{2+} sensitivity of molecules such as the contractile proteins in vascular smooth muscles is increased. The PKC has also been known to promote Ca^{2+} current through the channels sensitive to Ca^{2+} antagonists of the 1,4-dihydropyridine type (Vivaudou *et al.*, 1988). In contrast, PKC is also involved in a negative feedback mechanism; i.e., it decreases receptor-coupled responses (Nishizuka, 1984). The high concentrations of ET-1 or tumour promoting phorbol esters such as DPB and 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited the contractile responses of the pig coronary artery to various agonistic stimuli, such as 5-HT in the present study, and ACh (Itoh *et al.*, 1988) or mechanical stretch (our unpublished observations).

The combined effects of ET-1 and other agonists on the $[Ca^{2+}]_i$-tension relation were complex. In addition to the direct vasoconstriction effect on vascular smooth muscle cells, 5-HT enhances the contractile effects of a number of agonists (e.g. noradrenaline) (de la Lande *et al.*, 1966) and sympathetic stimulation *in vivo* and *in vitro* (Van Nueten *et al.*, 1981; Pott *et al.*, 1986). 5-HT also accelerates platelet aggregation when combined with low, subthreshold concentrations of other agonists (e.g. ADP, collagen, adrenaline, and thromboxane A_2 or its mimic U46619; De Clerck & De Courcelles, 1989). These amplifications occur biochemically at the post receptor stage of the signal transduction system: the turnover of PI and the rise in $[Ca^{2+}]_i$ are amplified (De Clerk & De Courcelles, 1989). Nishizuka (1984) and his colleagues (Kaibuchi *et al.*, 1983) have proposed an interesting idea concerning the role of PKC in the cellular signal transduction: they suggested synergistic interaction between the PKC and Ca^{2+}-calmodulin

pathways in a variety of cellular responses to external stimuli. The interaction between PKC-mediated phosphorylation and increased Ca^{2+}-calmodulin leads to full activation of a physiological cellular response such as release of 5-HT from platelets. Zawalich *et al.* (1983) also reported that two Ca^{2+} signalling systems, i.e., transmembrane Ca^{2+} mobilization and activation of PKC, are responsible for receptor-mediated release of insulin and aldosterone. On the other hand, Naka *et al.* (1983) showed that the Ca^{2+}-calmodulin system directly activates myosin light chain kinase in a $[Ca^{2+}]_i$-dependent manner, while the PKC system, which does not require an increase in $[Ca^{2+}]_i$, rather depresses the activation of actomyosin in platelets. Thus, the two pathways act antagonistically in the regulation of platelet function.

Our present study suggests that the simultaneous stimulation of two different receptors augments contraction in the porcine coronary arterial smooth muscle. It seems possible that the combined stimulation of 5-HT- and ET-1-receptors may amplify the intracellular signalling systems more effectively, and produce a full activation of contraction without a significant increase in $[Ca^{2+}]_i$. The activation of PKC may play an important role in this regard. In contrast, the present study showed that Bay K 8644 acted as a simple promoter of transmembrane influx of Ca^{2+}, for it potentiated the contractile response, along with an increase in $[Ca^{2+}]_i$, to all the pharmacological agents tested, as is also seen with other pharmacological, electrical, and mechanical stimuli (Nakayama *et al.*, 1983; Nakayama, 1986; Nakayama & Tanaka, 1989). It has been reported that Bay K 8644 potentiates 5-HT effects at the 5-HT_2 receptor in rabbit aorta in a manner consistent with a simple increase in efficacy (Barrett *et al.*, 1986). The present results with Bay K 8644 alone, Bay K 8644 plus 5-HT, or Ca^{2+} antagonists such as nisoldipine and diltiazem are not entirely consistent with such a second messenger system, but are similar to those reported by Barrett *et al.* (1986). Since ET-1 potentiated the 5-HT-induced contraction without increasing the $[Ca^{2+}]_i$ in the present study, it is unlikely that ET-1, even at a low concentration, acts solely as a promoter of Ca^{2+} influx in the coronary artery. We are now conducting experiments in order to obtain more direct evidence of combined effects of ET-1 and other agonists on PKC activation and contractile function.

Finally, the physiological and pathological significance of the present study should be mentioned: 5-HT released from platelets has been known as a strong coronary vasoconstrictor and platelet aggregator which may be a factor in coronary vasospasm (Ashton *et al.*, 1986). The plasma ET-1 level of normotensive and spontaneously hypertensive rats (Suzuki *et al.*, 1990) and of man (Ando *et al.*, 1989) is reportedly 1–2 pg ml^{-1} as measured by use of an enzymatic immunoassay, a value corresponding to about 0.5 pM, when the plasma volume is arbitrarily taken as 1/13 of body weight (about 60 kg). Thus, a small amount of ET-1 at the picomolar level released locally *in situ* may not only regulate physiologically vascular tone, but also promote contraction of vascular smooth muscles in the coronary as well as peripheral vessels. The recent study (Matsumoto *et al.*, 1990) reporting that ET-1 in combination with 5-HT activates platelets and accelerates aggregation and the results from the present study suggest the importance of the synergistic effects of ET-1 and other endogenous spasmogens, in particular 5-HT, on the coronary circulation.

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Increased endothelin excretion in rats with renal failure induced by partial nephrectomy

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1 Six weeks following partial nephrectomy in rats, significant increases in serum urea nitrogen and serum creatinine concentration and a significant decrease in creatinine clearance were observed.

2 Measurement of systolic blood pressure by tail plethysmography indicated that animals that had undergone partial nephrectomy were hypertensive.

3 Compared to sham-operated animals, there were 4 fold increases in both urinary protein excretion and urinary endothelin excretion.

4 There was a significant correlation between urinary protein and urinary endothelin excretion ($r = 0.77$). There was also a correlation ($r = 0.65$) between urinary endothelin excretion and systolic blood pressure.

5 Plasma endothelin concentrations were not different in sham-operated and partially nephrectomized rats.

6 The data indicate that there is an increased renal endothelin production in rats with chronic renal failure.

Keywords: Endothelin; chronic renal failure; hypertension; urinary excretion; partial nephrectomy

Introduction

Endothelin is a newly discovered peptide (Yanigasawa *et al.*, 1988) and is one of the most potent endogenous renal vasoconstrictors yet described (Goetz *et al.*, 1988; King *et al.*, 1989; Edwards *et al.*, 1990; Gardiner *et al.*, 1990). There is growing evidence that endothelin is involved in a variety of acute renal disorders including cyclosporine A nephrotoxicity. Thus, an endothelin antibody can attenuate cyclosporine A-induced renal vasoconstriction (Kon *et al.*, 1990; Perico *et al.*, 1990) and there is increased endothelin binding (Nambi *et al.*, 1990) and urinary excretion (Brooks *et al.*, 1991) in rats and man (Deray *et al.*, 1991) with cyclosporine A-induced nephrotoxicity. Since endothelin is a powerful renal vasoconstrictor and causes proliferation of mesangial cells in culture (Simonson *et al.*, 1989; Jaffer *et al.*, 1990), it may also be involved in chronic renal disease. Indeed, it has been suggested that endothelin has a possible role in glomerular inflammation (Simonson & Dunn, 1991) and it has been reported that there is increased renal endothelin production in rats with reduced renal mass (Benigni *et al.*, 1991). In the present study we have therefore measured the plasma concentration and urinary excretion of endothelin in rats with chronic renal failure induced by partial nephrectomy. The data indicate that the renal excretion of endothelin is increased and plasma endothelin concentration unchanged after partial renal ablation, suggesting that there is enhanced renal production of endothelin in chronic renal disease.

Methods

Male Sprague Dawley rats were anaesthetized with sodium pentobarbitone, the right kidney was removed and approximately 2/3 of the left kidney was infarcted by ligating two or three branches of the renal artery. Control rats underwent sham surgery. Before, and at weekly intervals after partial nephrectomy, rats were housed in individual metabolic cages for 48 h and urine collected for the second 24 h period. At the end of the collection period, systolic blood pressure was measured indirectly by tail plethysmography. Urine volume was measured and urinary protein excretion determined by the sulphosalicylic method (Davidsohn & Henry, 1969). Six weeks following ablation, animals were anaesthetized with sodium pentobarbitone and blood samples taken from the abdominal aorta for assay of serum creatinine, serum urea nitrogen and plasma endothelin concentration. Plasma for endothelin assays was prepared from 5 ml of blood drawn into a syringe containing a stop solution of 500 u ml^{-1} aprotinin and 5 mM EDTA (10:1, v/v, final concentration).

The extraction and radioimmunoassay for endothelin have been described previously (Brooks *et al.*, 1991). The antiserum used had 100% cross-reactivity with endothelin-1, -2 and -3 and <0.001% cross-reactivity with porcine proendothelin (1-39) or human proendothelin (1-38). Serum and urine samples were assayed for creatinine and urea nitrogen with a clinical analyzer (Synchron AS8) and 24 h creatinine clearance was calculated as ml min^{-1} 100 g^{-1} body weight. The body weights of the control rats ($n = 6$) and rats with partial renal ablation ($n = 6$) at six weeks after surgery were 513 ± 18 and 462 ± 9 g, respectively. Urinary endothelin excretion was corrected for creatinine clearance and expressed as fmol ml^{-1} creatinine clearance. Data are expressed as mean ± s.e.mean and were analyzed statistically using a t test. The correlations between urinary endothelin excretion, proteinuria and systolic blood pressure were determined by least squares analysis and statistical significance was determined by an analysis of variance.

Results

Partial renal ablation resulted in a significant reduction in renal function after 6 weeks (Figure 1) as indicated by significant increases in both serum urea nitrogen (+150%, $P < 0.01$) and serum creatinine concentration (+92%, $P < 0.01$) and by a significant decrease in creatinine clearance (−52%, $P < 0.01$). Systolic blood pressure was increased (61%, $P < 0.01$) and there were 4 fold increases in both urinary protein excretion ($P < 0.01$) and urinary endothelin excretion (Figure 2). Urinary protein excretion increased 7 fold if corrected for creatinine clearance (24.2 ± 6.2 as compared to 169 ± 6.5 μg ml^{-1} creatinine clearance). Plasma endothelin concentration in these animals, however, was unchanged

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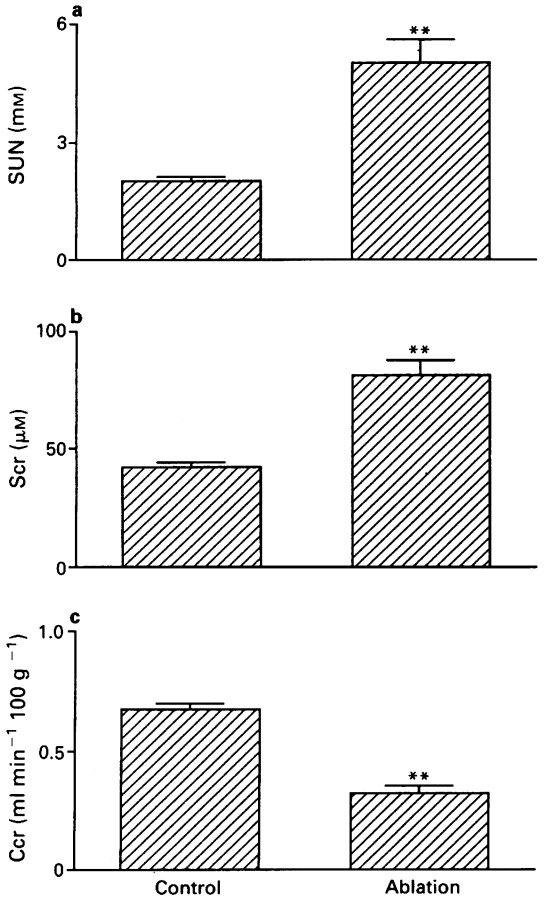


Figure 1 Serum urea nitrogen (SUN; a), serum creatinine concentration (Scr; b) and creatinine clearance (Ccr; c) in control rats ($n = 6$) and rats six weeks after partial renal ablation ($n = 6$). $^{**}P < 0.01$ vs. control.

being 0.88 ± 0.27 and 0.67 ± 0.05 fmol ml^{-1} in control rats and rats with chronic renal failure, respectively.

There was a significant correlation ($r = 0.77$, $P < 0.01$) between urinary protein and urinary endothelin excretion (Figure 3). The correlation was similar when both parameters were corrected for creatinine clearance ($r = 0.71$, $P < 0.01$). There was also a weak, but statistically significant ($r = 0.65$, $P < 0.05$), correlation between urinary endothelin excretion and systolic blood pressure (Figure 4).

Discussion

Partial renal ablation in rats leads to progressive renal failure and is characterized by proteinuria, hypertension and glomerulosclerosis. The mechanisms involved in the progression of chronic renal failure are unclear. Some investigators have suggested that glomerular hypertension is an important factor (Anderson *et al.*, 1985), whilst others have challenged this (Yoshida *et al.*, 1987), suggesting that factors such as mesangial cell proliferation may be important. In the present study we have investigated whether or not endothelin might play a role in chronic renal failure since it is a powerful vasoconstrictor (King *et al.*, 1989; Gardiner *et al.*, 1990), it results in mesangial cell proliferation (Simonson *et al.*, 1989; Jaffer *et al.*, 1990) and it has already been implicated in acute renal disorders (see Introduction). Furthermore, endothelin is synthesized by kidney cells in culture (Kosaka *et al.*, 1989; Ohta *et al.*, 1990; Bakris *et al.*, 1991) and a possible role for endothelin in glomerular inflammation has been proposed (Simonson & Dunn, 1991).

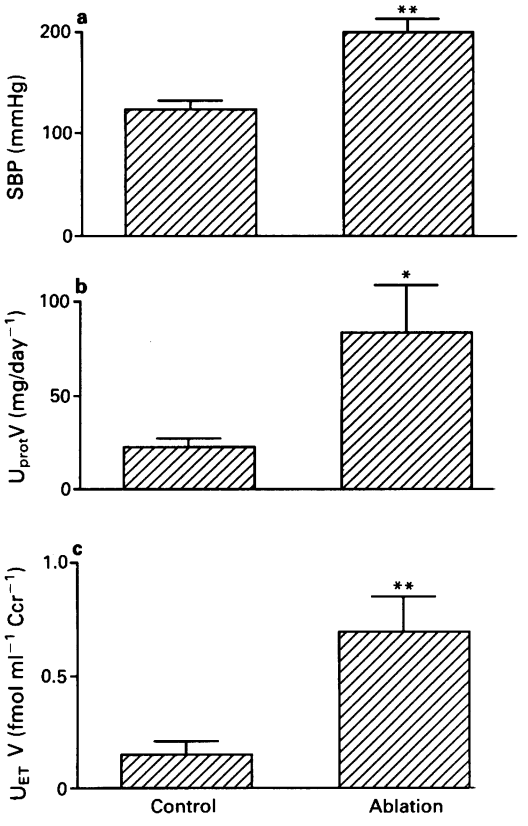


Figure 2 Systolic blood pressure (SBP; a), urinary protein excretion ($U_{prot}V$; b) and urinary endothelin excretion ($U_{Et}V$; c) in control rats ($n = 6$) and rats six weeks after partial renal ablation ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control.

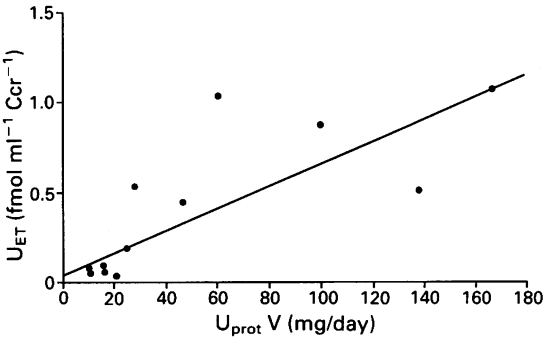


Figure 3 Correlation between urinary protein excretion ($U_{prot}V$) and urinary endothelin excretion ($U_{Et}V$) in control rats and rats six weeks after partial renal ablation; $r = 0.71$, $P < 0.01$.

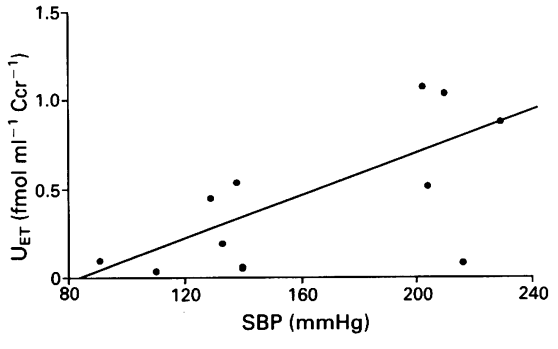


Figure 4 Correlation between systolic blood pressure (SBP) and urinary endothelin excretion ($U_{Et}V$) in control rats and rats six weeks after partial renal ablation; $r = 0.65$; $P < 0.05$.

In the present study we demonstrate that there is an increased urinary endothelin excretion associated with the reduction in renal function, the hypertension and the proteinuria induced by partial renal ablation. Since plasma endothelin concentration was similar in both control rats and rats with chronic renal failure, the increased urinary excretion of endothelin is likely to reflect increased renal production. These data confirm and extend the results of Benigni *et al.* (1991) who demonstrated that thrombin-induced endothelin production from the renal cortex *in vitro* is greater when the tissue is taken from rats with reduced renal mass. It has also been reported recently that there is increased urinary endothelin-1 excretion in patients with renal disease (Ohta *et al.*, 1991).

In the present study we have demonstrated, for the first time, that there is a significant correlation between urinary endothelin and urinary protein excretion. Since proteinuria is a good indicator of glomerular damage, our data suggest that there is a relationship between renal endothelin production and the progression of chronic renal failure. Whether or not this relationship indicates a role for endothelin in the pathogenesis of chronic renal failure is unclear. It is possible that the increased endothelin excretion is a marker for renal injury and reflects increasing endothelial cell damage. Since, however, there is increasing evidence that endothelin has actions on the renal vasculature and mesangial cells similar to those proposed to be involved in the progression of chronic renal failure, it is also possible that endothelin might indeed play an important role in the pathogenic process. Likewise, the relationship between urinary endothelin excretion and systolic blood pressure might indicate that the renal effects of endothelin are involved in the hypertension. The advent of selective endothelin receptor antagonists should clarify these issues.

In summary, the present study indicates that there is increased urinary excretion of endothelin in rats with chronic renal failure and this is not accompanied by changes in plasma endothelin concentration. The data, therefore, suggest that there is increased renal production of endothelin in rats with chronic renal failure.

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Effects of PAF-antagonists in mouse ear oedema induced by several inflammatory agents

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1 Several platelet activating factor (PAF)-antagonists of different chemical structures were tested in the arachidonic acid-, tetradecanoylphorbol acetate-, dithranol-, and benzoic acid-induced mouse ear oedema models.

2 Topical application of UR-10324, UR-11353, CV-6209 and WEB-2086 markedly inhibited ear oedema induced by the four irritants tested, mimicking the profile obtained with dexamethasone. YM-461 was highly effective only in the dithranol-induced ear oedema, while BN-52021 failed to inhibit ear oedema in all models tested.

3 Leukocyte recruitment into the inflamed ears was prevented by PAF-antagonists, as measured by myeloperoxidase activity in the supernatants of ear homogenates.

4 A relationship between PAF-antagonist and anti-inflammatory activities was found in some cases, but other mechanisms cannot be excluded to explain the topical anti-inflammatory effect of these compounds.

5 Our results suggest that topical formulations containing PAF-antagonists could be useful in the treatment of some inflammatory skin diseases and provide evidence on the involvement of PAF in these inflammatory processes.

Keywords: PAF-antagonists; mouse ear oedema; arachidonic acid; phorbol esters; dithranol; benzoic acid; myeloperoxidase

Introduction

Inflammation of the skin is characterized by increased vascular permeability, plasma protein exudation and migration of inflammatory cells. All the features of the inflammatory response are closely mimicked by platelet-activating factor (PAF) (Camussi & Brentjens, 1987). Thus, intradermal injection of PAF causes an acute wheal and flare response followed by delayed erythema in man (Pirotski *et al.*, 1985). Moreover, PAF and PAF-like materials have been isolated from psoriatic scales (Mallet & Cunningham, 1985) and from the blood of patients with primary acquired cold urticaria (Grandel *et al.*, 1985). All these observations support the role of PAF in the pathophysiology of the inflammatory skin responses, suggesting that PAF-antagonists could be useful in the treatment of some skin diseases.

The most commonly used animal model in the search for novel therapeutic agents useful in the topical treatment of inflammatory skin conditions is rodent ear oedema induced by application of an irritant agent (Young & De Young, 1989). In this work we have chosen some of the currently used primary irritant agents, such as arachidonic acid (AA), 12-O-tetradecanoylphorbol 13-acetate (TPA), dithranol (D), and benzoic acid (BA). Arachidonic acid produces an acute transient inflammatory reaction with immediate erythema, oedema and an elevated tissue concentration of prostaglandins and leukotrienes (Opas *et al.*, 1985). Although not very selective, this test has been used to evaluate 5-lipoxygenase inhibitors *in vivo* (Pignat *et al.*, 1986). TPA has been isolated from croton oil and identified as the active constituent. This substance has been reported as a tumour promoter and an inducer of epidermal hyperplasia (Young *et al.*, 1984). On the other hand, dithranol is one of the most important drugs used for the local treatment of psoriasis. The usefulness of dithranol is highly limited by its irritant effect on the surrounding skin (Keméry *et al.*, 1989). Topical administration of dithranol in the mouse ear produces a delayed irritant effect, which may be partly due to the generation of free radicals (Finnen *et al.*, 1984). Benzoic acid exposure provokes contact urticaria both in man and in guinea-pigs (Lahti & Maibach, 1984). We have therefore selected benzoic acid for testing in the mouse ear oedema model in this present study.

We have prepared a new series of ionic compounds with PAF-antagonist activity (Bartrolí *et al.*, 1990). Two of these compounds, together with standard PAF-antagonists of similar or different structure, were selected for testing in the above mentioned models of topical inflammation in the mouse ear. In addition to anti-inflammatory activity, we have evaluated PAF antagonist activity of the selected compounds, in order to verify a possible correlation between the two pharmacological activities. Standard anti-inflammatory agents were also tested, i.e. indomethacin (cyclo-oxygenase inhibitor), phenidone (lipoxygenase inhibitor) and dexamethasone (corticoid).

Methods

Dermatitis

Groups of 8 to 10 Swiss male mice, 6–8 weeks old, were used in the experiments. Animals had free access to food and water. Irritant dermatitis was elicited by painting both faces of the left ear of the mouse with 20 μl (10 μl each face) of the inflammatory agents. For screening purposes, concentrations (w/v) of the irritants were 10% arachidonic acid, 0.1% dithranol, 0.01% TPA, each dissolved in acetone, and 10% benzoic acid dissolved in ethanol. Total applied doses were 2, 0.02, 0.002, and 2 mg/ear, respectively. Treatment was effected by application of a total of 20 μl of the test compound dissolved in acetone to both faces of the left ear of the mouse 30 min before the application of the irritant agent. Control animals received only the vehicle. At 45 min (benzoic acid), 1 h (arachidonic acid), 6 h (TPA) and 24 h (dithranol) after administration of the irritant, animals were killed by cervical dislocation. An 8 mm diameter punch biopsy was performed on each ear, the

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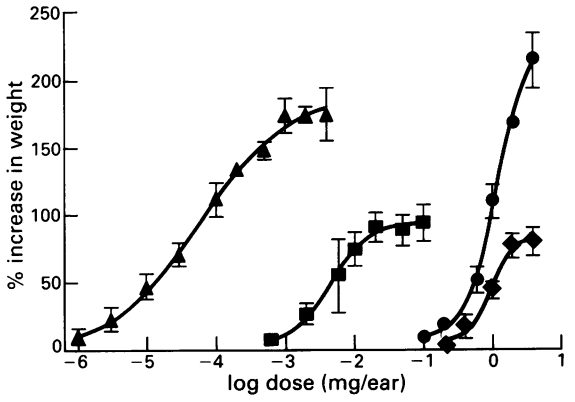


Figure 1 Inflammation caused by topical administration of various doses of 12-O-tetradecanoylphorbol 13-acetate (TPA) (▲); dithranol (■); arachidonic acid (●); and benzoic acid (◆) to both faces of the mouse ear, expressed as the percentage increase of the 8 mm-biopsy weight, compared to the biopsy-weight of the contralateral ear receiving the solvent alone. The application of the solvent alone caused no increase in ear weight. Values are means with s.d. shown by vertical bars (n = 8–10).

right (untreated) ear being the control for each animal. The swelling induced by the irritant was quantified as the percentage increase in the weight of left ear biopsy over that of the right ear biopsy. Percentage inhibition of oedema was calculated according to the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{wt. of inflamed-treated ear} - \text{wt. of control ear}}{\text{wt. of inflamed ear} - \text{wt. of control ear}}\right) \times 100$$

Activity of the inhibitors was expressed as the ID_{50} value, i.e. the dose required to inhibit the increase in weight by 50%, with respect to control animals.

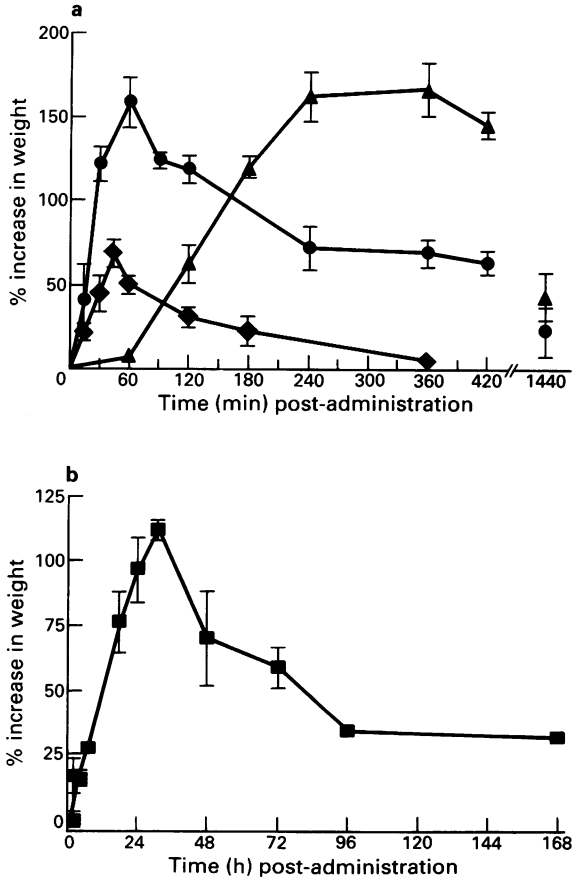


Figure 2 Time course of the ear oedema induced by (a) 12-O-tetradecanoylphorbol 13-acetate (TPA) 2 μg/ear (▲); arachidonic acid 2 mg/ear (●); and benzoic acid 2 mg/ear (◆); (b) dithranol 20 μg/ear (■). Results are expressed as the percentage increase of the 8 mm-biopsy weight compared to the biopsy weight of the contralateral ear receiving the solvent alone. The application of the solvent alone caused no increase in ear weight. Values are means with s.d. shown by vertical bars (n = 8–10).

Myeloperoxidase activity

Tissue biopsy specimens were homogenized in 0.5% hexadecyltrimethylammonium bromide with a Polytron apparatus. The entire process was performed in an ice bath to avoid warming of the sample. The homogenate was centrifuged at 6000 *g* for 30 min at 4°C. The supernatant was assayed for myeloperoxidase activity according to the method of Suzuki *et al.* (1983) with 3,3',5,5'-tetramethylbenzidine used as the substrate.

Platelet aggregation test

Blood was collected from male New Zealand rabbits (2–2.5 kg body weight) in 3.16% sodium citrate (1 volume to 9 volumes of blood) by cardiac puncture. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 250 *g* for 10 min at 4°C. The PRP was diluted with platelet-poor plasma, obtained by further centrifuging at 3000 *g* for 10 min. The platelet count was adjusted to 3.5×10^8 cells ml^{-1}. Platelet aggregation was induced by C_{18}-PAF (15 nM) and measured with a dual-channel aggregometer Chrono-log 500. Activity of the inhibitors was expressed as the IC_{50} value, i.e. the concentration required to inhibit platelet aggregation by 50%.

Hypotension test

Male Sprague-Dawley rats, weighing 180–220 g, were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Blood pressure was recorded from the left carotid artery with a Beckman pressure transducer coupled to a Beckman R611 polygraph. Right and left femoral veins were catheterized for injection of C_{18}-PAF (0.5 μg kg^{-1}) or the test compound. Test compounds were administered intravenously (1 ml kg^{-1}, dissolved in saline), 3 min before PAF injection. Control animals received only the vehicle. Blood pressure was monitored and percentage inhibition of PAF-induced hypotension with respect to controls was calculated. The results are expressed as ID_{50} values, i.e. the dose of the test compound required to inhibit PAF-induced hypotension by 50%.

Statistics

Results of percentage increase in weight are expressed as mean ± s.d. Regression analysis of not less than five data points was used to calculated ID_{50} and IC_{50} values, each point being the mean of the percentage inhibition at a given dose/concentration obtained from two or more independent experiments; 95% confidence limits were calculated with a standard calculation program (Tallarida & Murray, 1981).

Materials

Dexamethasone, dithranol, hexadecyltrimethylammonium bromide, phenidone, 3,3',5,5'-tetramethylbenzidine and TPA were obtained from Sigma (St. Louis, MO, U.S.A.) Arachidonic acid and benzoic acid were from Fluka (Buchs, Switzerland. Indomethacin was provided by Urquima (Sant

Table 1 Effect of topically applied PAF-antagonists on the different models of mouse ear oedema in comparison with their *in vitro* and *in vivo* PAF-antagonist activity

	PAF-induced platelet aggreg. IC_{50} (μM)	*PAF-induced hypotension* (ID_{50}, mg kg^{-1}, i.v.)	*Ear oedema induced by*			
			AA	*TPA*	*D*	*BA*
			ID_{50} (mg/ear)			
UR-10324	0.012 (0.009–0.017)	0.018 (0.016–0.020)	0.058 (0.040–0.083)	0.025 (0.019–0.040)	0.61 (0.47–0.75)	0.28 (0.19–0.45)
UR-11353	0.010 (0.008–0.014)	0.025 (0.022–0.029)	0.075 (0.038–0.14)	0.94 (0.69–1.3)	0.085 (0.052–0.14)	0.43 (0.25–0.76)
CV-6209	0.012 (0.010–0.014)	0.008 (0.006–0.011)	0.059 (0.037–0.088)	0.056 (0.036–0.086)	0.057 (0.042–0.076)	0.17 (0.12–0.24)
WEB-2086	0.091 (0.071–0.117)	0.17 (0.12–0.27)	0.76 (0.38–1.5)	0.39 (0.24–0.61)	0.29 (0.17–0.50)	0.47 (0.27–0.82)
YM-461	0.88 (0.78–0.98)	0.035 (0.023–0.053)	>2	1.52 (1.13–2.1)	0.12 (0.05–0.29)	>2
BN-52021	0.55 (0.38–0.77)	>5	>1	>1	>1	>1
Indomethacin	NT	NT	1.25 (0.61–2.6)	0.015 (0.007–0.029)	0.086 (0.053–0.14)	0.34 (0.21–0.57)
Phenidone	NT	NT	0.013 (0.005–0.034)	0.32 (0.22–0.46)	>2	0.57 (0.34–0.95)
Dexamethasone	NT	NT	0.12 (0.061–0.24)	0.023 (0.016–0.034)	0.14 (0.10–0.19)	0.33 (0.18–0.59)

The results are expressed as IC_{50} and ID_{50} values with their 95% confidence limits given below in parentheses. NT: not tested. AA: arachidonic acid; TPA: 12-O-tetradecanoylphorbol 13-acetate; D: dithranol; BA: benzoic acid.

Fost, Spain). C_{18}-PAF, UR-10324 (2-[[N-acetyl-N-[[2-[2-[heptadecylcarbamoyloxy] ethylthio]ethoxy] carbonyl]-amino] methyl]]-1-ethylpyridinium chloride), UR-11353 (2-[[N - acetyl - N - [[[2 - octadecyloxy]] - 4 - tetrahydrofuranyl] methoxy] carbonyl] amino]methyl] - 1 - ethylpyridinium chloride), CV-6209 (2-[N-acetyl-N-[[[2-methoxy-3-[(octadecylcarbamoyl)oxyl] propoxy] carbonyl] amino] methyl] - 1-ethylpyridinium chloride) (Takeda, Japan), and YM-461 (1-(3-phenylpropyl) - 4 - [2 - (3 - pyridyl) - thiazolidin - 4 - ylcarbonyl] - pipeazine fumarate) (Yamanouchi, Japan) were synthesized in our laboratories following published procedures. WEB-2086 (3 - (4 - (2 - chlorophenyl) - 9 - methyl - 6H - thieno - (3,2 - f)(1,2,4) - triazolo - (4,3 - a)(1,4) - diazepine - 2yl) - 1 - (4 - morpholinyl) - 1 - propanone) and BN- 52021 (3-[1,1-dimethylethyl] hexahydro-1,4,7b - trihydroxy - 8 - methyl - 9H - 1,7a (epoxymethanol) - 1H, 6aH - cyclopenta[c]furo[2,3 - 6] furo[3',2':3,4] cyclopenta-[1,2-d]furan- 5,9,12[4H]-trione) were generous gifts from Dr Heuer (Boehringer Ingelheim, Germany) and Dr Braquet (Henri Beaufour, France), respectively. All other chemicals used were of reagent grade or of the purest commercially available grade.

Results

The four irritants tested produced a marked and dose-dependent inflammatory reaction in the mouse ear (Figure 1). In the arachidonic acid-induced ear oedema, a dose of 2 mg/ear of arachidonic acid was selected for studying the activity of inhibitors for practical reasons, even though a maximal response was not achieved. In contrast, a dose of 20 μg/ear of dithranol was used, since higher doses did not induce a greater inflammatory response. TPA was the most potent irritant tested, showing an inflammatory response in the range of 0.01 to 4 μg/ear. Benzoic acid produced only mild oedema at doses above 1 mg/ear. The time-course of the oedema at the selected doses was also studied. A sharp rise in inflammatory response was observed in the arachidonic acid-induced ear oedema model, reaching a maximum at 1 h, after which the response declined slowly (Figure 2a). A similar time-profile was obtained with benzoic acid, with a maximal effect at about 45 min, returning to basal values at 6 h post-application (Figure 2a). In contrast, the maximal response in the TPA-induced oedema was reached at about 6 h after application of the irritant (Figure 2a). Dithranol-induced ear oedema showed a characteristic delayed-type inflammation, with a maximal effect at about 24–30 h after application of the irritant (Figure 2b). A certain degree of inflammation persisted even one week after dithranol administration.

The potency of PAF-antagonists in the *in vitro* PAF-induced platelet aggregation test and the *in vivo* PAF-induced hypotension test in normotensive rats is given in Table 1. UR-compounds showed a similar potency in the platelet aggregation test, with IC_{50} values of the order of 10^{-8} M, an activity

Table 2 Inhibition of leukocyte accumulation into the inflamed ear, measured as myeloperoxidase activity, in relation to the reduction of arachidonic acid-induced ear oedema

Product	*Dose* (mg/ear)	*% inhibition of AA-induced ear oedema*	*% inhibition of myeloperoxidase activity*
UR-10324	0.1	70.4	60.2
UR-11353	0.1	47.6	46.7
CV-6209	0.1	58.5	67.3
WEB-2086	0.3	37.7	37.8
Dexamethasone	0.1	48.8	35.8

Groups of 10 animals were used for the calculation of percentage inhibition of arachidonic acid (AA)-induced ear oedema. Data of myeloperoxidase activity are mean percentages of inhibition of two or more experiments in which sets of five ears were homogenized and processed as indicated in Methods.

comparable with that of CV-6209 and superior to that of WEB-2086, YM-461 and BN-52021. Potency of URs in the hypotension test was slightly lower than that of CV-6209, but higher than that of YM-461, WEB-2086 and BN-52021.

Topical administration of various PAF-antagonists produced an inhibition of ear oedema in mice. Inhibition was dose-related, and reached values greater than 70% when active compounds were considered, thus allowing calculation of ID_{50} values (Table 1). URs and CV-6209 showed a comparable and marked inhibition of arachidonic acid-induced ear oedema, whereas YM-461 and BN-52021 were practically ineffective and WEB-2086 showed an intermediate activity. Differences of activity between URs were more pronounced (ID_{50} values in the range from 0.025 to 0.94 mg/ear) when dithranol, TPA and benzoic acid were used as the inducers. CV-6209 exhibited a high degree of activity in the three models. WEB 2086 and YM-461 showed variable potency depending on the test, and BN-52021 was without significant activity. Among other reference compounds with no PAF-antagonist activity, only dexamethasone was highly effective in all models of oedema. Indomethacin was only slightly effective in the arachidonic acid-induced ear oedema, while it was very useful in suppressing TPA-, dithranol- and benzoic acid-induced ear oedema. Phenidone was particularly effective in the arachidonic acid-induced ear oedema test, but failed to inhibit dithranol-induced oedema. Inhibition of oedema was accompanied by a concomitant decrease of myeloperoxidase activity in the arachidonic acid-inflamed ears, as shown in Table 2. This result signifies that leukocyte recruitment into the damaged tissue can also be prevented by PAF-antagonists.

The PAF-antagonist and anti-inflammatory activities of structural analogues of UR-10324 are given in Table 3. The deacetylated compound and the non-ethylated neutral molecule showed a considerable reduction of both activities as compared with UR-10324. These results suggest a possible relationship between PAF-antagonist and anti-inflammatory activities.

Topical application of PAF-antagonists at pharmacologically effective doses did not provoke macroscopic changes in treated ears. Only at the highest dose tested, i.e. 3 mg, a mild irritative effect of the ionic compounds was observed, with a peak at about 45–60 min postadministration and a maximum increase of ear weight of about 10%.

Discussion

This study shows that PAF-antagonists of different chemical structures are capable of preventing the inflammatory response provoked by several primary irritants in the mouse ear. It is well known that arachidonic acid-induced ear oedema is accompanied by a rapid production of PGE_2 and leukotriene C_4 (LTC_4)/LTD_4 (Arner *et al.*, 1985), but there are no reports on the participation of PAF in this model of ear oedema. We have found that ionic and non-ionic PAF-antagonists, such as UR-10324, UR-11353, CV-6209 and WEB-2086 are able to inhibit arachidonic acid-induced ear oedema. Moreover, PAF-antagonists can also prevent leukocyte recruitment into the inflamed tissue, as shown by their ability to decrease myeloperoxidase activity in the supernatants of ear homogenates.

It has been well established that the inflammation observed in the TPA model is related to the activation of phospholipase A_2 (Furstenberger *et al.*, 1981) and is primarily mediated by prostaglandin E_2 (PGE_2) (Ashendel & Boutwell, 1979). In consequence, cyclo-oxygenase inhibitors (e.g. indomethacin) are very effective in this test. On the other hand, a relationship between TPA-stimulatory effects and PAF production has been found in some cases. Thus, TPA enhances PAF biosynthesis in human polymorphonuclear leukocytes (Sánchez-Crespo & Nieto, 1989), and CV-3988, a PAF-antagonist with PAF-like structure, suppresses pleurisy induced by TPA in rats (Ohishi *et al.*, 1986).

Kémery *et al.* (1989) demonstrated that dithranol-induced oedema was dose-dependently inhibited by intraperitoneal administration of BN-52021, but also showed the inhibitory activity of indomethacin, clemastine (H_1-antihistamine) and superoxide dismutase. These findings suggest a possible involvement of PAF, prostaglandins, histamine and reactive oxygen radicals in dithranol-induced dermatitis in mice. Nevertheless, in our studies, a unique topical treatment with a PAF-antagonist has managed to produce a near-total suppression of oedema (e.g., CV-6209 induced 87% inhibition at a dose of 0.2 mg/ear). Surprisingly, topical administration of BN-52021 was ineffective in preventing oedema in our conditions. The suppression of dithranol-induced dermatitis in the mouse by local administration of PAF-antagonists could be useful in developing antipsoriatic dithranol-formulations with improved local tolerance and an enhanced therapeutic profile.

No information is available with respect to the mediators involved in the benzoic acid-induced ear oedema. The inhibition caused by treatment with indomethacin and PAF-antagonists suggests a contribution by prostaglandins and PAF in this model.

PAF-antagonist activity may account, at least in part, for the topical anti-inflammatory activity. Indirect evidence in favour of PAF participation in these models of topical inflammation includes the following: (a) structurally different PAF-antagonists (i.e., ionic and non-ionic compounds) are able to suppress oedema and higher anti-inflammatory potency of ionic compounds correlates with their superior PAF-antagonist activity; (b) structural analogues of UR-10324, the deacetylated and the non-ethylated neutral molecule, which are much less potent PAF-antagonists than the parent compound, showed a markedly lower anti-inflammatory activity (Table 3). Nevertheless, we have also found that the PAF-antagonist YM-461 lacks effect in arachidonic acid- and benzoic acid-induced ear oedema, while this compound is highly effective in dithranol-induced oedema; UR compounds show differences in anti-inflammatory potency in spite of their similar PAF-antagonist activity, and we have reported that CV-6209 inhibits not only the rat paw oedema induced by PAF, but also the oedema induced by histamine, 5-hydroxytryptamine, compound 48/80 and carrageenin (Merlos *et al.*, 1990). All these data suggest that other mechanisms

Table 3 PAF-antagonist and anti-inflammatory activities of UR-10324 and its deacetylated and non-ethylated analogues

	PAF-induced platelet aggreg. IC_{50} (μM)	*PAF-induced hypotension* ID_{50} (mg kg^{-1}, i.v.)	*AA*	*Ear oedema induced by TPA* ID_{50} (mg/ear)	*D*	*BA*
UR-10324	0.012 (0.009–0.017)	0.018 (0.016–0.020)	0.058 (0.040–0.083)	0.025 (0.019–0.040)	0.61 (0.47–0.75)	0.28 (0.19–0.45)
Deacetylated analogue	6.1 (4.1–9.1)	>5	>1	>1	>1	>1
Non-ethylated analogue	6.4 (6.3–6.5)	>5	0.46 (0.41–0.52)	>1	>1	>1

The results are expressed as IC_{50} and ID_{50} values with their 95% confidence limits given below in parentheses. Abbreviations as in Table 1.

such as prostaglandin and/or leukotriene biosynthesis inhibition and/or phospholipase inhibition, may be involved in the action of PAF antagonists and explain their anti-inflammatory activity. In fact, preliminary studies have shown that both UR compounds inhibit phospholipase A_2 from snake venom with IC_{50} values of about 10 μM. This additional activity could explain, in part, the anti-inflammatory activity of UR compounds, but to date there is no well-founded explanation for the discrepant activities of UR-10324 and UR-11353 on ear oedema models. Studies to assess the relative contribution of other mechanisms to the overall anti-inflammatory effects of these compounds are now in progress.

In conclusion, the improvement observed in several models of irritant dermatitis in mice after local administration of PAF-antagonists provides evidence of the involvement of PAF in these topical inflammatory processes, and supports the use of PAF-antagonists in the treatment of some inflammatory skin diseases.

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Protective action of clonidine against the arrhythmogenic and lethal effects of ouabain in guinea-pigs

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1 Clonidine (1.25, 2.5 and 5.0 $\mu g\,kg^{-1}$) was studied for its effect on the cardiac arrhythmias and lethality induced by slow intravenous infusion of ouabain in guinea-pigs.

2 Clonidine produced significant delays in the onset of the arrhythmic stages and lethality. However, clonidine did not offer any such protection in reserpinised guinea-pigs, whereas its effects were unaltered in atropinized guinea-pigs.

3 Idazoxan (100 $\mu g\,kg^{-1}$, i.v.) abolished the antiarrhythmic effect of clonidine whereas corynanthine (1 $mg\,kg^{-1}$, i.v.) had no such effect.

4 Clonidine inhibited the rate of the ouabain-induced rise in blood pressure and the peak pressor response.

5 In isolated paced left atria of the guinea-pig, clonidine (3.75×10^{-4} M) did not offer any protection against rapid and/or irregular extrasystolic contractions induced by ouabain.

6 It is concluded that the antiarrhythmic effect of clonidine is due to its effects on the indirect neural components of digitalis toxicity mediated by the stimulation of α_2-adrenoceptors, without any direct anti-arrhythmic effect on the myocardium.

Keywords: Clonidine; arrhythmia; digitalis; ouabain-induced cardiotoxicity

Introduction

Digitalis, one of the most widely used drugs in the treatment of congestive heart failure, frequently produces a wide variety of cardiac arrhythmias, when high plasma concentrations are reached (Smith *et al.*, 1984). The pathophysiology of digitalis-induced arrhythmias involves glycoside-induced centrally mediated alterations of the autonomic nervous system (Roberts *et al.*, 1967; Saxena & Bhargava, 1975; Gillis & Quest, 1980) apart from the well established direct action on the myocardium (Ferrier, 1977). The role of the sympathetic nervous system in the genesis and perpetuation of cardiac arrhythmias induced by cardiac glycosides is receiving particular attention at the moment, in order to understand and prevent the occurrence of these life-threatening cardiac arrhythmias. Surgical or pharmacological procedures that decrease sympathetic nerve function also decrease digitalis toxicity. Even though many groups of drugs which decrease sympathetic tone, like β-adrenoceptor antagonists (Kelliher & Roberts, 1974; Hernandez & Serrano, 1982; Tripathi & Thomas, 1986), ganglion blocking agents (Gillis *et al.*, 1975), drugs interfering with catecholamine storage and release (Levitt & Roberts, 1967; Saito *et al.*, 1974) were studied and found effective against cardiac arrhythmias induced by various cardiac glycosides, there has been no serious attempt to study the effect of α_2-adrenoceptor agonists against digitalis-induced cardiotoxic effects. Numerous studies have proposed that clonidine reduces blood pressure by a reduction in sympathetic tone through activation of central α_2-adrenoceptors (Schmitt, 1977; Isaac, 1980; Van Zwieten & Timmermans, 1983). Relatively high doses of clonidine were reported to show antiarrhythmic properties against ouabain-induced arrhythmias (Pace & Gillis, 1976; Lechat & Schmitt, 1982; Thomas & Tripathi, 1986). The present study aimed to examine the effect of lower doses of clonidine (which act specifically on α_2-adrenoceptors) against ouabain-induced arrhythmias and lethality and to examine the nature of its antiarrhythmic action.

Methods

Arrhythmias and lethality induced by intravenous infusion of ouabain in guinea-pigs

Albino guinea-pigs of either sex in the weight range of 350–450 g were used in this study. The method described by Thomas & Tripathi (1986) was employed. The animals were anaesthetized by an intraperitoneal injection of pentobarbitone sodium (60 $mg\,kg^{-1}$). Positive pressure artificial respiration was maintained throughout the experiment by means of a rodent respiratory pump (Palmer) at the rate of 45 strokes min^{-1} and volume was adjusted at 1.0 ml 100 g^{-1} body weight. The left common carotid artery was cannulated and connected to a Bentley-Trantec physiological pressure transducer and the blood pressure was recorded on a Gemini Recorder (Ugo Basile, Model 7070). The right jugular vein was cannulated for the infusion of ouabain and administration of test drugs. Limb lead II ECG was recorded on a Grass Polygraph (Model 7D) and heart rate was calculated from ECG signals. Ouabain solution (80 $\mu g\,ml^{-1}$) was continuously infused at the rate of 100 $\mu l\,min^{-1}$. The amount of ouabain required per kg body weight for the onset of ventricular premature beats (VPB), ventricular tachycarrhythmias (VT) (denoted by ventricular tachycardia or ventricular fibrillation associated with a sudden fall in blood pressure) and lethality (L) was determined in control and clonidine-treated animals.

Pressor effect induced by ouabain

During the course of the experimental procedure described above, blood pressure was recorded every 2 min of ouabain infusion until the onset of VT. The rate of rise of blood pressure induced by ouabain and the peak pressor effect attained was noted for control and clonidine (2.5 $\mu g\,kg^{-1}$)-treated groups.

Interaction studies

In order to study the nature of the antiarrhythmic action of clonidine, the following sets of experiments were conducted using the method described above.

(1) Two groups of six guinea-pigs each were given reserpine (5 $mg\,kg^{-1}$, i.m.) 24 h before the experiment. The first group was administered clonidine at a dose of 2.5 $\mu g\,kg^{-1}$, i.v. and the rest given normal saline 10 min prior to the ouabain infusion.

(2) Corynanthine hydrochloride (1 $mg\,kg^{-1}$) was administered intravenously to six animals 10 min before clonidine (2.5 $\mu g\,kg^{-1}$), while another three groups of six animals each

were administered clonidine, corynanthine and normal saline respectively.

(3) Idazoxan (100 $\mu g\,kg^{-1}$, i.v.) was administered to six guinea-pigs and after 10 min, clonidine was given at a dose of 2.5 $\mu g\,kg^{-1}$, i.v. Another three groups of six animals each were also studied, one as control and the others were treated with clonidine and idazoxan.

(4) One group of five animals was pretreated with atropine sulphate (2 $mg\,kg^{-1}$, i.v.) and another five animals with atropine and clonidine (2.5 $\mu g\,kg^{-1}$) before the infusion of ouabain.

Ouabain-induced arrhythmias in paced guinea-pig left atria

Guinea-pigs of either sex (350–450 g) were killed by a hammer blow on the head and the heart was quickly excised and transferred to a Petri dish containing Ringer Locke solution (composition in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 6.0 and glucose 5.5). The left atrium was dissected free and hung vertically with the base anchored to a stimulating electrode and the other end tied to an isometric transducer (type DYO, Ugo Basile, Italy), in a 50 ml organ bath containing Ringer Locke Solution at 37 ± 1°C and buffered to pH 7.4 by saturation with oxygen. After a lapse of 10 min, the atrium was driven at a constant rate of 1 Hz at intervals of 1000 m and duration of 2 ms at twice the threshold voltage delivered by a square wave pulse generator (Grass, Model S44, U.S.A.).

After an initial stabilization period of 45 min during which the tissue was washed every 15 min and the threshold voltage checked, ouabain at a concentration of 1.0×10^{-6} M was added and the atrial contractions were recorded continuously on a Ugo Basile Microdynamometer. The time taken for the development of arrhythmia (spell I), defined as 30 s of continuous rapid and/or irregular extrasystolic contractions, was noted. The tissue was washed four times and allowed to stabilize for 60 min. During this period, the tissue was washed every 15 min and the threshold voltage checked. At the end of this stabilization period, ouabain (1.0×10^{-6} M) was once again added and the onset and duration of arrhythmic contractions (spell II) were noted ($n = 6$).

In another set of experiments, clonidine at concentrations of 3.75×10^{-6} M, 3.75×10^{-5} M and 3.75×10^{-4} M ($n = 5$ for each concentration in separate experiments) was added to the bath after spell I and the onset and duration of spell II were noted.

Drugs

The following drugs were used in this study: clonidine hydrochloride (Unichem Laboratories), corynanthine hydrochloride (Sigma), idazoxan (Reckitt & Colman), ouabain octahydrate (Sigma), reserpine and atropine sulphate (Boehringer Ingelheim). All drugs except reserpine were dissolved in normal saline for injection. Reserpine was dissolved in minimum amount of glacial acetic acid, the pH adjusted to 5.5 by 0.1 N NaOH and diluted with distilled water to give a concentration of 1 $mg\,ml^{-1}$.

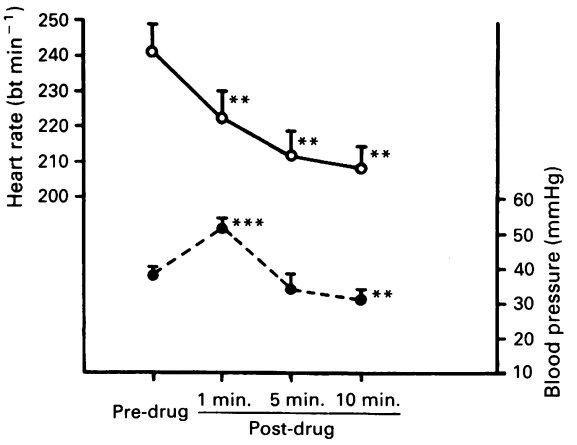


Figure 1 The effect of clonidine on heart rate (○) and blood pressure (●) in guinea-pig. Values are represented as mean of 5–6 experiments with s.e.mean by vertical bars. ** $P < 0.01$; *** $P < 0.001$. Clonidine 2.5 $\mu g\,kg^{-1}$.

Statistical analysis

Statistical analysis was performed by use of Student's t test. Significance was established when the probability value was less than 0.05. All values were expressed as mean ± s.e.mean.

Results

Haemodynamic effects of clonidine

Guinea-pigs anaesthetized with sodium pentobarbitone showed a basal mean arterial blood pressure of 38 ± 2 mmHg and heart rate of 241 ± 8 beats min^{-1}. Clonidine (2.5 $\mu g\,kg^{-1}$) on intravenous administration, produced an immediate significant reduction in the heart rate ($P < 0.01$). Further lowering was seen at the end of 5 and 10 min periods ($P < 0.01$). A biphasic response was observed on blood pressure. The initial statistically significant rise was transient and was followed by a gradual fall. The blood pressure value at the end of 10 min was significantly lower ($P < 0.01$) when compared to the control value (Figure 1).

Effect of clonidine on cardiac arrhythmias and lethality

Ouabain infusion produced initial bradyarrhythmias, ventricular premature beats, ventricular tachycardia (mostly associated with spells of ventricular fibrillation) and cardiac arrest in guinea-pigs. Initial bradyarrhythmias occurred almost at the same time as VPB and in some cases VPB was the first arrhythmic stage. Clonidine at a dose of 1.25 $\mu g\,kg^{-1}$ significantly increased the dose of ouabain required to cause VT. However, at this dose level, there was no significant alteration in the dose of ouabain required to produce VPB. At doses of 2.5 and 5.0 $\mu g\,kg^{-1}$ clonidine, there were significant increases in the dose of ouabain needed to elicit VPB and VT. Similarly, these doses caused a significant increase in the lethal dose of ouabain, whereas there was no significant difference at 1.25 $\mu g\,kg^{-1}$ dose level (Table 1).

Table 1 Effect of clonidine on ouabain-induced cardiac arrhythmias and lethality in guinea-pigs

Drug ($\mu g\,kg^{-1}$)	n	*VPB*	*VT*	*L*
Control	6	183.3 ± 11.2	209.4 ± 16.7	298.0 ± 6.0
Clonidine				
1.25	6	181.3 ± 5.2	282.0 ± 24.9*	337.9 ± 22.1
2.50	6	239.3 ± 11.6**	342.1 ± 13.9***	411.4 ± 14.4***
5.00	6	244.6 ± 13.6**	372.1 ± 10.9***	459.5 ± 15.8***

Unpaired 't' test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Values are expressed as mean ± s.e.mean of the doses of ouabain ($\mu g\,kg^{-1}$ body weight) required to cause ventricular premature beats (VPB), ventricular tachyarrhythmias (VT) and lethality (L).

Effect of clonidine on the ouabain-induced pressor response

A gradual rise in blood pressure was observed after intravenous infusion of ouabain. The peak pressor response was obtained just before the onset of VT. The rise in blood pressure induced by ouabain and its inhibition by clonidine is shown in Figure 2. The rate of rise of blood pressure was significantly ($P < 0.001$) lower in clonidine pretreated guinea-pigs than in controls. Similarly, the peak pressor response obtained in clonidine pretreated animals was significantly ($P < 0.05$) lower than that of controls (Figure 3). Corynanthine, the α_1-adrenoceptor antagonist could not influence the inhibition of the pressor effect caused by clonidine but idazoxan, the α_2-adrenoceptor antagonist completely abolished it.

Interaction studies

The dose of ouabain needed to induce VPB, VT and L were higher in reserpinised guinea-pigs than in normal controls. In reserpinsed animals, clonidine did not alter the arrhythmogenic and lethal effects of ouabain as the doses of ouabain needed to produce these effects were not significantly different in clonidine-treated, compared to control animals after reserpinization (Table 2). Corynanthine, an α_1-adrenoceptor blocking agent, *per se* did not have any significant effect on ouabain-induced arrhythmias in the guinea-pig. It also failed to modify the antiarrhythmic effect of clonidine significantly. Idazoxan, the specific α_2-adrenoceptor antagonist, showed significant inhibition ($P < 0.05$ for VPB, $P < 0.01$ for VT and L)

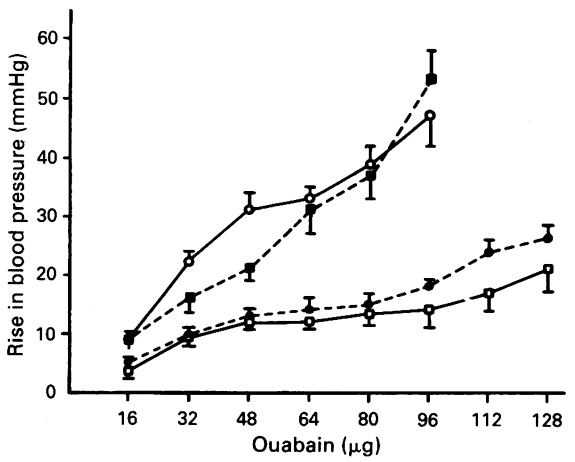


Figure 2 Effect of clonidine on the ouabain-induced rate of rise of blood pressure in guinea-pig. Values are expressed as mean of 5–6 experiments; vertical bars show s.e.mean. Control (○); clonidine ($2.5\,\mu g\,kg^{-1}$) (●) and clonidine after coryanthine ($1\,mg\,kg^{-1}$) (□) and idazoxan ($100\,\mu g\,kg^{-1}$) (■).

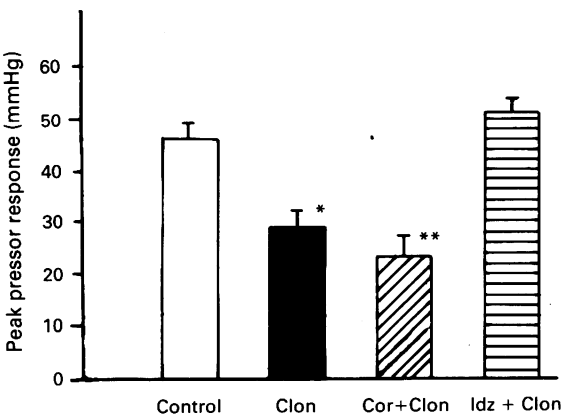


Figure 3 Effect of clonidine on the peak pressor response induced by ouabain: Clonidine (Clon) $2.5\,\mu g\,kg^{-1}$; coryanthine (Cor) $1\,mg\,kg^{-1}$; idazoxan (Idz) $100\,\mu g\,kg^{-1}$. * $P < 0.05$; ** $P < 0.01$.

of the antiarrhythmic effect of clonidine (Table 3). Atropinisation itself did not have any effect on the arrhythmogenic or lethal effect of ouabain whereas it potentiated the effect of clonidine against lethality caused by ouabain. The antiarrhythmic effect of clonidine was not altered significantly by atropine even though there was a general increase in the dose of ouabain needed to induce VPB and VT (Table 2).

Effect of clonidine on ouabain-induced arrhythmias in isolated paced left atria of guinea-pigs

Clonidine at concentrations of 3.75×10^{-6} M, 3.75×10^{-5} M and 3.75×10^{-4} M did not accord any protection against ouabain-induced arrhythmias in isolated paced left atria of guinea-pigs (Table 4), whereas propranolol at a concentration of 1.69×10^{-5} M and lignocaine at a concentration of 3.46×10^{-5} M completely protected the tissues from ouabain-induced arrhythmias.

Discussion

In the present study, clonidine at very low doses significantly increased the ouabain dose required to produce VPB, VT and L. The incidence and pattern of these stages remained unaltered. This result is in conformity with the previous studies with clonidine, even though used in higher doses. Pace & Gillis (1976) used $6–60\,mg\,kg^{-1}$ (i.v.), whereas Lechat & Schmitt (1982) employed $10–300\,\mu g\,kg^{-1}$ (i.v.) and Thomas & Tripathi (1986) used $500\,\mu g\,kg^{-1}$ (i.p.) and reported similar results. Dose-range is a vital factor as far as the specificity of clonidine for α_2-adrenoceptors is concerned. In higher doses, clonidine acts upon α_1-adrenoceptors also (Cavero & Roach, 1978) and exhibits membrane stabilizing properties (Hoefke & Kobinger, 1966). Hence, the present study was done with very low doses of clonidine in an effort to identify its antiarrhythmic activity through its α_2-adrenoceptor stimulant properties.

Central α_2-adrenoceptors in the brain stem have been identified as the target of clonidine-like centrally acting antihypertensive drugs (Kobinger, 1978). Clonidine in lower doses acts specifically on α_2-adrenoceptors and numerous studies have proposed that the drug reduces blood pressure by a reduction in sympathetic tone by activation of central α_2-adrenoceptors (Schmitt, 1977; Isaac, 1980; Van Zwieten & Timmermans, 1983).

Digitalis-induced arrhythmias are the outcome of a complex interplay of actions on the myocardium, the central nervous system and the autonomic nervous system. Since membrane stabilizing properties have been reported for clonidine (Hoefke & Kobinger, 1966) it could in principle act against the direct or indirect components of ouabain toxicity. Left atrial models of ouabain-induced arrhythmias are the result of the direct effect of ouabain as they exclude the indirect centrally mediated reflex effects (Thomas & Varma, 1990). In the present study, clonidine up to a concentration of 3.75×10^{-4} M failed to offer any protective effect in this tissue. In these concentrations, clonidine may be devoid of any membrane stabilizing action and this rules out any direct effect of clonidine on the myocardium as the cause of its antiarrhythmic action. In the same preparation, propranolol at a concentration of 1.69×10^{-5} M and lignocaine at 3.46×10^{-5} M afforded complete protection to the tissue against ouabain-induced arrhythmias. This also indicates that clonidine might be acting upon the indirect components of digitalis toxicity. In reserpinized guinea-pigs, clonidine was unable to alter the arrhythmogenic and lethal doses of ouabain. In reserpinized animals the effect produced by ouabain would be comparable to a situation where indirect (sympathetic) components of its toxic effects are removed or inhibited. Thus the inability of clonidine to alter the arrhythmogenic and lethal effects of ouabain in reserpinized guinea-pigs supports the assumption that it is the indirect

Table 2 Effect of clonidine on ouabine induced arrhythmias in atropinized and reserpinized guinea-pigs

Drug (Dose)	n	*VPB*	*VT*	*L*
Control	5	205.5 ± 17.0	238.4 ± 13.0	329.8 ± 18.7
Clonidine ($2.5\,\mu g\,kg^{-1}$)	5	269.1 ± 17.8*	339.0 ± 13.3***	387.8 ± 13.4*
Atropine ($2\,mg\,kg^{-1}$)	5	205.8 ± 7.7	251.4 ± 15.7	354.7 ± 10.2
Atr + clonidine ($2\,mg\,kg^{-1} + 2.5\,\mu g\,kg^{-1}$)	5	291.0 ± 16.6	385.4 ± 21.7	449.2 ± 8.6[a]
Reserpine ($5\,mg\,kg^{-1}$)	6	224.9 ± 8.3	292.2 ± 13.7	389.7 ± 5.1
Reserpine ($5\,mg\,kg^{-1}$) + clonidine ($2.5\,\mu g\,kg^{-1}$)	7	232.6 ± 13.1	324.8 ± 16.2	423.8 ± 10.6

Values are expressed as mean ± s.e.mean of the doses of ouabain ($\mu g\,kg^{-1}$ body weight) required to cause ventricular premature beats (VPB), ventricular tachyarrhythmia (VT) and lethality (L).
* $P < 0.05$; *** $P < 0.001$ compared to control. [a] $P < 0.01$ compared to clonidine (Student's t test).

Table 3 Effect of α-adrenoceptor antagonists on the anti-arrhythmic effect of clonidine

Drug (dose)	n	*VPB*	*VT*	*L*
Control	6	183.6 ± 6.1	232.3 ± 15.4	311.2 ± 14.0
Clonidine ($2.5\,\mu g\,kg^{-1}$)	6	230.8 ± 17.4*	330.8 ± 24.1**	392.8 ± 25.1*
Corynanthine ($1\,mg\,kg^{-1}$)	6	175.4 ± 9.4	253.2 ± 21.4	345.9 ± 26.5
Cor + Clon ($1\,mg\,kg^{-1} + 2.5\,\mu g\,kg^{-1}$)	6	249.1 ± 6.4***	363.1 ± 20.0***	428.8 ± 15.5***
Control	6	210.8 ± 10.5	250.2 ± 10.9	336.0 ± 16.9
Clonidine ($2.5\,\mu g\,kg^{-1}$)	6	287.9 ± 19.3**	369.9 ± 25.8**	426.3 ± 23.2**
Idazoxan ($100\,\mu g\,kg^{-1}$)	6	196.4 ± 14.8	231.4 ± 19.6	322.7 ± 28.9
Idazoxan + clonidine ($100\,\mu g\,kg^{-1} + 2.5\,\mu g\,kg^{-1}$)	6	227.6 ± 13.1[a]	265.0 ± 14.5[b]	324.8 ± 16.0[b]

Values are expressed as mean ± s.e.mean of the doses of ouabain ($\mu g\,kg^{-1}$ body weight) required to cause ventricular premature beats (VPB), ventricular tachyarrhythmia (VT) and lethality (L).
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to respective controls. [a] $P < 0.05$; [b] $P < 0.01$ compared to respective clonidine values (Student's t test)

components of digitalis toxicity that are acted upon by clonidine.

Clonidine has roughly ten times more affinity for α_2- than for α_1- adrenoceptors (Bousquet & Schwartz, 1983).However, many investigators have demonstrated the involvement of α_1-adrenoceptors in some of the pharmacological actions of clonidine in the periphery (Hoefke & Kobinger, 1966; Drew, 1976) and in the central nervous system (Cavero & Roach, 1978; Timmermans *et al.*, 1979; Hamilton & Longman, 1982). Corynanthine, a preferential α_1-adrenoceptor antagonist (Weitzell *et al.*, 1979; Van Zwieten & Timmermans, 1983) did not alter the antiarrhythmic effect of clonidine in this study, thereby excluding the involvement of α_1-adrenoceptors in its effect. Idazoxan, the highly selective α_2-antagonist (Clifford *et al.*, 1985; Al-Damluji *et al.*, 1988) completely abolished the protective effect of clonidine against the ventricular arrhythmias and lethality pointing to the involvement of α_2-adrenoceptors in this action.

Table 4 Effect of clonidine on ouabain-induced arrhythmia in isolated paced left atrium of guinea-pig

Drug (M)	n	*Pre-drug* *Onset* (min)	*Post-drug* *Onset* (min)	*Post-drug* *Duration* (min)
Control	6	6.13 ± 0.80	6.45 ± 0.59	>30
Clonidine				
3.75 ± 10^{-6}	5	6.43 ± 0.34	7.60 ± 0.41	>30
3.75 ± 10^{-5}	5	6.73 ± 0.61	5.89 ± 0.94	>30
3.75×10^{-4}	5	6.30 ± 0.84	6.59 ± 0.30	>30
Lignocaine				
3.46×10^{-5}	6	7.15 ± 0.81	—*	—*
Propranolol				
1.69×10^{-5}	6	6.90 ± 0.24	—*	—*

Values are expressed as mean ± s.e.mean of the time taken for the onset of extrasystolic contractions induced by ouabain (1.0×10^{-6} M).
* Complete protection.

Stimulation of central α_2-adrenoceptors by clonidine induces an enhanced vagal tone (Timmermans & Van Zweiten, 1977). There was no inhibition of its antiarrhythmic effect in atropinized guinea-pigs, ruling out enhanced vagal tone as the cause of its antiarrhythmic activity, whereas there was a significant increase in the lethal dose of ouabain in animals treated with atropine and clonidine compared to those treated with clonidine alone, indicating a potentiation of the effect of clonidine. It has been suggested that activation of muscarinic receptors by ouabain can cause the enhancement of catecholamine secretion (Yamada *et al.*, 1989). Whether the muscarinic blockade helped in the potentiation of the protective effect requires further examination and study.

Elevation in blood pressure induced by ouabain results primarily from activation of the sympathetic nervous system (Trzeciakowski, 1985). In the guinea-pig, digitalis glycosides induce the release of catecholamines by an action on the central nervous system and this is responsible for its pressor effects. This pressor effect is produced by the stimulation of

α_1-adrenoceptors and is suppressed by α_1-blockade and not altered by α_2-blockade (Lechat & Schmitt, 1982). In the present study, clonidine significantly inhibited the pressor effects induced by ouabain. The rate of rise of blood pressure and the peak pressor response induced by ouabain were significantly lower in clonidine pretreated guinea-pigs than in controls. The correlation between the inhibition of pressor effects and the reduction in the arrhythmogenic and lethal doses of ouabain indicates that both may be the result of the ability of clonidine to interfere with the neural components of ouabain action. In the interaction studies it was observed that idazoxan abolished the inhibition of the pressor effect caused by clonidine, whereas corynanthiane could not alter it. These results are parallel to their effects on the antiarrhythmic effect of clonidine and support the contention that these two effects are interrelated.

Clonidine causes a diminished release of noradrenaline from the nerve endings supplying the heart by stimulation of peripheral presynaptic α_2-adrenoceptors and decreases plasma catecholamines and peripheral sympathetic tone by stimulation of central α_2-adrenoceptors (Van Zwieten & Timmermans, 1984). Thus it is possible that the protective effect of clonidine against ouabain induced ventricular arrhythmias and lethality is brought about by the same mechanism which is responsible for its antihypertensive effect, i.e. the reduction in the sympathetic tone.

The results of the present study can be summarized as follows. Clonidine in relatively low doses demonstrated significant protection against ouabain-induced ventricular arrhythmias and lethality in guinea-pigs. In isolated paced left atria of the guinea-pig clonidine failed to produce any antiarrhythmic effect against ouabain. The pressor effect induced by ouabain was also inhibited by clonidine. In reserpinized animals, clonidine does not accord any further protection. α_2-Adrenoceptor blockade abolished the antiarrhythmic effect of clonidine whereas α_1-blockade was without any effect. Muscarinic blockade did not alter the antiarrhythmic efficacy of clonidine whereas it potentiated the clonidine-induced protection against lethality caused by ouabain.

It may be concluded that clonidine inhibits the indirect (neural) components of ouabain toxicity by a reduction in sympathetic tone caused by stimulation of α_2-adrenoceptors, without any direct antiarrhythmic actions on the myocardium.

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Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes

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1 The effects of noradrenaline (NA) were studied on vascular smooth muscle cells isolated from rat portal vein.

2 Two types of single-Ca^{2+} channel currents with conductances of 17 pS and 8 pS were obtained in cell-attached configuration. Bath application of NA increased the open probability of both channels during depolarizing pulses without a change of background membrane conductance. However, NA did not open Ca^{2+} channels when the membrane patch potential was held at −50 mV, which is about the resting potential in physiological conditions.

3 In the whole-cell configuration, studies of voltage-dependent Ca^{2+} channel currents showed that the peak conductance curve was not shifted to more negative potentials by NA.

4 Measurements of internal Ca^{2+}-concentration ($[Ca^{2+}]_i$) with Indo-1 indicated that NA increased $[Ca^{2+}]_i$ at a holding potential of −50 mV and evoked a Ca^{2+}-activated Cl^- current. These effects were blocked when heparin was included in the pipette solution.

5 A Cl^- channel blocker without effect on Ca^{2+} channels (anthracene-9-carboxylic acid) inhibited the contractions of portal vein strips induced by NA in a manner similar to that produced by a Ca^{2+} channel inhibitor (isradipine). The NA-induced contraction was completely suppressed in the presence of ryanodine which depletes intracellular Ca^{2+} stores.

6 The present study suggests that activation of Cl^- channels by Ca^{2+} release produces a membrane depolarization which is a prerequisite for enhanced opening of voltage-dependent Ca^{2+} channels in response to NA in venous smooth muscle.

Keywords: Noradrenaline; chloride channel; calcium channel; vascular smooth muscle cells; rat portal vein; anthracene-9-carboxylic acid; isradipine; ryanodine

Introduction

Noradrenaline (NA) has powerful effects on the mechanical and electrical activity of vascular smooth muscle cells (Bolton, 1979; Bülbring & Tomita, 1987). In portal vein smooth muscle, stimulation of α_1-adrenoceptors produces contraction that involves release of Ca^{2+} from intracellular stores and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Mironneau & Gargouil, 1979; Dacquet *et al.*, 1987). The NA-induced contraction is associated with a depolarization and an increase in membrane conductance (Takata, 1980; Nanjo, 1984). Recent results obtained from single venous smooth muscle cells indicated that the NA-induced depolarization is mainly mediated by an increase in chloride conductance (Byrne & Large, 1988a; Van Helden, 1988; Pacaud *et al.*, 1989). However, an increase in a non-specific cation channel (Byrne & Large, 1988b) and a decrease in potassium conductance (Suzuki, 1981) could also be involved.

In addition, NA enhances the amplitude of the Ca^{2+} channel current of vascular smooth muscle cells (Benham & Tsien, 1988; Pacaud *et al.*, 1989). We have recently shown the involvement of a G-protein regulating the phospholipase C activity in the electrophysiological effects of NA in portal vein smooth muscle cells (Loirand *et al.*, 1990). D-*myo*-Inositol 1,4, 5-trisphosphate ($InsP_3$) may be responsible for Ca^{2+} release and subsequent activation of Ca^{2+}-activated Cl^- current, while diacylglycerol may be responsible for the stimulation of the Ca^{2+} channel current, probably through activation of protein kinase C (Loirand *et al.*, 1990).

The present work was undertaken to determine whether NA acted by producing a shift to more negative voltages of the activation curve for Ca^{2+} channels so that the open state probability was greater at any given membrane potential and that depolarization would not be necessary to cause a substantial increase in Ca^{2+} entry through voltage-dependent channels. The alternative hypothesis is that NA induced only an increase in the open state probability without change in the voltage-dependence of the Ca^{2+} channels so that depolarization would be required to activate Ca^{2+} channels. It will be shown that NA activates an inward current which drives the membrane potential into the activation range of voltage-dependent Ca^{2+} channels as has already been suggested (Byrne & Large, 1988a), and that the contraction to NA was not brought about by direct activation of Ca^{2+} channels as was proposed by Nelson *et al.* (1988, 1990).

Methods

Single cell isolation and short term primary culture

Wistar rats (150 g) were stunned and then killed by cervical dislocation. Portal veins were dissected free of connective tissue and single cells were obtained by a dispersal procedure similar to that described previously (Loirand *et al.*, 1986). The cells were plated on collagen-coated cover-slips in medium M199 (Flow Laboratories) containing 10% foetal bovine serum, 2 mM glutamine, 20 u ml^{-1} penicillin and 20 $\mu g\,ml^{-1}$ streptomycin (Gibco) and kept in an incubator gassed with 95% O_2, 5% CO_2 at 37°C. The cells were used between 2 h and 20 h after isolation.

Contraction measurements

Contractions of portal vein longitudinal strips were measured with an isometric force transducer (Akers 801) as previously described (Dacquet *et al.*, 1987). The bath solution (reference solution) contained (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, HEPES 10, glucose 11, pH 7.4 with NaOH.

Electrophysiological recordings

Membrane currents were measured by use of standard patch-clamp techniques (Hamill *et al.*, 1981). Whole-cell Cl^- currents were recorded in response to 10^{-5} M NA applied from a glass pipette with a pressure ejector. The cells were bathed in the reference solution and the basic pipette solution contained (mM): CsCl 130, HEPES 10, pH 7.3 with NaOH. For the measurements of whole-cell Ca^{2+} channel current, the bath solution contained (mM): $BaCl_2$ 90, glucose 10, HEPES 10, pH 7.4 at 30°C and the pipette was filled with a solution containing (mM): CsCl 130, HEPES 10, EGTA 5, pH 7.3 with NaOH. Single Ca^{2+} channel currents were measured in the cell-attached patch configuration. The patch pipette solution was (mM): $BaCl_2$ 90, glucose 10, HEPES 10, pH 7.4. Under these experimental conditions, unitary Cl^- current could not be recorded since they could not be activated by Ba^{2+}. The unitary currents were filtered at 0.6 kHz, digitized at 10 kHz, stored and analysed with a Plessey 6200 computer. Capacitive and leakage currents were digitally subtracted using the average currents of blank sweeps. Records were displayed on an X-Y plotter (Hewlett-Packard 7470A).

Chemicals used were: noradrenaline and heparin (Sigma); anthracene-9-carboxylic acid (9-AC) and caffeine (Merck); Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer); isradipine (Sandoz); ryanodine (Calbiochem).

The values given in the text are means ± s.e.mean with n as the sample size. Significance was tested by means of Student's t test.

Fluorescence measurements

For these experiments, cells were plated on glass cover-slips and bathed in the reference solution. Combined microfluorimetric and electrophysiological experiments were performed by use of the whole-cell recording configuration. Cells were loaded passively with Indo-1 in the patch pipette filled with the basic pipette solution containing 0.06 mM Indo-1 pentasodium salt (Calbiochem). The dual emission microspectrofluorimeter was constructed from a Nikon Diaphot inverted microscope fitted with epifluorescence (× 40 oil-immersion objective). For excitation of the Indo-1, light from a 100 W mercury lamp (Osram) was reduced by use of two neutral density filters (ND 16, Nikon) and then filtered at 360 ± 5 nm and reflected off a dichroic mirror (380 nm). The emitted fluorescence signal was passed through a pinhole diaphragm slightly larger than an average cell and was directed to another dichroic mirror (455 nm). Transmitted light was filtered at 480 ± 10 nm and reflected light at 405 ± 5 nm. The intensities were recorded by two photometers (P1, Nikon), and single photon currents were converted to voltage signals. Before each experiment, the background fluorescence was determined in the cell-attached mode and cancelled by offsetting the output level of each photometer. Voltage signals at each wavelength were sampled at 17 Hz and stored in an IBM-PC computer for subsequent analysis. The ratio (R = F405/F480) was calculated on-line and displayed with the two voltage signals on a monitor. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated from the ratio R of the fluorescence measured at the two wavelengths by the equation $[Ca^{2+}]_i = K_D(F_o/F_s)(R - R_{min})/(R_{max} - R)$ (Grynkiewicz *et al.*, 1985) where K_D is the effective dissociation constant of Indo-1, F_o and F_s are the 480 nm signals in the absence and in the presence of saturating Ca^{2+} respectively. R_{min} and R_{max} are the 405/480 ratios in the absence and in the presence of saturating Ca^{2+} respectively. Since the values of R_{min} and R_{max} *in vitro* and *in vivo* were notably different (Almers & Neher, 1985) intracellular calibration was made. R_{min}, R_{max} and $K_D(F_o/F_s)$ were determined as described by Benham (1989) and Pacaud & Bolton (1991). The means value for R_{max}, R_{min} and $K_D(F_o/F_s)$ were 0.850 ± 0.007 ($n = 17$), 0.050 ± 0.001 ($n = 7$) and 1072 ± 29 nM ($n = 6$).

Results

Single-channel recordings

Figure 1 illustrates some features that distinguish two types of Ca^{2+} channel in isolated smooth muscle cells from rat portal vein in the same cell-attached patch. When the holding potential was held at −70 mV, single channel currents typified by brief openings of about −1 pA in amplitude were recorded upon stepping to a test potential of −32 mV in the presence of 1 μM Bay K 8644 (Figure 1a). At more depolarized test potentials a second type of channel activity of about −1.5 pA in amplitude was seen with long-lasting openings (Figure 1b). When the same patch was depolarized from a holding potential of −40 mV only large amplitude single-channel currents were observed (Figure 1c). Figure 1d shows the current-voltage relationships for both types of current. The slope conductance was 8 pS for the small conductance channel (T-type) and 17 pS for the large conductance channel (L-type). The extrapolated reversal potentials were near +80 mV for both Ca^{2+} channels. In Figure 1b separate L-type events (sweeps 1, 2, 4), T-type events (sweep 3), simultaneous occurrence of L-type and T-type events with a closing of the L-type channel (sweep 5) and simultaneous occurrence of L-type and T-type events with a closing of the T-type channel (sweep 6) can be observed in the same single L-type channel patch. In a total of 100 sweeps obtained in the absence of Bay K 8644, 9 sweeps only show L-type openings, in 24 sweeps only T-type openings are observed and 4 sweeps show both L-type and T-type events. Therefore the probability of the L-type channel and of the T-type channel being open are $P_f(L) = 0.13$ and $P_f(T) = 0.28$ respectively and the probability of the two types of channel being open in the same sweep is $P_f(L \wedge T) = 0.040$. The product $P_f(L) \times P_f(T) = 0.036$ is approximately equal to $P_f(L \wedge T)$, indicating that L-type and T-type currents appear to be independent.

Single-channel current recordings provide the most direct approach for the identification of the NA-sensitive Ca^{2+} channel and to characterize the modulatory effect. Ca^{2+} influx is proportional to $N_T.P_f.P_o.i$, where N_T is the total number of Ca^{2+} channel proteins per cell, P_f is the probability that the channel is functional i.e. that it responds to depolarization at all, P_o is the probability of a functional channel being open for a given time at a given membrane potential and i is the amplitude of the single-channel current. Figure 2a shows records of large conductance channel activity in the cell-attached mode at +10 mV from a holding potential of −70 mV in the absence (Figure 2a(i)) and in the presence of 10 μM NA (Figure 2a(ii)), without Bay K 8644 in the bath solution. NA did not significantly change single-channel current amplitude (+1 ± 0.5%, $n = 7$, $P > 0.05$). By contrast, there was a marked change in the probability of the large conductance Ca^{2+} channel being open (Figure 2b). This is seen as an increase of P_f from 0.12 ± 0.03 (control) to 0.22 ± 0.04 in 10 μM NA, and P_o from a mean value of 0.085 ± 0.004 (control) to 0.287 ± 0.011 in 10 μM NA ($n = 7$, $P < 0.01$). Similarly, NA increased the small conductance Ca^{2+} channel activity recorded at +10 mV from a holding potential of −70 mV (Figure 3a). The probability of the channel being open was enhanced by NA (Figure 3b) which acted by increasing P_f from 0.25 ± 0.02 (control) to 0.44 ± 0.04 and P_o from a mean value of 0.026 ± 0.005 (control) to 0.044 ± 0.009 ($n = 7$, $P < 0.01$).

These increases in the probability of the Ca^{2+} channels being open induced by NA could result from a shift in the activation curve of Ca^{2+} channels to more negative voltages and/or from an increase in the maximal open probability. In the absence of Bay K 8644, in cells where Ca^{2+} channel activity was recorded during depolarizing pulses, no evidence of Ca^{2+} channel activity was observed when the membrane patch potential was maintained at −50 and −30 mV ($n = 20$) without voltage steps, for recording times as long as 1 h. Under these conditions, addition of 10 μM NA in the bath

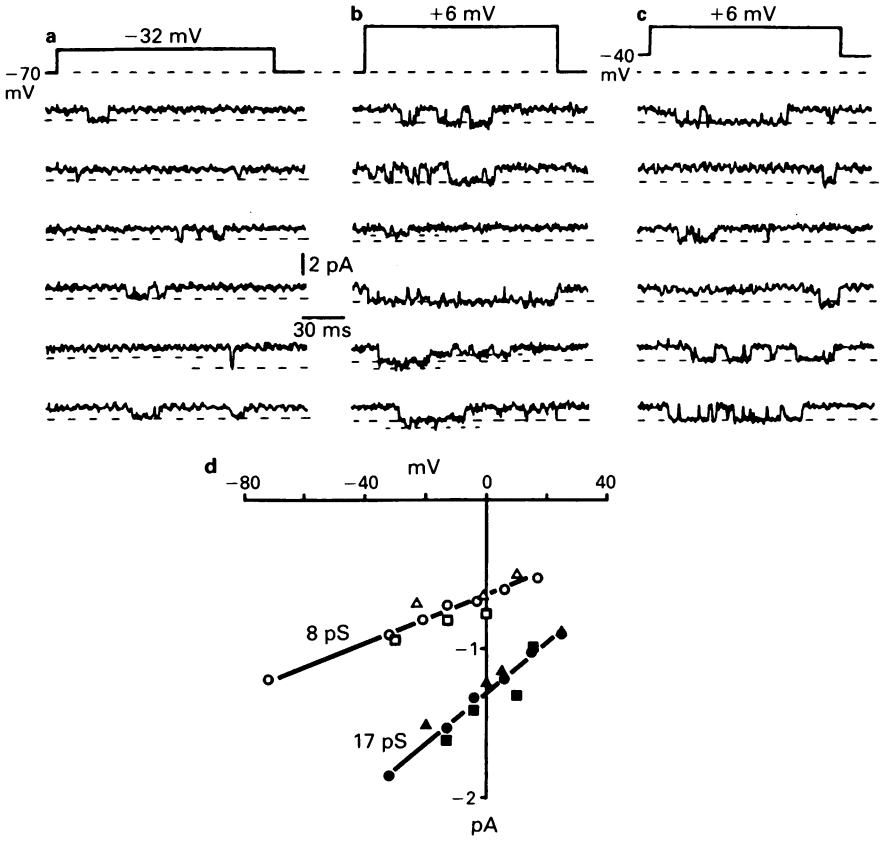


Figure 1 Voltage-dependence of single Ca^{2+} channels in a cell-attached patch. The holding potential was set to −70 mV (a,b) or −40 mV (c). The bath solution contained 1 μM Bay K 8644 and the pipette solution 90 mM Ba^{2+}. (a) At −32 mV, openings of the small conductance channel were evenly distributed throughout the test pulse whereas openings of the large conductance channel were very rare. (b) At + 6 mV, the open-state probability of the large conductance channels was increased. Separate large conductance channel (sweeps 1, 2, 4), small conductance channel (sweep 3), closing of large conductance channel with small conductance channel still open (sweep 5) and closing of the small conductance channel with large conductance channel still open (sweep 6) can be observed. (c) Only the large conductance channel was recorded upon stepping the patch potential from −40 mV to +6 mV. Sweeps with no detectable openings were not shown. Dashed lines indicate the mean single-channel current level obtained from amplitude histograms. (d) Single channel current-voltage relationships for the two channel types obtained from amplitude histograms (3 examples for each channels). Low conductance channel = 8 pS; high conductance channel = 17 pS.

never induced Ca^{2+} channel openings ($n = 15$), contrary to that expected if NA produced a change in the voltage-gating of Ca^{2+} channel activation. To confirm this result, Ca^{2+} conductance voltage relationship was established from macroscopic Ca^{2+} channel currents recorded in experimental conditions similar to those used for single channel recordings (90 mM Ba^{2+}) in the absence and in the presence of NA.

Whole-cell currents and $[Ca^{2+}]_i$ measurements

NA (10 μM) induced an increase in the Ca^{2+} channel current amplitude. Steady-state stimulatory effect of NA was obtained within 2–3 min. Figure 4 shows the mean peak conductance curves against voltage obtained in control conditions and in 10 μM NA. The two curves were superimposed and the potentials corresponding to half-activation were not significantly different: 15.31 ± 2.24 mV (control, $n = 8$) and 16.17 ± 1.66 mV (10 μM NA, $n = 6$). These results agreed with the absence of Ca^{2+} channel opening observed in the cell-attached condition at a holding potential of −30 mV, even in the presence of 10 μM NA. Under the ionic conditions of these experiments (90 mM external Ba^{2+}) the voltage-dependence of Ca^{2+}-channel activation will be shifted toward positive potentials by around 20 mV relative to its position under physiological conditions (Benham *et al.*, 1987; Fox *et al.*, 1987). The potential corresponding to half-activation, determined from the mean peak conductance curve obtained for the Ca^{2+} channel current recorded in the reference solution (Figure 4) was −5.7 ± 0.3 mV ($n = 3$), a value 21 mV more negative than that obtained in 90 mM Ba^{2+}. The foot of the activation curve was −50 mV, a value that corresponds to the resting potential of portal vein cells (Nanjo, 1984; Yamamoto & Hotta, 1985). Taken together, these results suggest that NA could not induce a significant Ca^{2+} influx through voltage-dependent Ca^{2} channels if the membrane potential was maintained at −50 mV. Changes in intracellular Ca^{2+} concentration induced by NA were estimated using the emission from the dye Indo-1 to verify this hypothesis.

In control conditions (Figure 5a), ejection of 10 μM NA at a holding potential of −50 mV in the reference solution evoked an initial peak in $[Ca^{2+}]_i$ followed by a smaller sustained rise. These changes in $[Ca^{2+}]_i$ were still observed in the presence of 10 μM desmethoxyverapamil (not shown). The initial increase in $[Ca^{2+}]_i$ induced activation of Ca^{2+}-dependent Cl^- channels. Heparin has been shown to block $InsP_3$ receptors (Hill *et al.*, 1987; Ghosh *et al.*, 1988) and to inhibit Ca^{2+} release via these receptors in smooth muscle cells (Komori & Bolton, 1990; 1991; Pacaud & Bolton, 1991). When 1 mM heparin was added to the pipette solution, the rise in $[Ca^{2+}]_i$ normally seen upon applying NA (10 μM) was completely inhibited as well as the Ca^{2+}-activated Cl^- current (Figure 5b) while activation of Ca^{2+} channels by a depolarization from −50 mV to +10 mV was still able to produce an increase in $[Ca^{2+}]_i$ (Figure 5c). Under these conditions, the amplitude of the Ca^{2+} channel current and of the corresponding $[Ca^{2+}]_i$ transient evoked by repetitive depolarizations were increased during NA (10 μM) application. This result indicated that NA did not produce a detectable Ca^{2+} entry through voltage-dependent Ca^{2+} channels at a holding potential of −50 mV.

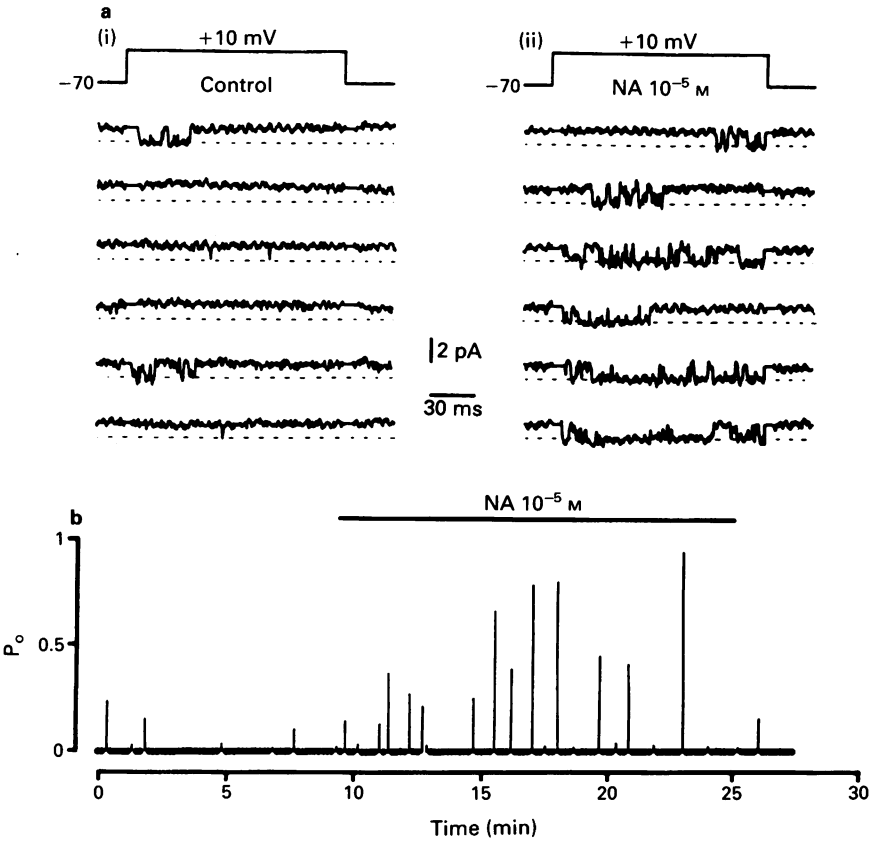


Figure 2 Effect of noradrenaline (NA) on the large conductance Ca^{2+} channel. (a) Activity of the large conductance Ca^{2+} channel was recorded from a holding potential of −70 mV to +10 mV, in the absence of Bay K 8644, before (i) and during bath application of 10 μM NA (ii). Dashed lines indicate the mean single-channel current level obtained from amplitude histograms. (b) Channel open probability for individual sweeps plotted against time. The open-state probability P_o was determined by dividing the time that the channel spends in the open state during a depolarization by the total time of the depolarization. Sweeps with no detectable openings were assigned a P_o value of 0. P_f corresponds to the ratio of the number of sweeps with at least one opening over the total number of sweeps in the ensemble. The NA effect is shown as an increase in P_o and P_f.

Contraction measurements

Our results suggest that NA does not alter the voltage-gating of Ca^{2+} channel activation. Consequently, opening of voltage-dependent Ca^{2+} channels has to be promoted by a membrane depolarization. Increase in Cl^- conductance can depolarize the membrane potential to between −20 and −30 mV (Pacaud *et al.*, 1989) at which values the open-state probability of Ca^{2+} channels is high. The role of both Ca^{2+} channel activation and membrane depolarization evoked by Cl^- current in NA-induced contractions is illustrated by the effects of isradipine (a dihydropyridine derivative), 9-AC and ryanodine. Isradipine (0.5 μM) completely blocked voltage-dependent Ca^{2+} channels in venous muscle (Loirand *et al.*, 1989). In contrast, 9-AC (5 mM) completely inhibited Ca^{2+}-activated Cl^- current but had no significant effects on Ca^{2+} channel current amplitude (Boton *et al.*, 1989; Baron *et al.*, 1991). Figure 6 shows that inhibition of Ca^{2+} channels by 0.5 μM isradipine (Figure 6a) or inhibition of Ca^{2+}-activated Cl^- channels by 5 mM 9-AC (Figure 6b(i)) led to a similar blockade of the NA-induced contraction: 57.1 ± 9% (n = 5) and 55 ± 7% (n = 4) respectively. The NA-induced contraction which is resistant to both 9-AC and isradipine is likely to be due to activation of agonist-sensitive intracellular Ca^{2+} stores since its amplitude is similar to that of NA-induced contraction obtained in Ca^{2+}-free solutions (Dacquet *et al.*, 1987). The contraction induced by 60 mM KCl was not significantly inhibited in the presence of 9-AC (5.3 ± 3.1%, n = 4, Figure 6b(ii)).

Ryanodine is known to deplete Ca^{2+} stores in smooth muscle cells (Rousseau *et al.*, 1987; Sakai *et al.*, 1988). In the presence of 10 μM ryanodine in the reference solution, the contractile response to 10 μM NA was completely suppressed (Figure 6c(i)). This effect seems to be specifically due to the depleting action of ryanodine since the caffeine-induced contraction was also completely inhibited whereas the contractile response induced by 130 mM KCl was only reduced by 9.0 ± 3.7% (n = 4; Figure 6c(ii)).

Discussion

The single channel data provide further insight into the cellular effects of NA. The fact that NA acts on Ca^{2+} channels after application to the bath in cell-attached mode agrees with the existence of an intracellular coupling mechanism between α_1-adrenoceptors and Ca^{2+} channels (G-protein, phospholipase C, protein kinase C; Loirand *et al.*, 1990). The stimulatory effect of NA on Ca^{2+} channels is brought about by two mechanisms. The first is through a decrease in the percentage of nulls; this means that the probability of the channel being in a closed state decreases. The second mechanism is an increase in open-state probability of the available channel. This effect is similar to that observed after β-adrenoceptor stimulation of neonatal rat cardiocytes (Tsien *et al.*, 1986), thrombin application on frog ventricular cells (Markwardt *et al.*, 1990), stimulation of mesenteric artery cells by NA (Nelson *et al.*, 1988) and action of endothelin on guinea-pig portal vein cells (Inoue *et al.*, 1990).

One mechanism by which NA increases the Ca^{2+} channel current could be a simple leftward shift of the steady-state activation curve. Our results do not provide any support for such a mechanism; NA cannot induce a significant Ca^{2+} influx through Ca^{2+} channels at a holding potential corresponding to the foot of the activation curve for Ca^{2+} channels and to the resting potential of portal vein cells (−50 mV), con-

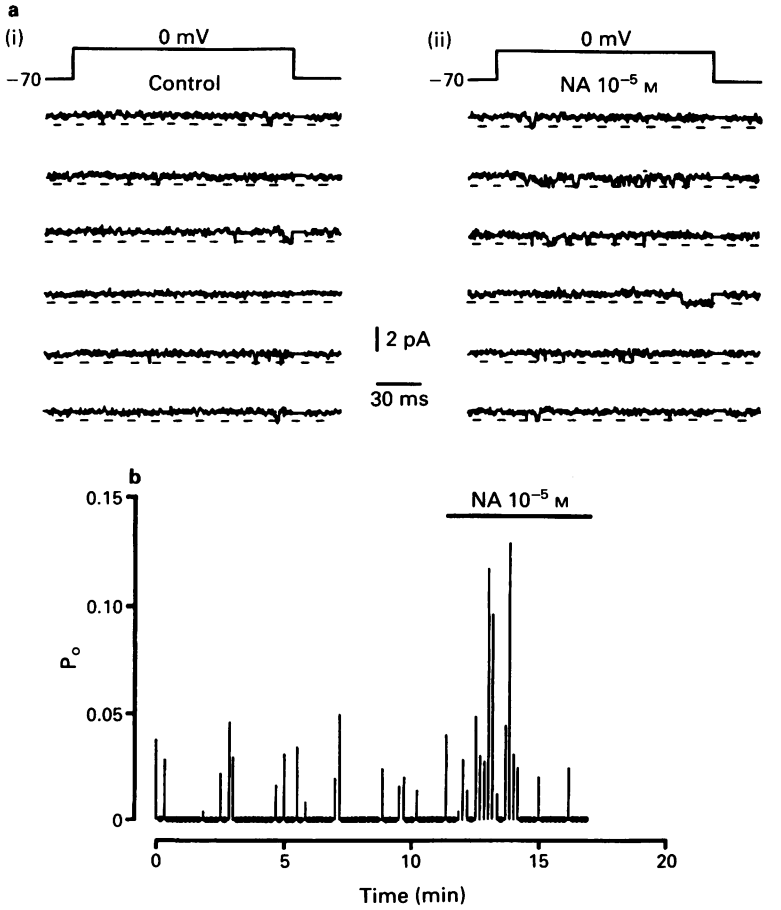


Figure 3 Effect of noradrenaline (NA) on the small conductance Ca^{2+} channel. (a) Activity of the small conductance Ca^{2+} channel was recorded from a holding potential of −70 mV to 0 mV, in the absence of Bay K 8644, before (i) and during bath application of 10 μM NA (ii). Dashed lines indicate the mean single-channel current level obtained from amplitude histograms. (b) Channel open probability for individual sweeps plotted against time. The open-state probability P_o and the probability that the channel is functional, P_f, were determined as in Figure 2. The NA effect is shown as an increase in P_o and P_f.

trary to that expected if a shift of the voltage-dependence of Ca^{2+} channel activation occurred upon NA application. Nevertheless, the strong inhibition produced by Ca^{2+} antagonists left no doubt about the important role played by the voltage-dependent Ca^{2+} channels in the NA-induced contraction of the portal vein (Figure 6a; Dacquet *et al.*, 1987).

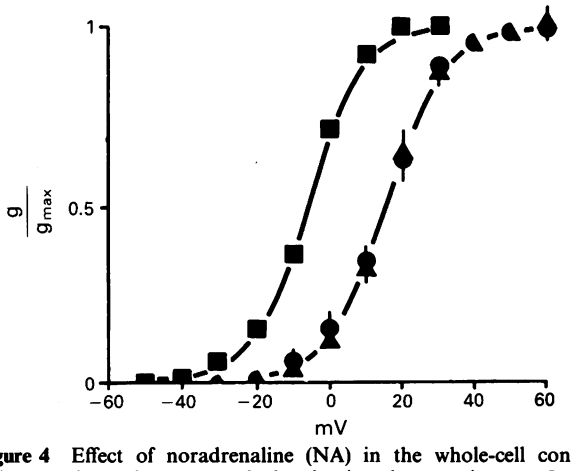


Figure 4 Effect of noradrenaline (NA) on the whole-cell configuration. Peak conductances calculated using the equation $g = I_{Ca}/(V_m - E_{rev})$ from whole-cell Ca^{2+} channel currents, recorded in the reference solution (■) and in a 90 mM Ba^{2+}-containing external solution before (●) and after bath application of 10 μM NA (▲). These are expressed as a fraction of maximal conductance and plotted against membrane potential.

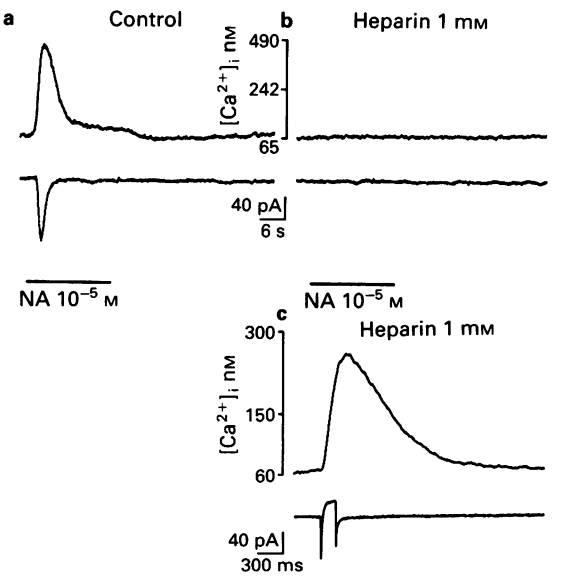


Figure 5 (a) In the reference solution, application of 10 μM noradrenaline (NA) evoked a transient increase in $[Ca^{2+}]_i$ followed by a smaller sustained component (upper trace). The internal Ca^{2+} concentration was determined using the emission from the dye Indo-1. This increase in $[Ca^{2+}]_i$ induced the activation of Ca^{2+}-dependent Cl^- channels (lower trace). (b–c) In the presence of 1 mM heparin in the pipette solution, no change in $[Ca^{2+}]_i$ and in whole-cell current was observed upon application of 10 μM NA; the holding potential was −50 mV (b). Activation of Ca^{2+} current by a depolarization to +10 mV from −50 mV produced an increase in $[Ca^{2+}]_i$ (c).

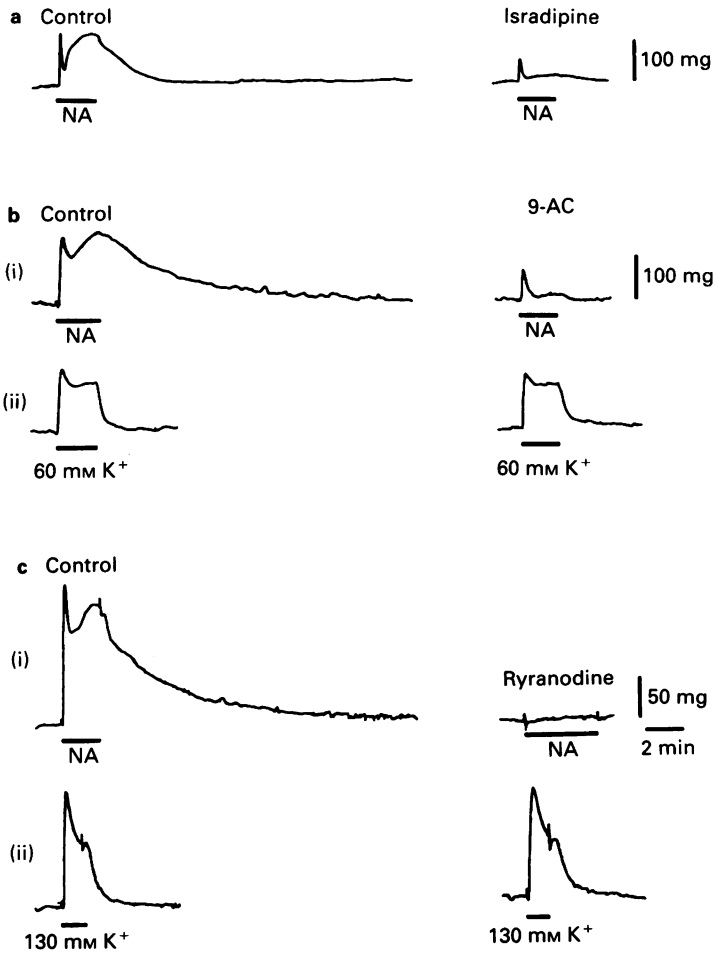


Figure 6 Effect of various pharmacological agents on noradrenaline (NA)-induced contractions. Contractions were induced by 10 μM NA (a, b(i), c(i)), by 60 mM KCl (b(ii)) and by 130 mM KCl (c(ii)) in the absence (left column) and in the presence (right column) of 0.5 μM isradipine (a), 5 mM anthracene-9-carboxylic acid (b) or 10 μM ryanodine (c).

Blockade of Ca^{2+}-activated Cl^- channels led to an inhibition of the NA-induced contraction similar to that produced by Ca^{2+} antagonists, suggesting that inhibition of Cl^- channels prevented the involvement of voltage-dependent Ca^{2+} channels during NA application. Experiments done with heparin and ryanodine also indicate that when Ca^{2+} release from intracellular store could not occur, NA was not able to induce Ca^{2+} influx through voltage-dependent Ca^{2+} channels. On portal vein smooth muscle cells, the NA-induced Ca^{2+} release is responsible for Ca^{2+}-dependent Cl-channel activation triggering membrane depolarization (Pacaud *et al.*, 1989). These results suggest that the depolarization produced by activation of Ca^{2+}-activated Cl^- channels is a prerequisite to opening of voltage-dependent Ca^{2+} channels in response to NA in portal vein cells. In addition, NA increased the open-state probability of both types of Ca^{2+} channels leading to an increased Ca^{2+} influx. For the large conductance Ca^{2+} channel corresponding to the slow calcium current (L-type), the change in the open-state probability was induced by NA through a protein kinase C-dependent pathway (Loirand *et al.*, 1990). For the small conductance Ca^{2+} channel, the intracellular mechanism involved in the increase in the open-state probability induced by NA, is not known and may use the same or a different pathway. However, the NA effect on the small conductance Ca^{2+} channel seems to be less sustained than on the large conductance Ca^{2+} channel, suggesting that the intracellular mechanisms involved may differ for the two channels. Thus, the sequence of events induced by NA could be summarized as follows: (i) NA releases intracellular Ca^{2+} stores through $InsP_3$ production; (ii) the Ca^{2+} released opens Cl^- channels producing inward current and membrane depolarization; (iii) this depolarization produces Ca^{2+} entry through voltage-dependent Ca^{2+} channels and their open probability is enhanced by NA.

It is possible that this mechanism of a required depolarization might represent a common mechanism involved in many agonist-mediated contractions of smooth muscle. However, the nature of the channels responsible for the depolarization could differ from one tissue to another. In vascular smooth muscle, Ca^{2+}-activated Cl^- channels seem to play the major role: in the portal vein in response to NA (Byrne & Large, 1988b; Pacaud *et al.*, 1989), in the mesenteric vein in response to NA (Van Helden, 1988), in the coronary and mesenteric arteries in response to endothelin (Klockner & Isenberg, 1991); whereas in visceral smooth muscle, Ca^{2+}-activated cation channels seem to be more important: in the ileum in response to acetylcholine (Inoue & Isenberg, 1990a, b) and in the jejunum in response to acetylcholine (Pacaud & Bolton, 1991).

Thus, Ca^{2+} store release probably has an important role in determining indirectly the final size of the contractile response to receptor activation by influencing strongly the size of the depolarization which occurs; this in turn will determine the extent of Ca^{2+} channel activation which is produced by the receptor agonist.

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Mechanisms of the anticholinergic effect of SUN 1165 in comparison with flecainide, disopyramide and quinidine in single atrial myocytes isolated from guinea-pig

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1 The mechanism of the anticholinergic effect of SUN 1165 on the acetylcholine (ACh)-induced K^+ current ($I_{K.ACh}$) was examined and compared with those of flecainide, disopyramide and quinidine in single atrial myocytes, in a whole-cell configuration by use of the concentration-jump technique. This technique combines an intracellular perfusion and a rapid exchange of external solution surrounding the voltage-clamped single myocyte within 2 ms.

2 In the cells loaded with guanosine-5'-triphosphate (GTP), 100 μM, the muscarinic ACh response, ($I_{K.ACh}$), was mediated by GTP-binding proteins. The concentrations of the test drugs that produced a half-maximal inhibition of ACh (1 μM)-induced $I_{K.ACh}$ (IC_{50}) were 29 μM for SUN 1165, 3.6 μM for flecainide, 1.7 μM for disopyramide, and 1.6 μM for quinidine. The blockade of $I_{K.ACh}$ by SUN 1165 and its recovery from the inhibition occurred within a few seconds. Disopyramide had a similar rapid action, while the effects of flecainide and quinidine occurred much more slowly within a few tens of seconds.

3 In cells loaded with 100 μM guanosine-5'-O-(3-thiotriphosphate) (GTPγS, a nonhydrolysable GTP analogue), the K^+ channel was uncoupled from the muscarinic receptors and activated irreversibly due to direct activation of GTP-binding proteins by GTPγS. SUN 1165 and disopyramide had a weak inhibitory effect (IC_{50} > 100 μM for both), while flecainide and quinidine depressed the GTPγS-induced K^+ current with similar potencies to the cases of ACh-induced currents; IC_{50} was 5.3 μM for flecainide and 4.4 μM for quinidine.

4 These results suggest that the mechanisms underlying the anticholinergic effects of these antiarrhythmic drugs are different; disopyramide and high concentrations of SUN 1165 mainly block muscarinic ACh receptors in atrial myocytes, while flecainide and quinidine inhibit the K^+ channel itself and/or GTP-binding proteins.

Keywords: Isolated atrial myocytes; concentration-clamp; anticholinergic effect; acetylcholine; K^+ channels; antiarrhythmic drugs; SUN 1165; flecainide; disopyramide; quinidine

Introduction

It is well known that class I antiarrhythmic drugs, such as quinidine and disopyramide, antagonize cholinergic actions in addition to directly depressing the fast inward Na^+ currents in cardiac myocytes (Mirro *et al.*, 1980). The anticholinergic action of antiarrhythmic agents determines their net effects on the heart, and is responsible for untoward effects, such as dryness of mouth, constipation, blurred vision and urinary hesitancy observed in patients treated with disopyramide (Bigger & Hoffman, 1985). Therefore, it is very important to examine and compare the anticholinergic effects of antiarrhythmic drugs from a clinical point of view.

It is known that the muscarinic acetylcholine (ACh)-receptor of the atrial myocytes affects the activation of GTP binding proteins and the K^+ channel is directly regulated by GTP binding proteins (Breitwieser *et al.*, 1985; Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a,b,c; Yatani *et al.*, 1987). Using an atrial myocyte, Nakajima *et al.* (1989) showed that the mechanisms underlying the anticholinergic effects of disopyramide and quinidine in cardiac cells were different: disopyramide may mainly block muscarinic ACh receptor while quinidine may inhibit the muscarinic K^+ channel itself and/or GTP binding proteins.

SUN 1165, N-(2,6-dimethylphenyl)-8-pyrrolizidinylacetamide hydrochloride hemihydrate, is a newly developed antiarrhythmic agent (Aisaka *et al.*, 1985; Hidaka *et al.*, 1985; Inomata & Ishihara 1988) which belongs to the class Ic antiarrhythmic drugs (Hattori *et al.*, 1988). It has been shown to block the fast Na^+ current with slow kinetics for both blocking and unblocking (Inomata *et al.*, 1987; 1989a).

In the present study, we compared the effect of SUN 1165 with those of other antiarrhythmic drugs such as flecainide, disopyramide and quinidine on the ACh-induced K^+ current ($I_{K.ACh}$) in single atrial cells of guinea-pig to clarify the mechanisms of their anticholinergic effects.

Methods

Single atrial cells were isolated by use of an enzymatic dispersion procedure similar to that described previously (Inomata *et al.*, 1989b). Briefly, male guinea-pigs, weighing 300–500 g, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.). The chest was opened under artificial respiration, the aorta was cannulated, and the heart was excised. The excised whole heart was perfused through the aorta with Tyrode solution which was equilibrated with 95% O_2 plus 5% CO_2. The composition of Tyrode solution was (in mM): NaCl 137, KCl 10.8, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.33, $NaHCO_3$ 11.9, glucose 5.5 and N-2-hydroxyethylpiperazine-N'-2-ethane- sulphonic acid (HEPES) 5.0. The pH was adjusted to 7.4. Thereafter the heart was perfused with 'nominally' Ca^{2+}-free Tyrode solution until cessation of the spontaneous contraction, and the heart was then perfused with a Ca^{2+}-free Tyrode solution containing 0.4% collagenase for about 45 min. The collagenase was washed out with 50 ml of a high-K^+ storage solution composed of (in mM): KCl 85, K_2HPO_4 30, $MgSO_4$ 5.0, Na_2ATP 5.0, pyruvate 5.0, creatine 5.0, taurine 20, glucose

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20 and ethylene glycol-*bis*(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 0.2; the pH was adjusted to 7.4. The temperature of all the solutions was kept constant at 37°C during coronary perfusion. The right atrium was dispersed in storage solution with forceps. The dispersed single cells were kept in storage solution at room temperature (26 ± 1°C) for later use.

To record the ACh-receptor-mediated K^+ current, experiments were done on internally perfused single atrial cells by use of a suction pipette technique (whole cell clamp mode) (Akaike *et al.*, 1978; 1987). A viable, rod-shaped cell was sucked into the tip of a suction pipette (inner diameter of the tip, 2 ~ 3 μm) and perfused intracellularly with a solution composed of (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1.0, Na_2ATP 3.0, $MgCl_2$ 1.0, and EGTA 5.0 (pH 7.3). Either guanosine-5'-triphosphate (GTP sodium salt; Sigma) 100 μM or guanosine-5'-O-(3-triphosphate) (GTPγS tetralithium salt; Boeringer) 100 μM was added to the solution. The cell-attached tip of the suction pipette was inserted into a plastic tube through a circular hole 500 μm in diameter (see Akaike *et al.*, 1986). The lower end of this tube was directly exposed to the external test solution by vertically moving a stage holding the dishes which contained test solutions. The external solution was as follows (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.33, $NaHCO_3$ 11.9, glucose 5.5 and HEPES 5.0 (pH 7.4). The external solution containing ACh and/or drugs was exchanged by a 'concentration-clamp' method (Akaike *et al.*, 1986; Inomata *et al.*, 1989a,b). Briefly, a negative pressure of −3 cmHg applied to the upper end of the plastic tube was controlled by an electromagnetic valve, driven by a stimulator (Dia-Medical System, DPS-160B). The speed of solution exchange by the concentration-clamp technique was estimated from the time constant of the liquid junction potential when the external K^+ concentration was changed from 85 to 2 mM. The value was 0.2 ~ 0.3 ms (Akaike *et al.*, 1987). However, in the cell-attached conditions of isolated atrial cells, the holding current shifted to a new value with a time constant of about 2 ms when the external K^+ concentration was changed. The membrane current was measured by means of an Ag-AgCl wire mounted on the suction pipette holder. The reference electrode was an Ag-AgCl wire in a Ringer plug on a solution exchange plastic tube. Both electrodes were led to a voltage-clamp circuit, and the membrane potential was controlled by a patch-clamp amplifier (List-Medical, EPC-7). The cell was held at −50 mV to examine the muscarinic ACh receptor-regulated K^+ channel current ($I_{K.ACh}$). Experiments were carried out at room temperature. The voltage and current were monitored on a storage oscilloscope (National, VP-5730A) and were simultaneously stored on an FM data recorder (Sony, A-45) for later analysis.

The relative amplitude of $I_{K.ACh}$ in the presence of various drugs was calculated as follows: in GTP-loaded cells, the difference between the 1 μM ACh-induced steady-state current and the current level in the absence of ACh was taken as 1.0; and in GTPγS-loaded cells, the difference between the persistent outward current in the absence of ACh and the current level after the blockade of outward K^+ current by 1 mM extracellular Ba^{2+} was taken as 1.0. Student's *t* test was used for statistical analysis of the data. A value of $P < 0.05$ was considered significant.

Drugs used were acetylcholine and collagenase Type I (Sigma), SUN 1165, flecainide acetate and disopyramide phosphate (Suntory), quinidine sulphate (Nakarai) and atropine sulphate (Merck).

Results

Effects of SUN 1165, flecainide, quinidine, disopyramide on $I_{K.ACh}$ in GTP-loaded atrial cells

On application of ACh, 1 μM, to the cell at −50 mV, a submaximal outward current flowing through the specific K^+ channel was induced (Kurachi *et al.*, 1986b; Inomata *et al.*, 1989b). After rapid activation with a latency of about 100 ms (Inomata *et al.*, 1989b), $I_{K.ACh}$ desensitized to reach a steady-state level within several tens of seconds in the continuous presence of ACh. This steady-state level of $I_{K.ACh}$ lasted constantly for more than a few minutes (data not shown) as reported by Nakajima *et al.* (1989). Therefore, the current was indeed sufficiently steady to allow measurements of blockade by the drugs in the continuous presence of ACh. $I_{K.ACh}$ was decreased to 10 ± 5% (mean ± s.e. of four experiments) by 10 nM atropine and inhibited completely by 1 mM Ba^{2+}, a K^+ channel blocker (Figure 1a). After $I_{K.ACh}$ reached a steady-state level, each antiarrhythmic drug was applied extracellularly. Flecainide, quinidine and disopyramide depressed $I_{K.ACh}$ effectively in a concentration-dependent manner, but the effect of SUN 1165 was much weaker than those of flecainide, quinidine and disopyramide (Figure 2a). The relative amplitude of $I_{K.ACh}$ in the presence of antiarrhythmic drugs is summarized in Figure 2b. Of the four drugs, SUN 1165 was the weakest inhibitor of the current. Disopyramide and quinidine were the most effective inhibitors of the current and suppressed the currents equipotently. The concentrations of drugs that produced a half-maximal inhibition of the current (IC_{50}) estimated from the concentration-response curve shown in Figure 2b were 1.6 μM for disopyramide, 1.7 μM for quinidine, 3.6 μM for flecainide and 29 μM for SUN 1165. Flecainide, 100 μM, reduced the outward current to below zero level. On application of 10 μM SUN 1165 or 1 μM disopyramide which blocked $I_{K.ACh}$ by about 30 ~ 40%, the blocking effect of $I_{K.ACh}$ reached the steady-state level within a few seconds; 2.0 ± 0.2 s (mean ± s.e. of eight experiments) for SUN 1165 and 1.8 ± 0.1 s (four experiments) for disopyramide. In addition, the recovery of $I_{K.ACh}$ after washing out SUN 1165 or disopyramide also occurred within a few seconds. On the other hand, with flecainide or quinidine at a concentration which blocked $I_{K.ACh}$ to the same degree, about 30 ~ 40%, the time-course of $I_{K.ACh}$ blockade was much slower than that obtained with SUN 1165 or disopyramide; the blocking effect of 3 μM flecainide and of 1 μM quinidine reached the steady-state level within 21.6 ± 5.1 s (five experiments) for flecainide and within 19.0 ± 2.7 s (three experiments) for quinidine, respectively. In addition, the onset of blockade by higher concentrations of quinidine (10 μM) or flecainide (30, 100 μM) occurred more rapidly. However, the recovery from $I_{K.ACh}$ blockade by

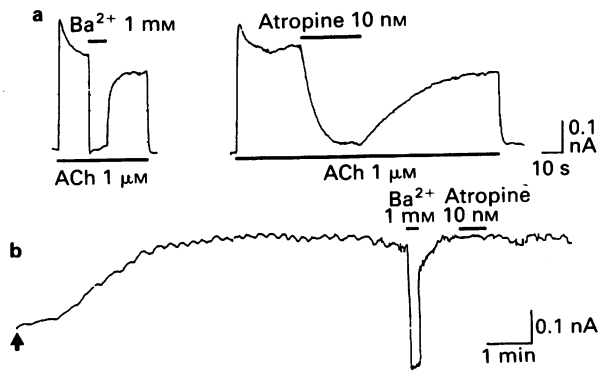


Figure 1 Effects of Ba^{2+} and atropine on acetylcholine (ACh)-induced outward K^+ current ($I_{K.ACh}$) in a single atrial cell. The cells were held at −50 mV. (a) In 100 μM guanosine 5'-triphosphate (GTP)-loaded myocyte, 1 mM Ba^{2+} or 10 nM atropine inhibited the 1 μM ACh-induced $I_{K.ACh}$. ACh, 1 μM, was applied during the period indicated by the bars under the current trace. Ba^{2+} or atropine was applied extracellularly during the time indicated by the bars above the current trace. (b) In 100 μM guanosine-5'-O-(3-thiotriphosphate) (GTPγS)-loaded cell, the GTPγS-activated $I_{K.ACh}$ was completely inhibited by 1 mM Ba^{2+} but not by 10 nM atropine. Arrow indicates the start of a whole-cell clamp configuration with patch-pipette filled with internal solution containing GTPγS.

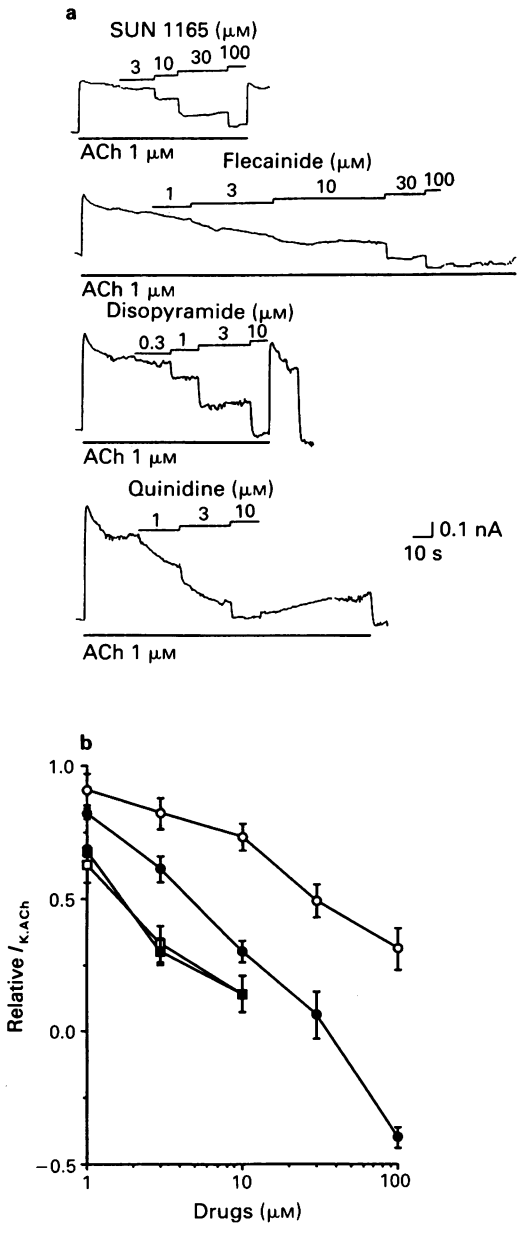


Figure 2 Effects of antiarrhythmic drugs on $I_{K.ACh}$ in 100 μM guanosine 5'-triphosphate (GTP)-loaded cells. The cells were held at −50 mV. (a) Original current traces: each drug was applied extracellularly during the time indicated by the bars above the current trace in the continuous presence of 1 μM acetylcholine (ACh). Note that the block of $I_{K.ACh}$ by either SUN 1165 or disopyramide and its recovery reached the steady-state level within a few seconds. On the other hand, in cells treated with flecainide or quinidine, the blockade of $I_{K.ACh}$ or its recovery occurred more slowly. (b) Relationship between relative amplitude of $I_{K.ACh}$ and the concentrations of SUN 1165 (○), flecainide (●), disopyramide (□) and quinidine (■) in GTP-loaded cells. Disopyramide and quinidine depressed the current more effectively than the other drugs. The blockade by SUN 1165 was the least effective. Each symbol represents the mean of 3 ~ 8 experiments; vertical bars show s.e.mean.

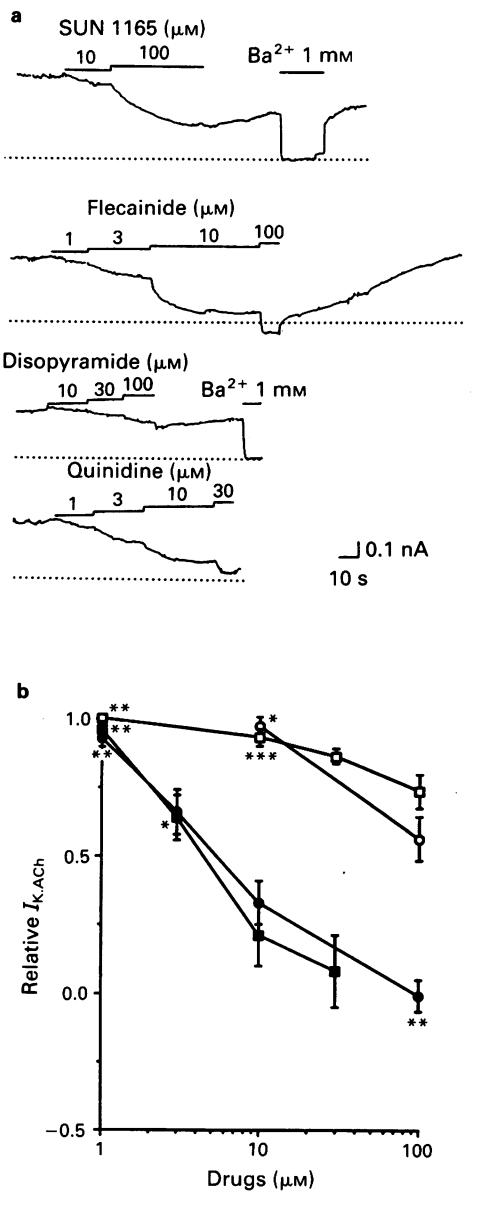


Figure 3 Effects of antiarrhythmic drugs on guanosine-5'-O-(3-thiotriphosphate) (GTPγS)-activated $I_{K.ACh}$ in 100 μM GTPγS-loaded cells. The cells were held at −50 mV. (a) Original current traces: each drug was applied extracellularly during the period indicated by the bars above the current trace. Each dotted line shows the current level in the presence of 1 mM Ba^{2+} extracellularly. (b) Relationship between the relative amplitude of $I_{K.ACh}$ induced by GTPγS and the concentration of SUN 1165 (○), flecainide (●), disopyramide (□) and quinidine (■) in GTPγS-loaded cells. Each symbol represents the mean of 3 ~ 8 experiments; vertical bars show s.e.mean. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$, versus relative $I_{K.ACh}$ in guanosine 5'-triphosphate (GTP)-loaded cells shown in Figure 2.

100 μM flecainide or 10 μM quinidine needed a much longer period than blockade induced by SUN 1165 or disopyramide.

Effects of drugs on the $I_{K.ACh}$ uncoupled from muscarinic acetylcholine receptors

The muscarinic ACh receptor of the auricle muscle involves the activation of GTP binding proteins and the K^+ channel is directly regulated by the GTP binding protein (Breitwieser *et al.*, 1985; Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a,b,c; Yatani *et al.*, 1987). Intracellularly applied GTPγS, a non-hydrolysable GTP analogue, directly activates the GTP binding proteins and causes an irreversible activation of the muscarinic K^+ channel (Kurachi *et al.*, 1986b,c; Nakajima *et al.*, 1989). Thus by using GTPγS-loaded cells, we studied the effects of SUN 1165, flecainide, quinidine, and disopyramide on $I_{K.ACh}$ uncoupled from the activation of the membrane receptor.

In 100 μM GTPγS-loaded cells, the outward K^+ current was induced gradually with a delay time of a few tens of seconds without applying ACh extracellularly (Figure 1b). In contrast

to the GTP-loaded cells, the outward K^+ current in GTPγS-loaded cells was not affected by 10 nM atropine, while extracellularly applied Ba^{2+} (1 mM) (a K^+ channel blocker) completely blocked this GTPγS-induced outward K^+ current and reduced the outward current below the initial level (Figure 1b). Therefore, these observations support the hypothesis that in GTPγS-loaded cells, the muscarinic K^+ channels are uncoupled from the muscarinic ACh receptors and that they are being irreversibly activated by GTP binding protein (Breitwieser *et al.*, 1985; Kurachi *et al.*, 1986b,c; Yatani *et al.*, 1987). Reduction of the outward current below the initial level by Ba^{2+} might indicate the blocking action of the background K^+ current, as reported by Carmeliet & Mubagwa (1986).

In GTPγS-loaded cells, the blocking action of SUN 1165 on $I_{K.ACh}$ was significantly smaller and occurred at a slower rate than that observed in GTP-loaded cells (Figure 3a). SUN 1165, 10 μM and 100 μM, reduced $I_{K.ACh}$ by 3% and 44% of the current, respectively (Figure 3b). Disopyramide inhibited $I_{K.ACh}$ to a much smaller extent in GTPγS-loaded cells than in GTP-loaded cells, as reported by Nakajima *et al.* (1989). On the other hand, flecainide blocked $I_{K.ACh}$ in a concentration-dependent manner ($IC_{50} = 5.3\,\mu M$), as in GTP-loaded cells. Quinidine also inhibited $I_{K.ACh}$ in a concentration-dependent manner ($IC_{50} = 4.4\,\mu M$), but the blocking effect was slightly smaller than that in GTP-loaded cells.

Discussion

The present study showed that SUN 1165, a newly developed class Ic antiarrhythmic agent, has the weakest anticholinergic effect among four kinds of antiarrhythmic drugs used.

Since it is known that the muscarinic ACh-receptor of the atrial myocytes affects the activation of GTP binding proteins and that the K^+ channel is directly regulated by the GTP-binding proteins (Breitwieser *et al.*, 1985; Pfaffinger *et al.*, 1985; Kurachi *et al.*, 1986a,b,c; Yatani *et al.*, 1987), it is considered that $I_{K.ACh}$ could be depressed by inhibiting (1) the muscarinic ACh receptors, (2) the GTP-binding proteins or (3) the K^+ channels. Regarding the anticholinergic effects of class I antiarrythmic agents, Nakajima *et al.* (1989) showed that disopyramide inhibits mainly the binding of muscarinic agonists to the receptor, while quinidine blocks mainly the K^+ channel itself and/or GTP binding proteins, as confirmed in the present study. Moreover, the present study showed that in GTP-loaded cells, blockade of $I_{K.ACh}$ by disopyramide and its recovery occurred very rapidly, within a few seconds, while it took much longer with quinidine. It is important to note that in GTP-loaded cells the blockade of $I_{K.ACh}$ by SUN 1165 and its recovery occurred within a few seconds just as in the case of disopyramide, while in the presence of flecainide and quinidine they were slow. In addition, in GTPγS-loaded cells, the IC_{50} values for SUN 1165 and disopyramide to depress the GTPγS-activated $I_{K.ACh}$ were significantly higher than those in GTP-loaded cells; while with flecainide the IC_{50} for blockade of $I_{K.ACh}$ was similar to that in GTP-loaded cells. These results suggest that the mechanism underlying the anticholinergic action of SUN 1165 especially at higher concentrations ($10 \sim 100\,\mu M$) is similar to that of disopyramide; i.e., blockade of agonist-binding to muscarinic receptors in atrial myocytes, and flecainide, another class Ic antiarrhythmic agent, is similar to that of quinidine; i.e., inhibition of the K^+ channel itself and/or GTP-binding proteins. It has already been reported that flecainide has a blocking action on a delayed rectifier K^+ current in addition to the fast inward Na^+ current at the clinically effective serum concentrations of 0.25 to 1.3 μM (Follmer & Colatsky, 1990). It is also well known that quinidine blocks various kinds of K^+ currents such as the transient outward current (Imaizumi & Giles, 1987) and the ATP-sensitive K^+ current in cardiac myocytes (Undrovinas *et al.*, 1990). Therefore, direct inhibition of the K^+ channel is the most likely mechanism for flecainide- or quinidine-mediated depression of $I_{K.ACh}$. In addition, as with Ba^{2+}, the fall below zero current with 100 μM flecainide observed in ACh-induced $I_{K.ACh}$ may be related to additional blocking actions at other channels, such as background K^+ channels. We have already reported that the antiarrhythmic action of SUN 1165 at effective serum concentrations in the coronary ligated arrhythmia model of dogs is primarily due to the blockade of the fast Na^+ current but not the slow Ca^{2+} current in the heart (Inomata *et al.*, 1987; 1989a). Therefore, it is also suggested that the net effect of SUN 1165 in the heart may mainly be due to blockade of the fast Na^+ channel, and that SUN 1165 may produce few untoward effects through its apparent anticholinergic action.

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Effects of vasoactive intestinal peptide, helodermin and galanin on responses of guinea-pig lung parenchyma to histamine, acetylcholine and leukotriene D_4

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1 The effect of vasoactive intestinal peptide (VIP) was studied on the contractile response of guinea-pig lung parenchymal strips (GPP) induced by bronchoconstrictor agonists, such as leukotriene D_4 (LTD_4), histamine and acetylcholine (ACh). This effect of VIP was compared with helodermin, a peptide that is structurally related to VIP, and galanin, another neuropeptide that is thought to co-exist with VIP.

2 VIP (10 nM) induced a potent and reversible inhibition of the contractions of GPP induced by LTD_4 (1–30 pmol) but did not affect those due to ACh (1–100 nmol) or histamine (1–30 nmol). A ten fold higher concentration of VIP (100 nM) did not further inhibit LTD_4-induced responses or reduce those induced by histamine or ACh.

3 Helodermin (10 nM) had a similar inhibitory effect on contractions of GPP induced by LTD_4 (3–30 pmol) but did not affect contractions induced by histamine (1–10 nmol).

4 Indomethacin (2.8 μM) and salbutamol (10 nM) significantly reduced responses elicited by LTD_4 and histamine but not those due to ACh. A ten fold higher concentration of salbutamol (100 nM) further inhibited the contractions due to LTD_4 and histamine and at this concentration responses induced by ACh were inhibited.

5 VIP (10 nM) and helodermin (10 nM) significantly reduced the LTD_4-induced release of thromboxane A_2 (TXA_2), measured as TxB_2 by radioimmunoassay, from GPP. The smaller release of TxA_2 induced by histamine was not significantly reduced in the presence of VIP.

6 In comparative studies, galanin (10–100 nM) did not affect contractions of GPP induced by either LTD_4, histamine or ACh. In contrast to VIP and helodermin, both at 0.1–3 nmol, which induced dose-related relaxations of guinea-pig trachea, galanin was inactive on this preparation in doses of up to 3 nmol.

7 In conclusion, our results show that contractions of GPP induced by LTD_4 are more sensitive to inhibition by VIP and helodermin than are contractions due to histamine or ACh. This inhibition appears to be associated with the different contribution of released TxA_2 to contractions evoked by the agonists. VIP and helodermin inhibit the cyclo-oxygenase-dependent component of the LTD_4-induced response, as in the case of indomethacin.

Keywords: Vasoactive intestinal peptide (VIP); helodermin; galanin; leukotriene D_4 (LTD_4); acetylcholine (ACh); histamine; guinea-pig airways

Introduction

Several lines of evidence implicate vasoactive intestinal peptide (VIP) as a neurotransmitter of the inhibitory component of non-adrenergic, non-cholinergic (NANC) nerves, which is the predominant inhibitory system in the respiratory tract of many species. VIP occurs in mammals in close association with submucosal glands, blood vessels and airway smooth muscle (Said, 1984). This peptide is a potent relaxant of respiratory smooth muscle *in vitro* but its activity depends on the size of the airways. In bovine and human airway preparations, responsiveness to VIP diminishes with decreasing airway size (Palmer *et al.*, 1985; 1986), suggesting that VIP is unlikely to influence small and peripheral airways. Inhaled VIP has been shown to protect against histamine- and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced bronchoconstriction in canine airways (Said *et al.*, 1982) while in man only a small protective effect against the bronchoconstrictor action of histamine was observed (Barnes & Dixon, 1984).

VIP is known to co-exist with acetylcholine (ACh) in cholinergic nerves, supplying certain exocrine glands, and in airway ganglia (Schultzberg *et al.*, 1982). Since VIP has been shown to reduce the contractile effect of exogenous ACh in bovine tracheal smooth muscle, it is therefore likely that a functional relationship exists between VIP and cholinergic nerve control (Palmer *et al.*, 1985).

More recently helodermin, another neuropeptide that is structurally related to VIP, has been identified (Hoshino *et al.*, 1984). It is a 35 amino acid peptide, originally isolated from the venom of the lizard *Heloderma suspectum*. Helodermin and VIP have 15 amino acids residues in identical positions but helodermin has an extended C-terminus. It is thought that this peptide acts by binding to VIP receptors, rather than to distinct and unique receptors (Robberecht *et al.*, 1988).

Galanin, a 29 amino acid neuropeptide isolated from porcine intestine, has been shown to be present in airway nerves and appears to be co-localized with VIP and ACh (Cheung *et al.*, 1985). The distribution of this peptide in the airways suggests that it could have some influence on airway function.

In the present study on guinea-pig lung parenchyma (GPP), a preparation of small and peripheral airways which also contains a large vascular component, the effects of VIP, helodermin and galanin were investigated on the contractile responses induced by bronchoconstrictor agents such as leukotriene D_4 (LTD_4), histamine and ACh. The effect of these peptides on the generation of thromboxane A_2 (TxA_2) induced by LTD_4 was examined, since the action of LTD_4 has been

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shown to be partly mediated by TxA_2 in guinea-pig lung (Piper & Samhoun, 1982). Comparative studies using salbutamol and indomethacin, a β_2-adrenoceptor agonist and cyclo-oxygenase inhibitor respectively, were also carried out. In addition, the actions of helodermin and galanin were compared with VIP on guinea-pig tracheal strips (GPT).

A preliminary account of part of this work was presented at the Sixth International Conference on Prostaglandins and Related Compounds (Samhoun *et al.*, 1987).

Methods

In all experiments male Dunkin-Hartley guinea-pigs (300–600 g) were used. Following cervical dislocation, the lungs with the tracheae intact were removed and placed in Tyrode buffer.

Lung parenchymal strips

Lungs were inflated via the trachea and perfused free from blood with oxygenated Tyrode solution (37°C) at 5 ml min^{-1}, via the pulmonary artery. The Tyrode solution was composed of (mM): NaCl 138, KCl 2.7, $CaCl_2 \cdot 6H_2O$ 1.8, $MgCl_2 \cdot 6H_2O$ 0.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 11.9, and D-glucose 5.6. Guinea-pig lung parenchymal strips (GPP), preparations of small airways which also contain blood vessels, were cut from the periphery of the major lobes and trimmed into strips. GPP were superfused in series in a cascade system with Tyrode solution (37°C) at 5 ml min^{-1}, under a tension of 1 g (Piper & Samhoun, 1981).

Tracheal strips

Guinea-pig isolated tracheae were opened by a longitudinal cut on the ventral side and cut in a zig-zag fashion with alternate incisions made every two to three cartilage rings (Emmerson & MacKay, 1979). These were suspended in a cascade system under a 1 g tension and superfused with oxygenated Tyrode solution (37°C) at 5 ml min^{-1}.

Preparation of drugs

Stock solutions of U-44069, [(15S)-hydroxy-9α,11α-(epoxymethano)prosta-5Z,13E-dienoic acid] (Wasserman, 1976) and indomethacin were prepared in ethanol, those of LTD_4 in methanol, and were diluted to the required concentration with Tyrode solution before use. VIP, helodermin, galanin, salbutamol and all other drugs used in this study were prepared in Tyrode solution.

Administration of drugs

Agonists (LTD_4, ACh, histamine and U-44069) were administered as bolus injections directly into the buffer superfusing the tissues. Dose-response curves to agonists were obtained before and during treatment with VIP, helodermin, galanin, indomethacin or salbutamol, which were administered as a continuous infusion (0.1 ml min^{-1}) into the superfusing fluid, using a Watson Marlowe Delta infusion pump, for 20 min before and then continuously throughout the experiment. In a separate series of experiments, the comparative activity of helodermin, galanin and VIP, administered as bolus injections, was also tested on GPT. U-44069, a stable prostaglandin endoperoxide which has TxA_2-like actions on smooth muscle preparations, was used as a standard agonist to test tissue sensitivity. Responses were measured with Harvard Smooth Muscle Transducers and auxotonic levers (Paton, 1957) and changes in tissue length following agonist administration were displayed on a multi-channel recorder (Graphtec Linearcorder MK VII). All points on the dose-response curves represent a height of tissue contraction, in mm, as measured on a 50 mV recorder setting, following administration of agonist.

Radioimmunoassay of thromboxane B_2

In some experiments, effluent was collected during responses induced by LTD_4 and histamine, in the presence and absence of VIP and helodermin (10 nM). Following partial purification with Sep-Pak C_{18} cartridges, the samples were assayed for TxB_2 (stable metabolite of TxA_2) by radioimmunoassay (RIA) (Jose *et al.*, 1976). The limit of detection was 0.02 pmol.

Materials

The following compounds were used: VIP (human, porcine, rat) and helodermin (Peninsula Laboratories), histamine acid phosphate (BDH), ACh and salbutamol (Sigma), LTD_4 (Merck Frosst Laboratories), U-44069 (The Upjohn Company), indomethacin (Merck, Sharp and Dohme), TxB_2 antiserum (Wellcome Research Laboratories) and [^{3}H]-TxB_2 (New England Nuclear). Porcine galanin was a gift from Professor S.R. Bloom.

Statistical analyses

The data were analysed by a paired Student's *t* test. A *P* value of less than 0.05 was considered to be statistically significant. In order to analyse data expressed as a percentage, a probit transformation was performed prior to Student's *t* test. Results are expressed as mean ± standard error of the mean (s.e.mean) where *n* represents the number of animals used.

Results

Effect of vasoactive intestinal peptide on contractions of guinea-pig lung parenchymal strips induced by leukotriene D_4, acetylcholine and histamine

LTD_4 (1–30 pmol, $n = 4$–17), histamine (1–30 nmol, $n = 5$–8) and ACh (1–100 nmol, $n = 4$–6) induced dose-related contractions of GPP (Figure 1). In the presence of VIP (10 nM), contractions induced by all doses of LTD_4 were significantly attenuated ($P < 0.05$), while those due to the other agonists were not significantly altered. This inhibition by VIP of LTD_4-induced contractile responses was readily reversible. Responses induced by 3 and 10 pmol LTD_4, re-examined at 10 and 30 min respectively, following termination of VIP infusion, were almost identical to those obtained before treatment of tissues with VIP ($n = 5$) (data not shown).

The effect of VIP (10 nM) was also evaluated on LTD_4-induced contractions of indomethacin-treated GPP. In the presence of indomethacin (2.8 μM) contractions induced by LTD_4 (3–30 pmol) were significantly reduced ($P < 0.05$); however, these responses were not further inhibited following additional treatment with VIP ($n = 4$) (data not shown).

In order to determine whether a higher concentration of VIP would inhibit contractions of GPP induced by histamine and ACh, and those induced by LTD_4 to a greater extent than that observed with VIP (10 nM), experiments were performed with a ten times higher concentration of VIP (100 nM). The contractile responses of GPP induced by histamine were not significantly attenuated in the presence of 100 nM VIP ($n = 4$–9) (Table 1). In contrast, indomethacin significantly inhibited contractions induced by histamine ($P < 0.05$). Contractions induced by ACh (3–30 nmol, $n = 3$–6) were not reduced in the presence of either VIP (100 nM) or indomethacin (data not shown).

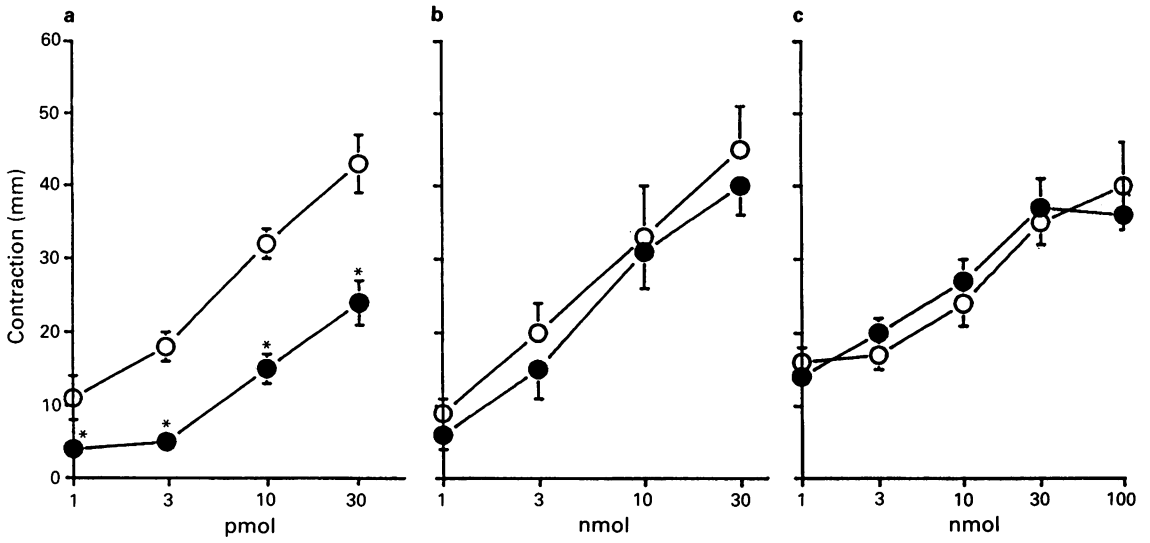


Figure 1 Dose-related contractions of guinea-pig lung parenchymal strips induced by (a) leukotriene D_4, $n = 4$–17, (b) histamine, $n = 5$–8 and (c) acetylcholine, $n = 4$–6 in the absence (○) and presence (●) of vasoactive intestinal peptide (10 nM). Each point represents mean height of tissue contraction (mm) with s.e.mean shown by vertical bars. * $P < 0.05$ when compared with untreated control (Student's t test for paired data).

VIP (100 nM) significantly attenuated the contractions of GPP induced by LTD_4 (3–30 pmol, $n = 4$–9, $P < 0.05$). This inhibitory effect of 100 nM VIP was not significantly greater than that evoked by 10 nM VIP (Table 2). In addition, the percentage inhibition of LTD_4-induced contractile response by both concentrations of VIP was similar to that observed in the presence of indomethacin.

In the presence of 1 nM VIP, the responses elicited by LTD_4 (1–30 pmol), ACh (3–100 nmol) or histamine (3–30 nmol) were unaltered ($n = 4$–6). In all experiments, VIP (1–100 nM) did not affect contractions of GPP induced by U-44069 (0.3–3 nmol) or reduce the basal tone of this preparation (data not shown).

Table 1 Effect of vasoactive intestinal peptide (VIP, 100 nM) and indomethacin (Indo, 2.8 μM) on contractile responses of guinea-pig lung parenchyma induced by histamine

Histamine (nmol)	*Control*	*VIP* (100 nM)	*Indo* (2.8 μM)
3	16.8 ± 3.0	14.0 ± 4.5	11.4 ± 3.4*
10	29.7 ± 4.5	27.7 ± 3.5	20.6 ± 2.9*
30	38.0 ± 6.5	35.8 ± 2.9	23.0 ± 3.2*

Results expressed as mean height of tissue contraction (mm) ± s.e.mean measured on a 50 mV scale. $n = 4$–9.
* $P < 0.05$ when compared with untreated control (Student's t test for paired data).

Table 2 Percentage inhibition by vasoactive intestinal peptide (VIP, 10 and 100 nM) and indomethacin (Indo, 2.8 μM) of leukotriene D_4 (LTD_4)-induced contractile responses of guinea-pig lung parenchyma

LTD_4 (pmol)	*VIP* (10 nM)	*VIP* (100 nM)	*Indo* (2.8 μM)
3	74 ± 5	69 ± 4	72 ± 3
10	55 ± 4	59 ± 5	60 ± 6
30	45 ± 2	44 ± 3	48 ± 6

Data from experiments with VIP (10 and 100 nM) and indomethacin (2.8 μM) were analysed as the difference in contraction after treatment, expressed as a percentage (%) of the control response. Results expressed as mean % inhibition of the contractile response ± s.e.mean. $n = 4$–9.

Differential effect of helodermin on contractions of guinea-pig lung parenchyma induced by leukotriene D_4, histamine and acetylcholine

In the presence of helodermin (10 nM) contractions of GPP induced by LTD_4 (3–30 pmol) were significantly reduced ($P < 0.05$), in contrast to responses induced by histamine (1000–10,000 pmol) which were not affected ($n = 4$–6) (Figure 2). Helodermin did not reduce the contractile responses induced by ACh (10 and 30 nmol, $n = 4$) (data not shown). There was no significant difference in the inhibition induced by either VIP or helodermin on responses elicited by all doses of LTD_4, e.g. contractions induced by 3 pmol LTD_4 were reduced by 73 ± 4% and 65 ± 5% in the presence of VIP and helodermin respectively.

Lack of effect of galanin on responses of guinea-pig lung parenchyma due to leukotriene D_4, histamine and acetylcholine

Contractions of GPP induced by LTD_4 (3–30 pmol), ACh (3–30 nmol) and histamine (3–30 nmol) were not significantly

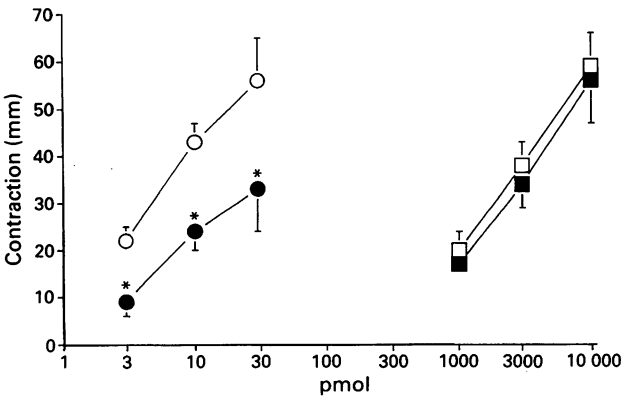


Figure 2 Contractions of guinea-pig lung parenchymal strips induced by leukotriene D_4 (3–30 pmol) in the absence (○) and presence (●) of helodermin (10 nM), and histamine (1000–10,000 pmol) in the absence (□) and presence (■) of helodermin (10 nM). Each point represents mean height of tissue contraction (mm) with s.e.mean shown by vertical bars. $n = 4$–6. * $P < 0.05$ when compared with untreated control (Student's t test for paired data).

reduced following treatment with galanin (10 and 100 nM, $n = 4$–6). In contrast, following treatment of these same GPP with VIP (10 nM), contractions induced by 10 pmol LTD_4 were inhibited by 55 ± 5% while those induced by ACh (30 nmol) and histamine (30 nmol) were unaffected ($n = 3$–6) (data not shown).

Effect of salbutamol on contractions of guinea-pig lung parenchyma induced by leukotriene D_4, acetylcholine and histamine

Contractions of GPP evoked by LTD_4 (1–30 pmol, $n = 7$–9) and histamine (1–30 nmol, $n = 7$–11) were significantly attenuated in the presence of 10 nM salbutamol ($P < 0.05$), while those due to ACh (1–100 nmol, $n = 7$) were unaffected (Figure 3). As in the case of indomethacin, salbutamol induced a greater inhibition of the responses due to LTD_4 than those induced by histamine, e.g. responses due to 10 pmol LTD_4 and 10 nmol histamine, which were of similar magnitude on GPP, were inhibited by 51 ± 4% and 19 ± 5% respectively. At this concentration, salbutamol did not affect responses due to U-44069 (0.3–3 nmol) or alter the basal tone of GPP.

In the presence of a ten fold higher concentration of salbutamol (100 nM), the basal tone of the tissues was reduced and contractions due to all agonists were inhibited. Contractions induced by LTD_4 (3–30 pmol) and histamine (3–30 nmol) were further attenuated by 100 nM salbutamol, while those induced by ACh (30 and 100 nmol) and U-44069 (1 and 3 nmol) were significantly inhibited ($n = 4$–12) (data not shown).

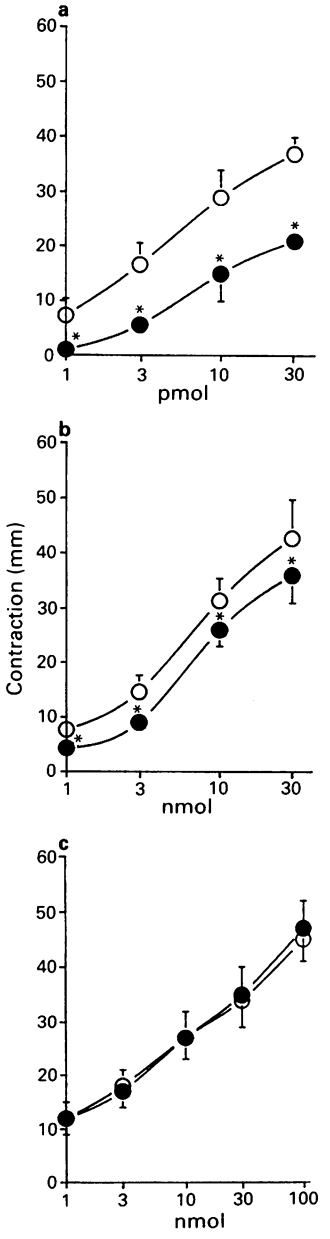


Figure 3 Dose-related contractions of guinea-pig lung parenchymal strips induced by (a) leukotriene D_4, $n = 7$–9, (b) histamine, $n = 7$–11 and (c) acetylcholine, $n = 7$ in the absence (○) and presence (●) of salbutamol (10 nM). Each point represents mean height of tissue contraction (mm) with s.e.mean shown by vertical bars. * $P < 0.05$ when compared with untreated control (Student's t test for paired data).

Inhibition by vasoactive intestinal peptide and helodermin of thromboxane B_2 release induced by leukotriene D_4 from guinea-pig lung parenchyma

LTD_4-induced contractions of GPP were associated with dose-related increases in levels of TxB_2. Following treatment of GPP with VIP (10 nM) both the contractions and TxB_2 release induced by LTD_4 (3–30 pmol, $n = 4$–7) were significantly inhibited ($P < 0.05$) (Figure 4). Helodermin (10 nM) induced a similar inhibition of the contractile responses of GPP and the associated TxB_2 release induced by LTD_4 (3–30 pmol, $n = 4$, $P < 0.05$) (Figure 4).

In contrast, in these experiments stimulation with histamine (3–30 nmol) resulted in the generation of low levels of TxB_2 which were not significantly inhibited by VIP (10 nM, $n = 3$) (data not shown). Histamine (3 nmol) and LTD_4 (10 pmol), doses which gave a similar height of contraction of GPP, induced the release of 66 ± 10 and 448 ± 64 pg min^{-1} TxB_2 respectively. In the presence of VIP (10 nM) this release of TxB_2 induced by histamine was 78 ± 10 pg min^{-1} while that induced by LTD_4 was below the limit of detection of the assay.

Comparative effects of vasoactive intestinal peptide, helodermin and galanin on guinea-pig trachea

VIP (10–3000 pmol) and helodermin (30–3000 pmol) induced dose-related relaxations of guinea-pig trachea ($n = 3$–6).

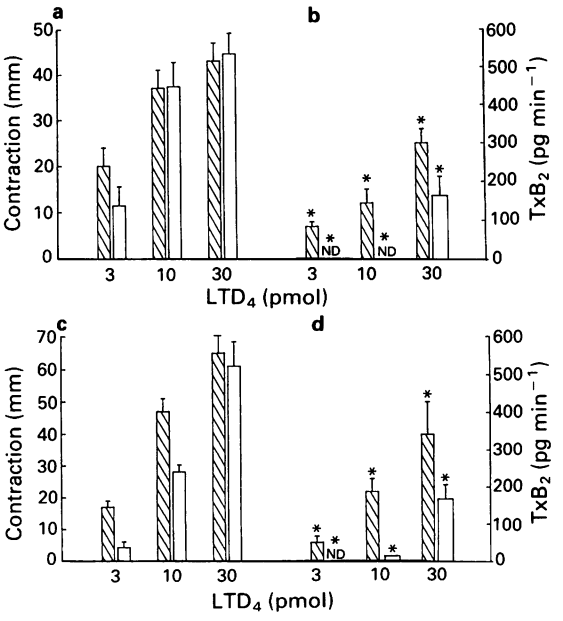


Figure 4 Contractions of guinea-pig lung parenchymal strips induced by leukotriene D_4 (LTD_4, 3–30 pmol) (hatched columns) and the associated thromboxane B_2 (TxB_2) release (open columns) in the absence (a) and presence (b) of vasoactive intestinal peptide (10 nM, $n = 4$–7); and in the absence (c) and presence (d) of helodermin (10 nM, $n = 4$). Results expressed as mean with s.e.mean shown by vertical bars. ND, none detected. * $P < 0.05$ when compared with untreated control (Student's t test for paired data).

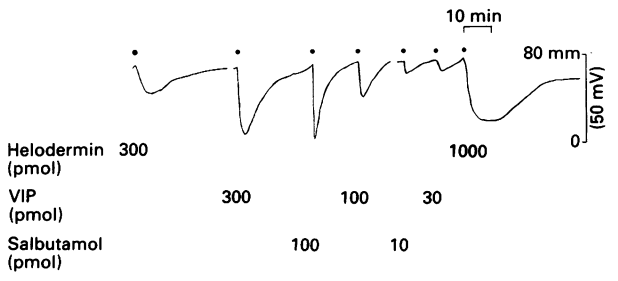


Figure 5 A representative tracing illustrating the comparative relaxant effects of vasoactive intestinal peptide (VIP, 30–300 pmol), helodermin (300 and 1000 pmol) and salbutamol (10 and 100 pmol) on a superfused strip of guinea-pig trachea. Vertical axis: relaxation (mm). Horizontal axis: time.

Figure 5 illustrates a representative tracing showing relaxation responses of a superfused GPT induced by VIP (30–300 pmol), helodermin (300 and 1000 pmol) and salbutamol (10 and 100 pmol). On this preparation, helodermin was approximately three times less active than VIP. However, its relaxant action was considerably more sustained than that induced by equiactive doses of VIP or salbutamol. Tracheal tone had returned to baseline within 26 min following a relaxant response induced by 300 pmol VIP, while that evoked by an equiactive dose of helodermin (1000 pmol) was not fully reversed at 45 min.

In contrast to VIP and helodermin, galanin was inactive on guinea-pig trachea, in doses ranging between 0.3–3.0 nmol ($n = 3$–6) (data not shown).

Discussion

In the present study, VIP and helodermin (10 nM) induced a potent and reversible inhibition of contractions of GPP evoked by LTD_4, suggesting an important modulatory role for these peptides in the peripheral airways of the guinea-pig. Both LTD_4 and histamine activate the cyclo-oxygenase pathway in guinea-pig lung, with the subsequent release of mainly TxA_2, a potent bronchoconstrictor (Piper & Samhoun, 1982; Dahlen *et al.*, 1983). TxA_2, released by agonists such as LTD_4, significantly augments the contractile response of GPP in the superfusion cascade system (Piper & Samhoun, 1982; Dahlen *et al.*, 1983). This is in contrast to the classical organ bath technique, where LTD_4-induced release of TxA_2 does not contribute to the contractile response (Dahlen *et al.*, 1983).

The effect of VIP and helodermin was similar to that observed with indomethacin, in that they inhibited LTD_4-induced responses. This suggests that these peptides, like indomethacin, could act by inhibiting the generation of TxA_2, which partly mediates the response of LTD_4 in GPP. This was further substantiated by radioimmunoassay of the effluents which showed that VIP and helodermin (10 nM) significantly attenuated LTD_4-induced release of TxB_2, the stable metabolite of TxA_2.

In contrast, neither the TxB_2 release induced by histamine stimulation, nor the contractions of GPP were significantly reduced by VIP (10 and 100 nM). Although the release of TxB_2 evoked by histamine was small in comparison with that associated with LTD_4 stimulation, inhibition of these contractile responses by indomethacin suggests that TxA_2 release contributes to the histamine-induced contraction in this preparation. Moreover, indomethacin reduced the duration of the contractile response induced by histamine suggesting that release of TxA_2 may contribute more to the time course of the contractile response rather than to the amplitude of the response. Dahlen *et al.* (1983) have shown, using a modified version of the superfusion cascade, that the release of TxA_2 following stimulation with histamine was about ten times less than that induced by an equipotent dose of LTC_4. In addition, the release of TxB_2 and the amplitude of the contractile response induced by LTC_4 were reduced by indomethacin, whereas in the case of histamine, indomethacin inhibited both TxA_2 release and the duration of the response but did not alter its amplitude. These results suggest that a large component of the contractile response of GPP due to LTD_4 can be accounted for by the released TxA_2 while the response to histamine is more direct and not as dependent on TxA_2 release.

The contractions induced by LTD_4 in the presence of indomethacin, were not further attenuated by the addition of VIP which shows that this peptide has only a partial inhibitory effect and acts via inhibition of the cyclo-oxygenase-dependent component of the LTD_4 response. In addition, a higher concentration of VIP (100 nM) did not further inhibit the contractions indicating that VIP does not affect the direct spasmogenic response (independent of cyclo-oxygenase activation) induced by LTD_4 on smooth muscle cells in guinea-pig lung.

Although VIP has been shown to reduce the contractile effect of exogenous ACh in bovine tracheal smooth muscle (Palmer *et al.*, 1985), it had no effect on contractions of GPP induced by ACh in the present study. In contrast to the action of both LTD_4 and histamine, ACh has a direct (cyclo-oxygenase-independent) contractile action in this preparation.

The inhibitory effect of VIP on LTD_4-induced release of TxB_2 may involve the activation of adenylate cyclase since stimulation of VIP receptors has been shown to activate this enzyme, thereby increasing the cellular concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in guinea-pig isolated lung and other tissues (Kitamura *et al.*, 1980). Indeed, by promoting cyclic AMP accumulation, the immunological release of histamine and slow-reacting substance of anaphylaxis were inhibited (Orange *et al.*, 1971). In addition VIP has been shown to inhibit the release of leukotrienes, induced by platelet-activating factor, from rat lung (Beaubien *et al.*, 1984) and that of histamine from guinea-pig lung following ovalbumin challenge (Undem *et al.*, 1983). VIP markedly attenuated acute inflammatory injury induced by xanthine/xanthine oxidase in rat lung and simultaneously abolished the generation of cyclo-oxygenase metabolites associated with oxidant injury (Berisha *et al.*, 1990). This could have resulted from selective inhibition of the cyclo-oxygenase or more likely from inhibition of phospholipase A_2 (PLA_2) activity since VIP has been shown to inhibit biosynthesis of lipoxygenase products (Beaubien *et al.*, 1984) and has recently been reported to inhibit PLA_2 activity *in vitro* (Trotz *et al.*, 1990).

VIP, by increasing cyclic AMP in lung, could act in a similar manner to that of the β-adrenoceptor agonist, salbutamol. In this study, 10 nM salbutamol inhibited contractions induced by both LTD_4 and histamine but did not affect those induced by ACh. As in the case of VIP, salbutamol was a much more effective inhibitor of the responses due to LTD_4 although a small, yet significant, inhibition of histamine-induced responses was observed. Salbutamol, at the higher concentration of 100 nM, inhibited contractions induced by all agonists tested, i.e. LTD_4, ACh, histamine and U-44069, indicating a non-selective effect of this β-adrenoceptor agonist which may be attributed to functional antagonism.

Agents which increase cyclic AMP such as forskolin, isoprenaline, salbutamol and dibutyryl cyclic AMP have been shown to inhibit phosphatidylinositol hydrolysis, in canine and bovine tracheal smooth muscle, in response to histamine but not methacholine or carbachol (Madison & Brown, 1988; Hall & Hill, 1988). More recently, VIP has been shown to inhibit histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle (Hall *et al.*, 1989). These findings suggest that cyclic AMP may selectively modulate receptor-mediated activation of cells. It is conceivable that VIP, by increasing cyclic AMP in guinea-pig lung, could inhibit LTD_4 receptor-mediated inositol lipid hydrolysis

(Mong *et al.*, 1987) and thus inhibit formation of two important intracellular messengers, diacylglycerol and inositol trisphosphate. These two second messengers activate protein kinase C and mobilize calcium respectively, and result in arachidonic acid release, with subsequent synthesis of cyclo-oxygenase metabolites, contraction of smooth muscle and various pharmacological effects (Berridge, 1984).

Helodermin, a peptide that is structurally related to VIP, induced dose-dependent relaxations of guinea-pig trachea and was approximately three times less active than VIP on this preparation. By contrast, the relaxant effect of helodermin was considerably more sustained. A similar finding was reported by Foda & Said (1989). This more sustained action of helodermin on airway smooth muscle may reflect the greater resistance of this peptide to peptidase degradation, after binding to its receptor, due to its extended C-terminal region since VIP and other neuropeptides are readily degraded by neutral endopeptidases (Matsas *et al.*, 1984). A synthetic variant of helodermin which lacked the C-terminal extension was also shown to lack the prolonged activity of the full-sequence peptide (Naruse *et al.*, 1986). The protection of peptide activity by an extended C-terminal region may offer an attractive approach to the design of peptides for therapeutic use in respiratory disease such as asthma. Galanin in this study had no relaxant effect on airway smooth muscle of guinea-pig which is in agreement with a previous report by Ekblad *et al.* (1985). This peptide was also ineffective in modulating the responses of GPP due to LTD_4, ACh or histamine. Recently, galanin was shown to inhibit the non-cholinergic contraction produced by field stimulation of the guinea-pig bronchus, suggesting that this peptide may be important in regulating neural function in the lungs (Giuliani *et al.*, 1989).

From the present experiments it can be concluded that VIP, helodermin and salbutamol, agents that increase cyclic AMP, antagonize contractions of GPP induced by LTD_4, ACh and histamine to different degrees. This differential effect appears to be associated with the different contribution of the released TxA_2 to contractions provoked by these agonists, Raised levels of cyclic AMP could have an effect at several levels, to inhibit either receptor-mediated PLC activation, PLA_2 activation or cyclo-oxygenase, but this is not currently known. However, the inhibitory effect of VIP in the guinea-pig perfused lung appears to be exerted on events preceding the mobilisation of arachidonic acid and not at the level of cyclo-oxygenase (Conroy *et al.*, 1988).

Until now it was thought that VIP, while regulating the calibre of large airways or pulmonary blood vessels, is unlikely to influence small airways. Our data, describing the inhibition by VIP of both the LTD_4-induced contractions and the associated generation of TxA_2, indicate a possible modulatory role for this peptide in guinea-pig peripheral airways. This, however, does not preclude the possibility that VIP may also act as a functional antagonist and that at higher concentrations could antagonize contractions induced by other constrictor agents by a bronchodilator mechanism. Our data suggest that this modulatory effect of VIP and helodermin may be important in bronchoconstriction induced by those agonists acting via the release of TxA_2.

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Characterization of the effects of the new inotropic agent BDF 9148 in isolated papillary muscles and myocytes of the guinea-pig heart

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1 The cardiotonic agent BDF 9148 (4-[3'-(1''-benzhydryl-azetidine-3''-oxy)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) is structurally related to DPI 201-106 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) which is known to modify cardiac sodium channels. In guinea-pig papillary muscles, both compounds increase force of contraction with similar concentration-response curves. Like DPI 201-106, BDF 9148 prolongs the action potential duration in a tetrodotoxin-sensitive manner. With high concentrations ($>3\,\mu$M), however, the action potential duration shortens again. In order to elucidate the underlying changes in membrane currents, we have investigated the effects of BDF 9148 in isolated ventricular myocytes of the guinea-pig heart.

2 In isolated cells, a concentration of $1\,\mu$M BDF 9148 prolonged the action potential duration and markedly enhanced unloaded cell shortening, indicating that the procedure of cell isolation does not abolish the effect of the drug.

3 Membrane currents were studied with the single electrode voltage clamp technique. With clamp steps from -80 mV to -40 mV, BDF 9148 ($1\,\mu$M) induced a slowly decaying inward current which was suppressed by tetrodotoxin. Therefore, like DPI 201-106, BDF 9148 slows the inactivation of the sodium channels.

4 In order to quantify the effects of BDF 9148 and DPI 201-106 on sodium current inactivation, we have measured the inward current amplitude still present at 100 ms after a depolarizing clamp step from -80 mV to -30 mV. Both drugs increased this current component in a concentration-dependent manner; however, BDF 9148 had a larger effect in the low concentration range.

5 The calcium current was inhibited by BDF 9148 and DPI 201-106 in a concentration-dependent manner; the pD_2 values were 5.70 and 5.95, respectively.

6 The two compounds are thought to produce similar positive inotropic effects by imposing a sodium load on the muscle cells via modification of the sodium channels. The differences in action potential duration could be due to different contributions of ionic currents other than sodium or calcium currents and of pump and exchange currents. At present, there is not sufficient data to identify clearly distinct current components responsible for the differences in action potential prolongation.

Keywords: Cardiotonic agent, BDF 9148; sodium channel modifying agents; DPI 201-106; papillary muscle; cardiac myocytes; action potential; whole cell patch clamp; calcium current; sodium current

Introduction

The positive inotropic effect of DPI 201-106 (4-[3'-(4''-benzhydryl-1''-piperazinyl)-2'-hydroxypropoxy]-1H-indole-2-carbonitrile) in isolated heart muscle is accompanied by a marked prolongation of action potential duration (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985). This *in vitro* effect is paralleled *in vivo* by a prolongation of the QT-interval of the electrocardiogram (Rüegg & Nüesch, 1987) which limits its clinical use because of the danger of developing torsades de pointes arrhythmias (for literature, see Roden, 1990). Therefore, a drug with a similar mechanism of action would be considered more favourable if it exhibited less prolongation of action potential duration and hence showed less prolongation of QT time.

DPI 201-106 prolongs the action potential duration due to a delay of the inactivation of the Na^+ channels (Buggisch *et al.*, 1985; Kohlhardt *et al.*, 1986). DPI 201-106 also inhibits the calcium current (I_{Ca}) as shown by its effects on slow action potentials in partially depolarized papillary muscles (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1985) and by voltage clamp experiments in guinea-pig cardiomyocytes (Holck & Osterrieder, 1988; Pfeifer *et al.*, 1989). The concentration which blocks calcium channels is one order of magnitude higher than that required for the positive inotropic effect (Scholtysik *et al.*, 1985).

The DPI 201-106 analogue, compound BDF 9148 (4-[3'-(1''-benzhydryl-azetidine-3''-oxy)-2'-hydroxypropoxy]-2-methyl-1H-indole-2-carbonitrile) also has a positive inotropic effect, prolongs the action potential duration, delays inactivation of the sodium current and blocks I_{Ca} (Armah *et al.*, 1990; Honerjäger *et al.*, 1990; Pfeifer & Ravens, 1990).

Since the prolongation of action potential duration accompanying a given positive inotropic effect seemed smaller for BDF 9148 than for DPI 201-106 (compare Buggisch *et al.*, 1985 and Armah *et al.*, 1990) the difference in the effects of BDF 9148 and DPI 201-106 on action potential duration can only be explained if either drug has additional effects on other membrane currents. Less prolongation in action potential duration could result if the BDF 9148 concentrations required for modification of I_{Na} and I_{Ca} were closer than those of DPI 201-106 or if the compounds have opposing effects on potassium conductance.

In a first series of experiments, we investigated concentration-response curves for DPI 201-106 and BDF 9148 in guinea-pig papillary muscles in order to quantify the differences in action potential prolongation and positive inotropic effects of these two agents under identical experimental conditions. We then examined the effects of BDF 9148 on membrane currents in isolated myocytes using the single electrode voltage clamp technique. The only difference between both compounds was a larger effect of BDF 9148 on sodium

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current (I_{Na}), particularly in the low concentration range ($< 1\,\mu M$); the effects on calcium current (I_{Ca}) were similar. Some of these results have been published in a preliminary form (Pfeifer & Ravens, 1990).

Methods

Experiments with papillary muscles

Right ventricular papillary muscles were carefully dissected from guinea-pig hearts and mounted horizontally in a 5 ml muscle chamber through which oxygenated modified Tyrode solution recirculated from a 100 ml reservoir kept at 35°C. A jet stream of carbogen simultaneously served for circulation and oxygenation of the solution. The preparations were stimulated via platinum electrodes at a frequency of 1 Hz (square wave pulses of 3 ms in duration 20% above threshold voltage). Action potentials were recorded with conventional glass microelectrodes (filled with 3 M KCl) and force of contraction was measured by an isometric force transducer (Statham UC2). Both signals were displayed on the screen of a dual beam oscilloscope, the screen of which was photographed at regular intervals for later analysis of the data. Drugs were added in a cumulative manner to the reservoir of the bathing solution, allowing at least 45 min for equilibration before the next concentration was tested.

Isolated cardiac myocytes

Single ventricular myocytes from guinea-pig hearts were prepared by enzymatic dissociation as described previously (Borchard & Ravens, 1986) and stored at room temperature (20–25°C) until use. A small drop of cell suspension was added to the investigation chamber placed on the stage of an inverted microscope (Olympus IMT-2). During the experiments, cells were superfused with modified Tyrode solution at a constant rate of $2\,ml\,min^{-1}$. Current recordings were made at room temperature, action potential and contractility recordings at 35–36°C. BDF 9148 and DPI 201-106 are poorly soluble in aqueous solutions and strongly adhere to surfaces; thus exact control of drug concentration in the superfusing solution is difficult to obtain. In order to avoid contamination of the chamber with agent from the previous experiment, a freshly cleaned chamber was used in each experiment and new connective tubings were employed. To clean the investigation chambers, they were left over night in detergent (Mucasol, Merz, Frankfurt, Germany) followed by rinsing several times with distilled water.

Action potentials were measured with a LIST EPC-7 patch-clamp amplifier (LIST medical instruments, Darmstadt, Germany) in the current clamp mode. The contractility of the cells was recorded as unloaded shortening via an array of photodiodes placed over the edge of the cell image on a television screen. An electronic device designed by H. Ehrler (Homburg/Saar, Germany) was used to record the light intensity changes due to the contraction of the cells. For current measurements, the single electrode voltage clamp circuit provided by the LIST EPC-7 amplifier was used in the voltage clamp mode with compensation made for series resistance (at least 50%) and membrane capacitance. Membrane currents were monitored and stored on a digital storage oscilloscope (NIC 4094A, Nicolet Instrument Corporation, Madison, Wisconsin, U.S.A.) and registered in parallel on a chart recorder (GOULD Brush 220, Gould Inc., Cleveland, Ohio, U.S.A.) for later evaluation. In some cells, the PClamp software (Axon Instruments, Foster City, CA, U.S.A.) was used for conducting the experiments and analyzing the results.

Patch electrodes were pulled from filament borosilicate glass (Hilgenberg, Malsfeld, Germany). Their tip resistances were in the range of 2–4 MΩ when filled with solution of the following composition (mM): KCl 140, $MgCl_2$ 4, EGTA (ethylene-glycol-*bis*-(β-aminoethylether-N,N,N',N'-tetraacetic acid) 10, HEPES (hydroxyethyl-piperazinyl-ethane-sulphonic acid) 10 and Na_2ATP 4. The pH was adjusted to 7.3 with KOH. EGTA was omitted in experiments in which contractility was studied. For recording of sodium and calcium currents, the electrode filling solution contained CsCl instead of KCl and the pH was adjusted with CsOH. Results from cells showing more than 30% decline of I_{Ca} during the initial 5 min of the experiment were discarded. For recording of potassium currents, the electrode filling solution contained (in mM): KCl 120, $MgCl_2$ 0.5, EGTA 10, HEPES 10 and Na_2ATP 4. The pH was adjusted to 7.3 with KOH.

Solutions and drugs

For experiments in guinea-pig papillary muscles, the composition of the modified Tyrode solution used was (in mM): NaCl 137, KCl 5.4, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.42, $NaHCO_3$ 22.0 and glucose 5. The solution was gassed with a mixture of 95% oxygen and 5% carbon dioxide, the pH was 7.4.

For experiments with myocytes, the modified Tyrode solution had the following composition (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 2.0, $CaCl_2$ 1.8, HEPES (hydroxyethyl-piperazinyl-ethane-sulphonic acid) 10 and glucose 10. In the experiments for the measurement of sodium current, CsCl was substituted for KCl (Cs-Tyrode) in order to reduce K^+ currents (Isenberg, 1976). The pH was adjusted with NaOH or CsOH to 7.4.

Racemic BDF 9148 was synthesized by Beiersdorf AG, Hamburg, Germany. Racemic DPI 201-106 was kindly provided by Sandoz Co., Basel, Switzerland. Stock solutions of BDF 9148 (1 mM) or DPI 201-106 (1 mM) were prepared in dimethylsulphoxide (DMSO), the final concentration was obtained by direct dilution in Tyrode solution. The final concentration of DMSO in the bathing solution never exceeded 1% (vol/vol) and did not influence the parameters under investigation. Tetrodotoxin was purchased from Boehringer-Mannheim, Mannheim, Germany. All other chemicals were purchased from Merck, Darmstadt, Germany.

Data analysis and statistics

All values are expressed as mean values ± s.e.mean. The data were analysed by Kruska-Wallis analysis and by Student's *t* test for paired of unpaired data where appropriate. Differences were considered statistically significant for $P < 0.05$.

Results

Concentration-response curves of DPI 201-106 and BDF 9148 in guinea-pig papillary muscles

After 60–90 min of equilibration, the control force of contraction of guinea-pig papillary muscles was 1.54 ± 0.31 mN and 1.39 ± 0.48 mN in the BDF 9148 and DPI 201-106 group, respectively ($P > 0.2$, not significant); the control values for the action potential duration were 225 ± 7 ms and 195 ± 8 ms. The muscles were then exposed to cumulatively increasing concentrations of BDF 9148 or DPI 201–106 and the changes induced by the compounds were expressed as a factor of the pre-drug control values (= 1). Under identical experimental conditions, both drugs augmented force of contraction and increased action potential duration in a monotonic time course. However, at the highest concentration of BDF 9148 investigated (10 μM), action potential duration declined again. Both drugs had similar concentration-response curves for the positive inotropic effect, but less prolongation of action potential duration was observed with BDF 9148 than with DPI 201-106 (Figure 1). The differences were statistically significant both in relative and absolute terms for concentrations equal to or larger than 1 μM. Mean action potential amplitude was 117.8 ± 1.5 mV, resting membrane potential was −81.6 ± 0.8 mV, and maximum rate of depolarization (dV/dt_{max}) was 240 ± 15 $V\,s^{-1}$. These values were not significantly influenced by either compound (data not shown).

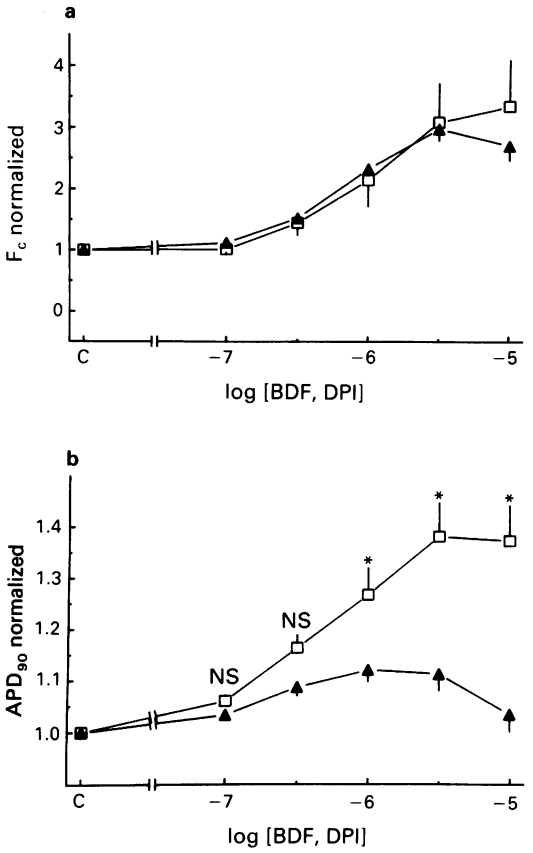


Figure 1 Concentration-response curves of the effects of BDF 9148 (▲) and DPI 201-106 (□) on force of contraction (a) and action potential duration (b) in guinea-pig papillary muscles. Drug concentration was increased cumulatively in steps of one half log unit. The experimental values were normalized to the control values, measured at the end of the equilibration period. Abscissa scale: molar concentration in log units. Mean values from 5 to 7 experiments; s.e.mean shown by vertical lines. The asterisks indicate statistically significant differences (Kruska-Wallis analysis for group comparison, $*P < 0.05$); NS, not significant.

Effects of BDF 9148 on action potentials and contractility in isolated myocytes

BDF 9148 (1 μM) also enhanced the contractility measured as unloaded shortening and prolonged the action potential duration (APD) of electrically stimulated cardiac cells (see Figure 2). After 14 min of washing in drug-free solution, the contraction amplitude declined below the pre-drug control value, whereas the prolongation in action potential duration was only slightly reversed. Neither resting potential nor action potential amplitude were changed; in 5 cells, the mean values of the action potential amplitude were 125.0 ± 2.1 mV and 125.5 ± 1.9 mV and the mean values of the resting membrane potential were −74.3 ± 0.3 mV and −75.5 ± 0.3 mV for the controls and in the presence of BDF 9148 (1 μM), respectively. These results demonstrate that after the isolation procedure, the cells remained responsive to BDF 9148. Therefore, they are a suitable model to study the effects of the compounds on membrane currents.

Effect of BDF 9148 on sodium current

Voltage clamp studies were carried out in order to identify the components of net membrane current responsible for the changes in APD. Because of the large amplitude of the Na^+ current in adult myocytes, adequate voltage control for quantitative analysis is difficult to achieve. At room temperature, however, small cells can be clamped with reasonable quality if low resistance electrodes (<2.5 MΩ) are used. Figure 3 shows tracings from a typical cell: a large, rapidly inactivating

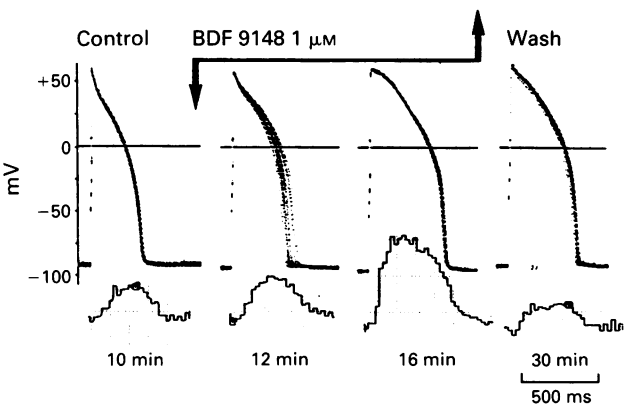


Figure 2 Action potentials and contraction amplitudes measured in a single myocyte (guinea-pig heart) before and after exposure to BDF 9148. After 6 min of exposure to 1 μM, the cell was washed with drug-free solution for 14 min. Time and voltage scales as indicated, cell shortening in arbitrary units. Stimulation frequency 0.5 s^{-1}, depolarizing current steps of 0.5 nA in amplitude and of 8 ms in duration.

inward current was measured during a potential step from −80 mV to −40 mV. Addition of BDF 9148 (1 μM) retarded inactivation of the inward current so markedly that the net membrane current remained inwardly directed for almost 500 ms. The effect was completely abolished by the selective Na^+ channel blocker tetrodotoxin (30 μM), suggesting that this current flows through modified Na^+ channels.

In order to quantify the drug effects on Na^+ current inactivation, we have established concentration-response curves (Figure 4a). Sodium currents were activated by clamp steps from −80 mV to −30 mV (clamp step duration 250 ms, frequency 0.2 s^{-1}). Under control conditions, the Na^+ current was completely inactivated 100 ms after the beginning of the depolarizing step. In the presence of various concentrations of BDF 9148 or DPI 201-106, however, an appreciable inward

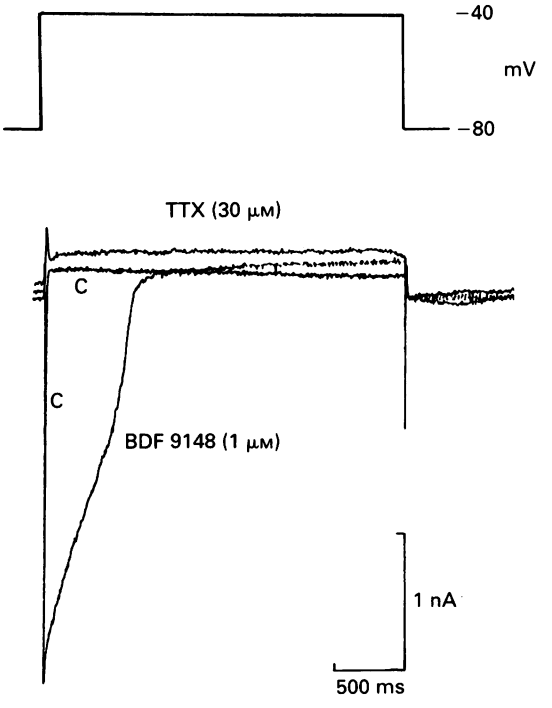


Figure 3 Effects of BDF 9148 (1 μM) and subsequent addition of tetrodotoxin (TTX, 30 μM) on net membrane current in response to 2.5 s long clamp pulses from −80 mV to −40 mV at a frequency of 0.1 s^{-1}. Calibrations as indicated by the bars. The pre-drug control tracing consists of a large, rapid downward deflection (inward current). After addition of BDF 9148, the inward current decays slowly and persists for almost 500 ms. It is blocked by TTX.

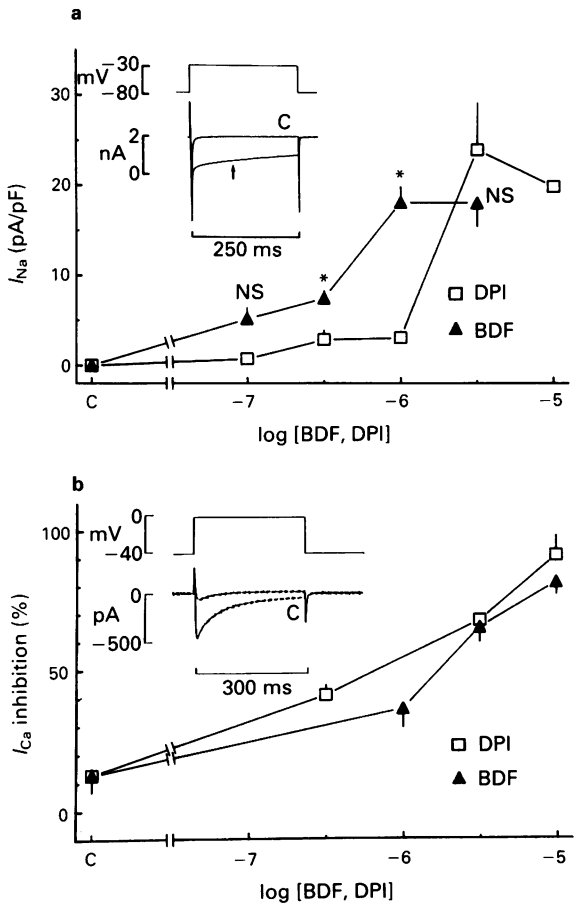


Figure 4 Concentration-response curves for the effects of BDF 9148 (▲) and DPI 201-106 (□) on membrane currents in cardiac myocytes. (a) Persisting I_{Na} after 6 min of drug exposure measured 100 ms after a step depolarization from −80 mV to −30 mV (arrow in inset), duration of test pulse 250 ms, frequency 0.2 s^{-1}. Only one drug concentration was studied in each cell (1 μM in inset). Mean values of at least 3 experiments in each group; vertical lines show s.e.mean. The asterisks indicate statistically significant differences (Kruska-Wallis analysis for group comparison, $P < 0.05$); NS, not significant. (b) Decrease in I_{Ca} expressed as percentage inhibition of the control value measured 8 min after the beginning of the experiment. The effect was measured after 7 min of drug exposure (one concentration only in each cell, 10 μM in inset). Clamp pulses were applied at a frequency of 0.2 s^{-1}, holding potential −40 mV, clamp potential 0 mV, duration of depolarization 300 ms. The control value (C) was time-matched and presents the spontaneous decline of I_{Ca} ('run-down') in the control cells. Mean values from 4–8 experiments; vertical lines show s.e.mean. pD_2 values of 5.70 and 5.96 for BDF 9148 and DPI 201-106, respectively, were estimated from the fit of a 4 parameter logistic equation. Insets: original tracings of membrane currents in response to the depicted clamp steps before (C) and after drug application (▲).

current was detectable at this time (see inset in Figure 4a), the amplitudes of which were taken as a measurement of the effects of the drugs on Na^+ current inactivation. Figure 4a clearly shows that BDF 9148 affected this parameter at lower concentrations than DPI 201-106. There was no statistically significant difference between the maximum effects.

Effect of BDF 9148 on calcium current

For the study of Ca^{2+} currents, cells were kept at a holding potential (V_h) of −40 mV to inactivate the Na^+ current and were depolarized by clamp pulses of 300 ms duration to 0 mV at a frequency of 0.2 Hz. BDF 9148 was added 8 min after the start of the experiment. All experiments were terminated after 15 min. Taking the value at the end of the 8 min control period as 100% I_{Ca} of control cells decreased to 87 ± 6% during the subsequent 7 min ($n = 6$; spontaneous 'run-down'), but BDF 9148 (10 μM) reduced I_{Ca} to 13 ± 2% ($n = 4$). The concentration-response curves for BDF 9148 and DPI 201-106 are depicted in Figure 4b. Both drugs inhibited I_{Ca} in a similar concentration range, the pD_2 values were 5.70 and 5.96, for BDF 9148 and DPI 201-106, respectively. For both drugs, inhibition of I_{Ca} was almost complete at the highest concentrations of 10 μM. Neither BDF 9148 nor DPI 201-106 shifted the current voltage relation of I_{Ca} along the voltage axis (data not shown).

Discussion

In the present study, we confirm that the new inotropic agent BDF 9148 enhances force of contraction in papillary muscles to a similar maximum as its parent compound DPI 201-106, but that this effect is accompanied by less prolongation in action potential duration (Armah *et al.*, 1990). Since the positive inotropic effect of the two compounds is abolished by the sodium channel blocking agent tetrodotoxin, it is concluded that it is intimately linked to their inhibitory effect on I_{Na} inactivation (Ravens, 1976; Honerjäger, 1982; Isenberg & Ravens, 1984; Buggisch *et al.*, 1985). BDF 9148- or DPI 201-106-modified sodium channels allow Na^+ influx to persist during membrane depolarization and the resulting increase in intracellular sodium concentration causes a rise in the cellular calcium content due to decreased calcium efflux via the Na^+/Ca^{2+} exchange (Ravens & Wettwer, 1989). This shift in intracellular ionic distribution is considered to be responsible for the positive inotropic effect (Honerjäger, 1982).

Changes in the shapes of the action potentials are more difficult to interpret because the action potential duration is determined by a delicate balance of ionic, pump and carrier currents directed both outward- and inwardly. The persisting Na inward current induced by both DPI 201-106 and BDF 9148 probably contributes directly to the APD prolonging effect, although it cannot be the only cause since we have found that BDF 9148 causes *less* APD increase in spite of the fact that it appears to have a greater influence on I_{Na}. Differential effects in I_{Ca} can be ruled out, because BDF 9148 and DPI 201-106 concentration-dependently reduced I_{Ca} without significant differences between the two compounds. The effect of BDF on APD could be explained if the concentration-response curves for modification of I_{Na} and I_{Ca} lay close together so that the APD-prolonging effect of persisting I_{Na} is counteracted by the APD-shortening effect of inhibition of I_{Ca}. This is, however, not the case; on the contrary, BDF 9148 is more potent in modifying I_{Na} than I_{Ca} whereas DPI 201-106 influences both currents with similar potencies. Therefore, either compound must have an additional effect on other membrane currents.

The inward rectifier, I_{K1}, or the delayed rectifier, I_K, could also be targets for BDF 9148 and DPI 201-106-induced changes in APD. In preliminary experiments, we were unable to observe clear effects on these currents, however, the changes may have been too small for detection yet still relevant for plateau prolongation (Trautwein, 1973). This point needs to be addressed in a further carefully designed study, because there is some evidence from the literature, that DPI 201-106 does indeed decrease I_{K1} (Holck & Osterrieder, 1988).

The concentration-response curves for the positive inotropic effects of BDF 9148 and DPI 201-106 were found to be superimposed. If we assume that the increase in force is mainly due to sodium load, both drugs should induce a similar load (estimated as sodium current integral corrected for the action potential duration). This corresponds well with the larger effect of BDF 9148 on I_{Na} combined with a shorter action potential, whereas DPI 201-106 increases I_{Na} to a lesser extent yet causes more AP prolongation.

In addition, the increased intracellular Na^+ concentration may modify physiological pump and exchanger currents which also affect the action potential duration. With BDF 9148, the (Na^+, K^+)-ATPase could contribute more outwardly directed pump current and therefore shorten the APD. In

the potential range of the plateau phase, the Na^+/Ca^{2+} exchange is also outwardly directed and an increase in exchange current caused by Na^+ load should also shorten the action potential duration (Hilgemann, 1988; Ravens & Wettwer, 1989; Wettwer & Ravens, 1991).

Each of the compounds used was the racemic mixture of a pair of enantiomers with opposing actions on I_{Na} (Scholtysik *et al.*, 1986; Romey *et al.*, 1987; Wang *et al.*, 1990) and similar effects on I_{Ca} (see Ravens *et al.*, 1991 for DPI 201-106 enantiomers; no data available for BDF 9148 enantiomers). Therefore, different contributions of the enantiomers in each racemic mixture to the various effects on membrane currents could also explain our observations. This problem must be solved in future experiments with the enantiomers.

In the present paper, we have shown that two new positive inotropic agents, DPI 201-106 and BDF 9148 enhance force of contraction by inducing a Na^+ load in cardiac tissue. Although both agents are equally efficient and potent with respect to their positive inotropic action, BDF 9148 induces much less prolongation in action potential duration which cannot be explained by a single mechanism. We can exclude the possibility that I_{Ca} plays an important role, but further experiments are required to obtain definite data on potassium conductance.

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Depression of miniature endplate potential frequency by acetylcholine and its analogues in frog

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1 Acetylcholine (ACh), 7.5×10^{-5} M, and carbachol, 5×10^{-6} M (CCh) depressed the frequency of miniature endplate potentials (m.e.p.ps) in the frog (*Rana temporaria*) sartorious neuromuscular junction with active acetylcholinesterase to about 50–55% of the controls.

2 A similar depression was produced by the nicotinic agonists, nicotine, suberyldicholine and tetramethylammonium.

3 The muscarinic agonists, oxotremorine, methylfurmethide and methacholine were without effect on m.e.p.p. frequency. The muscarinic antagonist, atropine and the nicotinic antagonist, (+)-tubocurarine, had no effect on the depression of m.e.p.p. frequency evoked by CCh.

4 The ganglionic blockers, benzhexonium and IEM-1119, were also without effect on the CCh-evoked depression of m.e.p.p. frequency.

5 Pretreatment of muscles with anticholinesterases did not prevent the CCh-induced drop in m.e.p.p. frequency.

6 The effect of CCh was proportionally the same as in the controls in preparations where the m.e.p.p. frequency was changed by elevation of K^+ and in the presence of theophylline, noradrenaline, dibutyryl adenosine 3′:5′-cyclic monophosphate (db cyclic AMP) and db cyclic GMP.

7 An inhibitor of Na^+,K^+-ATPase, ouabain, 5×10^{-5} mol l^{-1}, prevented or reversed the depression of m.e.p.p. frequency by CCh. However, the depression was present in a nominally K^+-free medium. Insulin and adrenaline, which are considered to be Na^+,K^+-ATPase activators, were without effect on depression of m.e.p.p. frequency.

8 The depression of m.e.p.p. frequency by 5×10^{-6} M CCh was the same at temperatures between 5 and 30°C with a Q_{10} near to 1.0. When threshold amounts of CCh were used (6×10^{-7} and 3×10^{-7} M), the depression was less at higher temperatures.

9 The receptive structures responsible for the CCh (or ACh)-evoked depression of m.e.p.p. frequency differ pharmacologically from muscarinic, nicotinic ganglionic and neuromuscular junction ACh-receptors as well as from the synaptic cholinesterase, in contrast to previous reports (Duncan & Publicover, 1979). The low temperature-dependence points to the possibility that physical rather than biochemical processes are limiting in this presynaptic effect of cholinomimetics.

Keywords: Acetylcholine; carbachol; miniature endplate potentials; neuromuscular junction; presynaptic receptors

Introduction

Acetylcholine (ACh) and its analogues increase the frequency of miniature endplate potentials (m.e.p.p.) recorded at the neuromuscular junctions of rats due to activation of presynaptic receptors of the nicotinic type (Miyamoto & Volle, 1974; Nikolsky, 1982; 1984; Bierkamper & Aizenman, 1984). In contrast, the m.e.p.p. frequency in frog skeletal muscle is depressed significantly by ACh and its non-hydrolysable analogue, carbachol (CCh) (Duncan & Publicover, 1979; Bukharaeva *et al.*, 1986). Duncan & Publicover (1979) proposed that the cholinesterase in frog nerve terminals might mediate the inhibitory effect of cholinomimetics. In some of our experiments we observed that not only ACh and CCh but also specific nicotinic cholinomimetics such as nicotine itself lowered the m.e.p.p. frequency at the amphibian endplate (Magazanik & Nikolsky, 1979; Bukharaeva *et al.*, 1986); with respect to the effect of anticholinesterase some discrepancy was found between our preliminary observations (Bukharaeva *et al.*, 1986) and those of Duncan & Publicover (1979).

In the present paper, the presynaptic action of cholinomimetics was therefore re-examined and the m.e.p.p. frequency was followed in the presence of several cholinolytic substances, during Na^+, Cl^-, Ca^{2+} and K^+ changes and under conditions when the activity of membrane Na^+,K^+-ATPase was altered. No simple pharmacological or ionic basis for the CCh depression was found and the negligible temperature-dependence of the presynaptic action of cholinomimetics may indicate that physical rather than biochemical processes determine this phenomenon.

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Methods

The experiments were performed on the sartorius muscle of the winter frog *Rana temporaria* at temperatures of 5–30°C. After rapid decapitation of animals anaesthetized with ether, muscles were dissected and placed in Ringer solution (in mM: NaCl 113.0, KCl 2.5, $CaCl_2$ 1.8 and $NaHCO_3$ 3.0; pH = 7.3) in a 1.7 ml translucid perfusion chamber with Peltier cooling elements. When Br^-, NO_3^- and SO_4^{2-} were substituted for Cl ions, osmotically identical amounts of these salts were used. Drugs were applied to the muscle bath in the perfusion (5 ml min^{-1}). When the ionic composition of the standard medium was changed, isosmolarity was maintained by changes in NaCl concentration or by adding the corresponding amount of sucrose. Acetylcholinesterase (AChE) was inhibited by treating muscles outside the perfusion chamber with the 5×10^{-6} M anti-AChE drug, armin (diethoxyparanitrophenylphosphate), dissolved in Ringer solution, for 30 min and then washing extensively three times with Ringer for 10 min to remove free anti-AChE. In other experiments, the reversible anti-AChE drug, proserin, was present in the bath continually at a concentration of 1×10^{-6} M.

Synaptic m.e.p.ps and resting membrane potentials (RMP) were measured by standard microelectrode techniques. Occasionally a standard two microelectrode voltage-clamp technique was used to measure endplate currents. The microelectrodes were filled with 2.5 M KCl and had resistances ranging from 3–7 MΩ.

M.e.p.ps were processed on a digital analyser-computer system. Spontaneous quantal release was estimated as the mean frequency of at least 100 m.e.p.ps. Postsynaptic effects of cholinergic agents were measured from the change in m.e.p.p. amplitude and resting membrane potential in the synaptic zone of each muscle fibre.

Attention was paid to the m.e.p.p./noise ratio to avoid a possible loss of small amplitude m.e.p.ps during sampling or, at depolarized RMP, due to the action of cholinomimetics or the presence of cholinolytics, as well as to avoid inclusion of some random deflections of the RMP. Only those experiments were evaluated in which the m.e.p.p. amplitude histogram shape was not changed markedly after application of cholinergic agents (see Figure 1).

The results are expressed as the mean of seven or more experiments ($n \pm$ s.e.mean). The statistical significance was evaluated by Wilcoxon's non-parametric test at an indicated probability level, usually 0.05, unless otherwise stated.

The following chemicals were used: acetylcholine chloride, nicotine, oxotremorine and adrenaline (Serva), carbachol chloride (Koch-Light), tetramethylammonium chloride (TMA) (Fluka), (+)-tubocurarine (TC), atropine sulphate (Burroughs-Wellcome), dibutyryl cyclic AMP (db cyclic AMP), dibutyryl cyclic GMP (db cyclic GMP), proserine, metacholine, insulin, ouabain and theophylline (Sigma). Methylfurmethide, benzhexonium, 4-(isopropylammonium butyl) trimethylammonium dibromide (IEM-1119) and diethoxyparanitrophenylphosphate (armin) were synthesized and kindly provided by the Institute of Organic Chemistry, Acad. Sci., U.S.S.R.

Results

Presynaptic effects of cholinomimetics of the nicotinic and muscarinic type

ACh and CCh reduced the frequency of m.e.p.p. which usually ranged from 1.5 to 3 s^{-1} ($2.75 \pm 0.74\,s^{-1}$, Figure 1, Table 2) and depolarized the muscle membrane in a dose-dependent manner (Figure 2a and b). The effects of nicotine, suberyldicholine and TMA were similar, but in concentrations chosen to affect m.e.p.p. frequency similar to CCh, their effects on membrane potential were weaker. Most of experiments in the following sections were therefore done with CCh and no anticholinesterase was used, unless otherwise stated.

The muscarinic agonists oxotremorine, methylfurmethide and metacholine were without effect on m.e.p.p. frequency. Typical muscarinic antagonists, atropine 1×10^{-6} M (Table 1) and pirenzepine 1×10^{-5} M (data of five experiments not given) also did not prevent the depression of m.e.p.p. frequency evoked by CCh (Table 1). In addition the nicotinic antagonist, TC (at 4.5×10^{-7} M), was without effect on CCh-induced m.e.p.p. frequency depression (Figure 2a). Note that this concentration of TC decreased both the postsynaptic action of CCh on the RMP (Figure 2b, see also Pennefather & Quastel, 1981) and the m.e.p.p. amplitude to about 50% (data

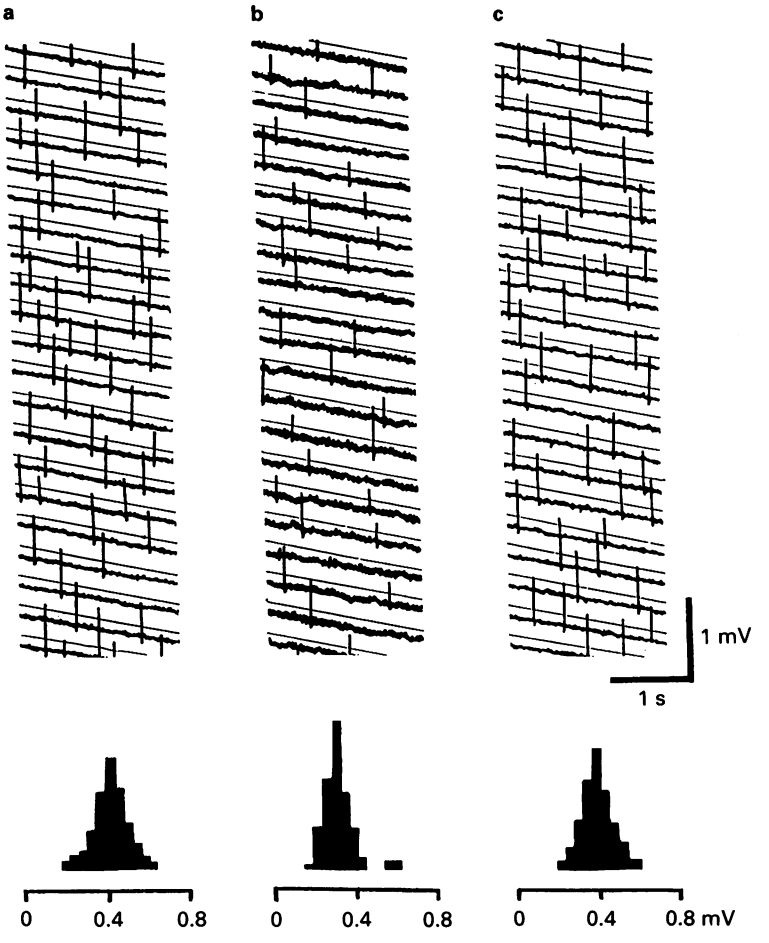


Figure 1 The effect of carbachol (CCh) on the amplitude and frequency of miniature endplate potentials (m.e.p.ps): (a) control; (b) 20 min after bath application of 5×10^{-6} M CCh; (c) 30 min after washing out of the drug. Upper section: E.C. oscilloscopic records of m.e.p.ps on continuously moving film. Upper traces in each record indicate the threshold level for computer counting of m.e.p.ps. Lower section: amplitude histograms constructed from 100 m.e.p.ps.

Table 1 Effects of several cholinomimetics and cholinolytics on the frequency of miniature endplate potentials (m.e.p.ps)

	Concentration (M)	*RMP* (%)	*Amplitude of m.e.p.ps* (%)	*m.e.p.p. frequency* (%)	n
Acetylcholine	7.5×10^{-5}	81.8 ± 3.8*	64.3 ± 5.1*	55.8 ± 5.6*	6
Carbachol (CCh)	5×10^{-6}	91.2 ± 2.5*	78.6 ± 8.7*	52.4 ± 7.6*	16
Nicotine	5×10^{-6}	89.8 ± 2.4*	49.9 ± 4.5*	79.7 ± 7.3*	5
Tetramethylammonium	5×10^{-5}	90.7 ± 2.4*	56.0 ± 9.7*	75.5 ± 3.2*	6
Suberyldicholine	1×10^{-6}	78.6 ± 3.1*	48.2 ± 9.9*	78.3 ± 7.0*	5
Metacholine	5×10^{-5}	99.3 ± 4.3	95.6 ± 4.6	94.3 ± 4.5	5
Oxotremorine	5×10^{-5}	100.1 ± 1.8	97.0 ± 5.4	93.5 ± 6.9	5
Methylfurmethide	5×10^{-5}	100.3 ± 3.1	98.6 ± 3.9	95.4 ± 6.5	5
CCh + atropine 1×10^{-6}	5×10^{-6}	91.0 ± 1.8*	75.6 ± 4.6*	54.6 ± 6.8*	6
CCh + benzhexonium 1×10^{-5}	5×10^{-6}	94.8 ± 2.3*	82.6 ± 4.5*	58.7 ± 5.0*	5
CCh + IEM-1119 4×10^{-5}	5×10^{-6}	92.1 ± 3.0*	81.8 ± 5.0*	54.8 ± 6.9*	5

Resting membrane potential (RMP), amplitude of m.e.p.ps and m.e.p.p. frequency are expressed as percentage of control values (100%) measured at the beginning of each experiment. n = number of experiments.
Asterisks indicate the values which are significantly different from controls ($P < 0.05$).
All values in the Table and in the text are the mean ± s.e.mean, significance tested by t test.

not given). Ganglionic blockers (Brown, 1980) benzhexonium, hexamethonium and IEM-1119 were also without effect on the depression of m.e.p.p. frequency evoked by CCh (Table 1). Thus, the presynaptic structures involved in the CCh-evoked depression of m.e.p.p. frequency differ pharmacologically from nicotinic and muscarinic endplate ACh-receptors and as well as from those of the ganglionic postjunctional membrane.

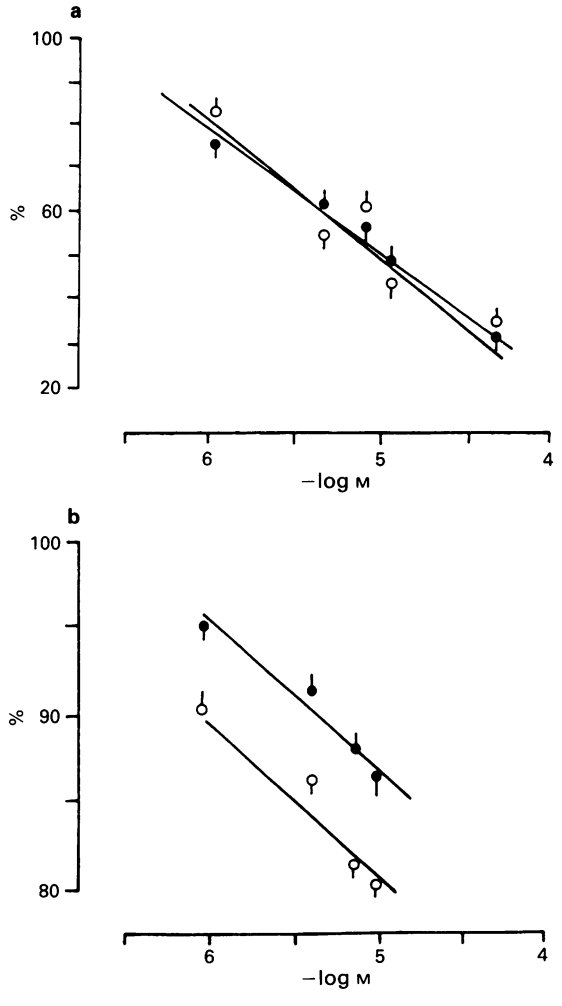


Figure 2 Effects of different concentrations of carbachol (−log M) on the frequency of miniature endplate potentials (a) and on the resting membrane potential (b) expressed as a percentage of the average values obtained 30 min before carbachol application. The average value of frequency was 1.32 ± 0.41 imp s^{-1} and resting membrane potential was 85.3 ± 1.2 mV in control experiments: (○)without (+)-tubocurarine; (●) in presence of (+)-tubocurarine, 4.5×10^{-7} M. Each point represents the mean value (s.e.mean shown by vertical lines) obtained from 5 experiments. Temperature 21°C.

Presynaptic action of cholinomimetics on anticholinesterase-treated preparations

It has been suggested that the presynaptically localized cholinesterase might be the target for the CCh and ACh effects on m.e.p.p. frequency in the frog (Duncan & Publicover, 1979). Therefore the effects of a reversible anticholinesterase, proserin, and an irreversible drug, armin, on CCh effects were examined. Armin, 5×10^{-6} M, and proserin, 1×10^{-5} M, inhibit the specific as well as the non-specific cholinesterase (Aluf, 1955; Prozorovskij & Savatjeev, 1976); both drugs increased the m.e.p.p. amplitude by about 80%, as expected. However, pretreatment of muscles with 5×10^{-6} armin or 1×10^{-6} M proserin had no measurable effect on m.e.p.p. frequency. Carbachol, 5×10^{-6} M, when added 30 min after pretreatment of muscles with armin or 45 min after application of proserine, decreased m.e.p.p. frequency by 46.3 ± 5.3% ($n = 4$, $P < 0.05$) and by 50.3 ± 6.7% ($n = 7$, $P < 0.05$). The decreases in m.e.p.p. frequency were similar to the values obtained without cholinesterase inhibition. Thus, the absence of an effect of inhibition of cholinesterase on the CCh-induced depression of m.e.p.p. frequency suggests that cholinesterase does not directly regulate the cholinolytic-induced modulation of spontaneous quantal release at the frog endplate.

Presynaptic action of carbachol during variation in the ionic composition of the medium

Changes in ion permeabilities, ion gradients and corresponding shifts in membrane potential of the nerve terminal might be involved in the cholinergic modulation of m.e.p.p. frequency. To check this possibility, we measured the effect of ionic composition changes, namely in Na^+, Cl^-, Ca^{2+} and K^+ on m.e.p.p. frequency influenced by CCh.

Na^+ and Cl^- variations First, sucrose was substituted for 2/3 of NaCl in the medium (Table 2A). This led to an increase of initial m.e.p.p. frequency (cf. Duncan & Publicover, 1979). The new level was stable for at least 90 min; the depressive effect of CCh on m.e.p.p. frequency was, however, the same as in a normal Ringer solution.

Similar results were also obtained in muscles bathed in a solution where NaCl (Statham & Duncan, 1977) was exchanged for NaBr and $NaNO_3$ in a molar ratio 1:1 or Na_2SO_4 in a molar ratio 3:2 (Table 2B). The substituted anions differ in their membrane permeabilities but they are less permeable than chlorides in all cases (Saint *et al.*, 1987). In these solutions, the m.e.p.p. frequency was almost identical

Table 2 Effects of decreases in Cl^- and in NaCl concentrations on presynaptic action of 5×10^{-6} M carbachol (CCh)

	Original ion or solution	*Exchanged for*	*% of NaCl exchange*	*Concentration of exchanged ion* (mM)	*m.e.p.p. frequency without CCh* (control)	*m.e.p.p. frequency with CCh*	*Decrease of m.e.p.p. frequency* (% of control)	*Number of experiments* n
A	Control solution	—	0	118.6	2.75 ± 0.74	1.28 ± 0.42	48.8 ± 6.5	6
	Na^+	Sucrose	66.7	43.2	4.62 ± 1.22	2.10 ± 0.52	49.1 ± 7.4	6
B	Control solution	—	0	119.1	0.97 ± 0.23	0.41 ± 0.10	51.1 ± 6.6	5
	Cl^-	$NaNO_3$	100	6.1	0.50 ± 0.13	0.50 ± 0.13	46.2 ± 7.6	5
	Cl^-	Na_2SO_4	100	6.1	0.39 ± 0.09	0.39 ± 0.09	58.7 ± 7.2	6
	Cl^-	NaBr	100	6.1	0.43 ± 0.11	0.43 ± 0.11	51.2 ± 6.4	5

Miniature endplate potential (m.e.p.p.) frequency expressed as number of events per s. Chlorides of KCl and $CaCl_2$ in Ringer solution were not exchanged. A, B – experiments with lowered Na^+ or Cl^- respectively. Mean ± s.e.mean are given. Temperature 20°C.

and no statistically significant difference was observed as far as the action of CCh on m.e.p.p. frequency was concerned. These experiments indicate that the CCh-induced drop in frequency is not directly connected with ionic channels for Na^+ and Cl^- in the nerve terminal membrane.

Effect of Ca^{2+} and K^+-depolarization Duncan & Publicover (1979) proposed that Ca^{2+} permeability of the nerve terminal membrane is decreased during the action of cholinomimetics. We therefore followed the CCh effect on m.e.p.p. frequency during changes in extra- and intracellular Ca^{2+} concentration and also in the presence of agents which are known to modify Ca^{2+} action within the terminal.

First of all, the extracellular Ca^{2+} ($[Ca^{2+}]_o$) was removed from the Ringer solution. This decreased m.e.p.p. frequency from 1.4 ± 0.4 ($n = 4$) in 1.8 mM $[Ca^{2+}]_o$ to 0.5 ± 0.2 imp s^{-1} ($n = 7$, $P < 0.05$) after 30 min in a nominally Ca^{2+}-free medium. Subsequent addition of 3.5×10^{-6} M CCh further decreased the m.e.p.p. frequency. This decrease was, however, smaller in nominally Ca^{2+}-free solution (by 29.3 ± 3.5%, $n = 7$, $P < 0.05$) than in the controls with 1.8 mM $[Ca^{2+}]_o$ (by 46.0 ± 3.5). An increase of $[Ca^{2+}]_o$ above 1.8 mM (Figure 3) increased m.e.p.p. frequency which may well be explained by a larger influx of Ca^{2+} into the terminals. In parallel, a greater presynaptic effect of CCh was observed (Figure 3, open symbols), namely when $[Ca^{2+}]_o$ exceeded 6 mM. At a concentration of 7.2 mM $[Ca^{2+}]_o$, for example, CCh depressed the m.e.p.p. frequency by 62.4 ± 3.4% ($n = 6$, $P < 0.05$), i.e. by about 16% more than in the controls with 1.8 mM $[Ca^{2+}]_o$.

Further experiments were done during potassium depolarization which is known to increase the Ca^{2+} entry into the terminals due to activation of the voltage-dependent Ca^{2+}-channels (Liley, 1956; Gardos, 1958; Elmquist & Feldman, 1965) and to stimulate the spontaneous quantal release of the transmitter (for review, see Cohen & Van der Kloot, 1985).

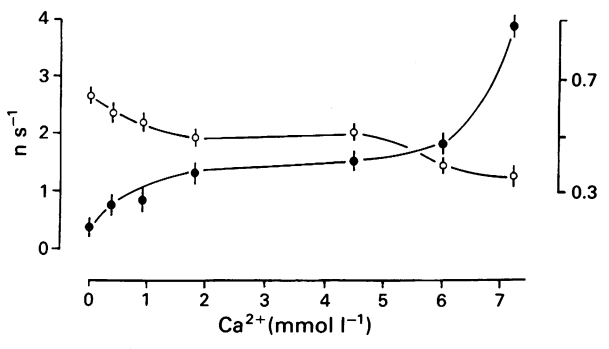


Figure 3 Dependence of miniature endplate potential (m.e.p.p.) frequency (●) and ratio of frequencies before and after 5×10^{-6} M carbachol (right ordinate scale, ○) on calcium concentration in the bath. Each point is mean from 7 experiments; s.e.mean shown by vertical lines.

An increase of extracellular K^+ above 0.5 mM produced a concentration-dependent increase in m.e.p.p. frequency (Figure 4) but there was no change in the percentage decrease of the frequency with CCh at any K^+ concentration (Figure 4, open symbols). Moreover, if the m.e.p.p. frequency was already decreased by CCh in normal potassium, the percentage increase of frequency produced by elevation of external K^+ was similar to that in the absence of CCh (data not given). Interestingly, the percentage reduction of m.e.p.p. frequency by CCh was independent of the original frequency in individual muscle fibres (Table 3). The effect of CCh or ACh on m.e.p.p. frequency was therefore not connected directly with the K^+-induced depolarization and seemed not to depend on the intraterminal calcium level.

This conclusion is supported by experiments with magnesium and manganese which inhibit Ca^{2+} entry (for review see Silinsky, 1985). The addition of 6 mM Mg^{2+} or 3 mM Mn^{2+} to the muscle led to a small increase (by about 8%) in m.e.p.p. frequency. The decrease produced by subsequent application of 5×10^{-6} M CCh was not significantly different from the control (61.1 ± 7.8, $n = 6$ for Mg^{2+}, 62.1 ± 3.2, $n = 7$ for Mn^{2+}).

Effects of db cyclic AMP, db cyclic GMP and theophylline

Regulatory mechanisms of transmitter release might be connected with shifts in cyclic nucleotides (Goldberg & Singer,

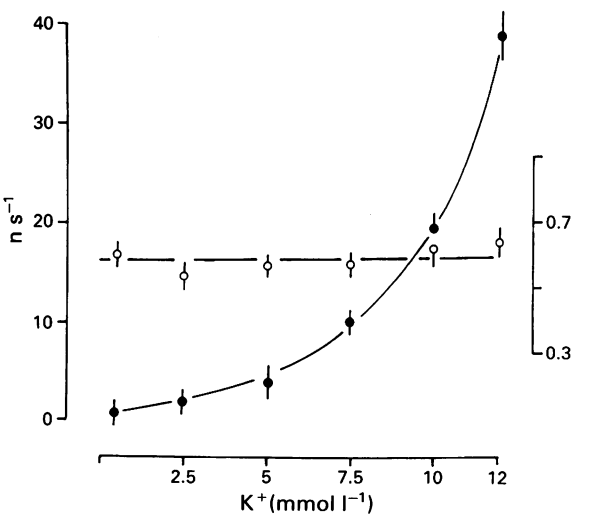


Figure 4 Dependence of miniature endplate potential (m.e.p.p.) frequency (the number, n, of m.e.p.ps per 1 s, ●) on the concentration of potassium ions (K^+) in the bath; (○) the ratio of the frequency of m.e.p.ps after 20 min of 5×10^{-6} M carbachol (right ordinate scale) to that before carbachol. Average values from 5–7 experiments.

Table 3 Effects of changes in miniature endplate potential (m.e.p.p.) frequency on the effect of 5×10^{-6} M carbachol (CCh) on m.e.p.p. frequency

Range of m.e.p.p. frequency without CCh (control)	*% of m.e.p.p. frequency in CCh*	*Number of experiments* (n)
0.3–0.5	54.6 ± 2.6	11
0.6–0.8	56.2 ± 4.5	11
0.8–1.1	57.9 ± 5.5	9
1.1–1.4	49.4 ± 3.8	11
1.4–2.0	52.5 ± 6.3	9
2.0–2.5	49.6 ± 4.8	7
2.5–3.0	47.9 ± 6.8	5
3.0–4.5	53.9 ± 7.9	8

m.e.p.p. frequency = number of events per s. Mean ± s.e.mean. Temperature 20°C.

1969; for review, see Greengard, 1979). Agents known to decrease the hydrolysis of cyclic AMP by phosphodiesterase, e.g. theophylline, increase the m.e.p.p. frequency and the quantum content of evoked ACh released during stimulation (Goldberg & Singer, 1969). Catecholamines which stimulate the production of cyclic AMP (Sutherland & Robison, 1966) also increase the m.e.p.p. frequency (Kuba, 1970) as well as the permeable analogue of cyclic AMP, db cyclic AMP (Goldberg & Singer, 1969).

In our experiments the application of theophylline or noradrenaline, db cyclic AMP or db cyclic GMP did not influence significantly the effect of CCh on m.e.p.p. frequency (Table 4).

Presynaptic action of carbachol during inhibition and activation of Na^+-K^+-ATPase

The Mg^{2+}-dependent, Na^+,K^+-activated ATPase (E.C. 3613) has been shown to participate in the regulation of transmitter release (Baker & Crawford, 1975; Vizi, 1978; 1979; Vyskočil, 1979; Vizi & Vyskočil, 1979) and can be influenced by cholinomimetic and cholinolytic drugs (Kometiani *et al.*, 1975; Dlouhá *et al.*, 1979). Moreover, there is considerable homology between the nicotinic ACh receptor and a catalytic subunit of Na^+,K^+-ATPase (Elman *et al.*, 1982).

Ouabain (5×10^{-5} M) blocked the inhibitory action of CCh on m.e.p.p. frequency (Figure 5); it even reversed the depression when added to the bath after CCh. The presence of ouabain in the bath was restricted to only 15–20 min to avoid the increase in m.e.p.p. frequency usually found after 30 min, when ionic gradients are already distorted and RMP of terminals decreased (Baker & Crawford, 1975). The effect of ouabain on the dose-response curves for CCh showed no signs

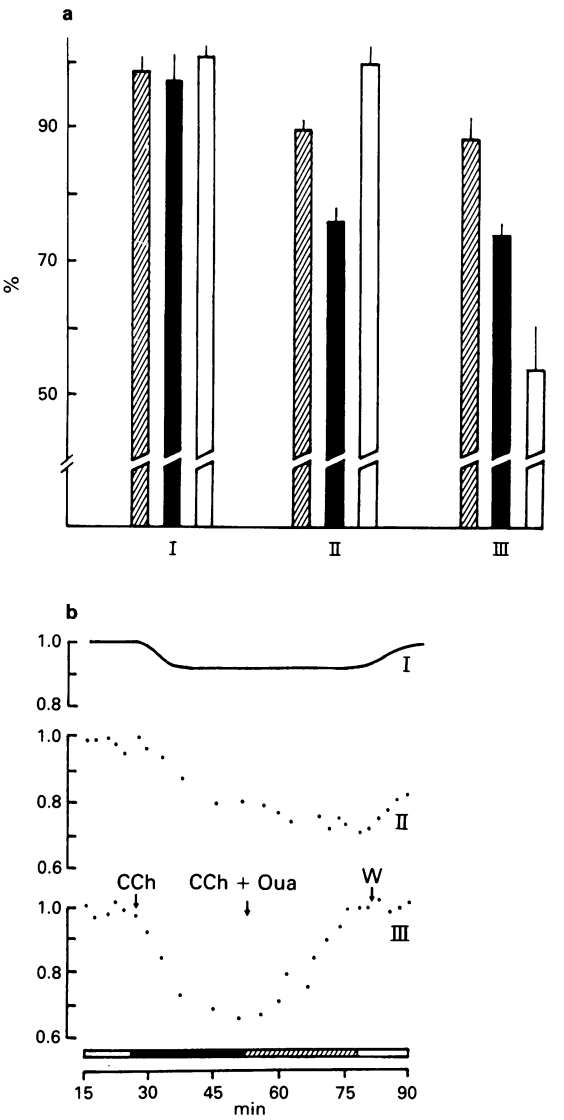


Figure 5 Pre- and postsynaptic effects of 5×10^{-6} M carbachol (CCh) in the absence and presence of 5×10^{-5} M ouabain (Oua). (a) Percentage changes of membrane potential (hatched columns), miniature endplate potential (m.e.p.p.) amplitude (closed columns) and frequency (open columns) in muscles treated for 20 min with (I) ouabain, (II) ouabain plus carbachol and (III) carbachol only. Seven fibres were measured in each group between 15 and 20 min of the treatment. (b) Single fibre membrane potential (I), m.e.p.p. amplitude (II) and frequency (III) before CCh, after CCh, CCh + Oua and washing (W) respectively. Ordinates: ratio of experimental and control values.

Table 4 The effects of theophylline, dibutyryl cyclic AMP (db cyclic AMP) and dibutyryl cyclic GMP (db cyclic GMP) on the frequency of miniature endplate potentials (m.e.p.ps) in the absence and presence of carbachol (CCh)

	A	B	C	D
Drug	*Concentration* (M)	*Increase without CCh* (%)	*Drug + CCh* (%)	n
Theophylline	1×10^{-3}	198.3 ± 4.6	58.8 ± 3.4	8
Noradrenaline	1×10^{-5}	189.2 ± 3.6	61.3 ± 2.9	7
db cyclic AMP	1×10^{-6}	217.1 ± 6.1	63.3 ± 3.7	10
db cyclic GMP	1×10^{-6}	262.4 ± 5.3	57.5 ± 5.0	10

Muscles (*n*) were bathed first without drugs for 20 min; then the fibre was impaled with a microelectrode and m.e.p.p. frequency was measured. Then drug was added (column A) and a new series of m.e.p.ps was collected during 20–30th min of drug action (column B). Carbachol was then added and during the next 20 min the last series of m.e.p.ps was measured.
Values in column B were taken as 100% for column C.
Temperature 20°C.

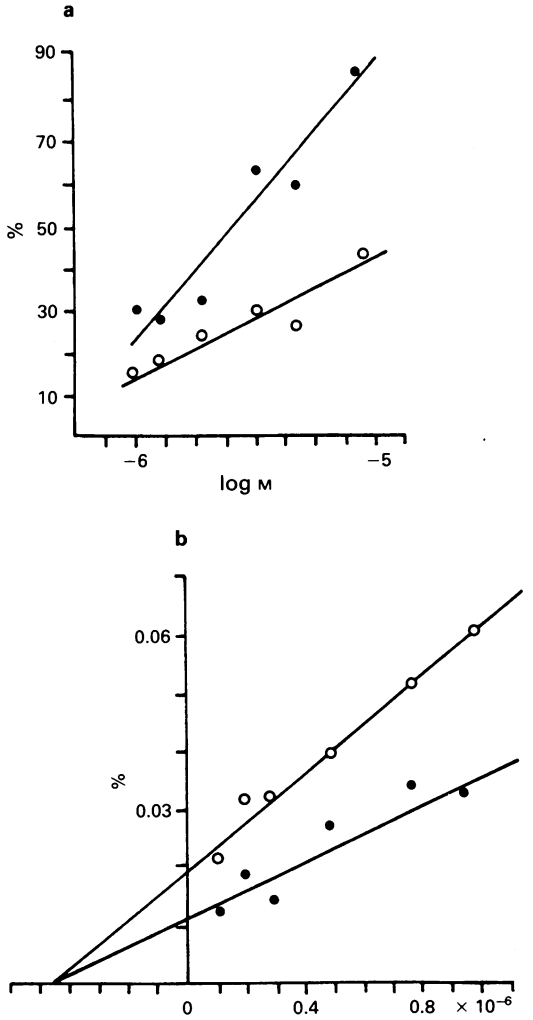


Figure 6 Logarithmic (a) and double-reciprocal (b) plots of the depressive effect of carbachol at several concentrations on the frequency of miniature endplate potentials (m.e.p.ps) without (●) and with 3.5×10^{-5} M (○) ouabain (20 min) in the perfusion bath. (a) Ordinate scale percentage inhibition

$$\left[\left(1 - \frac{\text{m.e.p.p. frequency in carbachol}}{\text{m.e.p.p. frequency in control}}\right) \times 100\right]$$

Abscissae: log of carbachol concentration in mol l^{-1} (M). (b) Ordinate scale: reciprocal values of m.e.p.p. frequency depression by carbachol; abscissae: reciprocal values of carbachol concentration. Each point represents a mean from 3 independent experiments.

of competitive interaction between ouabain and CCh which suggests, that the drugs interact with different sites on the enzyme molecule (Figure 6).

Na^+,K^+-ATPase can also be inhibited by removing potassium from the bath solution (Kernan, 1962; Vyskočil & Illés, 1977; Vizi & Vyskočil, 1979; Marunaka, 1986). Application of 5×10^{-6} M CCh to muscles bathed for 30 min in K^+-free solution, decreased the m.e.p.p. frequency by 41.6 ± 5.2% ($n = 6$, $P < 0.05$). To reduce the likelihood of K^+ release from the muscle fibres, which could activate the Na^+,K^+-ATPase, several experiments were done on fibres voltage-clamped at a holding potential of −110 mV to prevent, or at least drastically reduce K^+ efflux. The results were similar. After addition of 5×10^{-6} M CCh, the clamp current increased as expected, but the frequency of miniature endplate currents was decreased by 36.5 ± 0.5% ($n = 5$, $P < 0.05$). Thus, under conditions where the Na^+,K^+-ATPase was blocked in a K^+-free solution, no significant inhibition of the CCh-induced effect on m.e.p.p. frequency was observed, in contrast to ouabain. Apparently, the blocking effect of ouabain on CCh-induced frequency inhibition is not connected with Na^+,K^+-ATPase function.

Adrenaline and insulin activate Na^+,K^+-ATPase and the electrogenic Na^+,K^+ pump (cf. Clausen, 1986). The m.e.p.p. frequency was increased slightly 30 min after adding 1×10^{-5} M adrenaline to 111.3 ± 2.3% ($n = 6$, $P > 0.05$) of the control and muscle fibre RMP was hyperpolarized by 3.3 ± 1.1 mV ($n = 6$, $P < 0.05$). CCh application reduced the m.e.p.p. frequency by 34.3 ± 3.5% ($n = 5$, $P < 0.05$). Insulin (0.5 iu ml^{-1}) also hyperpolarized the resting membrane by 3.3 ± 1.1 mV ($n = 12$, $P < 0.05$), and reduced m.e.p.p. frequency by 18.9 ± 5.0% to 81% ($n = 12$, $P < 0.05$). A higher dose (2 iu ml^{-1}) hyperpolarized the resting membrane by 6.3 ± 2.8 mV ($n = 3$, $P < 0.05$) and decreased the m.e.p.p. frequency by 20.5 ± 5.2% ($n = 3$, $P < 0.05$). Three iu ml^{-1} of insulin were without further effect. In the presence of insulin, the percentage depression of frequency by 5×10^{-6} M CCh was similar to that in the controls (to 43.1 ± 22%).

Temperature and presynaptic action of carbachol

If an enzyme or receptor were the target for the CCh presynaptic action one would expect a significant effect of temperature on the response. A pronounced temperature-dependence is characteristic for many enzymatic and ion channel functions as well as for processes of transmitter release. Temperature coefficients (Q_{10}) range between 1.5–3.0 (e.g. Frankenheuser & Moore, 1963, for Na^+ and K^+ permeability; Anderson & Stevens, 1973, for mean open time of ACh-operated channel) and 10–12 (e.g. Thesleff *et al.*, 1983, for

Table 5 The temperature (t) dependence of different concentrations of carbachol (CCh) action on frequency of miniature endplate potentials (f.m.e.p.ps)

	Carbachol (M)	t (°C)	*Control f.m.e.p.ps*	*f.m.e.p.ps with CCh*	*% of control*	*Number of experiments* (n)
A	1×10^{-5}	10	0.35 ± 0.11	0.18 ± 0.04	51.4 ± 7.8	5
		20	2.25 ± 0.86	0.97 ± 0.25	43.1 ± 5.8	6
		25	11.20 ± 2.76	5.10 ± 2.16	45.5 ± 9.8	5
		30	19.17 ± 4.33	8.71 ± 1.70	45.4 ± 6.6	6
B	5×10^{-6}	5	0.36 ± 0.11	0.18 ± 0.03	50.0 ± 5.4	6
		10	0.14 ± 0.024	0.08 ± 0.01	57.1 ± 7.8	8
		15	0.39 ± 0.10	0.20 ± 0.05	51.3 ± 8.0	6
		20	2.63 ± 0.71	1.31 ± 0.40	49.8 ± 6.5	6
		25	4.72 ± 0.95	2.04 ± 0.29	43.2 ± 6.9	6
		30	23.50 ± 5.53	11.54 ± 4.02	49.1 ± 4.0	6
C	6×10^{-7}	8.5	0.12 ± 0.01	0.072 ± 0.01	60.0 ± 4.7	6
		30	21.26 ± 4.24	17.71 ± 2.11	83.3 ± 3.2	6
D	3×10^{-7}	6	0.103 ± 0.010	0.073 ± 0.010	56.2 ± 2.1	6
		26	9.72 ± 2.16	9.45 ± 1.91	97.2 ± 4.3	6

f.m.e.p.ps = number of events per s. Mean ± s.e.mean.

calcium insensitive and slow m.e.p.p.; Hartzel *et al.*, 1977, for muscarinic ACh receptors).

Therefore, the effects of CCh were examined at temperatures between 5° and 30°C (Table 5). The basal m.e.p.p. frequency increased from about $0.14\,s^{-1}$ at 10°C to about $23\,s^{-1}$ at 30°C as shown by Fatt & Katz (1952). Surprisingly, the inhibition of m.e.p.p. frequency by 5×10^{-6} and 1×10^{-5} M CCh was the same throughout the temperature-range with a Q_{10} close to 1.0. These concentrations of CCh were maximal in that a further increase of the CCh in the bath did not produce a greater depression of m.e.p.p. frequency. On the other hand, with lower (threshold) concentrations (6×10^{-7} and 3×10^{-7} M) of CCh, the depression of frequency was much less at 26° and 30°C (97% and 84% respectively) than at 6° and 8.5°C (71 and 62% respectively, Table 5A,B).

Discussion

Several possibilities should be taken into account when discussing the observed effect of acetylcholine analogues on the frequency of m.e.p.p. in the frog neuromuscular junction.

Receptor hypothesis

The depression of m.e.p.p. frequency has already been studied by Duncan & Publicover (1979). This depression is not caused by a loss of small-amplitude m.e.p.ps in the presence of the agonists because the histograms of m.e.p.p. amplitude retained a normal Gaussian character (Figure 1). The fact that current and voltage clamp experiments were similar suggests that this phenomenon was not caused by ionic or metabolic influences from depolarized muscle fibres (Magazanik & Nikolsky, 1979; Hohlfeld *et al.*, 1981) and point to a direct action on the nerve terminal. It was not mediated by muscarinic autoreceptors which ensure negative feed-back in other nerve tissue, including poikilothermic vertebrates (Michaelson *et al.*, 1979; Kilbinger & Kruel, 1981), since different specific muscarinomimetics such as methylfurmethide, oxotremorine and metacholine did not affect m.e.p.p. frequency. Moreover, atropine, which blocks muscarinic ACh receptors, did not prevent the depression of m.e.p.p. frequency evoked by cholinomimetics. Our data are thus not in accordance with the results of Duncan & Publicover (1979) who reported that muscarinic antagonists have a depressant effect on m.e.p.p. frequency. The discrepancy might be ascribed to differences in the experimental condition. In the above mentioned paper, the preparation was first cooled to 10°C and then warmed to 22.5°C. During this temperature change, a decrease of m.e.p.p. frequency was reported in the controls as well as in the presence of muscarine, atropine and anticholinesterase. Lowering of temperature to or below 10°C may well switch off a temperature-dependent system, e.g. the Na^+,K^+-ATPase, which is then activated after warming the bath (Vizi, 1978; Vizi & Vyskočil, 1979). Our finding that other nicotinomimetics depress m.e.p.p. frequency in a similar way to ACh and CCh supports the 'autoreceptor' hypothesis. Furthermore, the reduction of the quantal content of the evoked e.p.p. after application of cholinomimetics in the frog (Ciani & Edwards, 1963; Nikolsky & Giniatullin, 1979) supports the possibility that quantal release is modulated by receptors. However, in contrast to evoked e.p.ps, which curare blocks (Nikolsky & Giniatullin, 1979), the depression of spontaneous m.e.p.p. frequency was not affected by this drug (Figure 2). Other pharmacological tests (Table 1) of the depression in frequency of m.e.p.p. showed that the responsible structures were different from the pharmacologically well-characterized ACh receptors of the muscle fibre and autonomic ganglia.

Ca^{2+} hypothesis

Because of the dependence of transmitter release on membrane potential, it is possible that hyperpolarization mediates the cholinomimetic action. However, the hyperpolarization induced by potassium removal and potassium depolarization did not change the effect of cholinomimetics and a simple connection between nerve polarization and m.e.p.p. frequency depression probably does not exist.

Quantal release depends on the external level of Ca^{2+} ions (Elmquist & Feldman, 1965). It is also possible that the Ca^{2+} regulating systems in the nerve terminal are connected with m.e.p.p. frequency depression. If cholinomimetics simply blocked Ca^{2+} entry, their effect should be dependent on external Ca^{2+}. This was true, however, only at very low or relatively high (6–7 mM) concentrations. The CCh inhibition of m.e.p.p. frequency was well developed in a Ca^{2+}-free solution. Furthermore, the fact that the calcium antagonists, Mg^{2+} and Mn^{2+}, had no effect on the CCh-induced depression shows that Ca^{2+} entry was not a limiting step. This was most clearly supported by the experiments with potassium depolarization where the depression of m.e.p.p. frequency was always the same.

The entry of Ca^{2+} from the outside is not the only mechanism by which the intracellular Ca^{2+} concentration might be altered. Changing the intracellular Ca-buffering processes e.g. via the cyclic nucleotide system would also alter the free intracellular Ca^{2+}. However, we did not find any alternation in the CCh effect even when m.e.p.p. frequency was increased by db cyclic AMP or db cyclic GMP or when cyclic AMP splitting was inhibited by theophylline. Thus, the effect of CCh is similar to the Ca^{2+}-independent stimulation of m.e.p.p. frequency in frog muscle by trinitrobenzene sulphonic acid (Kijima & Tanabe, 1988) which acts by an as yet unknown mechanism even in a Ca^{2+}-free medium, when Ca^{2+} channels were blocked by ω-conotoxin and internal calcium was buffered with 1,2-*bis*(2-aminophenoxy)ethane tetraacetic acid (BAPTA). It also resembles the effect of ethanol and dimethyl sulphoxide described by Quastel *et al.* (1971) and McLarnon *et al.* (1986).

Na^+-K^+-ATPase

Because ouabain, a well-known inhibitor of membrane Na^+,K^+-ATPase, decreased the effect of cholinomimetics on m.e.p.p. frequency, this enzyme was thought to be a candidate for this cholinergic modulation. However, the absence of an effect of K^+-free solution on the CCh-induced depression, together with the ambiguous effects of adrenaline and insulin suggested that this enzyme is not directly involved in the CCh presynaptic effect. The protective effect of ouabain might be connected with its rather nonspecific mode of action. It can, for example, increase Na permeability (Robbins, 1977) or modulate the vesicle recycling (Haimann *et al.*, 1985) and non-quantal ACh release (Zemková *et al.*, 1990).

Acetylcholinesterase

Our observation that anticholinesterase drugs had little or no effect on the depression of m.e.p.p. frequency by carbachol indicates that cholinesterases do not mediate the carbachol effect, in contrast to the conclusions of Duncan & Publicover (1979).

Temperature

The absence of temperature-dependence at saturating concentrations of CCh was surprising. Either two parallel processes exist, both temperature-dependent, acting under CCh in an opposite direction on m.e.p.p. frequency, or the limiting step in presynaptic CCh action is purely of a physical nature. According to the classical physical concept of adsorption (Bikerman, 1948; Cassidy, 1951), not only would the temperature-dependence in the case of saturating concentrations be negligible, but the temperature-dependence, when threshold concentrations are used, would be opposite. Free presynaptic hypothetical sites for CCh might be occupied to a greater extent at lower temperatures. Experiments with

6×10^{-7} and 3×10^{-7} M CCh are in agreement with this, showing that the CCh effect is much smaller at higher temperatures than at lower ones. Because the effective doses of ACh as well as of CCh are in the micromolar range, it is probably not the whole surface of the nerve terminal membrane but only some specific parts (e.g. active zones of ACh release; Heuser *et al.*, 1974) which are affected by CCh. Despite the fact that the percentage drop in m.e.p.p. frequency was not temperature-dependent, the presence of CCh or ACh did not prevent the increase or decrease of m.e.p.p. frequency as such e.g. by external K^+ changes, application of cyclic nucleotides or by temperature itself.

It should also be mentioned that the same degree of inhibition by CCh was observed in the case of stimulation-evoked ACh release, where a drop of endplate potentials to about one half was found with 5×10^{-6} M CCh (Nikolsky & Giniatullin, 1979) irrespective of quantum content size.

Whatever the most probable explanation, the temperature independence of this process in the frog might reflect the fact that for amphibians living in a broad range of temperatures, the cholinergic depression is of physiological significance. For example, one can speculate that the post-tetanic increase in m.e.p.p. frequency (Magleby & Zengel, 1976) is compensated for, to some extent, by the feedback action of the released acetylcholine.

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Endothelium-independent relaxation of rabbit coronary artery by 17β-oestradiol *in vitro*

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1 We assessed the relaxant effect of 17β-oestradiol (10^{-7}, 10^{-6} and 10^{-5} M) on rabbit isolated coronary arteries precontracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M), high extracellular potassium (30 mM) and Bay K 8644 (10^{-6} M) plus high extracellular potassium (15 mM) by measuring isometric tension. 17β-Oestradiol (10^{-6} and 10^{-5} M) induced significant relaxation in coronary arteries from male and female rabbits. No differences were seen between arteries with or without endothelium. There were also no differences between coronary arteries isolated from male and female rabbits.

2 Inhibitors of endothelium-derived relaxing factor and vasodilator prostanoids, namely, reduced haemoglobin, N^{ω}-nitro-L-arginine methyl ester and indomethacin, did not affect the relaxation induced by 17β-oestradiol in endothelium-intact coronary arteries.

3 Methylene blue, an inhibitor of guanylate cyclase, did not affect the coronary artery relaxation induced by 17β-oestradiol.

4 The calcium concentration-dependent contraction curve in potassium-depolarization medium was shifted to the right by 17β-oestradiol (10^{-6} and 10^{-5} M) in the rabbit coronary artery and rat aorta. The $-\log EC_{50}$s of calcium in control and after incubation with 17β-oestradiol (10^{-6} and 10^{-5} M) were 3.7 ± 0.09, 3.1 ± 0.10 and 2.8 ± 0.08 respectively in rabbit coronary arteries and 3.8 ± 0.11, 3.3 ± 0.14 and 2.9 ± 0.15 in rat aorta.

5 The results indicate that 17β-oestradiol induces rabbit coronary artery relaxation by an endothelium-independent mechanism *in vitro*. A calcium antagonistic property may be involved in the mechanism of rabbit coronary arterial relaxation by 17β-oestradiol.

Keywords: 17β-Oestradiol; coronary relaxation; vascular endothelium; calcium antagonism; vasodilator prostanoid

Introduction

Oestrogen replacement therapy in postmenopausal women reduces the risk of cardiovascular and cerebrovascular disease (Godsland *et al.*, 1987). Recently, large-scale epidemiological studies indicate that postmenopausal women who receive oestrogens appear to have one-third to one-half the cardiovascular mortality rate and one-half the cerebrovascular mortality rate when compared to untreated women (Colditz *et al.*, 1987; Bush *et al.*, 1987). These findings are supported by the evidence that oestrogen affects cholesterol metabolism and deposition, inhibiting atherosclerotic plaque formation in the liver and arterial walls (Sarrel, 1990), and by angiographic studies that show a protective effect of postmenopausal oestrogens on coronary occlusion (Gruchow *et al.*, 1988; Sullivan *et al.*, 1988). However, analyses of lipid changes in postmenopausal women taking oestrogens show they do not fully account for the cardiovascular protective effects (Adams *et al.*, 1990). It is important to study other mechanisms by which oestrogens may provide cardiovascular benefits to women. Another important mechanism may be the modulation of coronary vasomotion.

17β-Oestradiol increases cardiac output and arterial flow velocity, but decreases vascular resistance, systolic and diastolic blood pressure (Magness & Rosenfeld, 1989). 17β-Oestradiol causes coronary artery vasodilatation in isolated perfused hearts of rabbits (Raddino *et al.*, 1986) and increases myocardial perfusion in ewes (Magness & Rosenfeld, 1989). Recent reports demonstrate that 17β-oestradiol treatment modulates responses to acetylcholine in coronary arteries in cynomolgus monkeys (Williams *et al.*, 1990) and femoral arteries in rabbits (Miller *et al.*, 1988), suggesting an endothelium-dependent mechanism *in vivo* in the long term. It has also been reported that oestrogen decreases calcium entry into the vascular smooth muscle cell of pig uterine arteries (Stice *et al.*, 1987a,b). However, the effect of 17β-oestradiol on the coronary artery and its mechanism of action is not well understood.

The purpose of this study was to investigate the possible relaxant effect of 17β-oestradiol on the coronary artery *in vitro* and the role of endothelial modulation and calcium antagonism in this relaxation by use of rabbit coronary arterial smooth muscle preparations.

Methods

Animals and tissues

Adult male or non-pregnant female New Zealand white rabbits (2.5–3 kg) were killed by an overdose of pentobarbitone (60 mg kg^{-1}) and heparin (150 units kg^{-1}). The heart was removed and epicardial coronary arteries were dissected free of connective tissue. Male Wistar rats (5–6 weeks old) were killed by pentobarbitone (30 mg kg^{-1}) and the thoracic descending aortae were removed and dissected free of connective tissue. Arterial rings were prepared and in alternate rings, the endothelium was removed by gentle rubbing with a wooden probe. Each ring (2–3 mm length) was suspended horizontally between two stainless steel parallel hooks for the measurement of isometric tension in individual organ baths containing 7 ml modified Krebs solution at 37°C, bubbled with 95% O_2 and 5% CO_2. The composition of modified Krebs solution was as follows (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, K_2PO_4 1.2 and glucose 11.1.

Relaxant effect of 17β-oestradiol on precontracted coronary arteries

Coronary arterial rings with or without endothelium from male and female rabbits were allowed to stabilize for 90 min under a resting tension of 1 g before the rings were contracted

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with potassium (K^+, 30 mM), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 3×10^{-6} M) or Bay K 8644 (Bay K, 10^{-6} M) plus K^+ (15 mM). 17β-Oestradiol (10^{-7}, 10^{-6} and 10^{-5} M) or equivalent ethanol solvent (1:2000) was added 7 min after the addition of constrictor agents. In another group of coronary arteries from male or female rabbits, testosterone (dissolved in ethanol, 10^{-7}, 10^{-6}, 10^{-5}, 3×10^{-5} and 10^{-4} M) was added 7 min after the addition of constrictor agents.

Effect of reduced haemoglobin, N^{ω}-nitro-L-arginine methyl ester and indomethacin on relaxation induced by 17β-oestradiol

Reduced haemoglobin was prepared as previously described and is an inhibitor of endothelium-dependent vasodilatation mediated by endothelium-derived relaxing factor (EDRF) (Martin *et al.*, 1985). N^{ω}-nitro-L-arginine methyl ester (L-NAME) is an inhibitor of EDRF synthesis from L-arginine in vascular endothelial cells (Rees *et al.*, 1990). Reduced haemoglobin (10^{-5} M) or L-NAME (10^{-4} M) was added to precontracted coronary arterial rings with endothelium. Indomethacin was dissolved by sonication in a Na_2CO_3 solution. Indomethacin (10^{-5} M), an inhibitor of prostanoid synthesis, was incubated with endothelium-intact rings for 20 min before they were precontracted with the agonists. 17β-Oestradiol (10^{-6} and 10^{-5} M) was subsequently added 7 min after the addition of constrictor agents. In addition, effects of indomethacin (10^{-5} M) incubation on relaxation induced by arachidonic acid (3×10^{-5} M) were assessed in endothelium-intact tissues.

Effect of methylene blue on relaxation induced by 17β-oestradiol

To determine the possible involvement of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the relaxation induced by 17β-oestradiol, coronary artery rings without endothelium were incubated with methylene blue (10^{-5} M) for 20 min before being contracted with $PGF_{2\alpha}$ (3×10^{-6} M). 17β-Oestradiol (10^{-6} and 10^{-5} M) was subsequently added.

Calcium antagonistic effect of 17β-oestradiol on coronary artery and rat aorta

Rabbit coronary artery and rat aortic rings without endothelium were incubated in calcium-free solution containing 0.5 mM EGTA for 10 min. Calcium concentration-dependent contraction curves were then performed in K^+-depolarization medium (K^+ = 100 mM). The rings were readjusted in modified Krebs solution for 20 min before being incubated in calcium-free solution containing 0.5 mM EGTA for another 10 min. These rings were preincubated with 17β-oestradiol or solvent 30 mm before the calcium concentration-dependent contraction curves were obtained.

Drugs

The following drugs were used: 17β-oestradiol (dissolved in ethanol, Sigma); indomethacin (Sigma); L-NAME (Sigma); testosterone (Sigma); arachidonic acid (Sigma); Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)- pyridine-5-carboxylate, Bayer UK); prostaglandin $F_{2\alpha}$ (Sigma); pentobarbitone (Sigma); haemoglobin (Sigma); methylene blue (Sigma). All drugs were Analar grade.

Statistics

All results are expressed as mean ± s.e.mean. Relaxation was expressed as percentage relaxation of contraction induced by agonists and K^+. The results were analysed with Student's *t* test for paired and unpaired observations. Each group was compared to the time-matched ethanol solvent control. A probability level of less than 0.05 was considered significant; *n* indicates the number of animals.

Results

Relaxant effect of 17β-oestradiol on precontracted coronary arteries

K^+ (30 mM), $PGF_{2\alpha}$ (3×10^{-6} M) or Bay K (10^{-6} M plus 15 mM K^+) induced comparable contractile responses in rings with and without endothelium (0.8 ± 0.1, 0.7 ± 0.2 and 0.9 ± 0.1 g; $P > 0.05$). 17β-Oestradiol (10^{-7} M) had no effect on the contraction evoked by these agents. However, 10^{-6} M and 10^{-5} M 17β-oestradiol induced significant concentration-related relaxation of contracted rings with or without endothelium from male or female rabbits (compared to time-matched ethanol solvent controls, all $P < 0.01$). There were no differences between arteries from male and female rabbits (all $P > 0.05$, $n = 8$ in male group and $n = 6$ in female group). There were no differences between groups with and without endothelium in K^+ precontracted rings ($P > 0.05$, Figure 1). No differences were seen between endothelium-denuded rings precontracted with the 3 agents ($P > 0.05$, $n = 8$ in each group). In another group of rabbits (male = 5, female = 2) testosterone (10^{-7}, 10^{-6} and 10^{-5} M) did not induce significant relaxation (all $P > 0.05$ in comparison with time-matched ethanol controls) in either endothelium-intact or -denuded coronary arteries precontracted with three constrictor agents. Higher doses of testosterone (3×10^{-5} M and 10^{-4} M) caused a slight relaxation in endothelium-intact and -denuded rings precontracted with K^+ (30 mM) by 14 ± 1.8% and 16 ± 2.6%; 19 ± 2.1% and 18 ± 3.4% respectively (compared with time-matched ethanol controls, all $P < 0.05$). Similar results were obtained in endothelium-denuded rings precontracted with $PGF_{2\alpha}$ (3×10^{-6} M) or Bay K (10^{-6} M plus 15 mM K^+).

Effect of reduced haemoglobin, L-NAME and indomethacin on 17β-oestradiol-induced relaxation

Reduced haemoglobin (10^{-5} M) and L-NAME (10^{-4} M) did not inhibit the relaxation induced by 17β-oestradiol (10^{-6} and 10^{-5} M) in rings with endothelium ($P > 0.05$, Figure 2). Indomethacin (10^{-5} M) significantly reduced the transient relaxation induced by arachidonic acid (3×10^{-5} M) by 93 ± 2.1% ($n = 5$) in endothelium-intact coronary arteries precontracted

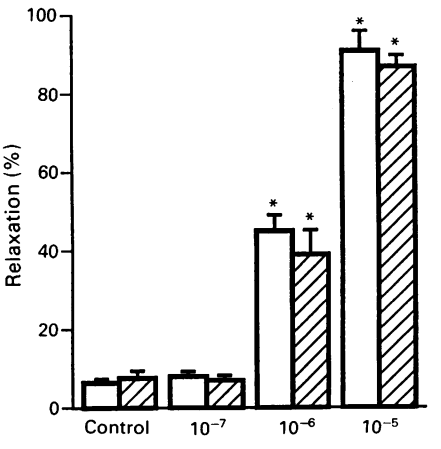


Figure 1 The relaxant effect of 17β-oestradiol (10^{-7}, 10^{-6} and 10^{-5} M; 10^{-7}, 10^{-6} and 10^{-5} respectively) on rabbit coronary arteries with and without endothelium (open and hatched columns respectively) precontracted by K^+ (30 mM). Data are expressed as percentage relaxation of contraction induced by K^+ (mean with s.e.mean shown by vertical bars, $n = 8$ in each group). Control = time-matched ethanol solvent controls.
Significant differences in comparison with corresponding controls * $P < 0.01$.

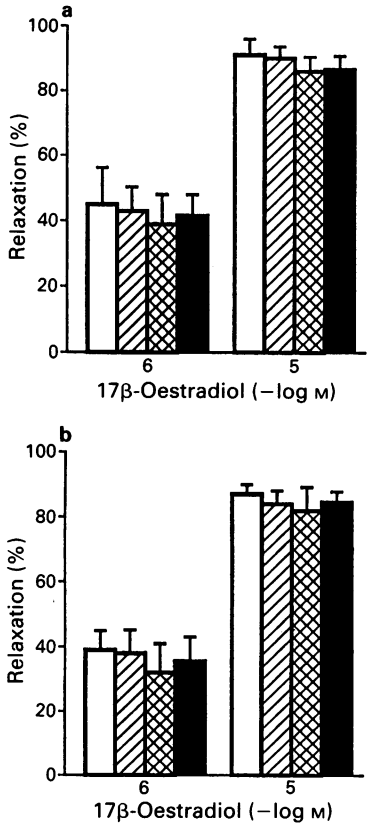


Figure 2 The effect of reduced haemoglobin (10^{-5} M, hatched columns), indomethacin (10^{-5} M, cross-hatched columns) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M, solid columns) on the relaxation induced by 17β-oestradiol (10^{-6} and 10^{-5} M, open columns) in rabbit coronary artery with endothelium, precontracted by K^+ (30 mM, a) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 3×10^{-6} M, b). Data are expressed as a percentage relaxation of contraction induced by K^+ or $PGF_{2\alpha}$ (mean with s.e.mean shown by vertical bars, $n = 8$ in each group).

with K^+ (30 mM). However, the same dose of indomethacin did not affect the relaxation induced by 17β-oestradiol (10^{-6} and 10^{-5} M) in rings with endothelium ($P < 0.05$, Figure 2).

Effect of methylene blue on 17β-oestradiol induced relaxation

Incubation with the guanylate cyclase inhibitor methylene blue (10^{-5} M), for 20 min before contraction with $PGF_{2\alpha}$ (3×10^{-6} M) had no effect on relaxation induced by 17β-oestradiol (10^{-6} and 10^{-5} M) in rings without endothelium. Relaxation induced by 17β-oestradiol (10^{-6} and 10^{-5} M) was $34 \pm 4\%$ and $84 \pm 3\%$ in the presence of methylene blue, and $35 \pm 10\%$ and $88 \pm 4\%$ in controls ($P > 0.05$).

Calcium antagonistic effects on rabbit coronary artery and rat aorta

The calcium concentration-dependent contraction curves in K^+-depolarization medium were shifted to the right after incubation with 17β-oestradiol (10^{-6} and 10^{-5} M) in rabbit coronary arterial and rat aortic rings, without endothelium. Maximal contraction was also reduced by $19 \pm 1\%$ and $36 \pm 2\%$ in rabbit coronary arteries and by $12 \pm 1\%$ and $29 \pm 1\%$ in rat aorta respectively. The $-\log EC_{50}$s of calcium in control and after incubation with 17β-oestradiol (10^{-6} and 10^{-5} M) were 3.7 ± 0.09, 3.1 ± 0.10 and 2.8 ± 0.08 in rabbit coronary arterial rings (Figure 3). Similar results were obtained from rat aorta preparations ($n = 6$) and the $-\log EC_{50}$s of calcium in control and after incubation with 17β-oestradiol (10^{-6} and 10^{-5} M) were 3.8 ± 0.11, 3.3 ± 0.14 and 2.9 ± 0.15 respectively.

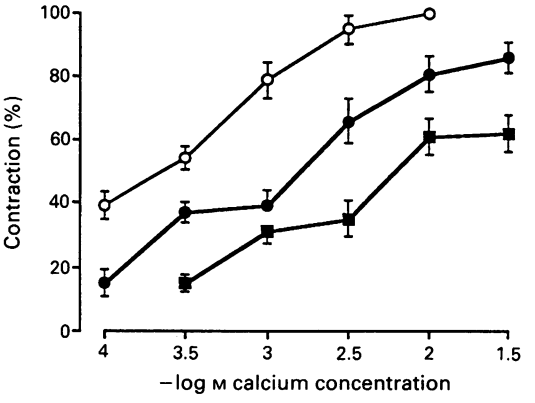


Figure 3 The effect of 17β-oestradiol (10^{-6} and 10^{-5} M) on the calcium concentration-dependent contraction curves in rabbit coronary arteries without endothelium: (○) control; (●) 10^{-6} M 17β-oestradiol; (■), 10^{-5} M 17β-oestradiol. Data are expressed as percentage of maximal contraction induced by calcium in controls (mean with s.e.mean shown by vertical bars, $n = 6$ in each group).

Discussion

We have demonstrated that 17β-oestradiol (10^{-6} M and 10^{-5} M) induces relaxation in precontracted rabbit coronary arteries with or without endothelium. No difference was seen between arteries from male and female rabbits. Reduced haemoglobin, L-NAME, indomethacin and methylene blue did not affect this relaxation. 17β-Oestradiol shifted the calcium concentration-dependent contraction curve to the right, in high K^+ (100 mM) solution, in rabbit coronary and rat aortic preparations. In addition, testosterone up to a dose of 10^{-5} M did not induce any relaxation in similar preparations.

Oestrogen receptors are present in both vascular and cardiac tissues of male and female rats (Stumpf *et al.*, 1977). Administration of oestrogen has been shown to induce haemodynamic changes in male human subjects after a transsexual operation (Slater *et al.*, 1986). However, it is well known that oestradiol has different effects on bioenergetic enzymes in the female and male. In the present study, we have demonstrated that 17β-oestradiol induces an identical relaxation in coronary arteries from female and male rabbits. Therefore, we suggest that the acute relaxant effect of 17β-oestradiol in the rabbit coronary artery *in vitro* is independent of sex. The other possible sex-related effects of this steroid hormone on vascular tissues are not excluded.

The heart is also a target for androgens in female monkeys (McGill *et al.*, 1980), although their effects on coronary vascular tissue are not well understood. In the present study, however, testosterone, which is the most active androgen, up to a concentration of 10^{-5} M, did not cause any relaxation in coronary arteries from either male or female rabbits, whilst 3×10^{-5} M and 10^{-4} M testosterone induced a small relaxation. We conclude that the acute relaxant effect of 17β-oestradiol is not a property shared by testosterone in our preparations.

Recently, Gisclard *et al.* (1988) reported that femoral arteries from rabbits treated with 17β-oestradiol show an enhanced endothelium-dependent relaxation to acetylcholine. Infusion of acetylcholine in ovariectomized guinea-pigs caused relaxation only after treatment with oestrogen (Bell, 1973; Bell & Coffey, 1982). Furthermore, the infusion of acetylcholine into atherosclerotic coronary arteries of ovariectomized, hypercholesterolaemic cynomolgus monkeys caused contraction while a comparison group of 17β-oestradiol-treated, animals showed a relaxation response to acetylcholine (Williams *et al.*, 1990). Acetylcholine induces endothelium-dependent vascular relaxation mediated by the release of EDRF (Furchgott & Zawadzki, 1980). These studies may therefore indicate that 17β-oestradiol is affecting vascular tone by an EDRF-dependent mechanism. However, we have demonstrated that 17β-oestradiol induced an equal degree of

relaxation in rabbit coronary arteries with and without endothelium. Haemoglobin, a well-known inhibitor of EDRF activity (Martin *et al.*, 1985), did not affect 17β-oestradiol-induced relaxation in endothelium-intact coronary arteries. L-NAME, a novel inhibitor of EDRF synthesis (Rees *et al.*, 1990), markedly inhibited acetylcholine-induced endothelium-dependent relaxation in rabbit coronary artery preparations (data not shown). However, the same dose of L-NAME did not affect the relaxation by 17β-oestradiol. Methylene blue, an inhibitor of EDRF-induced increase of cyclic GMP (Martin *et al.*, 1985), also had no effect on relaxation induced by 17β-oestradiol. Our results suggest that the *in vitro* acute relaxation of rabbit coronary arteries by 17β-oestradiol is independent of EDRF. The effect of 17β-oestradiol on EDRF-mediated relaxation *in vivo* over a long time course may still occur.

The possibility that relaxation of smooth muscle by 17β-oestradiol may be via prostacyclin release is suggested by the increased production of a prostacyclin metabolite in the lower uterine artery of postmenopausal hypooestrogenic women induced by administration of 17β-oestradiol (Steinleitner *et al.*, 1989). Increased prostacyclin production activity in rat aortic smooth muscle cells in culture with 17β-oestradiol stimulation has also been reported (Chang *et al.*, 1980). However, indomethacin does not affect 17β-oestradiol-induced enhancement of endothleium-dependent vasorelaxation and the authors concluded that a major role of prostanoid-induced vasorelaxation was unlikely (Gisclard *et al.*, 1988). Indomethacin inhibits synthesis of prostaglandins (Ferreira *et al.*, 1971; 1973). In the present study, indomethacin markedly inhibited the transient relaxation induced by arachidonic acid which has been shown to induce prostanoid-like action via transformation to prostaglandins in various tissues (De Mey *et al.*, 1982). However, indomethacin did not affect 17β-oestradiol-induced relaxation in endothelium-intact coronary arteries. These results indicate the release of vasodilator prostanoids is not involved in 17β-oestradiol-induced coronary relaxation *in vitro*.

Oestrogens have calcium antagonistic properties in uterine arteries. Stice *et al.* (1987a,b) reported that oestradiol decreases calcium entry into uterine vascular smooth muscle cells. Downing *et al.* (1988) observed that the inhibitory effect of nifedipine, a calcium entry blocker, was enhanced by oestradiol in the rat uterine artery. Sandahl *et al.* (1978) reported in the rat that nifedipine, a potential-sensitive calcium channel blocker, caused an increase in uterine arterial flow. However, this was not the case in rats pretreated with 17β-oestradiol possibly suggesting they were already maximally dilated by 17β-oestradiol. Calcium antagonistic properties of 17β-oestradiol have also been reported in isolated smooth muscle cells from uterine arteries and veins (McCalden, 1975; Takayama, 1986). Potential-sensitive calcium channels are activated by depolarization of the plasma membrane when the extracellular K^+ concentration is increased. The uterine arterial smooth muscle tone of gilts is reduced in oestrous when endogenous oestrogen is increased, suggesting that oestrogen may reduce intracellular calcium via a potential-operated calcium channel mechanism (Ford *et al.*, 1984). Bay K is a specific potential-sensitive calcium channel agonist (Brown *et al.*, 1984). In the present study, we found that 17β-oestradiol induced relaxation of coronary arterial contraction evoked by Bay K. This may be evidence that 17β-oestradiol has an inhibitory effect on potential-sensitive calcium channel activation. We also demonstrated that 17β-oestradiol induced relaxation of contraction evoked by $PGF_{2\alpha}$, an agonist of receptor-operated calcium channels, in the presence of extracellular calcium. These results indicate that 17β-oestradiol has similar relaxant effects on contraction induced by activation of both receptor-operated and potential-operated calcium channels. Furthermore, incubation with 17β-oestradiol shifted the calcium concentration-dependent contraction curves to the right in high K^+-depolarization medium in rabbit coronary arteries and rat aorta without endothelium. The curves shifted by incubation with different 17β-oestradiol concentrations were parallel, in rabbit coronary arteries and rat aorta, although the maximal contractions were also affected. These results suggest that 17β-oestradiol may have a partial calcium antagonistic property. This calcium antagonistic property could be one of the mechanisms of 17β-oestradiol-induced endothleium-independent relaxation in rabbit isolated coronary arterial preparations. Animal studies have shown that calcium-channel blockers such as nifedipine can alter the progression of atherosclerosis in animals fed a cholesterol-rich diet (Henry & Bentley, 1981). Recent clinical studies demonstrate the beneficial effect of nifedipine (Lichtlen *et al.*, 1990) and nicardipine (Water *et al.*, 1990) on the development of early atheromatous lesions seen at coronary angiography. Similar results have been obtained in cynomolgus monkeys (Williams *et al.*, 1990) where oestrogens inhibit the progression of atherosclerosis in the coronary arteries of these animals. If 17β-oestradiol does have a calcium antagonistic effect in man then its protective effect in atherosclerotic disease may also involve this mechanism.

The risk of coronary heart disease is lower in premenopausal women compared with men of the same age (Godsland *et al.*, 1987). Sex hormone withdrawal associated with the menopause substantially increases the incidence of myocardial infarction in postmenopausal women (Kannel *et al.*, 1976). Oestrogen replacement therapy in postmenopausal women reduces the cardiovascular mortality rate (Matthews *et al.*, 1989). The beneficial effects of oestrogen on menopausal symptoms (Campbell & Whitehead, 1977) and skeletal health (Ettinger *et al.*, 1985) have been increasingly recognised. However, the beneficial effects of oestrogen on the cardiovascular system would now appear to be the most important (Ettinger, 1988).

In conclusion, we have demonstrated that 17β-oestradiol induces endothelium-independent relaxation in rabbit isolated coronary artery preparations. 17β-Oestradiol-induced relaxation may play an important role in the regulation of coronary tone and this may be one of the explanations as to why oestrogen replacement therapy reduces the risk of coronary heart disease in postmenopausal women. 17β-Oestradiol has effects on contraction evoked by activation of both receptor- and potential-operated calcium channels. 17β-Oestradiol may also have calcium antagonistic properties. If 17β-oestradiol does have a calcium antagonistic effect in the human then its protective effect in atherosclerotic disease may also involve this mechanism. Many studies have demonstrated the effect of calcium antagonists on animal experimental atheroma causing regression. Some such studies emphasise the requirement for long term therapy.

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Activation of the 5-HT_{1C} receptor expressed in *Xenopus* oocytes by the benzazepines SCH 23390 and SKF 38393

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1 A cloned 5-HT_{1C} receptor expressed in *Xenopus laevis* oocytes was used to characterize the action of four dopamine D_1-selective benzazepines at the 5-HT_{1C} receptor. Additionally, the apparent binding of the D_1-selective benzazepines to 5-HT_{1C} receptors was measured in the choroid plexus of the pig.

2 In voltage-clamped oocytes expressing the cloned 5-HT_{1C} receptor, 5-hydroxytryptamine (5-HT) elicited a characteristic inward current response with an EC_{50} of 13 nM. SCH 23390 acted as a stereoselective agonist (or partial agonist) with an EC_{50} of about 550 nM. SKF 38393 (1 μM–1 mM), SKF 77434 (100 μM), and SKF 82958 (100 μM) also acted as agonists (or partial agonists) at the cloned 5-HT_{1C} receptor. SKF 38393 was not stereoselective at the 5-HT_{1C} receptor.

3 The response to SCH 23390 activated slowly and, although the response contained many oscillations characteristic of the activation of the phosphatidylinositol signal transduction system, SCH 23390 rarely elicited the rapid spike-like response seen routinely in response to 5-HT. However, the responses to SKF 38393, SKF 77434, and SKF 82958 were identical in appearance to the response to 5-HT, except that the responses to the benzazepines were smaller. These comparisons were made by applying both a benzazepine and 5-HT to each individual oocyte expressing the cloned 5-HT_{1C} receptor.

4 Consistent with the responses measured in oocytes, SCH 23390 bound stereoselectively to 5-HT_{1C} receptors in the choroid plexus of the pig (K_i = 6.3 nM), and SKF 38393 bound non-stereoselectively with lower affinity (K_i = 2.0–2.2 μM).

5 It is concluded that while these benzazepines demonstrate selectivity for the dopamine D_1 receptor, they also can act as agonists or partial agonists at the 5-HT_{1C} receptor *in situ* and as expressed in *Xenopus* oocytes. The oocyte expression system is useful for studies of the functional pharmacology of these 5-HT_{1C} receptors. Information about the pharmacological actions and variations in stereoselectivity among dopamine and 5-HT receptors should be of interest in modelling the interactions of ligands with these G-protein coupled receptors, and in the testing of such models through receptor mutagenesis.

Keywords: Oocytes; *Xenopus laevis*; choroid plexus; 5-hydroxytryptamine; benzazepines; dopamine receptor; 5-HT_{1C} receptor; dopamine; cloning, molecular; pharmacology; molecular biology

Introduction

Benzazepines such as SKF 38393 and SCH 23390 appear to be selective, although not entirely specific, for the D_1-dopamine receptor, and these compounds have been the standards used in many experiments to define responses mediated by D_1 receptors. However, at higher concentrations these benzazepines also interact with 5-hydroxytryptamine (5-HT) receptors, particularly 5-HT_{1C} and 5-HT_2 (Hicks *et al.*, 1984; Dawson *et al.*, 1986; Hess *et al.*, 1986; Bischoff *et al.*, 1986; 1988; De Keyser *et al.*, 1988; 1989; Hoyer & Karpf, 1988; Nicklaus *et al.*, 1988; Skarsfeldt & Larsen, 1988; Hoyer *et al.*, 1989; Yamamoto & Kebabian, 1989). Indeed, the affinity of SCH 23390 for the 5-HT_{1C} receptor is only about 10–30 fold less than for the D_1 receptor. Thus, in heterogeneous systems with multiple receptors, the effects of the D_1 'benchmark' compounds SKF 38393 and SCH 23390, especially at micromolar doses, are not necessarily due to the activation and inhibition of dopamine D_1-receptors.

While there have been several publications concerned with the binding of benzazepines at 5-HT_{1C} receptors, there has been less work on the functional consequences of this interaction (see Hoyer *et al.*, 1989). We thought it of value to characterize the action and stereoselectivity of such benzazepines at the 5-HT_{1C} receptor. Are, for example, agonists at the D_1 receptor also agonists at the 5-HT_{1C} receptor? It was also thought that such comparative information would be valuable in the molecular modelling of these receptors and their interactions with ligands. Both the dopamine D_1 and the 5-HT_{1C} receptors are members of the larger class of G-protein coupled receptors. While D_1 and D_2 dopamine receptors clearly are related, in terms restricted to benzazepine binding the D_1 receptor appears more similar to the 5-HT_{1C} receptor than to the D_2 receptor.

In the brain, recent evidence suggests that 5-HT_{1C} receptors are expressed outside the choroid plexus (Conn & Sanders-Bush, 1985; Hoyer *et al.*, 1986a; Peroutka, 1986; Hoffman & Mezey, 1989; Molineaux *et al.*, 1989; Mengod *et al.*, 1990), raising questions about the role of 5-HT_{1C} receptors in the CNS. However, assessing 5-HT_{1C} function pharmacologically could be difficult because of the presence of other 5-HT and dopamine receptors, the lack of highly selective 5-HT_{1C} agonists and antagonists, and the fact that 5-HT_2 and perhaps D_1 receptors in addition to the 5-HT_{1C} receptor are coupled to inositol phospholipid hydrolysis (Hoyer, 1988; Peroutka, 1988; Felder *et al.*, 1989a,b; Hartig, 1989; Schmidt & Peroutka, 1989; Dyck, 1990; Rubinstein & Hitzemann, 1990; Undie & Friedmann, 1990).

One approach would be to utilize *Xenopus laevis* oocytes where the 5-HT_{1C} receptor can be expressed and coupled to the oocyte's endogenous inositol phospholipid system (Lübbert *et al.*, 1987a,b; Hoyer, 1988; Julius *et al.*, 1988; Hartig, 1989). Responses can be measured electrophysiologically through a multi-step signal transduction pathway involving inositol trisphosphate generation from phosphatidylinositol, release of internal Ca^{2+}, and activation of a Ca^{2+}-dependent Cl^- current which is the measured response (for reviews see Sumikawa *et al.*, 1986; Lester, 1988; Snutch, 1988; Sigel, 1990). A significant advantage of this approach, using clonal mRNA, is the ability to deal with an individual

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selected receptor, assuming that the protein expressed in the oocyte is similar to the protein expressed *in situ*. Additionally, there is the potential for further studies of the receptor, its interaction with ligands, and its interaction with the signal transduction pathway through site-directed mutagenesis and expression of mutated receptors in oocytes.

This paper describes studies on benzazepine functional pharmacology in oocytes expressing the 5-HT$_{1C}$ receptor. Some of the results have been published in an abstract (Briggs *et al.*, 1990).

Methods

5-HT$_{1C}$ receptor cloning

Oligonucleotides were synthesized that encode sequences which flank the coding region of the rat brain 5-HT$_{1C}$ receptor (Julius *et al.*, 1988). cDNA was synthesized from total brain RNA using reverse transcriptase. The oligonucleotides and the cDNA were then used in a polymerase chain reaction (PCR) to generate a fragment that encoded the complete 5-HT$_{1C}$ coding sequence. The fragment was cloned into M13mp19 and completely sequenced to confirm identity with the known 5-HT$_{1C}$ receptor. Three PCR-generated base changes were correced by site-directed mutagenesis by use of a kit (Biorad, Richmond, CA, U.S.A.).

5-HT$_{1C}$ receptor mRNA

An oligonucleotide (tgtaatacgactcactatagtaaaacgacggccagtg) was synthesized that contained both the promoter sequence for T7 RNA polymerase and sequences complimentary to the M13 'universal primer' site. This oligonucleotide and a second M13 complimentary oligonucleotide (commonly referred to as 'reverse primer') were used to amplify the 5-HT$_{1C}$ insert in M13 by PCR. This amplified DNA contained the coding sequence of the 5-HT$_{1C}$ receptor downstream from the T7 promoter and served as a template for *in vitro* transcription producing the 5-HT$_{1C}$ mRNA. *In vitro* transcription for large scale synthesis was performed according to a protocol provided by Promega, except that a cap analogue was included at the start of the reaction (m7G(5')ppp(5')G, 1 mM final concentration; Pharmacia, Piscataway, New Jersey, U.S.A.). After synthesis, the sample was treated with DNAse (Promega, Madison, Wisconsin, U.S.A.) and the mRNA was extracted with phenol:chloroform (1:1), precipitated with ethanol, and resuspended in RNAse-free water. A portion was analysed by gel electrophoresis and the quantity was estimated by comparison of the ethidium bromide staining intensity with that of RNA size markers (BRL, Gaithersburg, Maryland, U.S.A.).

Oocyte preparation and RNA injection

Oocyte-positive *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI, U.S.A.) or Xenopus-1 (Ann Arbor, MI, U.S.A.), fed a commercial diet thrice weekly (Nasco or Carolina Biological Supply, Burlington, North Carolina, U.S.A.), and maintained in recirculating, filtered, dechlorinated water at 19–20°C under controlled, full-spectrum lighting (Vita-lite, on 14 h daily). Ovaries were removed from animals after anaesthesia in ice and decapitation followed by spinal pithing, and the oocytes were freed after digestion with collagenase (Sigma Type 1A, 2–2.5 mg ml^{-1}) for 1.5–2.0 h in a low-Ca^{2+} solution (composition mM: NaCl 87.5, KCl 2.5, MgCl 1.0, sodium HEPES buffer 10, pH 7.6) at room temperature (23–25°C). The preparation was washed with the low-Ca^{2+} solution followed by the culture solution (see below), and Stage V-VI oocytes (Dumont, 1972) were selected and maintained for 1–5 days at 15°–17°C. In most experiments the culture solution was modified Leibovitz L-15 culture medium, diluted to 67% and supplemented with 2.4 mM $NaHCO_3$ and 10 mM sodium HEPES buffer (pH 7.55, 233–242 mOsm). In other experiments, the oocytes were maintained in a modified Barth's solution containing (mM) NaCl 90, KCl 1.0, $NaNO_3$ 0.66, $CaCl_2$ 0.74, $MgCl_2$ 0.82, $NaHCO_3$ 2.4, sodium HEPES buffer 10, and sodium pyruvate 2.5 (pH 7.55, 196–205 mOsm). These solutions also contained antibiotics (100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and were sterilized by 0.2 μm filtration. The culture solution was changed daily and any shrunken or mottled oocytes were discarded.

The 5-HT$_{1C}$ mRNA dissolved in RNAse-free water was injected by pneumatic pressure into the selected State V-VI oocytes within 24 h of their preparation. Typically 10–50 nl was injected per oocyte; the injection volume was estimated by monitoring the injection micropipette with a calibrated video system (see also Smith *et al.*, 1987). As a control, some oocytes were injected with a sample of the RNAse-free water.

Response measurement and analysis

Oocytes were used 1–4 days after injection and, during the experiment, were superfused at 2 ml min^{-1} in a chamber of approximately 550 μl volume. In experiments using oocytes cultured in the modified Barth's solution, the superfusate also was modified Barth's solution (composition mM: NaCl 90, KCl 1.0, $NaNO_3$ 0.66, $CaCl_2$ 0.74, $MgCl_2$ 0.82, $NaHCO_3$ 2.4, sodium HEPES buffer 10, and sodium pyruvate 2.5, pH 7.55; 196–205 mOsm). In most experiments, however, the oocytes were cultured in the higher-osmolarity modified L-15 medium, and recordings were obtained in a modified Ringer solution of similar osmolarity (mM: NaCl 110, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 2.4, sodium HEPES buffer 10, and sodium pyruvate 2.5; pH 7.55; 233–242 mOsm).

Each oocyte was voltage-clamped at −70 mV with two intracellular electrodes and a virtual ground circuit (Axoclamp-2A, Axon Instruments Inc., Foster City, CA, U.S.A.). The intracellular electrodes in some initial experiments were filled with hypertonic 3.0 M KCl (0.8–3 MΩ resistance). In most experiments, however, the electrodes were filled with a nearly-isoosmolar concentration of KCl (5–10 mOsm hypertonic with respect to the bathing solution; e.g., 135 mM KCl electrolyte for use with the modified Ringer's solution). These electrodes (0.5–2.0 MΩ resistance) were made from borosilicate omega-dot glass tubing, 1.5 mm o.d. by 1.17 mm i.d., pulled in two stages, and bevelled. In some cases it was necessary to allow several minutes for the oocyte-electrode seal to improve and reach a steady state, but afterwards the recording usually was stable for many hours and there was no evidence of swelling of the oocyte as with the hypertonic electrodes. Overall, in these and previous studies (not shown), oocyte whole cell resistances obtained after impalement with the larger isoosmolar electrodes were similar to those obtained with the sharper electrodes filled with 3 M KCl (1.31 ± 0.06 MΩ, $n = 188$ compared to 1.30 ± 0.14 MΩ, $n = 58$ respectively).

Most receptor agonists, except for SCH 23390 and SCH 23388, were applied for 1 min. Under these conditions, in most oocytes 5-HT could be applied repeatedly at 30 min intervals without an apparent prolonged 'desensitization'. However, because SCH 23390 elicited a response only slowly, SCH 23390 and its enantiomer SCH 23388 were applied for 5 min. This longer exposure to SCH 23390 was followed by a prolonged 'desensitization' of the response to 5-HT, and therefore oocytes generally were discarded after exposure to SCH 23390 and other benzazepines that elicited an agonist-like response. On the other hand, if the oocyte did not respond to benzazepine (e.g., SCH 23388), there was no apparent 'desensitization' of the response to 5-HT.

Agonists produced an inward current response which was captured, stored, and analysed on a digital oscilloscope (Nicolet model 440, Nicolet Instrument Corp., Madison, Wisconsin, U.S.A.). Responses to 5-HT and other agonists applied for 60 s were quantified by measurement of the maximal or peak inward current and by integration of the response over a time base of 60 s from the initiation of the response. These integrals included the initial spike-like

response and the subsequent plateau when present, but not recovery from the plateau during washout of the agonist. Responses to SCH 23390 and SCH 23388 (applied for 5 min) were quantified by integration over a time base of 5 min.

The cell potential was also recorded. Current responses accompanied by a voltage loss of more than 2 mV during any part of the response were discarded. Typically the peak voltage loss was very brief and held to 1 mV or less.

Normalized responses In experiments with benzazepines, each oocyte was first exposed to 5-HT at 30-min intervals to confirm a stable response. Subsequently, the response to a benzazepine was obtained. Data show the ratio, computed for each oocyte, of the benzazepine response integral divided by the maximal 5-HT response integral. The purpose of this normalization was to correct for variability in the expression of 5-HT_{1C} receptors among ooctyes.

Dose-response relationships Estimates of the EC_{50} or IC_{50} for various drug actions in oocytes were obtained by non-linear curve fitting (Marquardt-Levenberg algorithm; SigmaPlot software, Jandel Scientific, Corte, Madera, California). Similar results were obtained by logit-log analysis when both types of analysis were used to estimate the EC_{50} for 5-HT from the same set of data.

5-HT_{1C} receptor binding (choroid plexus)

Porcine choroid plexus was obtained from a local slaughterhouse and kept frozen. Membranes were prepared from the choroid plexus essentially as described by Pazos *et al.* (1985a, b) with minor modifications. The frozen choroid plexus was homogenized for 20 s in 10 volumes of 0.32 M sucrose at 4°C (Polytron homogenizer; Brinkman Instruments, Westbury, New York, U.S.A.), centrifuged at 6,000 *g* for 10 min at 4°C, and the membranes were then pelleted from that supernatant by centrifugation at 65,000 *g* for 15 min. The pellets were resuspended to the original volume in Tris-Ca buffer (50 mM Tris-Cl, pH 7.4, and 4 mM $CaCl_2$), and incubated at 37°C for 15 min. Finally, ascorbic acid (0.2% w/v) was added to the membrane suspension, and the preparation was stored at −70°C.

Ligand binding was measured by displacement of [^{125}I]-SCH 23982 in Tris-Ca buffer at 37°C for 60 min. Each tube contained 120 pM [^{125}I]-SCH 23982, non-labelled ligand ($\leq 10\,\mu$M), and 100 μl (10 mg) of the choroid plexus membrane preparation in a total volume of 200 μl. Tissue containing bound ligands was separated by vacuum filtration and washed with cold Tris-Ca buffer. The radioactivity trapped on the glass-fibre filters was quantified (Beta-Plate; LKB, Gaithersburg, Maryland). Nonspecific binding was defined as that remaining in the presence of 10 μM SCH 23390. Binding affinities were estimated by computer analysis ('Accucomp', Lundon Software, Chagrin Falls, Ohio or 'Ligand' software kindly supplied by Dr Peter J. Munson, National Institutes of Health, Bethesda, Maryland, U.S.A.). Statistical comparisons were made by the Newman-Keuls procedure.

Materials

5-Hydroxytryptamine (5-HT) hydrochloride, 3-hydroxytyramine (dopamine) hydrochloride, collagenase Type 1A, Leibovitz L-15 powder with L-glutamine, penicillin/streptomycin solution and sodium pyrivate were purchased from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). SCH 23390 ((R(+) > 7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride), SCH 23388 ((S(−)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride), R(+)-SKF 38393 ((R(+)-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol) hydrochloride), S(−)-SKF 38393, SKF 77434 (N-allyl-SKF 38393), SKF 82958 (6-chloro-N-allyl-SKF 38393), and spiperone were purchased from Research Biochemicals Incorporated (Natick, Massachusetts, U.S.A.). [^{125}I]-SCH 23982 ((+)-8-iodo-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol) was obtained from DuPont-NEN (Boston, Massachusetts, U.S.A.).

Results

Studies were conducted with 5-HT_{1C} mRNA synthesized from a cloned plasmid containing the complete 5-HT_{1C} receptor coding region. This receptor could be studied pharmacologically following injection of the clonal mRNA into oocytes. In non-injected oocytes or oocytes injected with a portion of the RNAse-free water used to dissolve the mRNA, 5-HT and other compounds used in this study did not elicit inward current responses exceeding 10 nA (data not shown).

5-Hydroxytryptamine

The dose-response relationship for 5-HT in one oocyte that had been injected with clonal 5-HT_{1C} mRNA is shown in Figure 1a. The oocyte was exposed repeatedly to 5-HT at various concentrations. The EC_{50} determined in this oocyte was 7.8 nM. The combined results from four additional similar experiments are shown in Figure 1b, indicating an average EC_{50} of 13 nM for 5-HT. This is in good agreement with our measurement of the 5-HT binding to 5-HT_{1C} receptors in porcine choroid plexus, which had an apparent K_i of 19 nM ($pK_i = 7.7 \pm 0.1$, mean ± s.e.mean, $n = 3$). These values also

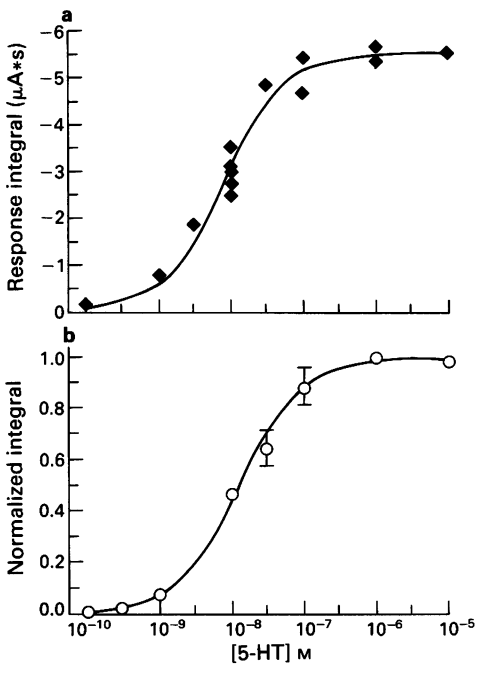


Figure 1 Dose-response relationship for 5-hydroxytryptamine (5-HT) in oocytes injected with clonal 5-HT_{1C} MRNA. (a) Data are from one oocyte injected with 5-HT_{1C} mRNA. 5-HT at various concentrations was applied for 1 min at 30 min intervals, in the following sequence: 10 μM, 10 nM, 10 nM, 0.1 nM, 1 nM, 1 μM, 10 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 10 nM, 100 nM, and 1 μM. Each response (inward current) was integrated and the individual response integrals are plotted. The fitted curve indicates an $EC_{50} = 7.8 \pm 1.1$ nM (mean ± s.d.) in this oocyte. (b) Data are from four additional oocytes, each exposed repeatedly to 5-HT at various doses. Each response was integrated, and the integral was normalized to the integral of the response to 1 μM 5-HT measured in the same oocyte in order to account for variability in the expression of the injected 5-HT_{1C} mRNA. The responses to 1 μM 5-HT were −12.7 to −122 μA*s (−45 ± 26 μA*s), and −1.78 to −9.15 μA (−3.9 ± 1.8 μA) at the peak of the response. The fitted curve indicates an $EC_{50} = 13 \pm 17$ nM (mean ± s.d.).

are in reasonably good agreement with other studies of 5-HT interaction with the 5-HT$_{1C}$ receptor in mammalian cells (Hoyer *et al.*, 1985; Yagaloff & Hartig, 1985; Conn *et al.*, 1986; Peroutka, 1986; Hoyer *et al.*, 1989), although the 5-HT EC_{50}s determined in the choroid plexus (Conn *et al.*, 1986; Hoyer *et al.*, 1989) and in oocytes injected with brain RNA (Lübbert *et al.*, 1987b) are about 3–6 fold higher.

Methysergide (0.1 μM) inhibited the response to 10 nM 5-HT by 90 ± 3% (n = 3). Spiperone had little or no effect at a concentration of 1.0 μM (4 ± 9% inhibition, n = 4), and at 10 μM inhibited the response to 10 nM 5-HT by only 31 ± 4% (n = 4). Thus, methysergide was a potent antagonist while spiperone was a weak antagonist of the response to 5-HT in oocytes injected with clonal 5-HT$_{1C}$ mRNA, similar to the 5-HT$_{1C}$ receptor found in mammalian cells (Pazos *et al.*, 1985b; Yagaloff & Hartig, 1985; Conn *et al.*, 1986; Hoyer *et al.*, 1986b).

SCH 23390 and SCH 23388

SCH 23390 acted as an agonist in oocytes injected with clonal 5-HT$_{1C}$ mRNA. However, the response to SCH 23390 developed much more slowly than the response to 5-HT, usually requiring exposure for 3 min or more rather than 1 min or less (Figure 2). Additionally, the response to SCH 23390 usually lacked the initial spike-like phase although many oscillations were present as is typical for this signal transduction system (Parker & Ivorra, 1990). Application of SCH 23390 (1 μM) for 5 min did elicit a response in every oocyte tested, but that application also caused a prolonged reduction in the sensitivity to SCH 23390 and to 5-HT that lasted for several hours in some oocytes. Such 'desensitization' of similar responses in oocytes has been commonly observed and may be related to depletion of internal Ca^{2+} or other aspects of the signal transduction process (Berridge, 1989; Vasilets *et al.*, 1990). With 5-HT, we found that the 'desensitization' could be reduced by limiting the exposure to 1 min, allowing reproducible responses to be obtained at 30 min intervals.

Because of the need to apply SCH 23390 for several minutes and the ensuing hours-long desensitization, it was not generally practical to obtain reproducible responses to repeated application of SCH 23390 in individual oocytes. It was anticipated that there may be considerable variability among oocytes in the response to SCH 23390 due, at least in part, to variability in the expression of the 5-HT$_{1C}$ receptors. To reduce such variability, the response to SCH 23390 (applied for 5 min) was normalized to the response to 5-HT (applied for 1 min) determined in each oocyte before the application of SCH 23390.

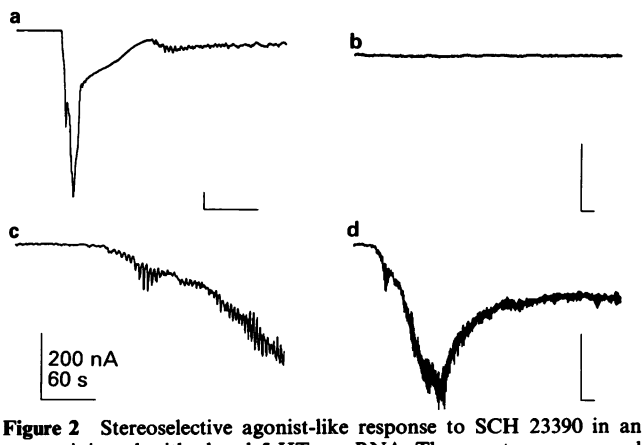


Figure 2 Stereoselective agonist-like response to SCH 23390 in an oocyte injected with clonal 5-HT$_{1C}$ mRNA. The oocyte was exposed to 30 nM 5-hydroxytryptamine (5-HT) for 1 min (a, one of 2 similar responses is shown), followed by 1 μM SCH 23388 for 5 min (b, little or no response), followed by 1 μM SCH 23390 for 5 min (c and d, each from the same response). The responses to SCH 23388 and SCH 23390 are shown at a higher gain than is the response to 5-HT. At (c), the response to SCH 23390 is shown on the same time scale as is the response to 5-HT above it. At (d), the response to SCH 23390 is shown on a longer time scale, identical to that for SCH 23388 above it. Calibration marks represent 200 nA and 60 s for each recording.

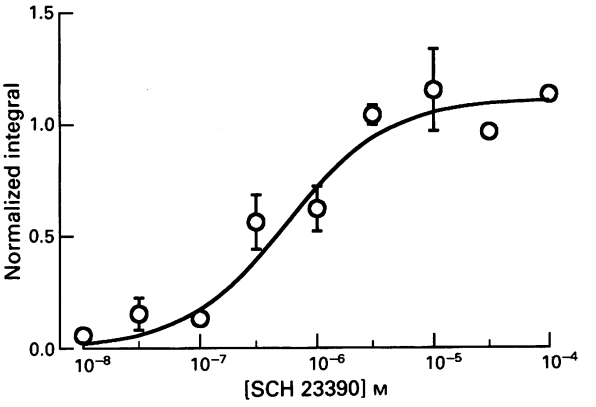


Figure 3 SCH 23390 dose-response relationship in oocytes injected with clonal 5-HT$_{1C}$ mRNA. Oocytes (n = 26) were exposed at 30 min intervals repeatedly to 5-hydroxytryptamine (5-HT) (for 1 min) and then finally exposed once to one concentration of SCH 23390 for 5 min. To reduce variability among oocytes due to differences in the number or activity of expressed receptors, the response to SCH 23390 (integrated over 5 min) was normalized to the maximal response to 5-HT (integrated over 1 min) in each oocyte. The maximal responses to 5-HT were −2.72 to −69.12 μA*s (−22.6 ± 4.2 μA*s). The fitted curve indicates an EC_{50} = 550 ± 240 nM (mean ± s.d.).

Figure 3 shows the dose-response relationship of the normalized integrals for SCH 23390, determined after one application of one dose of SCH 23390 per oocyte. The apparent EC_{50} was 550 nM. The effect of SCH 23390 was clearly stereoselective because the enantiomer SCH 23388 (1 μM) produced little or no response (Figures 2 and 5a). The stereoselectivity also was seen in the binding to 5-HT$_{1C}$ receptors in porcine choroid plexus, where the apparent K_i for SCH 23390 was 6.3 nM (pK_i = 8.20 ± 0.08, n = 14) compared to an apparent K_i for SCH 23388 of 1,100 nM (pK_i = 5.96 ± 0.2, n = 3).

SKF 38393, SKF 77434 and SKF 82958

Other benzazepines were tested in a paradigm similar to that used for SCH 23390, except that 1-min applications were sufficient. As shown for SKF 38393 in Figure 4, at micromolar doses the dopamine D_1-selective agonists SKF 38393, SKF 77434, and SKF 82958 each elicited a response in oocytes injected with clonal 5-HT$_{1C}$ mRNA. In individual oocytes, the responses to these benzazepines were smaller than the

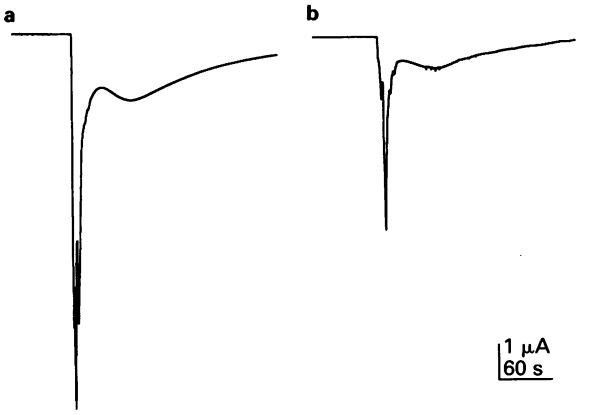


Figure 4 Responses to SKF 38393 and 5-hydroxytryptamine (5-HT) in one oocyte injected with clonal 5-HT$_{1C}$ mRNA. The oocyte was exposed to 1 μM 5-HT for 1 min (a) followed by 100 μM R(+)-SKF 38393 for 1 min (b). As in other oocytes, the responses to SKF 38393, SKF 77434, and SKF 82958 were similar in appearance but smaller than the response to 5-HT. The calibration marks represent 1 μA and 60 s for each recording.

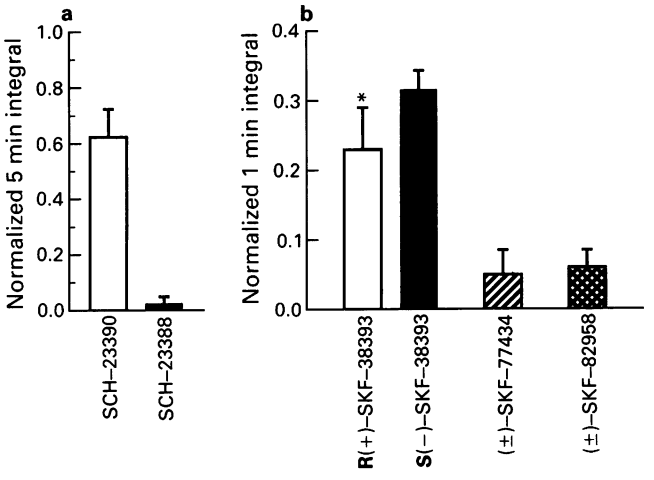


Figure 5 Benzazepine responses in oocytes injected with clonal 5-HT_{1C} mRNA. In each oocyte, the responses to the benzazepines were determined and normalized to the 5-hydroxytryptamine (5-HT) response in Figure 3, except that the SKF compounds were applied for 1 min and their responses were integrated over 1 min. The normalized responses were averaged among oocytes and are presented as mean ± s.e.mean. (a) Shown are normalized response to 1 μM SCH 23390 (open column, 7 oocytes) and the lack of response to its enantiomer 1 μM SCH 23388 (solid column, 7 oocytes). In 6 of these 7 oocytes there was no discernible response to 1 μM SCH 23388. (b) Shown are the normalized responses to 100 μM **R**(+)-SKF 38393 (open column, 5 oocytes), 100 μM S(−)-SKF 38393 (solid column, 4 oocytes), 100 μM (±)-SKF 77434 (hatched column, 4 oocytes) and 100 μM (±)-SKF 82958 (cross-hatched column, 4 oocytes). There was no apparent stereoselectivity for **R**(+)-SKF 38393 (100 μM), which is the more active enantiomer at the dopamine D_1-receptor.
* **R**(+)-SKF 38393 significantly different from (±)-SKF 77434 and (±)-SKF 82958, $P < 0.03$ (1-way ANOVA with *post-hoc* comparison using the method of contrasts).

response to 5-HT, but otherwise appeared similar to the response to 5-HT itself. The relative amplitudes of the responses to SKF 38393, SKF 77434 and SKF 82958 are shown in Figure 5b.

This effect of SKF 38393 was not stereoselective. The response to 100 μM **S**(−)-SKF 38393 was as large as the response to 100 μM **R**(+)-SKF 38393 (Figure 5b). Similarly, in the porcine choroid plexus the binding of SKF 38393 to 5-HT_{1C} receptors was not stereoselective; the apparent K_i for **R**(+)-SKF 38393 was 2.2 μM ($pK_i = 5.67 \pm 0.1$, $n = 3$) while that for S(−)-SKF 38393 was 2.0 μM ($pK_i = 5.70 \pm 0.06$, $n = 3$).

The responses to SKF 38393 in oocytes injected with 5-HT_{1C} mRNA were reasonably consistent with the binding parameters determined in choroid plexus. The response to 10 μM **R**(+)-SKF 38393 appeared to be maximal, but only 29 ± 10% of the maximal response to 5-HT in the same oocytes ($n = 4$). Higher concentrations of **R**(+)-SKF 38393 did not elicit larger effects after normalization to the 5-HT response; the responses to 100 μM and 1 mM SKF 38393 were 23 ± 6% ($n = 5$) and 27 ± 8% ($n = 5$) of the maximal response to 5-HT. The response to 1 μM (+)-SKF 38393 was, on average, approximately half-maximal (13 ± 5% of the response to 5-HT, $n = 5$). Furthermore, the binding of SKF 38393 in choroid plexus, like the response to SKF 38393 in oocytes injected with clonal 5-HT_{1C} RNA, was not stereoselective.

Derivatives of SKF 38393, (±)-SKF 77434 and (±)-SKF 82958 (100 μM), also elicited 5-HT-like inward current responses in oocytes injected with 5-HT_{1C} mRNA although these responses were smaller than the responses to SKF 38393 after normalization to the 5-HT response (Figure 5b). Additionally, (±)-SKF 77434, and (±)-SKF 82958 bound to 5-HT_{1C} receptors in porcine choroid plexus with apparent K_is of 4.1 μM ($pK_i = 5.39 \pm 0.06$, $n = 3$) and 1.3 μM ($pK_i = 5.89 \pm 0.10$, $n = 3$) respectively. These micromolar K_is should be considered estimates, since the highest ligand concentration used was 10 μM. However, the data do indicate a good correspondence between the binding of these benzazepines to 5-HT_{1C} receptors in choroid plexus and their activation of the clonal 5-HT_{1C} receptor expressed in *Xenopus* oocytes.

Thus, it would appear that the stereoselective D_1-dopamine antagonist SCH 23390 is, at higher concentrations, a stereoselective 5-HT_{1C} agonist, while the stereoselective D_1-dopamine agonist is, at higher concentrations, a non-stereoselective 5-HT_{1C} agonist. Indeed, dopamine itself (100 μM), like SKF 82958 and SKF 77434, also elicted a response in oocytes injected with 5-HT_{1C} RNA (not shown).

Discussion

In oocytes injected with clonal 5-HT_{1C} mRNA ('5-HT_{1C} oocytes'), SCH 23390 acted as a stereoselective agonist or partial agonist, and bound to choroid plexus 5-HT_{1C} receptors stereoselectively. Similarly, SCH 23390 and SKF 83566 have been found to be partial agonists (about 40% efficacy) in the choroid plexus, presumably by actions at the 5-HT_{1C} receptor (Hoyer *et al.*, 1989); stereoselectivity was not tested for this choroid plexus action. The response to SCH 23390 in 5-HT_{1C} oocytes, unlike the responses to the other benzazepines and 5-HT, activated slowly and often required several minutes of exposure to the compound. The rate of association of SCH 23390 with the dopamine D_1-receptor is relatively slow (Billard *et al.*, 1984; Andersen *et al.*, 1985; Niznik *et al.*, 1986). A similar slow association with the 5-HT_{1C} receptor could explain the need for longer application and the slow activation of the response observed in oocytes.

The EC_{50} for SCH 23390 in 5-HT_{1C} oocytes was nearly two orders of magnitude greater than its binding K_i in porcine choroid plexus. This is consistent with other studies of SCH 23390 interaction with 5-HT_{1C} receptors and does not indicated an artefact of the cloned receptor expressed in oocytes. Hoyer *et al.* (1989) observed a similarly large difference between the SCH 23390 EC_{50} (2.0 μM) and binding K_i (5.5 nM) when both apparent 5-HT_{1C} receptor binding and functional activity (phosphatidylinositol hydrolysis) were measured in one tissue (choroid plexus). The reason for these EC_{50}/K_i differences is not known, but slow association of SCH 23390 with the receptor and desensitization of the response might be contributing factors. In any case, the EC_{50} for SCH 23390 estimated in oocytes injected with 5-HT_{1C} mRNA is reasonably close to the SCH 23390 EC_{50} determined in choroid plexus, and in other comparisons, the actions of 5-HT and benzazepines in 5-HT_{1C} oocytes appeared to correlate well with the binding of these substances in porcine choroid plexus.

It is important to realize that as a partial agonist, SCH 23390 can act as an antagonist as well as an agonist at 5-HT_{1C} receptors (see Hoyer *et al.*, 1989). Indeed, in other experiments using oocytes injected with rat brain RNA, we have found that SCH 23390 can appear to be a stereoselective 5-HT_{1C} antagonist (data not shown). Thus, it should not be assumed that antagonism of a response by SCH 23390 necessarily distinguishes an action at D_1-dopamine versus 5-HT_{1C} receptors.

Three benzazepines regarded as selective D_1-dopamine agonists, SKF 38393, SKF 77434, and SKF 82958, at relatively high concentrations (1–100 μM) also acted as agonists at the clonal 5-HT_{1C} receptor expressed in oocytes. Unlike the slow response to SCH 23390, these benzazepines elicited responses that were very similar in appearance to the response to 5-HT. The only apparent difference was that the responses to these SKF compounds were smaller in amplitude than the response to 5-HT recorded in the same oocytes. The responses to various concentrations of SKF 38393 were consistent with its binding K_i (2.0–2.2 μM) to 5-HT_{1C} receptors in choroid plexus; the large difference between binding affinity and EC_{50} noted with SCH 23390 was not apparent with SKF 38393.

However, SKF 77434 (100 μM) and SKF 82958 (100 μM) elicited responses that were smaller than those elicited by SKF 38393 (100 μM). This was not explained by differences in the apparent binding affinity estimated in the choroid plexus. A likely explanation is that these benzazepines, at the 5-HT$_{1C}$ receptor, are partial agonists with various degrees of efficacy. This hypothesis needs to be tested by measurement of complete dose-response curves.

Thus, at micromolar concentrations these benzazepines, which have been used as standard dopamine D$_1$-receptor agonists and antagonists, also act as 5-HT$_{1C}$ agonists. Additionally, SCH 23390 has been found to interact with the related 5-HT$_2$ receptor (Hyttel, 1983; Hicks *et al.*, 1984; Ohlstein & Berkowitz, 1985; Hess *et al.*, 1986; Bischoff *et al.*, 1986; 1988; McQuade *et al.*, 1988; De Keyser *et al.*, 1989). Clearly, caution must be exercised in attributing all actions of SKF 38393, SKF 77434 and SKF 82958, particularly at micromolar concentrations, to activation of the dopamine D$_1$-receptor.

In oocytes injected with whole brain or striatal RNA, other investigators have reported responses to dopamine (10 μM–1 mM) and to SKF 38393 (100 μM) similar to the responses observed here in oocytes injected with clonal 5-HT$_{1C}$ mRNA (Sumikawa *et al.*, 1984; Mahan *et al.*, 1990; Sakai *et al.*, 1990). In one report (Sumikawa *et al.*, 1984) it was found that 1 μM methysergide blocked the response to 1 mM dopamine, as would be expected if dopamine acted through stimulation of a 5-HT$_{1C}$ or 5-HT$_2$ receptor. Others, however, reported that neither mianserin nor ketanserin inhibited the response to dopamine or SKF 38393 (Mahan *et al.*, 1990; Sakai *et al.*, 1990), and it was suggested (Mahan *et al.*, 1990) that the responses may be due to activation of D$_1$-receptors thought to be coupled to phosphatidylinositol hydrolysis in mammalian cells (Undie & Friedman, 1990; Felder *et al.*, 1989a,b; but see Dyck, 1990; Rubinstein & Hitzemann, 1990).

Interestingly, although SKF 38393 is stereoselective as a dopamine-D$_1$ agonist, SKF 38393 was not stereoselective as an agonist at the clonal 5-HT$_{1C}$ receptor expressed in oocytes, nor in its binding to the 5-HT$_{1C}$ receptor in choroid plexus. Thus, stereoselectivity may be one means of determining whether an effect of SKF 38393 is due to activation of D$_1$ versus 5-HT$_{1C}$ (or 5-HT$_2$?) receptors. Indeed, there have been reports of behavioural responses to SKF 38393 which were not stereoselective (Molloy & Waddington, 1987; LaHoste & Marshall, 1990). It may be of interest to determine if such responses were due to the activation of 5-HT$_{1C}$ or 5-HT$_2$ receptors, particularly since the 5-HT$_{1C}$ receptor recently has been found to occur in many regions of the brain outside of the choroid plexus (Hoyer *et al.*, 1986a; Hoffman & Mezey, 1989; Molineaux *et al.*, 1989; Mengod *et al.*, 1990). The roles of these 5-HT$_{1C}$ systems are not known (but see Jenck *et al.*, 1990).

The actions of benzazepines at both dopamine and 5-HT receptors is not only of pharmacological interest, but also may be useful in the molecular modelling of this G-protein-linked receptor family. For example, how can one benzazepine (SCH 23390) be a stereoselective antagonist at the D$_1$ receptor and a stereoselective agonist at the 5-HT$_{1C}$ receptor, while another benzazepine (SKF 38393) is an agonist at both receptors but stereoselective at the D$_1$ and not the 5-HT$_{1C}$ receptor? These functional variations coupled with the ability to create and examine mutant receptors should help elucidate the pharmacology and biophysics of such ligand-receptor interactions.

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Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception

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1 Capsazepine has recently been described as a competitive capsaicin antagonist. We have used this compound to test the hypotheses that the *in vitro* and *in vivo* effects of capsaicin are due to interactions with a specific receptor.

2 In an *in vitro* preparation of the neonatal rat spinal cord with functionally connected tail, the activation of nociceptive afferent fibres by the application of capsaicin, bradykinin or noxious heat (48°C) to the tail could be measured by recording a depolarizing response from a spinal ventral root. Application of capsaicin or substance P to the spinal cord also evoked a depolarizing response which was recorded in a ventral root.

3 When capsazepine (50 nM–20 μM) was administered to the tail or spinal cord it did not evoke any measurable response. However on the tail, capsazepine reversibly antagonized ($IC_{50} = 254 \pm 28$ nM) the responses to capsaicin but not to heat or bradykinin administered to the same site. Similarly capsazepine administration to the spinal cord antagonized the responses evoked by capsaicin ($IC_{50} = 230 \pm 20$ nM) applied to the cord but not responses evoked by substance P on the cord or by noxious heat and capsaicin on the tail.

4 In halothane anaesthetized rats, C-fibre responses evoked by transcutaneous electrical stimulation of the receptive field were recorded from single wide dynamic range neurones located in the spinal dorsal horn. C-fibre evoked discharges were consistently reduced by the systemic administration of capsaicin (20 μmol kg^{-1}, s.c.) and this action of capsaicin was antagonized by capsazepine (100 μmol kg^{-1}) administered by the same route. In addition the systemic effect of capsaicin was antagonized by a spinal intrathecal administration of capsazepine (5–50 nmol).

5 Intradermal injections of capsaicin, localized to the peripheral receptive field, usually one toe of the ipsilateral hind-paw, produced a transient increase in C-fibre-evoked activity followed by a prolonged period of localized insensitivity to transcutaneous C-fibre stimulation. These effects of capsaicin were significantly reduced by the concommitant administration of capsazepine to the same site.

6 These data demonstrate that capsazepine is a selective antagonist of capsaicin on nociceptive neurones *in vitro* and *in vivo* and suggest that the effects of capsaicin were mediated by activation of a specific receptor. Since the antinociceptive effect produced by systemically administered capsaicin was antagonised by spinal intrathecal capsazepine this further supports the hypothesis that capsaicin exerts its antinociceptive effect by acting on specific receptors localized to sensory nerve fibres in the spinal cord.

Keywords: Capsaicin; capsazepine; peripheral nociceptors; spinal cord; antagonist

Introduction

Capsaicin (8-methyl N-vanillyl-6-nonenamide) produces a number of effects by selective actions on mammalian polymodal nociceptive neurones and warm thermoceptors (Fitzgerald, 1983; Szolcsanyi, 1985; 1990; Buck & Burks, 1986; Bevan *et al.*, 1987). In man, local applications of capsaicin produce a sensation of burning-pain due to the activation of primary afferent C- and probably Aδ-neurones. However, with repeated exposure to capsaicin both the C-fibre activation and the algesic effect desensitize (Petsche *et al.*, 1983; Marsh *et al.*, 1987). On the other hand acute systemic administration of capsaicin produces antinociception which has been shown by several conventional tests (Hayes & Tyers, 1980; Hayes *et al.*, 1984; Campbell *et al.*, 1989). The basis for the reversible antinociceptive effect of capsaicin is unclear though recent studies (Dickenson *et al.*, 1990a,b) have indicated that sensory nerve terminals in the spinal cord are important.

It has been suggested that the effects of capsaicin on mammalian sensory neurones are mediated through an interaction with a specific membrane receptor. However, the evidence for this has been rather indirect. Thus a capsaicin recognition site has been proposed from structure-activity studies (Szolcsanyi & Jancso-Gabor, 1975; 1976; Hayes *et al.*, 1984) and by the use of a capsaicin-like photoaffinity probe (James *et al.*, 1988). Somewhat more directly, capsaicin has been shown to displace the binding of [^{3}H]-resiniferatoxin (Szallasi & Blumberg, 1990), a highly potent, naturally occurring capsaicin analogue (Szallasi & Blumberg, 1989; Winter *et al.*, 1990) with a similar mechanism of action (Winter *et al.*, 1990). Recently however, a selective capsaicin antagonist, capsazepine, has been described (Bevan *et al.*, 1991; Dray *et al.*, 1991) which has provided direct pharmacological evidence for a capsaicin receptor localized on the mammalian sensory neural membrane.

In the present experiments we have used capsazepine to indicate whether the capsaicin-mediated activation of nociceptors *in vitro* or *in vivo* is a consequence of specific receptor interactions. Furthermore we have sought additional evidence for sensory elements in the spinal cord as primary targets for the acute analgesic action of systemic capsaicin. Parts of this study have been published as abstracts (Dickenson *et al.*, 1991; Dray *et al.*, 1991).

Methods

The in vitro *preparation*

The intact spinal cord and the functionally connected tail were removed from 1–2 day old rats following decapitation

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and prepared by the method described by Otsuka & Yanagisawa (1988). The skin was carefully removed from the distal four fifths of the tail. This procedure was thought to expose cutaneous fibres and their endings to facilitate activation of nociceptors by capsaicin and other algesic agents (Dray *et al.*, 1990a,b). Damage to the underlying tissue was avoided as this severely compromised responsiveness to peripheral stimuli. We cannot be certain however that our tests were made in an environment entirely free of tissue damage. Histology was not routinely performed. Indeed electron microscopy would have been necessary to confirm the structural integrity of fine afferent nerve endings and quantification of this would have presented considerable difficulty. However bradykinin, which was used routinely as an algesic chemical stimulus, is not known to activate axons of nociceptors. It is more likely to stimulate nociceptors via the signal transducing elements localized to the terminations of primary afferent fibres. The efficacy of bradykinin thus suggested that nociceptors were preserved in our viable preparations. This would strongly indicate minimal nerve fibre damage. In addition the effects of our peripheral stimuli were robust and reproducible over many hours. This also would be unlikely in the face of significant tissue damage.

The preparation was placed in a chamber such that the cord and tail could be separately superfused (2–4 ml min^{-1}) with a physiological salt solution (composition mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10) at 24°C and gassed with 95% O_2/5% CO_2. Peripheral nociceptive fibres were activated by superfusion of the tail with noxious chemicals (capsaicin, bradykinin) and by superfusate heated to 48°C. Each stimulus was applied for 10 s with an intervening period of 15 min between stimuli. Bradykinin doses were separated by at least 40–60 min to avoid tachyphylaxis.

The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root (L_3–L_5). The ventral root depolarization was recorded d.c. (with respect to the spinal cord which was earthed) with a low impedence glass pipette. This was placed in an electrolyte-filled well which contained the selected ventral root. The signals were amplified by conventional means and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder.

Reproducible ventral root responses were obtained to peripheral stimuli including noxious heat and submaximal concentrations of capsaicin and bradykinin. Following this capsazepine was administered in the tail superfusate for 15 min prior to retesting capsaicin and other stimuli. The antagonist potency (IC_{50}) of capsazepine was estimated by cumulatively increasing the concentration to produce an incremental reduction of the response to capsaicin. Three or more submaximal concentrations of capsazepine were used in each experiment to determine the IC_{50} concentration. Ventral root responses were also used to measure the activation of afferent fibres and postsynaptic elements in the spinal cord by spinal administrations of capsaicin and substance P respectively. The effect of capsazepine administered to the spinal cord was determined as described above against responses evoked by administration of substance P and capsaicin to the spinal cord and to responses evoked by noxious heat and capsaicin administration to the tail.

In vivo *preparation*

Single unit recordings were made from dorsal horn neurones in the intact halothane-anaesthetized rat (Dickenson & Sullivan, 1987). Following induction of anaesthesia (3% halothane in O_2/N_2O) a tracheal cannula was inserted and a laminectomy was made to expose the spinal L_1–L_3 area. The animal was then mounted in a frame so that the vertebrae rostral and caudal to the recording site were securely clamped. Anaesthesia was then maintained by 1–1.5% halothane. A tungsten-glass microelectrode was inserted into the dorsal horn and extracellular recordings were made, by conventional techniques, from single neurones located in the superficial and deep laminae. All cells responded to innocuous peripheral stimuli (touch/brush/pressure) and to noxious thermal and mechanical (pinch) stimuli. Cells were characterized by their responses to the natural peripheral stimuli mentioned above and then two fine needles were placed in the center of the hindpaw receptive field for transcutaneous stimulation. Both Aβ and C-fibre responses, based on latency and threshold could always be elicited in the neurones, and on occasion Aδ fibre responses were observed. Following at least two stable control responses (3 times threshold for the C-fibre and then Aβ-fibre responses), capsaicin or the vehicle (10% Tween/10% ethanol (10%); physiological saline) were administered at the following sites: (1) subcutaneously into the scruff of the neck, (2) intrathecally in a volume of 50 μl onto the exposed surface of the spinal cord and (3) locally, in a 10 μl volume into a discrete region of the receptive field. Administration at these sites was repeated with the co-administration of capsazepine or with capsazepine alone. The effect of intrathecal capsazepine was also tested against the effect of systemic capsaicin. Responses to transcutaneous stimulation were measured at 5 min post injection and then at 10 min intervals for between 45 and 180 min. Responses were quantified by use of post stimulus histograms, analysed and built with a CED 1401 interface and software (Dickenson & Sullivan, 1990). Only one cell was used per animal for each systemic drug study. Data were statistically evaluated by the unpaired *t* test (95% confidence limits).

The following drugs were used: capsaicin (Sigma), capsazepine (2-[2-4-chlorphenyl)ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro- 1H-2-benzazepine; bradykinin (Bachem, CRB).

Results

In vitro *study*

As in previous studies (Dickenson *et al.*, 1990; Dray *et al.*, 1990a,b), a brief administration of capsaicin at a submaximal concentration (usually 0.5–0.7 μM), in the tail superfusate, evoked a short lived (45–90 s) depolarizing response in a ventral root. These responses could be reproduced over several hours. In addition stable and reproducible responses could be evoked by brief noxious heat stimulation of the tail. In a number of experiments responses evoked by bradykinin (100–350 nM) were used to test further the selectivity of the antagonist action of capsazepine.

Continuous superfusion of the tail with capsazepine (50 nM–20 μM) for 15 min preceding and during the administration of capsaicin or other stimuli did not itself evoke any response. However capsazepine consistently and reversibly (within 30–60 min) attenuated the effect of capsaicin in a concentration-related manner (Figure 1). The capsazepine IC_{50} concentration was 254 $\pm$ 28 nM (n = 8). However, even at the highest concentration tested (20 μM), capsazepine did not affect the responses evoked by heat (n = 8) or bradykinin (n = 5) (Figure 1).

In a separate series of experiments, brief administration (10 s) of a submaximal concentration of capsaicin (200 nM), in the spinal cord superfusate, evoked reproducible depolarizing responses following the activation of nociceptive afferent nerve terminals. A selective effect of capsaicin on afferent terminals had been indicated from previous studies showing (a) capsaicin-evoked release of neuropeptide specifically located in fine afferent fibres (Theriault *et al.*, 1976; Dickenson *et al.*, 1990b), (b) lack of effect of capsaicin on spinal neurones following neurotoxic doses (Yaksh *et al.*, 1979) and (c) lack of a direct effect of capsaicin on spinal dorsal horn neurones measured electrophysiologically (Urban & Dray, 1990). Responses were also evoked by administration of a submaximal concentration of substance P (25 nM), presumably through activation of receptors on postsynaptic spinal elements (Akagi *et al.*,

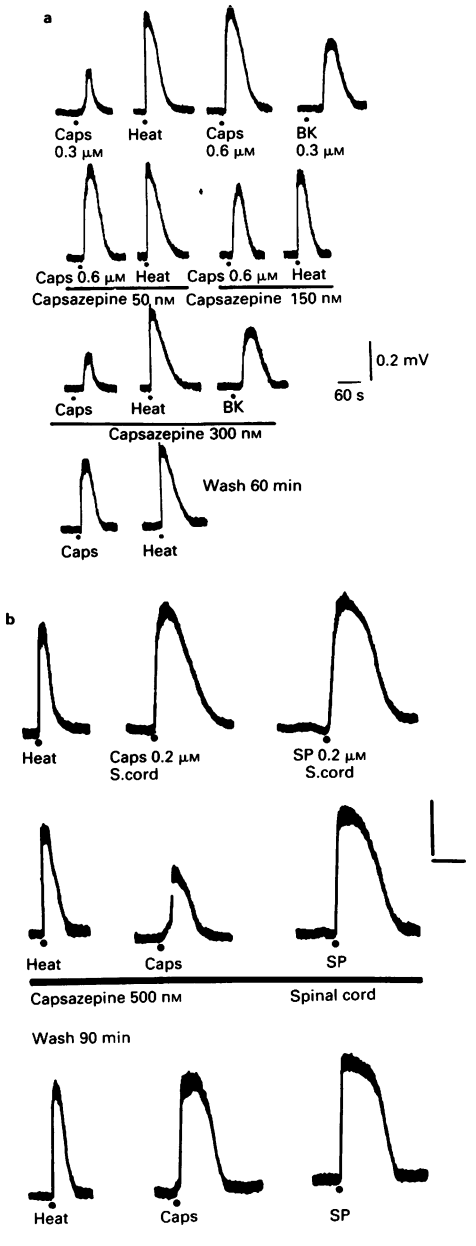


Figure 1 (a) Concentration-related antagonism of capsaicin (Caps) by capsazepine in the spinal cord/tail *in vitro*. The top traces show ventral root responses produced by application of capsaicin (0.3 and 0.6 μM), heat (48°C) and bradykinin (BK, 0.3 μM) to the tail. The second and third row of traces show concentration-related reduction of the response to capsaicin (0.6 μM) by capsazepine (50, 150 and 300 nM) but not the responses to heat or bradykinin (0.3 μM). The application of capsazepine was started 15 min before capsaicin was tested and the concentration was increased cumulatively. The response to capsaicin recovered following a 60 min wash of the tissue (bottom traces). The calibration bars of 60 s and 0.2 mV are indicated in (a) and (b). (b) Selective antagonism of capsaicin on the spinal cord by capsazepine. The top traces show control responses to heat administered to the tail and to capsaicin (0.2 μM) and substance P (SP, 0.2 μM) administered in the spinal cord superfusate. During the continuous administration of capsazepine to the spinal cord (500 nM), begun 15 min before the agonists were retested, the effect of capsaicin was selectively reduced. The bottom traces show complete recovery of capsaicin responses after washing the tissue for 90 min.

1985). Such responses, together with the ventral root responses evoked by peripheral application of heat or capsaicin, served as controls to check for selectivity of the effect of capsazepine.

As before, prolonged superfusions of the spinal cord with capsazepine (50 nM–5 μM) did not produce any response or change the responses evoked by spinal administration of substance P. In addition, responses evoked by peripheral noxious heat or capsaicin applications were unaffected (Figure 2). However, the responses evoked by capsaicin administration to the spinal cord were consistently attenuated in a concentration-related manner (IC_{50} = 230 ± 20 nM, n = 5). In each case recovery from the effect of capsazepine occurred within 30–60 min.

In vivo *study*

Administration into the receptive field Eight dorsal horn neurones were studied. All had receptive fields in two or more toes. Each neurone served as its own control. Thus the effect of capsaicin alone (200 pmol) and capsaicin plus capsazepine (1 nmol) was tested on each cell. Each cell responded to C-fibre stimulation (3 × threshold) with a mean response of 20 ± 6 spikes per stimulus. After capsaicin, none of the cells responded to C-fibre stimulation for at least 1 h afterwards. Each cell was activated by Aβ-fibre stimulation but these responses were unchanged by capsaicin. After the co-administration of capsazepine with capsaicin the effect of capsaicin was completely abolished. Thus the mean C-fibre evoked responses of the population was 17 ± 5 spikes per stimulus and this was not significantly different from values obtained following vehicle injections. The same concentration of capsazepine also prevented the activation of the neurones by capsaicin (Figure 2). Capsazepine alone (n = 4), had no effect on cell firing or on C-fibre evoked responses.

Systemic capsazepine versus systemic capsaicin Capsaicin (20 μmol kg^{-1}) injected subcutaneously into the scruff of the neck at a dose found to produce antinociception both in behavioural and electrophysiological studies (Campbell *et al.*, 1989; Dickenson *et al.*, 1990a), produced a maximal 66% inhibition of the C-fibre evoked responses of dorsal horn cells at 40 min post injection (n = 7). In three other animals the administration of capsaicin was immediately preceded by a s.c. injection of 100 μmol kg^{-1} capsazepine into the same or another s.c. site. In each of these studies the inhibitory effect of

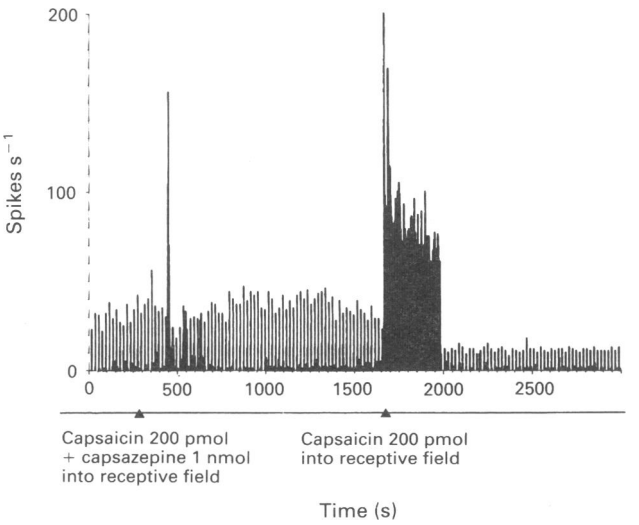


Figure 2 Example of a ratemeter recording of a dorsal horn nociceptive neurone in response to transcutaneous electrical stimulation of the receptive field (two toes on the ipsilateral hindpaw). The combined Aβ and C-fibre evoked response is plotted as spikes s^{-1} (vertical axis) against time (s). The combined intradermal injection of capsaicin (200 pmol) and capsazepine (1 nmol) produced minimal C-fibre activation and little evoked effect on the neurone (injected into toe 1), whereas capsaicin alone (injected into toe 2) activated the cell and then abolished the C-fibre evoked response. The residual activity seen in the post-stimulus histogram after capsaicin was due entirely to the Aβ-fibre evoked activity.

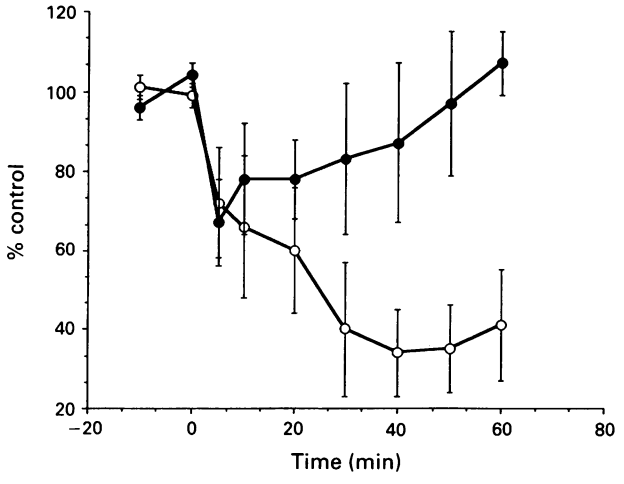


Figure 3 The C-fibre-evoked responses of dorsal horn neurones, expressed as percentage (with s.e.mean shown by vertical bars) of the pre-injection control responses, were inhibited by s.c. capsaicin ($20\,\mu mol\,kg^{-1}$, $n = 7$, ○). Co-administration of capsazepine ($100\,\mu mol\,kg^{-1}$, s.c., $n = 3$, ●) together with s.c. capsaicin produced a significant reduction ($P < 0.05$), at the 30, 40, 50 and 60 min time points. The control readings prior to the application of the agents at time 0 are shown.

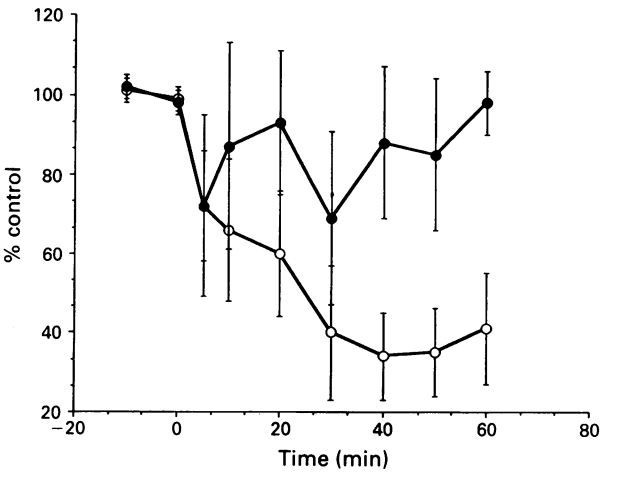


Figure 4 The inhibition of the C-fibre responses of dorsal horn nociceptive neurones produced by s.c. capsaicin ($20\,\mu mol\,kg^{-1}$, s.c., $n = 7$, ○) was attenuated by intrathecal capsazepine (50 nmol) applied just prior to the capsaicin ($n = 5$, ●). The capsaicin response was significantly reduced ($P < 0.01$) at the 40, 50 and 60 min time points. Applicatoin of the drugs was at time zero.

capsaicin was now transient and at 30 min post injection C-fibre evoked activity was depressed by only 13% (Figure 3). The response of cells to Aβ fibre stimulation was unaffected by capsaicin, capsazepine or by the combination of these substances.

Intrathecal capsazepine and systemic capsaicin Neuronal activity was recorded for at least 60 min following the intrathecal administrations of capsazepine. No significant change in spontaneous firing of dorsal horn cells was observed and no effects on responses evoked by C- or Aβ-fibre stimulation were produced by 5 nmol, (4 cells) or 50 nmol (3 cells) capsazepine.

In control animals ($n = 7$), s.c. capsaicin ($20\,\mu mol\,kg^{-1}$) produced a gradual reduction of the discharges evoked by C-fibre stimulation. This effect plateaued some 40 min after the injection and reached a maximum of 66% inhibition. The Aβ-fibre evoked responses of the same cells were maximally reduced by only 25% at 30 min after the adminstration of capsaicin. Intrathecal capsazepine (50 nmol; $n = 5$) significantly ($P < 0.05$) reduced (at the 40, 50 and 60 min time points) the inhibitory effect of systemic capsaicin (Figure 4). Following capsazepine the responses of these cells to Aβ fibre input was maximally reduced by capsaicin by only 8%. When the C-fibre evoked responses were analysed, taking into account the firing frequency and duration of the response, the intrathecal capsazepine-induced reduction of capsaicin (by 58%, $P < 0.01$, Figure 4) was highly significant. Also the antagonism of capsaicin by capsazepine appeared to be dose-related since the inhibition of C-fibre responses produced by capsaicin ($20\,\mu mol\,kg^{-1}$, s.c.) was unaffected by a lower dose (5 nmol, i.t.) of capsazepine ($n = 4$).

Discussion

Indirect evidence obtained from a number of previous studies has supported the suggestion that the unique effects of capsaicin on mammalian sensory neurones was due to an interaction with a specific membrane receptor (Szolcsanyi & Jancso-Gabor, 1975; 1976; Hayes *et al.*, 1984; James *et al.*, 1988; Szallasi & Blumberg, 1989; 1990). This hypothesis now receives direct support following the discovery of capsazepine, a capsaicin analogue with selective antagonist properties. Capsazepine antagonized a number of the effects of capsaicin *in vitro*, including the evoked whole cell membrane currents and the flux of a number of cations (Bevan *et al.*, 1991). The antagonistic effect was both selective and competitive.

The present *in vitro* experiments further extend these observations. We have thus shown, as in previous studies (Yanagisawa & Otsuka, 1984; Dray *et al.*, 1990a,b) that physiological stimulation of peripheral nociceptive fibres by capsaicin and a number of other noxious stimuli, can evoke reproducible responses in the spinal cord, maintained *in vitro* with the functionally connected tail. Capsazepine, at concentrations which itself produced no discernible effect on peripheral nerves or on the excitability of the spinal cord, consistently and reversibly antagonized the effects of capsaicin when both substances were administered to the same peripheral or central elements of sensory neurones. Capsazepine did not reduce the responses evoked by capsaicin when each substance was administered to different regions (i.e. spinal vs. peripheral) of sensory neurones.

Although the present experiments did not test whether capsazepine was a competitive antagonist, they clearly demonstrated that capsazepine was selective, since responses produced by noxious heat, bradykinin or substance P administered to the periphery or spinal cord respectively, were unaffected. The potency of capsazepine, under the present conditions, was similar to that measured in dorsal root ganglion neurones or vagal afferent fibres maintained in vitro (Bevan *et al.*, 1991) where competitive interactions were also demonstrated. This supports the possibility that the capsaicin receptive site is similar in a variety of *in vitro* preparations of sensory neurones.

In addition we have tested capsazepine against a number of effects mediated by capsaicin *in vivo*: specifically against the effects produced by local and systemic capsaicin administrations. Thus capsazepine antagonized the initial capsaicin-induced C-fibre activation and the subsequent capsaicin-induced inactivation of C-fibres following the administration of capsaicin into the peripheral receptive field, (usually a single toe) of dorsal horn neurones. These effects were clearly selective and well localized as a subsequent administration of capsaicin into another part of the receptive field (another toe), evoked both an initial activation and a subsequent blockade of C-fibre evoked activity. In keeping with our previous observations (Dickenson *et al.*, 1990a,b), systemic capsaicin also reduced C-fibre evoked responses of dorsal horn nociceptive neurones. This antinociceptive effect of capsaicin was also reduced by the concomitant administration of capsazepine by the same route. Finally and significantly, spinal intrathecal administration of capsazepine prevented the reduction of the C-fibre evoked input produced

by systemic capsaicin. This was considered to be due to an antagonism of a capsaicin-induced effect exerted at the level of the spinal cord since (a) capsazepine itself did not produce a response or alter nerve excitability but acted only as a selective capsaicin antagonist and (b) the amount of capsazepine administered i.t. was insufficient to antagonize the systemic effect of capsaicin should diffusion out of the spinal cord have occurred.

In summary, the present experiments have demonstrated that capsazepine is a selective antagonist against a number of capsaicin-induced effects on nociceptive neurones *in vitro* and *in vivo*. In addition, the effectiveness of capsazepine following spinal intrathecal administration further supports the likelihood that the systemic antinociceptive effect of capsaicin is produced by an action within the spinal cord as we have previously suggested (Dickenson *et al.*, 1990a,b).

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Endothelin-1 inhibits pre-stimulated tracheal submucosal gland secretion and epithelial albumin transport

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1 Endothelin-1 potently contracts smooth muscle, including that in the airways. However, its effect on airway mucosal function has not so far been studied.

2 We have used the ferret whole trachea *in vitro* to examine the effect of endothelin-1 on tracheal smooth muscle tone, transepithelial potential difference (p.d.), submucosal gland secretion (including lysozyme secretion from serous cells) and active epithelial albumin transport. In addition we have examined the effects of endothelin on submucosal gland secretion and albumin transport pre-stimulated with the muscarinic agonist methacholine and the α-adrenoceptor agonist phenylephrine. The effects of the Ca^{2+} channel blocker nifedipine on the responses to endothelin have also been assessed.

3 Endothelin (0.1–100 nM) produced concentration-dependent increases in intraluminal tracheal pressure indicating smooth muscle contraction, and in the negativity of the transepithelial p.d. These effects were partially inhibited by nifedipine (10 μM).

4 Endothelin (0.01–100 nM) had no significant effect on baseline rates of mucus, lysozyme or albumin outputs, but produced concentration-dependent reductions in maintained methacholine- and phenylephrine-induced mucus, lysozyme and albumin outputs. In general endothelin was more potent against methacholine-induced effects. All of the concentration-response curves for endothelin were shallow and some appeared to be biphasic, suggesting the possibility of more than one mechanism of action of endothelin.

5 The effects of endothelin (at concentrations >1 nM) on phenylephrine-induced mucus volume, lysozyme and albumin outputs were significantly inhibited by nifedipine. Similarly the effect of endothelin (>1 nM) on methacholine-induced mucus volume and albumin outputs (but not lysozyme output) was attenuated by nifedipine. The effects of endothelin (at concentrations <1 nM) on methacholine and phenylephrine-induced responses were generally not affected by nifedipine.

6 Thus, endothelin contracts ferret tracheal smooth muscle and increases transepithelial p.d. at least in part by opening dihydropyridine-sensitive Ca^{2+} channels. Endothelin does not directly stimulate submucosal gland secretion or epithelial albumin transport, but inhibits methacholine- and phenylephrine-induced secretion and transport. The inhibitory effects produced by higher concentrations of endothelin may be mediated partially by activation of dihydropyridine-sensitive Ca^{2+} channels, although the explanation for this is not clear. The mechanism of action of endothelin in attenuating stimulated secretion and epithelial transport at lower concentrations is unknown.

Keywords: Endothelin; methacholine; phenylephrine; lysozyme; albumin; epithelium; submucosal glands

Introduction

The endothelins are a family of 21-amino acid peptides recently discovered in the human genome (Inoue *et al.*, 1989). Of the three identified peptides, namely endothelin-1, endothelin-2 and endothelin-3, endothelin-1 is identical to porcine endothelin which was originally isolated from procine cultured aortic endothelial cells (Yanagisawa *et al.*, 1988). The endothelins, including endothelin-1, are potent vasoconstrictors both *in vitro* and in intact animals (Lippton *et al.*, 1988; Walder *et al.*, 1989; Rodman *et al.*, 1989). The endothelins not only contract vascular smooth muscle but also bronchial smooth muscle from several species, with endothelin-1 being the most potent (Advenier *et al.*, 1990; Hay, 1990; Hye-Kyung *et al.*, 1990; McKay *et al.*, 1991).

Immunofluorescent staining for endothelin-1 occurs in epithelial cells from the hilum to the periphery of the conducting airways of mice (Rozengurt *et al.*, 1990). In addition, epithelial cells from human bronchi (Mattoti *et al.*, 1990), and canine and porcine trachea (Black *et al.*, 1989) form and release endothelin-like material. However, little is known about the effects of endothelins on airway epithelial function and the function of other mucosal tissues such as the submucosal glands. Therefore we have used the ferret trachea *in vitro* to examine the effect of endothelin-1 on transepithelial potential difference (p.d.) (indicating epithelial function and integrity), submucosal gland secretion (using lysozyme as a marker for serous cell secretion) and the active transport of albumin across the tracheal epithelium (Webber & Widdicombe, 1989), as well as on tracheal smooth muscle tone.

There is some debate as to the mode of action of endothelins on smooth muscle. In initial studies it was suggested that, since endothelin had marked structural similarities to sodium channel openers and its contractile effect on vascular smooth muscle was blocked by nicardipine, it may be an endogenous agonist of dihydropyridine-sensitive voltage-dependent calcium channels (Yanagisawa *et al.*, 1988). However, more recent studies have indicated that this may not be the case (Gu *et al.*, 1989). Contraction of airway smooth muscle by endothelin may be mediated by dihydropyridine-sensitive calcium channels (Advenier *et al.*, 1990), by other calcium channels (Sarria *et al.*, 1990), by release of cyclo-oxygenase products such as thromboxane A_2 (Battistini *et al.*, 1990) or by none of these mechanisms (Hay, 1990). We have assessed the role of dihydropyridine-sensitive voltage-dependent calcium channels in the responses to endothelin in the present study by using the Ca^{2+} channel antagonist nifedipine.

Methods

The ferret trachea in vitro

The preparation of the ferret trachea has previously been described in detail (Webber & Widdicombe, 1987). Briefly, the trachea was removed, cannulated at both ends, and mounted

laryngeal end down in an organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the buffer was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2/5% CO_2. The lumen of the trachea remained air-filled. Secretions carried by gravity and mucociliary transport to the lower cannula were withdrawn periodically into catheters and washed out into vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the albumin and lysozyme assays. Frozen storage for up to 6 months does not affect the lysozyme activity or albumin content of the samples. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and secretion rates were expressed as $\mu l\,min^{-1}$ (assuming 1 g of secretion is equivalent to 1 ml).

The carinal cannula was attached to a digital manometer. Changes in smooth muscle tone produced changes in intraluminal tracheal pressure which were registered by the manometer. The electrical potential difference (p.d.) across the trachea was measured by two calomel reference electrodes filled with 3.8 M KCl and placed in separate beakers of the same solution. Electrical contact was made across the preparation with two agar bridges constructed from polyethylene tubing (0.5 mm internal diameter) and filled with 3.8 M KCl in 2.5% w/v agar solution. One bridge was placed in the submucosal buffer and the second inserted into a second hole in the cannula used to collect the mucus. Electrical contact between this bridge and the tracheal luminal wall was maintained by the mucus collecting in the perspex cannula. Output from the two electrodes passed into a high input impedance buffer amplifier and was then displayed on a digital voltmeter. The two agar bridges were initially placed together in 0.15 M NaCl to confirm that this produced a stable p.d. close to 0 V. Any residual voltage measured here was subtracted from subsequent measurements of p.d. made in the preparation. Before the start of an experiment each trachea was allowed to equilibrate for 20 min, with changes of bathing medium every 5 min.

Assay for lysozyme

Addition of lysozyme to a solution of the bacterium *Micrococcus lysodeikticus* reduced the turbidity of the solution leading to a fall in optical density (o.d.) at 450 nm. A suspension of *M. lysodeikticus* ($3\,mg\,ml^{-1}$) was prepared which when diluted 10 fold (the dilution in the assay) gave an $o.d._{450}$ of approximately 0.6. Hen egg white lysozyme (0.5 to $100\,ng\,ml^{-1}$) was incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* ($0.3\,mg\,ml^{-1}$), sodium azide ($1\,mg\,ml^{-1}$) and bovine serum albumin (BSA, $1\,mg\,ml^{-1}$). BSA is a protein stabilizer and sodium azide prevents bacterial growth in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C and the $o.d._{450}$ measured with potassium phosphate buffer pH 7.4 containing BSA ($1\,mg\,ml^{-1}$) as a blank. The concentration of lysozyme in a mucus sample was estimated by incubating 20 μl of sample exactly as for the known concentrations of lysozyme. The rate of output of lysozyme equalled the total amount of lysozyme in a mucus sample divided by the time over which the sample accumulated.

Albumin transport

BSA ($4\,mg\,ml^{-1}$) was added to the buffer bathing the submucosal surface of the trachea together with fluorescent BSA ($0.02–0.03\,mg\,ml^{-1}$). The fluorescent albumin concentration (excitation 490 nm and emission 550 nm) of the mucus samples was estimated from a standard curve (range $25\,ng\,ml^{-1}$ to $3\,\mu g\,ml^{-1}$). Total albumin concentration was obtained by multiplying the fluorescent albumin concentration by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin equalled the total amount of albumin in a mucus sample divided by the time over which that sample accumulated.

Experimental protocol

Effect of endothelin on baseline variables After a 30 min control, three or four concentrations (0.1–100 nM) of endothelin were added randomly to the submucosal buffer. Each concentration was left in contact with the trachea for 30 min and any change in smooth muscle tone was recorded. After 30 min any secretion produced was withdrawn and processed. One or two control periods of 30 min were allowed between additions of endothelin. Measurements of p.d. were taken 5 min before the end of each 30 min period. Changes in mucus volume output produced by endothelin were calculated as the difference in output between the control period immediately before the endothelin was added and the period when endothelin was present.

Effect of endothelin on responses to methacholine and phenylephrine Methacholine and phenylephrine increase mucus volume, lysozyme and albumin outputs from the ferret trachea in a concentration-dependent manner (Webber & Widdicombe, 1987; 1989). After a 30 min control, either methacholine (20 μM) or phenylephrine (100 μM) was added to the buffer. These concentrations of methacholine and phenylephrine produced 70–80% of their respective maximum responses for mucus volume, lysozyme and albumin outputs. Mucus samples were taken every 30 min until a steady 'maintained' mucus volume output was obtained (2.5–3 h). Every 30 min the buffer was replaced with buffer containing either methacholine or phenylephrine. When a maintained mucus volume output had been obtained, five concentrations of endothelin (0.01–100 nM) were added in ascending order to the buffer containing the secretagogue. Each concentration of endothelin was left in contact with the trachea for 30 min, when the secretion produced was withdrawn and processed. The buffer was then replaced with buffer containing the secretagogue and the next concentration of endothelin. Changes in mucus volume output produced by endothelin were calculated as the difference in output obtained in the period immediately before the peptide was added and the period when the peptide was in the organ bath, expressed as a percentage.

Effect of nifedipine on responses to endothelin

Baseline responses to endothelin Tracheae were set up in pairs, in separate organ baths; one acted as a control (not exposed to nifedipine) and one as a test (nifedipine present throughout experiment). Both tracheae were exposed to three concentrations of endothelin (0.1–10 nM) given randomly. At least two control periods of 30 min were allowed between additions of peptide. The maximum change in intraluminal pressure and p.d. produced by each concentration of endothelin was recorded.

Effects of endothelin on methacholine and phenylephrine Tracheae, again set-up in pairs, were exposed to methacholine or phenylephrine until a maintained mucus volume output was achieved. The buffer surrounding the test trachea was then replaced with buffer containing the secretagogue and nifedipine (1 μM). After 30 min this was replaced with buffer containing the secretagogue, nifedipine and endothelin (0.1 nM). This procedure was repeated for two further concentrations of endothelin (1 and 10 nM). The test trachea was then exposed to buffer containing secretagogue and nifedipine but no endothelin for two further 30 min periods. The same protocol was used for the control trachea except that nifedipine was absent throughout.

Analysis of results

Concentration-response curves for endothelin were obtained by pooling results from a number of experiments. The effects

of endothelin on baseline, and on methacholine- and phenylephrine-induced responses were analysed for significance by use of repeated measures analysis of variance followed by Student's two-tailed t test for paired observations, comparing the response to a particular concentration of endothelin to the previous control. Differences between methacholine- or phenylephrine-induced responses in control and test tracheae, and the effect of nifedipine on maintained responses to methacholine and phenylephrine were analysed by a paired t test. Differences between concentration-response curves for endothelin in the presence and absence of nifedipine were analysed for significance by use of a repeated measures analysis of variance followed by paired t tests at each concentration of endothelin. In all cases $P < 0.05$ was taken as significant.

Materials

Materials used were acetyl-β-methylcholine chloride (methacholine), phenylephrine hydrochloride, nifedipine, bovine serum albumin (BSA), FITC-BSA, lysozyme (hen egg white), *Micrococcus lysodeikticus* (all from Sigma); sodium azide (BDH) and endothelin-1 (Bachem).

Results

Effects of endothelin on baseline variables

The mucus volume output in control periods before the addition of any drugs was zero, i.e. no mucus was produced. As there was no mucus, lysozyme and albumin assays could not be carried out and so the output of these markers was also considered to be zero in these initial control periods. Endothelin (0.1–100 nM) had no significant effect on baseline mucus volume output (no mucus was produced by any concentration of endothelin). As with the control samples lysozyme and albumin assays could not be performed so it was assumed that endothelin has no effect on baseline lysozyme or albumin output.

Endothelin produced a concentration-dependent increase in intraluminal pressure indicating tracheal smooth muscle contraction (Figure 1a). The baseline p.d. before the addition of endothelin was -8.8 ± 1.1 mV. Endothelin (0.1–100 nM) produced small but significant concentration-dependent increases in the negativity of this p.d. (Figure 1b).

Effects of endothelin on responses to methacholine and phenylephrine

Addition of methacholine (20 μM) or phenylephrine (100 μM) to the bathing medium produced significant increases in mucus volume (1.64 ± 0.27 and 1.38 ± 0.33 μl min^{-1}, respectively), lysozyme (978 ± 227 and 773 ± 169 ng min^{-1}) and albumin (4.37 ± 1.61 and 3.64 ± 0.28 μg min^{-1}) outputs from previous control levels where the outputs were zero. On continued application of methacholine or phenylephrine the mucus volume, lysozyme and albumin outputs all fell but reached maintained levels (0.73 ± 0.22 and 0.47 ± 0.18 μl min^{-1}; 526 ± 69 and 429 ± 137 ng min^{-1}; 2.22 ± 0.52 and 2.04 ± 0.31 μg min^{-1}) at about the same time (2.5–3.5 h after the first addition of methacholine or phenylephrine).

Endothelin produced concentration-dependent reductions in maintained methacholine and phenylephrine-induced mucus volume, lysozyme and albumin outputs (Figure 2a,b,c). In the case of mucus volume and lysozyme output, endothelin at concentrations greater than 0.01 nM was significantly more potent at inhibiting the responses to methacholine than to phenylephrine (Figure 2a,b). In the case of albumin output, endothelin was approximately equipotent against methacholine- and phenylephrine-induced responses (Figure 2c). In all cases the concentration-response curves to endothelin were shallow occurring over a 10,000 fold concentration range. For inhibition of methacholine- and phenylephrine-induced mucus volume and albumin outputs the curves for endothelin showed a tendency to be biphasic with a first maximum between 0.1 and 1 nM and a further inhibition at concentrations greater than 1 nM.

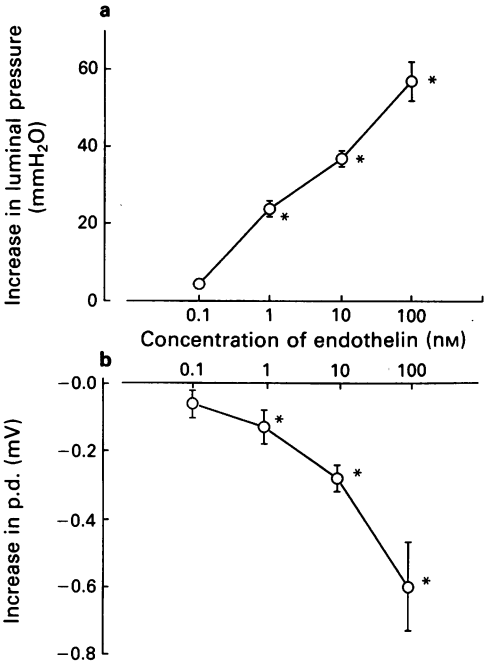


Figure 1 Concentration-response curves for the effect of endothelin-1 on (a) intraluminal tracheal pressure and (b) transepithelial p.d. in the ferret trachea *in vitro*. Points are means of 6 determinations and the vertical bars show s.e.means. * Significant ($P < 0.05$) increase in pressure or negativity of p.d. by Student's two-tailed t test for paired observations.

Effects of nifedipine

Baseline responses to endothelin Endothelin produced concentration-dependent increases in intraluminal pressure and in the negativity of p.d. in the absence and presence of nifedipine (Figure 3). However, for both pressure and p.d. the response to each concentration of endothelin was significantly less in the presence of nifedipine (Figure 3).

Responses to methacholine and phenylephrine In control and nifedipine-treated (test) tracheae methacholine (20 μM) and phenylephrine (100 μM) significantly increased mucus volume, lysozyme and albumin outputs (Table 1). There were no differences between these initial responses to methacholine and phenylephrine in control and test tracheae. On continued application of methacholine or phenylephrine the mucus volume, lysozyme and albumin outputs in control and test tracheae fell but reached maintained outputs significantly higher than baseline levels which were zero (Table 1). Again, there was no significant difference between the maintained outputs in control and test tracheae for methacholine or phenylephrine. Hence the addition of nifedipine to test tracheae had no significant effect on the mucus volume, lysozyme and albumin outputs produced by methacholine or phenylephrine.

Effects of endothelin on responses to methacholine and phenylephrine

Endothelin inhibited methacholine- and phenylephrine-induced mucus volume, lysozyme and albumin outputs in a concentration-dependent manner both in the presence and absence of nifedipine (Figure 4). With the exception of the effect of 1 nM endothelin on the albumin output response to methacholine, the inhibition by endothelin at 1 and 10 nM of

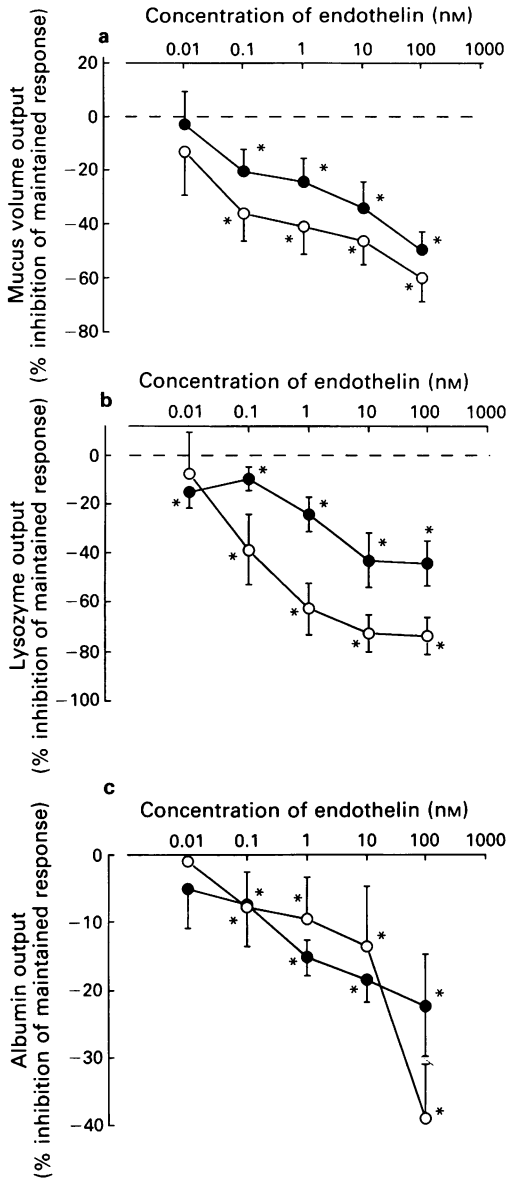


Figure 2 Concentration-response curves showing the effects of endothelin-1 on maintained (a) mucus volume output, (b) lysozyme output and (c) albumin output produced by (○) methacholine, 20 μM and (●) phenylephrine, 100 μM. Points are means of 4–6 determinations with s.e.means shown by vertical bars. * Significant ($P < 0.05$) reduction in methacholine or phenylephrine-induced output as assessed by Student's two-tailed t test for paired observations.

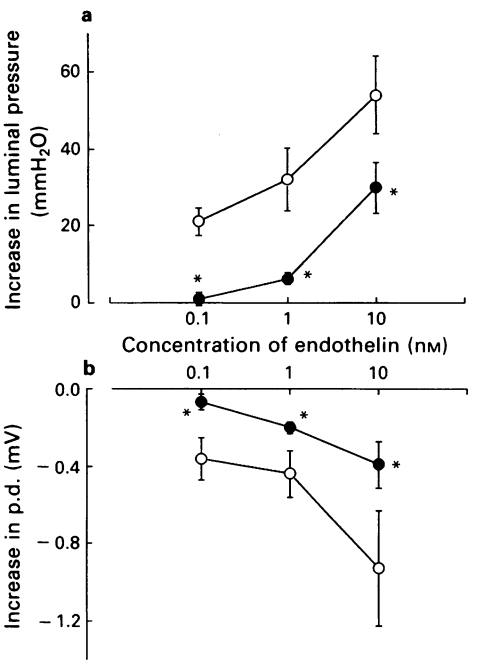


Figure 3 The effect of nifedipine (10 μM) on the concentration-response curves for endothelin-induced increases in intraluminal pressure (a) and negativity of p.d. (b). Responses to endothelin in the absence (○) and presence (●) of nifedipine. Points are means of 4–6 determinations with s.e.means shown by vertical bars. * Significant ($P < 0.05$) inhibition of endothelin-induced response as assessed by Student's two-tailed t test for paired observations.

the mucus volume and albumin output responses to both agonists was significantly reduced in the presence of nifedipine (Figure 4a,c), although the responses at 0.1 nM were not significantly affected. The inhibition by endothelin (all concentrations) of phenylephrine-induced lysozyme output was also reduced in the presence of nifedipine, but the inhibition of methacholine-induced output was not affected (Figure 4b).

Discussion

Endothelin produced a concentration-dependent increase in intraluminal tracheal pressure, indicating contraction of the tracheal smooth muscle. These results are consistent with previous studies showing endothelin to be a potent bronchoconstrictor *in vivo* (Matsuse *et al.*, 1990) and *in vitro* on isolated preparations from a number of species, including man

Table 1 Initial and maintained responses to methacholine and phenylephrine in control and test (nifedipine-treated) tracheae

	Control tracheae		*Nifedipine-treated test tracheae*		
				Maintained response	
	Initial response	*Maintained response*	*Initial response*	*Before nifedipine*	*With nifedipine*
Methacholine					
Mucus volume (μl min^{-1})	1.82 ± 0.27	0.71 ± 0.22	1.76 ± 0.19	0.65 ± 0.22	0.59 ± 0.18
Lysozyme (ng min^{-1})	1026 ± 173	772 ± 64	994 ± 104	743 ± 73	753 ± 61
Albumin (μg min^{-1})	3.96 ± 0.81	2.62 ± 0.47	4.22 ± 0.73	2.75 ± 0.76	2.58 ± 0.72
Phenylephrine					
Mucus volume (μl min^{-1})	1.27 ± 0.42	0.42 ± 0.18	1.16 ± 0.32	0.33 ± 0.05	0.27 ± 0.05
Lysozyme (ng min^{-1})	807 ± 93	631 ± 59	822 ± 103	620 ± 146	581 ± 132
Albumin (μg min^{-1})	3.39 ± 0.52	1.17 ± 0.18	3.52 ± 0.29	1.06 ± 0.10	0.97 ± 0.31

In test tracheae, responses to methacholine and phenylephrine immediately before and during exposure to nifedipine are shown. There are no significant differences ($P > 0.05$, paired t test) in initial and maintained responses between control and test tracheae.

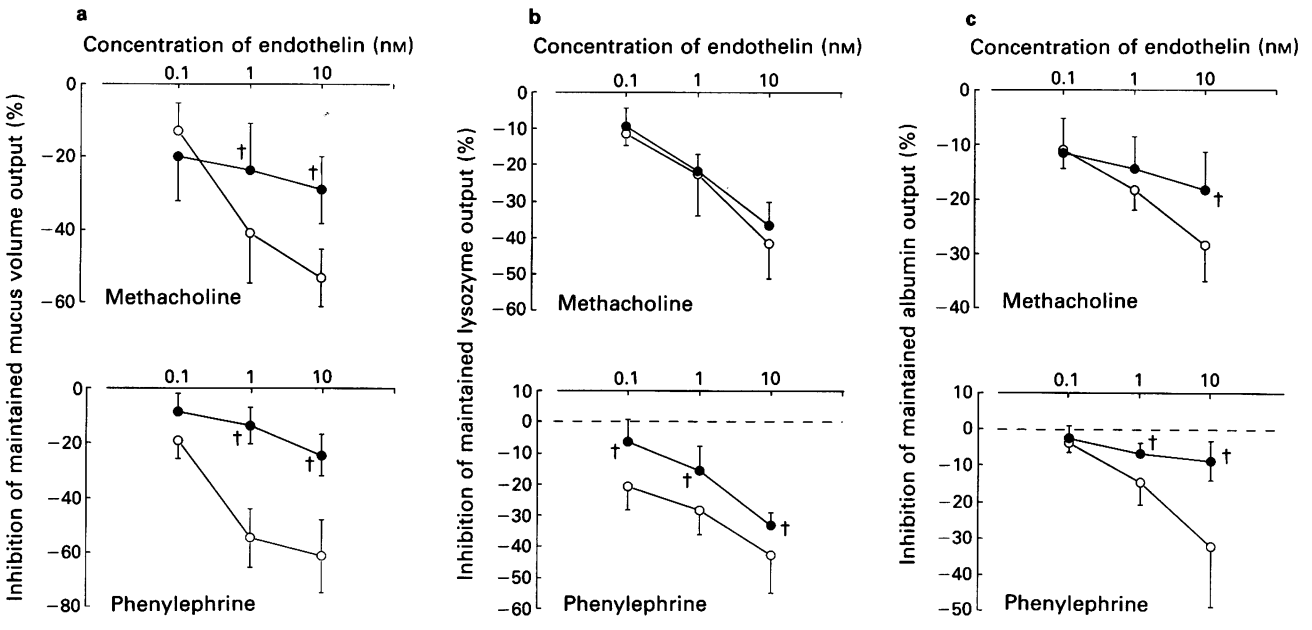


Figure 4 The effect of nifedipine (10 μM) on the endothelin-induced inhibition of (a) maintained mucus volume, (b) lysozyme and (c) albumin outputs produced by methacholine (20 μM) and phenylephrine (100 μM). Responses to endothelin in the absence (○) and presence (●) of nifedipine. Points are means of 4–6 determinations with s.e.means shown by vertical bars. † Significant ($P < 0.05$) inhibition of endothelin-induced response as assessed by Student's two-tailed t test for paired observations.

(Uchida *et al.*, 1988; Lee *et al.*, 1990; McKay *et al.*, 1991). Furthermore, the concentrations of endothelin required to induce contraction in the present study are similar to those used previously on other *in vitro* preparations (Hay, 1990; McKay *et al.*, 1991). The contraction of tracheal smooth muscle produced by endothelin in the present study was significantly reduced by nifedipine; the inhibition being about 45% at the highest concentration of endothelin (10 nM). These results suggest that the contraction to endothelin is mediated in part by activation of dihydropyridine-sensitive Ca^{2+} channels in the smooth muscle cell membranes. However, the concentration of nifedipine used (10 μM) should block almost all of these Ca^{2+} channels (Hering *et al.*, 1988), suggesting that endothelin can induce contraction of the smooth muscle by a mechanism which is independent of dihydropyridine-sensitive channels. A similar partial inhibition of endothelin-induced contraction with blockers of this type of channel has previously been shown with guinea-pig (Uchida *et al.*, 1988; Sarria *et al.*, 1990) and human (Advenier *et al.*, 1990) isolated airway preparations. In contrast, other studies have suggested no involvement of dihydropyridine-sensitive Ca^{2+} channels in the contractile response to endothelin (Hay, 1990; McKay *et al.*, 1991) and it was suggested that other Ca^{2+} channels, or mobilization of intracellular Ca^{2+} stores, may be responsible for the effects of endothelin (Hay, 1990). Alternatively, endothelin can release a contractile cyclo-oxygenase product from guinea-pig lung parenchyma (Conroy *et al.*, 1990). The contraction to endothelin that is not inhibited by nifedipine in the present study may be mediated by one or more of these other mechanisms.

Endothelin also produced a concentration-dependent increase in the negativity of the p.d. across the tracheal wall. The size of the transepithelial p.d. is determined in the main by the degree of active ion transport across the epithelium (Na^+ reabsorption from or Cl^- secretion into the lumen) and the size of the paracellular pathways (conductance) between epithelial cells. The increased negativity of p.d. produced by endothelin could be due to stimulation of Na^+ reabsorption or of Cl^- secretion into the lumen, or to a reduction in the size of the paracellular pathways. It is not clear which of these effects or combination of effects endothelin is having. The effects of endothelin on epithelial ion transport have not been studied. The increase in p.d., like the smooth muscle contraction to endothelin, is only partially blocked by nifedipine and the same arguments apply. Thus, this effect is partially due to activation of dihydropyridine-sensitive Ca^{2+} channels and partly due to some other mechanism, which may involve other types of Ca^{2+} channel, intracellular Ca^{2+} stores or release of other mediators.

Endothelin had no significant effect on baseline mucus volume, lysozyme or albumin outputs, suggesting that it does not stimulate basal submucosal gland secretion, including secretion from serous cells, or active epithelial albumin transport. In contrast endothelin increased the output of radiolabelled glycoconjugates from isolated tracheal submucosal glands of the cat (Shimura *et al.*, 1991). However, in this study very high concentrations of endothelin were used and even at 1 μM it only induced a 60% increase in secretion. Alternatively there may be a species difference in the effects of endothelin on submucosal gland secretion, or endothelin may stimulate the output of glycoprotein from mucous cells without increasing the volume output from these cells or from serous cells.

As baseline secretion from the ferret trachea was not significantly different from zero, any inhibitory effect of endothelin on secretion would not be observed unless secretion was prestimulated. Therefore we examined the effect of endothelin on secretion produced by methacholine and phenylephrine. Endothelin produced concentration-dependent reductions in methacholine- and phenylephrine-induced mucus volume, lysozyme and albumin outputs, suggesting it inhibits submucosal gland secretion (including secretion from serous cells) and epithelial albumin transport produced by muscarinic and α-adrenoceptor stimulation. In the case of mucus volume and lysozyme output, but not albumin output, endothelin was more potent at inhibiting the response to methacholine than that to phenylephrine. The reason for this difference is not clear; however, methacholine and phenylephrine act through different intracellular pathways to contract airway smooth muscle and may do the same on gland cells, while working through similar mechanisms on epithelial cells. Endothelin may more readily interfere with the signal transduction mechanisms involved in the glandular response to methacholine than those associated with the response to phenylephrine.

The effects of nifedipine on the lysozyme secretion and albumin transport responses to methacholine and phenylephrine, and on the endothelin-induced inhibition of responses to methacholine and phenylephrine are difficult to interpret. The

main reason for this is that so little is known about the intracellular mechanisms regulating muscarinic and α-adrenoceptor-mediated effects on secretion and epithelial transport. We can only assume that the mechanisms are similar to those for cholinergic and α-adrenoceptor-mediated effects on smooth muscle. Nifedipine, even at the high concentration used (10 μM), had no effect on methacholine- or phenylephrine-induced lysozyme secretion or albumin transport. Therefore, both methacholine and phenylephrine must be acting by mechanisms that do not involve influx of Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels, and are also not affected by any non-specific effects of nifedipine. Thus, it is unlikely that nifedipine is affecting muscarinic or α-adrenoceptors directly, or affecting the release of intracellular Ca^{2+} via the phospholipase C/IP_3 pathway, or any step in the adenylate cyclase pathway.

The concentration-response curves for endothelin-induced inhibition of mucus volume, lysozyme and albumin outputs were all very shallow, occurring over a 10,000 fold concentration range. Furthermore, in many cases, the curves appeared to be biphasic. This suggests that the action of endothelin in inhibiting glandular secretion and epithelial albumin transport to methacholine and phenylephrine is complex and involves at least two different mechanisms. This hypothesis is supported by the results with nifedipine. The inhibition of methacholine- and phenylephrine-induced mucus volume and albumin outputs by high concentrations of endothelin, and the inhibition of phenylephrine-induced lysozyme output by endothelin, were all attenuated by nifedipine, suggesting that it is working, at least in part, by activating dihydropyridine sensitive Ca^{2+} channels. These effects are unusual, as one would expect a rise in intracellular Ca^{2+}, such as that produced by opening dihydropyridine-sensitive Ca^{2+} channels, to produce a positive response (Roger, 1985). It is possible that endothelin is increasing the influx of Ca^{2+} through dihydropyridine-sensitive channels to cause a rise in intracellular Ca^{2+} which is big enough to cause a feedback inhibition and close the Ca^{2+} channels, preventing any further influx of Ca^{2+}. Additionally, the increased intracellular Ca^{2+} may activate guanosine 3′:5′-cyclic monophosphate (cyclic GMP), which is known to cause sequestration of Ca^{2+} into the intracellular stores (Schultz *et al.*, 1973). These effects will tend to reduce the intracellular Ca^{2+} concentration and therefore, reduce the response to methacholine or phenylephrine. However, this should lead to the same degree of inhibition by nifedipine of the endothelin-induced attenuation of methacholine and phenylephrine-induced lysozyme output, which did not occur in the present study.

Another possible interpretation of the results is that nifedipine, at the high concentration used, is having a non-specific effect on the responses to endothelin, which does not affect the responses to methacholine and phenylephrine. This is unlikely to be an effect on the phospholipase C/IP_3 pathway, or the adenylate cyclase pathway, as this would produce changes in the response to methacholine and phenylephrine. It is more likely that nifedipine is having a non-specific effect on the endothelin receptors themselves, thereby affecting responses to endothelin but not to the other agonists. The nature of any such effect is not clear. Furthermore, if this were the case, one would expect the endothelin-induced inhibition of methacholine and phenylephrine-stimulated lysozyme output to be attenuated equally by nifedipine.

The effects of endothelin on methacholine- and phenylephrine-induced responses which are resistant to nifedipine may be due to an effect on Ca^{2+} channels insensitive to dihydropyridines, on intracellular Ca^{2+} stores, or be due to release of other mediators such as prostanoids. Whatever the mechanism of action of endothelin in producing these effects, it is not sensitive to any non-specific action of nifedipine.

In summary, endothelin contracts ferret tracheal smooth muscle and increases the negativity of the transepithelial p.d., at least in part by activating dihydropyridine-sensitive Ca^{2+} channels. Endothelin has no effect on baseline submucosal gland secretion or epithelial albumin transport but inhibits the glandular and epithelial responses to muscarinic and α-adrenoceptor stimulation. At high concentrations of endothelin these inhibitory effects may be partially mediated by activation of dihydropyridine-sensitive Ca^{2+} channels, although the explanation of these results is not clear, whereas at lower concentrations other mechanisms are involved. These mechanisms may involve other Ca^{2+} channels, intracellular Ca^{2+} stores or release of other mediators.

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Estimation of the efficacy and affinity of the β_2-adrenoceptor agonist salmeterol in guinea-pig trachea

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1 We have attempted to estimate the affinity and efficacy of the new β_2-adrenoceptor agonist salmeterol in the guinea-pig isolated trachea and to compare these estimates with those obtained for salbutamol.

2 The kinetics of the relaxation to salmeterol were considerably slower than those to salbutamol and the responses were poorly defined. Nevertheless, in experiments employing a cumulative curve design both salmeterol and salbutamol exhibited partial agonist behaviour. Operational model-fitting of these data indicated that the intrinsic efficacies of the two agonists were indistinguishable but that salmeterol had an affinity ($pK_A = 7.4$) some 30 fold greater than salbutamol ($pK_A = 5.9$).

3 Single dose experiments, using maximally effective concentrations of salmeterol or salbutamol were carried out in an attempt to quantify more reliably the relative efficacies of the two agonists. In these experiments salbutamol was found to have an efficacy (2.5) approximately 3 fold greater than that of salmeterol (0.8).

4 Full/partial agonist interaction studies were carried out to provide another estimate of the affinity of salmeterol. Isoprenaline and adrenaline were employed as full agonists in these experiments and the affinity (pK_B) estimates obtained for salmeterol against these agonists were 7.3 and 7.4 respectively. These affinity estimates were similar to the pK_A value (7.4) obtained by operational model-fitting.

5 Salmeterol has slightly lower efficacy (3 fold) but markedly greater affinity (30 fold) than salbutamol at β_2-receptors. The slightly lower efficacy of salmeterol is unlikely to have clinical implications for this drug as a bronchodilator β_2-adrenoceptor agonist in asthma.

Keywords: Salmeterol; salbutamol; affinity; efficacy, guinea-pig trachea

Introduction

Salmeterol (4-hydroxy-α[[[6-[4-(phenyl(butyl)oxy]-hexyl] amino]methyl]-1,3-benzene dimethanol) is a new β_2-adrenoceptor agonist developed for the treatment of asthma. It has been shown to be both potent and long acting *in vitro* (Bradshaw *et al.*, 1987; Ball *et al.*, 1987a) and *in vivo* (Ball *et al.*, 1987b). Much emphasis has been placed on its long duration of action, and the mechanism by which it achieves such duration has been extensively investigated (Bradshaw *et al.*, 1987; Nials & Coleman, 1988; Coleman *et al.*, 1990). In contrast, the pharmacological characterization of its agonism in terms of affinity and efficacy has not been addressed, despite the obvious importance of these drug-receptor parameters in its therapeutic action.

The purpose of this study was therefore to characterize quantitatively the agonist action of salmeterol in a functional β_2-receptor system. The results of this study are discussed with regard to the therapeutic action of salmeterol.

Methods

Guinea-pig isolated trachea

Male albino Dunkin-Hartley guinea-pigs (400–600 g) were killed by a blow to the back of the head and the whole trachea removed. After removing the adherent connective tissue, the trachea was cut into six ring segments each three cartilage bands wide and then suspended in 20 ml organ baths containing Krebs solution of the following composition (mM): NaCl 117.56, KCl 5.36, NaH_2PO_4 1.15, $MgSO_4$ 1.18, glucose 11.10, $NaHCO_3$ 25.00 and $CaCl_2$ 2.55. This was maintained at 37°C and continually gassed with 5% CO_2 in O_2. Indomethacin (2.8 μM), cocaine (30 μM), corticosterone (10 μM), ascorbate (1 mM), CGP20712A (1 μM) and phentolamine (3 μM) were added to the Krebs solution: indomethacin to prevent development of smooth muscle tone due to the synthesis of cyclooxygenase products, cocaine and corticosterone to inhibit uptake$_1$ and uptake$_2$ processes respectively, ascorbate to prevent catecholamine oxidation and CGP20712A and phentolamine to avoid any complicating effects of β_1- and α-adrenoceptor activation respectively.

The tracheal rings were suspended between two wire hooks (tungsten or stainless steel) one attached to an Ormed Beam isometric force transducer and the other to a stationary support in the organ bath. Changes in isometric force were recorded on 2-channel Advance Bryans flat bed recorders.

Experimental protocols

General At the beginning of each experiment a force of 1.0 g was applied to the tissues and this was reinstated over a 60 min equilibration period until it remained steady. Tissues were then exposed to 100 μM of the muscarinic agonist, methacholine, to assess tissue viability. All subsequent contractions were with methacholine and relaxant agonist concentration-effect, E/[A], curves were constructed by cumulative (except for single dose studies, see below) additions of isoprenaline, salbutamol or salmeterol at 0.5 $\log_{10}$ unit increments. In all experiments a paired curve design was used. Responses were recorded as percentage relaxations of the methacholine-induced contraction.

Estimation of the relative efficacies of salbutamol and salmeterol

Cumulative curve design Each tissue was contracted with 3 μM methacholine and when a steady response was obtained an isoprenaline E/[A] curve constructed. After washing, tissues were left for 60 min and then re-contracted with 3 μM methacholine and a second E/[A] curve performed to either isoprenaline, salbutamol or salmeterol.

Single dose design This method was as for the cumulative curve design except that instead of performing a second E/[A]

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curve, a single, maximally effective concentration of either isoprenaline (10 μM), salbutamol (100 μM) or salmeterol (10 μM) was added.

Full/partial agonist interactions: estimation of affinity of salmeterol

Each tissue was contracted with 10 μM methacholine and when a steady response was obtained an isoprenaline or adrenaline E/[A] curve constructed. After washing, tissues were left for 60 min and then re-contracted with 10 μM methacholine. Subsequently, tissues were incubated with salmeterol or vehicle for 60 min before construction of a second isoprenaline or adrenaline E/[A] curve.

Drugs

The following drugs were used: methacholine chloride (Sigma Chemical Company), indomethacin (Sigma Chemical Company), cocaine hydrochloride (May & Baker), corticosterone 21-acetate (Sigma Chemical Company), ascorbic acid (Fisons Scientific Apparatus), phentolamine mesylate (Ciba-Geigy Laboratories), CGP20712A (ICI Laboratories), (±)-isoprenaline hydrochloride (Sigma Chemical Company), (±)-adrenaline hydrochloride (Sigma Chemical Company), (±)-salbutamol hemisulphate (Sigma Chemical Company) and salmeterol free base (Glaxo Laboratories).

Indomethacin was dissolved in 10% w/v Na_2CO_3, corticosterone 21-acetate in ethanol and isoprenaline and adrenaline in 100 μM ascorbate. Salmeterol was made up as a stock solution of 2 mM in distilled water with the addition of a few drops of dilute hydrochloric acid. All other drugs were dissolved in distilled water.

Data analysis

Operational model-fitting In order to estimate agonist efficacies and affinities experimental E/[A] curve data were fitted using the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (1)$$

in which E and [A] are the pharmacological effect and the concentration of the agonist, respectively; E_m is the maximum possible effect; K_A is the agonist dissociation constant (this was estimated as the negative logarithm, that is pK_A); τ is the efficacy of the agonist (estimated as a logarithm); n determines the steepness of the occupancy-effect relation.

In the case of the cumulative curve design the partial agonist (salbutamol or salmeterol) curve data were analysed according to the comparative method (Barlow *et al.*, 1967) by fitting them to the operational model (equation 1) simultaneously to fitting the full agonist (isoprenaline) data to a logistic function of the form (Leff *et al.*, 1989):

$$E = \frac{E_m [A]^n}{[A_{50}]^n + [A]^n} \quad (2)$$

where E_m and n are as defined above and $[A_{50}]$ is the location parameter for the full agonist curve. (Location parameters were estimated as negative logarithms ($p[A_{50}]$)). This allows K_A and τ for the partial agonist to be estimated as well as E_m and n from each pair of curves.

In the case of the single dose experiments, each individual isoprenaline E/[A] curve was fitted to equation 2 to give estimates of E_m and n. These values were used to estimate the corresponding τ values using the following relationship, which is the operational model definition of the intrinsic activity, α, of the agonist (Black *et al.*, 1985):

$$\frac{\alpha}{E_m} = \frac{\tau^n}{1 + \tau^n} \quad (3)$$

where E_m, τ and n are as defined above and α is the response produced by the single dose of partial agonist. It is not possible to estimate the affinity of the partial agonists from such experiments.

Logistic curve fitting In control experiments designed to determine any time-dependent changes in tissue sensitivity and in full/partial interaction experiments, individual E/[A] curves were fitted to a logistic of the form:

$$E = \frac{\alpha [A]^m}{[A_{50}]^m + [A]^m} \quad (4)$$

where α, $[A_{50}]$ and m are the asymptote (or intrinsic activity), location and slope parameters respectively.

Full/partial agonist interaction analysis

Dose-ratios (r) were calculated from the $[A_{50}]$ estimates for each pair of curves and fitted to equation 5 (Arunlakshana & Schild, 1959). The $[A_{50}]$ of the full agonist curve in the presence of the partial agonist was estimated as the concentration that produced half the maximum increment that could be obtained. (This maximum increment was the difference between the response to the partial agonist alone and the maximum response of which the tissue was capable).

$$\log_{10}(r - 1) = n \log_{10}[B] + pK_B \quad (5)$$

If n, the Schild slope parameter, was not significantly different from unity it was constrained to this value in order to estimate $pK_B (-\log_{10} K_B)$.

All data fitting procedures were carried out using the statistical package, BMDP, and a Vax 11/780 mainframe computer. Differences were assessed by Student's t test and considered significant at the level of $P < 0.05$.

Results

Estimation of the relative efficacies of salbutamol and salmeterol

Figure 1 shows the relaxant effects of isoprenaline, salbutamol and salmeterol on tracheal rings contracted with 3 μM methacholine. The kinetics of the relaxations were similar for isoprenaline and salbutamol but those of salmeterol were considerably slower and the curves poorly defined. Indeed in many instances the curves were so poorly defined as to be unmeasurable. This was particularly noticeable at low concentrations of salmeterol, responses at high concentrations being reasonably well-defined. However, it was evident from such experiments that both salbutamol and salmeterol produced smaller maximum relaxations than isoprenaline.

The apparently partial agonist behaviour of salbutamol and salmeterol were analysed by applying the comparative method (Barlow *et al.*, 1967). Firstly, control experiments showed that paired isoprenaline E/[A] curves were not significantly different in terms of maximum (α) and slope (m). A small but significant rightward shift in the location ($p[A_{50}]$) of the 2nd curve occurred. This time-dependent change in potency was not corrected for as it would have little effect on the results. The mean logistic parameters (±s.e.; $n = 4$) of the curves were as follows: 1st curve: $\alpha = 97.0$ (±0.8); m = 1.3 (±0.1); $p[A_{50}] = 7.4$ (±0.1). 2nd curve: $\alpha = 93.7$ (±1.5); m = 1.4 (±0.1); $p[A_{50}] = 7.2$ (±0.1).

Results of applying the comparative method to salbutamol and salmeterol are illustrated in Figure 2a and b respectively. The lines drawn through the data are the results of operational model-fitting. Analysis of 4 experiments gave average estimates for efficacy (τ) and affinity (pK_A) of 2.0 ($\log \tau = 0.3$ (±0.1)) and 5.9 (±0.1) for salbutamol and 1.3 ($\log \tau = 0.1$

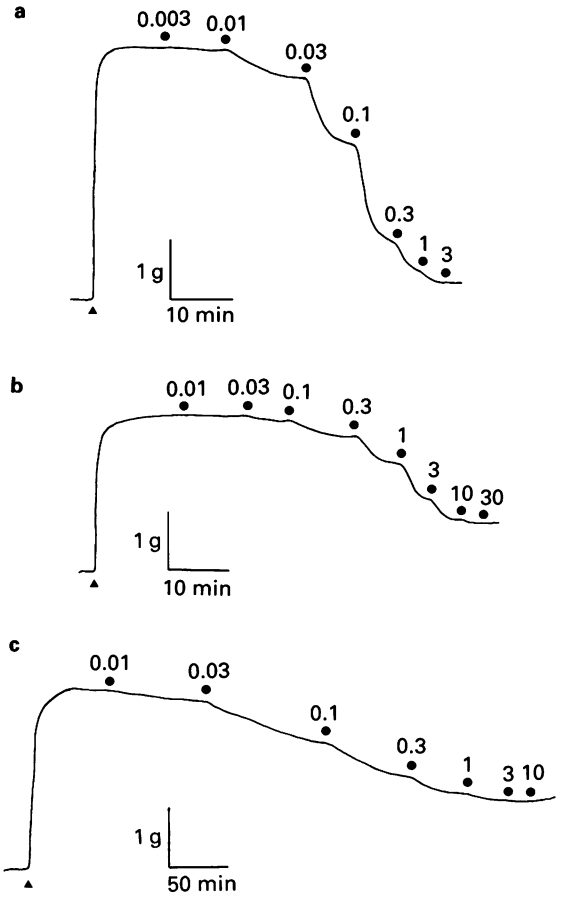


Figure 1 Representative experimental recordings of isoprenaline (a), salbutamol (b) and salmeterol (c) E/[A] curves. Tissues were contracted with 3 μM methacholine (▲). Applied concentrations of agonist are given in μM units. Note the different time scale in (c).

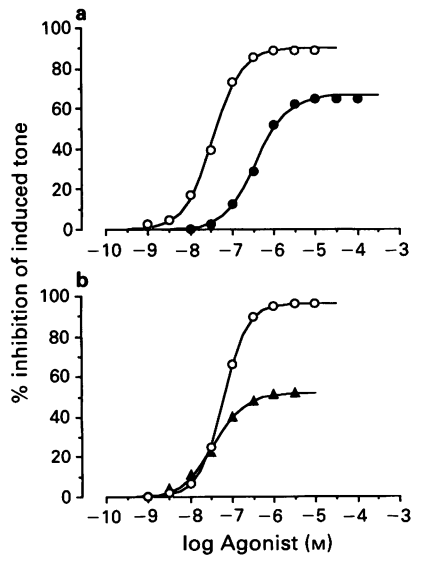


Figure 2 Analysis of salbutamol and salmeterol E/[A] curve data by operational model-fitting. In both (a) and (b) the data shown are from single tissues and the lines drawn through the points are the results of operational model-fitting. (a) Illustrates the analysis of salbutamol (●) with isoprenaline (○) used as the reference full agonist. The model parameter estimates were as follows: $E_m = 90.1$; $n = 1.3$; $\tau = 1.3$; $pK_A = 6.0$; $p[A_{50}]$ (isoprenaline) = 7.2. (b) Illustrates the analysis of salmeterol (▲). The model parameter estimates were as follows: $E_m = 96.2$; $n = 1.5$; $\tau = 1.1$; $pK_A = 7.4$; $p[A_{50}]$ (isoprenaline) = 7.2. See Table 1 for full results.

Table 1 Mean parameter estimates obtained from operational model-fitting of cumulative E/[A] curve data

	Salbutamol	*Salmeterol*
$p[A_{50}]$(Isop)	7.3 ± 0.1	7.3 ± 0.1
n	1.3 ± 0.1	1.4 ± 0.1
E_m	96.4 ± 3.1	98.2 ± 1.3
log τ	0.3 ± 0.1	0.1 ± 0.1
pK_A	5.9 ± 0.1	7.4 ± 0.1
Replicates	4	4

(±0.1)) and 7.4 (±0.1) for salmeterol respectively. The efficacy estimates were not significantly different (see Table 1 for complete results).

Single dose experiments

Although the above experiments gave estimates of the efficacy and affinity of salmeterol, we felt that the slow kinetics of the salmeterol responses may have compromised them due to possible difficulties with response definition and measurement. In the time period needed to complete the E/[A] curve it was likely that the methacholine-induced contraction would have faded significantly. In fact, control experiments indicated that the contraction faded by 19% ± 2% (±s.e.; $n = 4$, data not shown) over the time period needed to complete a salmeterol E/[A] curve. Such fade would result in the efficacy of salmeterol being overestimated. The single dose experiments were therefore conducted to give a more reliable estimation of the efficacy of salmeterol (affinity estimates cannot be determined with this experimental design). Isoprenaline E/[A] curves were constructed in each tissue and this was followed by a single concentration of isoprenaline (10 μM), salbutamol (100 μM) or salmeterol (10 μM). These concentrations were previously determined to be maximally effective (see Figure 2). The results of these experiments are shown in Figure 3. The data in Figure 3(a) indicate that there was not a time-dependent change in maximum response to isoprenaline. As was the case with the cumulative curve design, both salbutamol (Figure 3(b) and salmeterol (Figure 3(c)) produced smaller maximum relaxations than isoprenaline. Salbutamol produced on average 80% (±10%; $n = 6$) and salmeterol 40% (±10%; $n = 6$) of the maximum relaxation to isoprenaline; that is, their respective intrinsic activities were 0.8 and 0.4. Analysis of the data as described in the Methods section gave efficacy estimates of 2.5 (log $\tau = 0.4$ (±0.1)) for salbutamol and 0.8 (log $\tau = -0.1$ (±0.1)) for salmeterol (see Table 2 for complete results). These efficacy estimates were significantly different.

Full/partial agonist interactions: estimation of the affinity of salmeterol

Since it was not possible to estimate the affinity of salmeterol from the single dose experiments we adopted another approach in which the ability of salmeterol to antagonize the effects of higher efficacy agonists (isoprenaline and adrenaline) was investigated. In these experiments tracheal rings were

Table 2 Mean parameter estimates obtained from single dose experiments

	Isoprenaline	*Salbutamol*	*Salmeterol*
$p[A_{50}]$(Isop)	7.1 ± 0.2	7.2 ± 0.1	7.1 ± 0.1
n	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
E_m	92.1 ± 4.3	92.9 ± 2.6	92.5 ± 1.9
α	88.6 ± 1.7	69.1 ± 5.7	41.3 ± 7.6
α/E_m	1.0 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
log τ	—	0.4 ± 0.1	−0.1 ± 0.1
Replicates	6	6	6

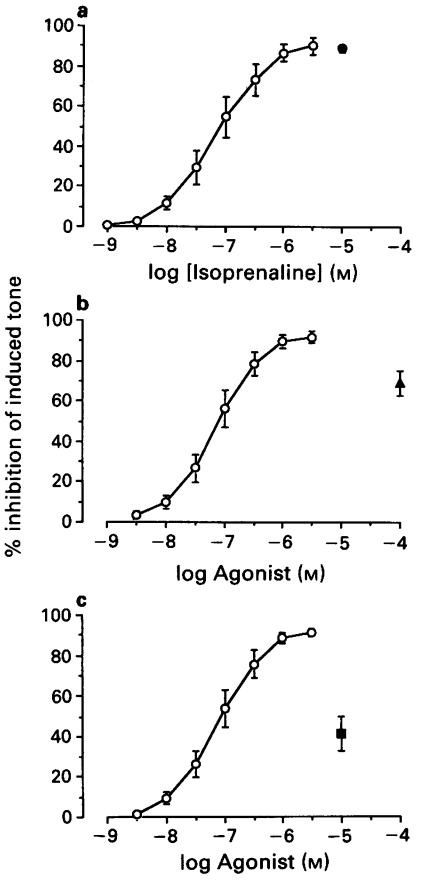


Figure 3 Effect of single doses of isoprenaline, salbutamol and salmeterol. The data are the average of 6 replicates with vertical lines indicating s.e. In each panel the E/[A] curve data are for isoprenaline. The single points represent the effects produced by 10 μM isoprenaline (●) (a), 100 μM salbutamol (▲) (b) and 10 μM salmeterol (■) (c). See Table 2 for model parameter estimates.

contracted with 10 μM methacholine rather than 3 μM as used previously. Under these conditions of greater functional antagonism, salmeterol produced smaller relaxations allowing better defined full agonist E/[A] curves to be constructed. In this instance control experiments indicated small but significant changes in the maximum response (α) and location ($p[A_{50}]$) of consecutive isoprenaline E/[A] curves. As above, these changes were considered to have minimal effect on the subsequent analyses. Adrenaline control pairs were not significantly different. The mean logistic parameters ($\pm$s.e.; $n = 6$ (isoprenaline) or $n = 4$ (adrenaline)) were as follows: isoprenaline; 1st curve $\alpha = 77.6$ (± 3.3); $m = 1.3$ (± 0.1); $p[A_{50}] = 7.1$ (± 0.1). 2nd curve: $\alpha = 71.4$ (± 4.6); $m = 1.3$ (± 0.1); $p[A_{50}] = 7.0$ (± 0.1). Adrenaline: 1st curve $\alpha = 88.2$ (± 3.6); $m = 1.3$ (± 0.1); $p[A_{50}] = 6.0$ (± 0.1). 2nd curve: $\alpha = 79.2$ (± 4.6); $m = 1.3$ (± 0.1); $p[A_{50}] = 6.0$ (± 0.1).

Figures 4a and 5a show typical antagonist effects of 1 μM salmeterol on isoprenaline and adrenaline E/[A] curves respectively. The raised baseline of the second E/[A] curves indicates the amount of relaxation produced by 1 μM salmeterol in these tissues. It should be noted that E/[A] curves performed in the presence of salmeterol were significantly less steep (i.e. the midpoint slope parameter, m, was smaller) than control curves. The reason for this is unclear but it indicated that the interactions did not conform with simple competition. Nevertheless, the calculated dose-ratios from several of these experiments are displayed in Schild Plot form in Figures 4b and 5b. Analysis of these curve displacements using equation (5) yielded Schild slope parameter (n) estimates of 1.2 ± 0.1 (s.e., 22 d.f.) and 1.1 ± 0.1 (s.e., 18 d.f.) for isoprenaline and adrenaline respectively. Neither value was significantly different from unity and the resulting pK_B estimates were 7.3 ± 0.1 (s.e., 23 d.f.) and 7.4 ± 0.1 (s.e., 19 d.f.). These estimates were not significantly different from each other.

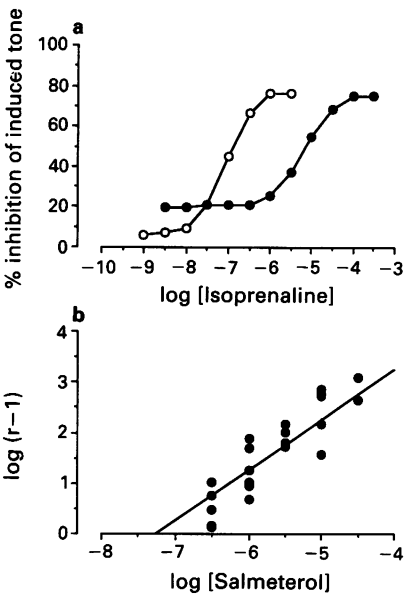


Figure 4 Antagonism of isoprenaline by salmeterol: (a) shows E/[A] curve data from a single tissue for isoprenaline in the absence of (○) and the presence of 1 μM salmeterol (●); (b) illustrates the results of several such experiments in Schild plot form. The slope of the plot was not significantly different from unity (1.2 ± 0.1) (22 d.f.) and the estimated pK_B was 7.3 ± 0.1 (23 d.f.).

Discussion

The purpose of this study was to quantify the activity of the β_2-adrenoceptor agonist, salmeterol, in terms of its affinity and efficacy and to compare it with salbutamol.

Previous studies (Ball *et al.*, 1987a,b; Bradshaw *et al.*, 1987) have shown that salmeterol has a slower onset of action than salbutamol and a longer duration of action. The slow onset

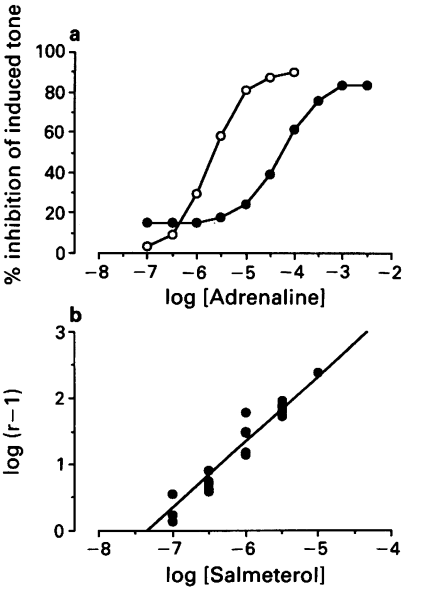


Figure 5 Antagonism of adrenaline by salmeterol: (a) shows E/[A] curve data from a single tissue for adrenaline in the absence of (○) and the presence of 1 μM salmeterol (●); (b) illustrates the results of several such experiments in Schild plot form. The slope of the plot was not significantly different from unity (1.1 ± 0.1) (18 d.f.) and the estimated pK_B was 7.4 ± 0.1 (19 d.f.).

was also evident in the present study (see Figure 1); the time course of salmeterol E/[A] curves was considerably slower than those of isoprenaline and salbutamol. In many instances the responses to salmeterol were so poorly defined as to preclude accurate measurement. Even in the cases that were measurable it was impossible to assess how much of the relaxant response was due to a β_2-mediated effect and how much was the result of time-dependent fade of the methacholine-induced contraction (control experiments indicated that the average degree of fade was 19% but this is obviously tissue-dependent). The estimates of efficacy and affinity for salmeterol obtained by operational model-fitting of the E/[A] curve data must therefore be treated with some caution. Such experiments indicated that salbutamol and salmeterol had efficacies that were indistinguishable ($\tau = 2.0$ and 1.3 respectively) but that salmeterol ($pK_A = 7.4$) had approximately 30 fold higher affinity for β_2-receptors than salbutamol ($pK_A = 5.9$). However, if the maximum response to salmeterol were overestimated as a result of a time-dependent loss of tone, as suggested by control experiments then efficacy would be overestimated.

The purpose of the 'single dose' experiments (Figure 3) was to provide a more reliable estimate of the relative efficacies of salmeterol and salbutamol. Although a maximally effective concentration of salmeterol takes significantly longer than that of salbutamol to equilibrate, any time-dependent loss of tone would be expected to be markedly less than that occurring in the course of construction of a salmeterol E/[A] curve. These experiments yielded τ estimates of 2.5 for salbutamol and 0.8 for salmeterol; that is salbutamol has approximately 3 fold higher efficacy than salmeterol. In terms of intrinsic activity these efficacy estimates correspond to 0.8 and 0.4 respectively (see Table 2). Under the experimental conditions used, therefore, salmeterol was capable of generating 50% of the relaxation produced by salbutamol. The important consideration from the therapeutic point of view is whether such a difference would be relevant in terms of bronchodilator activity. In principle, partial agonist behaviour in man would be undesirable. However, the difference between salmeterol and salbutamol, at least on the scale operating in the present experiments, is of a size which is unlikely to have clinical implications.

Having obtained what we felt was a reliable estimate of the relative efficacies of salbutamol and salmeterol, we then attempted to estimate the affinity of salmeterol by performing full/partial agonist interactions. Full agonist E/[A] curves obtained in the presence of salmeterol were significantly less steep than control curves. The reason for this is unclear but it may be a consequence of the slow offset of salmeterol limiting the establishment of new equilibrium with the full agonist. Strictly speaking this observation precludes Schild analysis of the data as one of the criteria for simple competitive behaviour is violated. However, in order to obtain estimates of the affinity of salmeterol we fitted the data to equation 5 and estimated pK_B values. With isoprenaline and adrenaline as the full agonists, the pK_B values obtained were 7.3 and 7.4 respectively. These values were not significantly different and thus confirm the agonist-independence of salmeterol's antagonism.

Although these affinity estimates cannot be considered as true pK_B's (for the reason stated above) their similarity to the pK_A estimate (7.4) obtained by operational model-fitting increases confidence in their reability. It should also be noted that in full/partial agonist interaction experiments the affinity of the partial agonist obtained by Schild analysis is an overestimate. The overestimation is a consequence of the ability of the partial agonist to produce a response, the higher the efficacy of the partial agonist the bigger the error in the affinity estimate (Kenakin, 1987). In the case of a system in which the agonist E/[A] curves are hyperbolic the affinity of a partial agonist with an intrinsic activity of 0.5 is overestimated by 0.3 $\log_{10}$ units. In the above experiments the error would be expected to be less than 0.3 $\log_{10}$ units since the intrinsic activity of salmeterol was less than 0.5.

Salmeterol and salbutamol differ only in the N-substituent on their ethanolamine side-chain, salmeterol having a long lipophilic chain and salbutamol having a tertiary butyl group. It has been suggested that the long duration of action of salmeterol results from the binding of its lipophilic side-chain to an exo-site within the β_2-receptor but distinct from the physiological receptor (Bradshaw *et al.*, 1987). If an exo-site does exist the affinity of a compound which binds to it and to the physiological β_2-receptor would be expected to be greater than that of structurally similar compounds which bind only to the latter. Indeed, binding studies have indicated that the affinity of salmeterol is approximately 50 fold greater than that of salbutamol (Coleman *et al.*, 1990). In contrast, such exo-site binding would be expected to have little effect on the response-generating capacity (efficacy) of the agonist. The results of this study which show that salmeterol has markedly higher affinity (approx. 30 fold) but only slightly lower efficacy (3 fold) than salbutamol lend support to these suggestions.

We wish to thank Glaxo Group Research Limited for their gift of salmeterol.

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Amelioration of cisplatin-induced acute renal failure with 8-cyclopentyl-1,3-dipropylxanthine

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1 The effect of the selective adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (CPX), on the development of cisplatin-induced acute renal failure was investigated in the rat.

2 CPX at doses of 0.03, 0.1 and 0.3 mg kg^{-1}, i.v. caused increasing degrees of antagonism of adenosine-induced bradycardia in anaesthetized rats. The magnitude of antagonism was not directly proportional to the increment in dose, but for each dose, it was similar in rats injected with either saline or cisplatin. CPX at a dose of 0.03 mg kg^{-1} significantly antagonized adenosine-induced bradycardia for up to 2.5 h, while doses of 0.1 and 0.3 mg kg^{-1} produced significant blockade for periods longer than 5 h.

3 Administration of cisplatin (6 mg kg^{-1}, i.v.) caused acute renal failure characterized by decreased inulin and *p*-aminohippurate clearances, increased urine volume but decreased excretion of Na^+, K^+ and Cl^- ions and by increased plasma levels of urea and creatinine. Kidney weight was increased in cisplatin-treated rats and renal tubule necrosis occurred.

4 Administration of CPX (0.03 mg kg^{-1}, i.v.; twice daily for two days) to rats given cisplatin did not reduce the severity of the resultant renal failure. However, treatment with 0.1 mg kg^{-1} CPX attenuated the increases in plasma creatinine/urea levels observed in rats on days 3 and 7 after induction of renal failure. In addition, this dose significantly reduced renal tubule damage and increased inulin and *p*-aminohippurate clearances. A similar pattern of protection was noted with CPX at a dose of 0.3 mg kg^{-1} although the increase in inulin clearance was not statistically significant. However, this higher dose of CPX significantly increased Na^+ and K^+ excretion compared to vehicle-treated rats.

5 CPX at doses of 0.03, 0.1 and 0.3 mg kg^{-1} produced blockade of an A_1-receptor mediated response i.e. adenosine-induced bradycardia, but only treatment with the higher doses of CPX (0.1 and 0.3 mg kg^{-1}) ameliorated nephrotoxicity produced by cisplatin. The lack of any protective effect afforded by the lowest dose of CPX could be a result of its shorter duration of action.

6 This study indicates that adenosine plays a significant role in the pathophysiology of cisplatin-induced acute renal failure.

Keywords: Cisplatin; acute renal failure; adenosine antagonism; 8-cyclopentyl-1,3-dipropylxanthine

Introduction

Cisplatin (cis-diamminedichloroplatinum II) is an inorganic complex of platinum whose cytotoxicity was first discovered by Rosenberg *et al.* in 1965. Cisplatin is one of the most potent anti-cancer drugs currently in use and is effective against a wide spectrum of human tumours. Its efficacy appears to increase with dose (Ozols *et al.*, 1988), but high doses of cisplatin are associated with severe adverse effects and in particular, with nephrotoxicity. This latter condition is often the dose-limiting factor in therapy. In order to reduce renal damage, and thereby enhance the efficacy of cisplatin, the pathophysiological mechanisms which underlie cisplatin-induced nephrotoxicity need to be investigated.

Winston & Safirstein (1985) have shown that renal blood flow, superficial nephron glomerular filtration rate, transglomerular pressure and effective filtration pressure at the afferent portion of the glomerulus were all decreased 72 h after a single dose of cisplatin. The reduction of renal blood flow was substantial (about 36%) and occurred even though mean arterial blood pressure was the same in control and cisplatin-injected rats (Winston & Safirstein, 1985). Consequently diminished renal blood flow must stem from increased renal vascular resistance. Moreover, the reductions in transglomerular pressure and effective filtration pressure at the afferent portion of the glomerulus, suggest that the increase in vascular resistance is of pre-glomerular origin (Winston & Safirstein, 1985). These observations of reduced renal blood flow and increased vascular resistance are also early features of cisplatin-induced renal failure in both dog and man (Offerman *et al.*, 1984; Daugaard *et al.*, 1987).

The mediator(s) of increased vascular resistance in cisplatin-induced acute renal failure is unknown, but one possible candidate is adenosine. This purine has been proposed to modulate renal haemodynamics under physiological conditions (Osswald, 1984) and in pathophysiological states such as acute renal failure (Churchill & Bidani, 1982). Steady-state infusion of adenosine reduces glomerular filtration rate (GFR) and the mechanism for this appears to be sustained afferent arteriolar constriction in the outer renal cortex (Osswald, 1984). These effects of exogenous adenosine are analogous to the changes in GFR and vascular resistance found in cisplatin-induced renal failure (Winston & Safirstein, 1985). Recently, Heidemann *et al.* (1989) have shown that theophylline, a non-selective adenosine antagonist and phosphodiesterase inhibitor, was able to reduce the severity of cisplatin-induced acute renal failure in the rat. They attributed this effect to the ability of theophylline to block adenosine receptors in the renal vasculature.

8-Cyclopentyl-1,3-dipropylxanthine (CPX; Figure 1) has been shown both *in vitro* (Haleen *et al.*, 1987) and *in vivo* (Kellett *et al.*, 1989) to be an A_1-selective adenosine antagonist. Since there is evidence that activation of A_1-receptors evokes constriction of the afferent arteriole (Holz & Steinhausen, 1987), we have studied the potential of this compound to protect kidney function from the deleterious effects of cisplatin. We have previously shown that CPX ameliorates glycerol-induced acute renal failure and that the selective A_1-adenosine receptor blockade produced by CPX, might be more beneficial in renal failure than non-selective antagonism

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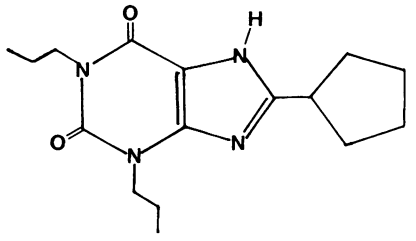


Figure 1 Structure of the selective A_1-adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (CPX).

of both A_1- and A_2-receptors (Kellett *et al.*, 1989). If adenosine is an important mediator of the changes in pre-glomerular vascular resistance which occur in cisplatin-induced renal failure, then treatment with CPX at doses which block the effects of adenosine, should abrogate this form of renal dysfunction. A preliminary account of this work has been given (Knight *et al.*, 1990).

Methods

Evaluation of 8-cyclopentyl-1,3-dipropylxanthine as an adenosine antagonist

This was carried out by determining the blockade of adenosine-induced changes in heart rate (predominantly mediated by A_1-receptors; Kellett *et al.*, 1989). Male Wistar albino rats (200 to 250 g) were anaesthetized with thiobutabarbitone (180 mg kg^{-1}, i.p.) and cannulae were inserted into: the trachea to allow artificial respiration (80 strokes min^{-1}, 10 ml kg^{-1} stroke volume); the left jugular vein for drug delivery and the right carotid artery for measurement of blood pressure. Body temperature was monitored with a rectal thermometer and maintained at 37–37.5°C by heat lamps. Systemic blood pressure was recorded via a pressure transducer (Druck PDCR 75) and heart rate was obtained electronically from the blood pressure signal. Thirty minutes after the completion of surgery, experiments were conducted to: (1) determine dose-response curves for the negative chronotropic effects of adenosine in the presence of CPX and (2) assess the duration of blockade produced by various doses of CPX on adenosine-induced falls in heart rate. These experiments were performed on rats 48 h after an i.v. injection of either 0.9% w/v NaCl (saline, 3 ml kg^{-1}) or cisplatin (6 mg kg^{-1}, 2 mg ml^{-1} in saline).

Dose-response curves Dose-response curves to adenosine dissolved in saline (0.02 to 12 mg kg^{-1}, i.v. administered every 3 min) were repeated in the presence of 0.03, 0.1 and 0.3 mg kg^{-1} CPX; dissolved in 1% v/v dimethyl sulphoxide, 0.75% v/v 1 M NaOH in saline. The vehicle for CPX had no significant effect on adenosine-induced changes in heart rate. Doses of CPX were achieved by cumulative addition every 30 min i.e. an initial dose of 0.03 mg kg^{-1} followed by doses of 0.07 and 0.2 mg kg^{-1}, respectively. In recent experiments we have administered [^{3}H]-CPX i.v. and determined the subsequent decline in plasma levels of radioactivity 2 days after administration of saline or cisplatin. These pilot experiments showed that the elimination half-life for radioactivity was 9.6 $\pm$ 2.2 h (n = 4) in saline-injected rats and 4.9 $\pm$ 1.6 h in rats injected with cisplatin (n = 4) (unpublished data). These data suggest that 30 min after the administration of one dose of CPX the fraction remaining of the original dose is 0.96 in saline-injected rats and 0.93 in cisplatin-injected rats. In view of this, the doses of CPX were given in a cumulative manner as indicated above.

Duration of adenosine blockade Rats received 3 injections of adenosine (1.2 mg kg^{-1}, i.v.) with a 3 min interval between doses. The mean response to these injections was taken as the control response. Following recovery from the third dose of adenosine, CPX (0.03, 0.1 or 0.3 mg kg^{-1}) or vehicle (1.0 ml kg^{-1}) was administered i.v. after which the same dose of adenosine (1.2 mg kg^{-1}) was repeatedly administered every 30 min for up to 5 h after injection of CPX or vehicle.

Evaluation of 8-cyclopentyl-1,3-dipropylxanthine in cisplatin-induced acute renal failure

Experimental protocol Male Wistar albino rats (250–350 g) were placed in metabolic cages for 24 h and urine was collected. At the end of this period rats were lightly anaesthetized with ether and a control (day 0) blood sample (about 0.75 ml) was taken from the tail vein. Rats were then injected i.v. with either cisplatin (6 mg kg^{-1}, 2 mg ml^{-1}) or its vehicle (saline, 3 ml kg^{-1}). Immediately after injection of cisplatin, rats received i.v. one of four treatments: CPX (0.03, 0.1 or 0.3 mg kg^{-1}) or the vehicle for CPX (1 ml kg^{-1}; 1% v/v dimethyl sulphoxide, 0.75% v/v 1 M NaOH in saline). This first injection of CPX or vehicle was made between 09 h 00 min to 10 h 00 min and additional i.v. doses of CPX or its vehicle were given 12, 24 and 36 h later. One group of cisplatin-injected rats received no treatment. Animals given saline were treated i.v. with either saline (1 ml kg^{-1}) or CPX (0.1 or 0.3 mg kg^{-1}) using the same dosage regimen employed for the cisplatin-injected groups. The saline-injected saline treated rats are referred to as normal animals.

A second blood sample (0.75 ml) was taken from the tail vein 3 days after injection of cisplatin or saline and rats were then placed in metabolic cages for a second 24 h urine collection. Seven days after the initial injection, rats were anaesthetized and the clearances of [^{3}H]-inulin (C_{IN}) and *p*-[^{14}C]-aminohippuric acid (C_{PAH}) determined. At the end of the experiment, a final blood sample (1 ml) was taken from the carotid artery and rats were killed with an overdose of anaesthetic. The kidneys were removed and prepared for histological examinination.

Estimation of [^{3}H]-inulin and p-[^{14}C]-aminohippuric acid clearances Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and cannulae were inserted into the trachea, left jugular vein and right carotid artery. Animals were injected with heparin (500 units kg^{-1}) and the clearances of [^{3}H]-inulin (100 mg kg^{-1}, 20 μCi kg^{-1}, i.v.) and *p*-[^{14}C]-aminohippuric acid acid (40 mg kg^{-1}, 4 μCi kg^{-1}, i.v.) dissolved in saline, were then determined simultaneously by the single injection method of Hall *et al.* (1977).

Measurement of urinary electrolytes Sodium and potassium concentrations were measured by use of a Corning 480 flame photometer whilst chloride levels were estimated with a Corning 925 chloride analyzer.

Measurement of plasma creatinine and urea concentrations Levels of these nitrogenous metabolites were assayed by standard colorimetric procedures: creatinine by reaction with picrate in alkaline solution and urea by reaction with diacetyl monoxime.

Kidney histology At the end of the clearance experiment, both kidneys were removed, cleared of adipose tissue and weighed. Kidneys were washed in saline, bisected longitudinally and preserved in formal saline (BDH). A longitudinal section was cut from one kidney of each rat and stained with haematoxylin and eosin. Sections were evaluated by a pathologist who was unaware of the treatment each donor animal had received. Renal damage was largely confined to proximal tubule necrosis (S_3 segment) and the extent of damage was scored out of four using the following scoring system:
0 = normal; 1 = mild tubular basophilia and dilatation; 2 = mild tubular necrosis, evidence of extensive regeneration and some hyaline and granular casts in the tubular lumen; 3 = moderate tubular necrosis with evidence of regeneration,

numerous casts; 4 = severe late stage tubular and papillary necrosis, little evidence of regeneration and numerous casts.

Statistical methods

Results are given as mean ± s.e.mean. Statistical analyses of functional and biochemical data were done by use of either Student's non-paired t test or where appropriate by one way analysis of variance (ANOVA) with means compared by Scheffe's test. Analysis of the histology scores was by a two-sided Mann-Whitney test. Dose-ratios for adenosine-induced bradycardia were calculated at a response level equivalent to a fall in heart rate of 140 beats min^{-1}.

Materials

Adenosine (free base) and cisplatin were purchased from Sigma Chemical Co. [^{3}H(G)]-inulin (355.5 mCi g^{-1}) and p-[glycyl-1-^{14}C]-aminohippuric acid (60 mCi $mmol^{-1}$) were obtained from NEN Research Products. The stated radiochemical purity of each isotope was greater than 98%. CPX was synthesized by Dr R. James of ICI and reagent kits for the assay of creatinine and urea were bought from Pierce & Warriner and BDH Ltd, respectively.

Results

Evaluation of adenosine antagonism

There was no significant ($P > 0.05$) difference in basal heart rate or mean arterial blood pressure between saline-injected rats (374 ± 8 beats min^{-1}; 71 ± 7 mmHg, $n = 5$) and cisplatin-injected rats (370 ± 5 beats min^{-1}; 80 ± 3 mmHg, $n = 5$). Administration of CPX at any dose (0.03 to 0.3 mg kg^{-1}) had no significant ($P > 0.05$) effects on basal heart rate or mean arterial blood pressure in either saline- or cisplatin-injected rats. All three doses of CPX (0.03, 0.1 and 0.3 mg kg^{-1}, i.v.) antagonized the bradycardia induced by adenosine (0.02 to 12 mg kg^{-1}, i.v.) in both groups of animals and Figure 2 illustrates the results for saline-injected rats. The mean log dose-ratios in saline-injected animals were similar to those obtained for rats given cisplatin (Table 1). In both groups of rats, however, the displacement of the agonist dose-response curve in the presence of CPX was not proportional to the increment in antagonist dose (Table 1, Figure 2). Adenosine at doses from 0.02 to 12 mg kg^{-1} evoked decreases in mean arterial blood pressure of 6 to 40 mmHg in both saline- and cisplatin-injected rats. As found previously (Kellett *et al.*, 1989), CPX was less potent in antagonizing the hypotensive responses produced by adenosine than the reductions in heart rate. There was no antagonism of the hypotensive responses to lower doses of adenosine with statistically significant ($P < 0.05$) reductions in response only occurring at doses of adenosine > 0.60 mg kg^{-1}, when the dose of CPX was either 0.03 or 0.1 mg kg^{-1}, and >0.40 mg kg^{-1} when the dose of CPX was 0.3 mg kg^{-1}.

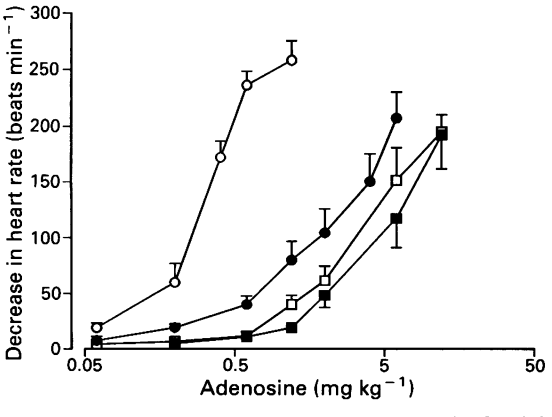


Figure 2 Adenosine-induced bradycardia in rats 48 h after injection with saline (3 ml kg^{-1}, i.v.) in the absence (○) and presence of 8-cyclopentyl-1,3-dipropylxanthine (CPX) 0.03 mg kg^{-1} (●), 0.1 mg kg^{-1} (□) and 0.3 mg kg^{-1} (■), i.v. Results are given as mean and vertical lines indicate s.e.mean ($n = 5$).

Table 1 Log dose-ratios for antagonism by 8-cyclopentyl-1,3-dipropylxanthine (CPX) of adenosine-induced bradycardia in anaesthetized rats

	Log dose-ratios	
CPX dose (mg kg^{-1})	*Saline-injected rats* ($n = 5$)	*Cisplatin-injected rats* ($n = 5$)
0.03	0.92 ± 0.09	0.90 ± 0.09
0.1	1.2 ± 0.1	1.1 ± 0.1
0.3	1.4 ± 0.1	1.3 ± 0.1

Mean ± s.e.mean and number of rats in parentheses.

The duration of blockade of adenosine-induced bradycardia produced by the various doses of CPX in saline-injected animals is shown in Figure 3. The blockade produced by a dose of 0.03 mg kg^{-1} of CPX lasted for 2.5 h since by 3 h the fall in heart rate produced by 1.2 mg kg^{-1} of adenosine was not significantly different ($P > 0.05$) from the response to adenosine following vehicle administration. By contrast, the blockade produced by 0.1 and 0.3 mg kg^{-1} of CPX persisted for longer than 5 h as the responses to adenosine showed little recovery over this period (Figure 3). The duration of adenosine blockade produced by the various doses of CPX in cisplatin-injected rats was identical to that in saline-injected animals.

Effect of CPX treatment on saline-injected rats

In saline-injected rats given 0.1 mg kg^{-1} CPX every 12 h for 2 days, there were no significant ($P > 0.05$) changes in plasma creatinine/urea concentrations (data not shown), C_{IN}/C_{PAH} (Figure 4), histology score (Table 2) or 24 h urine output/inorganic ion excretion (data not shown) compared to data for normal animals. These results agree with previous work (Kellett *et al.*, 1989). Similar results were obtained when the dose was increased to 0.3 mg kg^{-1}. However, in comparison to normal rats, there was a small but significant ($P < 0.05$) elevation of plasma creatinine levels on day 3 (0.73 ± 0.07 vs. 0.59 ± 0.04 mg 100 ml^{-1}; $n = 8$) and day 7 (0.73 ± 0.10 vs.

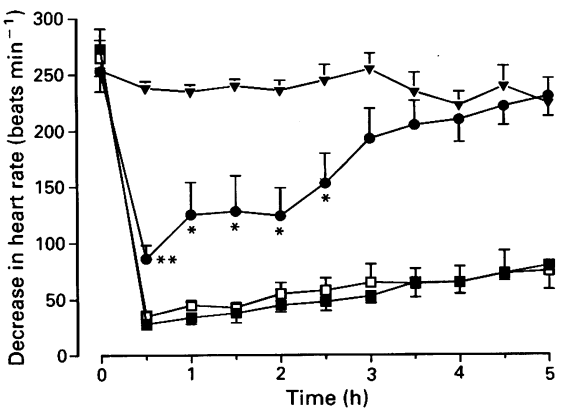


Figure 3 The bradycardia produced by repeated bolus i.v. administration of adenosine (1.2 mg kg^{-1}) in saline-injected rats before (0 h) and at various times after the i.v. administration of 8-cyclopentyl-1,3-dipropylxanthine (CPX) 0.03 mg kg^{-1} (●), 0.1 mg kg^{-1} (□) and 0.3 mg kg^{-1} (■) or vehicle (1.0 ml kg^{-1}, ▼). Results are given as mean and vertical lines indicate s.e.mean ($n = 4$). *$P < 0.05$, **$P < 0.001$, compared to the response in the vehicle-injected group at the same time (t test). All the responses to adenosine following administration of 0.1 and 0.3 mg kg^{-1} of CPX were significantly different ($P < 0.001$ t test) from the response in the vehicle-injected group at the equivalent time point.

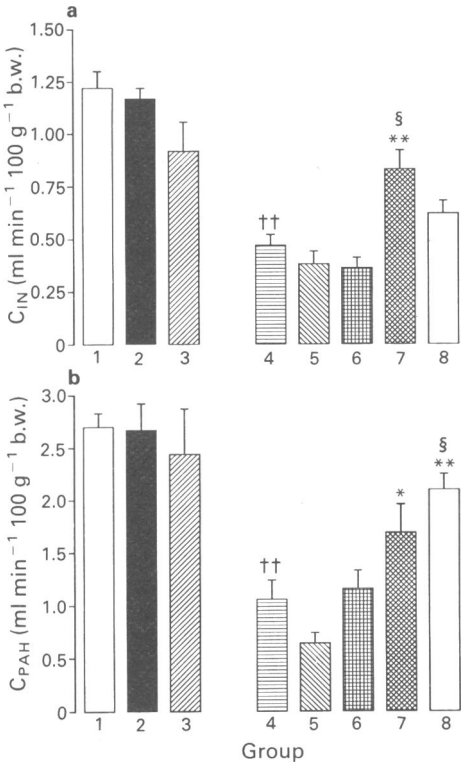


Figure 4 Clearances of (a) [^{3}H]-inulin (C_{IN}) and (b) *p*-[^{14}C]-aminohippuric acid (C_{PAH}) determined in rats seven days after i.v. injection of either saline or cisplatin. Rats were treated with either 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.03, 0.1 or 0.3 mg kg^{-1}, i.v.) or its vehicle (1.0 ml kg^{-1}, i.v.) twice daily for two days. Key to group: (1) saline-injected saline-treated ($n = 8$); (2) saline-injected + CPX 0.1 mg kg^{-1} ($n = 8$); (3) saline-injected + CPX 0.3 mg kg^{-1} ($n = 8$); (4) cisplatin-injected ($n = 15$); (5) cisplatin-injected vehicle treated ($n = 15$); (6) cisplatin-injected + CPX 0.03 mg kg^{-1} ($n = 15$); (7) cisplatin-injected + CPX 0.1 mg kg^{-1} ($n = 15$) and (8) cisplatin-injected + CPX 0.3 mg kg^{-1} ($n = 25$). Columns represent mean and vertical bars s.e.mean. ††$P < 0.001$ relative to group 1 (*t* test). *$P < 0.05$, **$P < 0.001$ relative to group 5 (ANOVA). §$P < 0.01$ relative to group 6 (ANOVA).

0.44 ± 0.04 mg 100 ml^{-1}; $n = 8$). Moreover, there was a 25% decrease in C_{IN} (Figure 4a), although this latter change was not statistically significant ($P > 0.05$).

Effect of cisplatin on renal function

A single intravenous dose of cisplatin (6 mg kg^{-1}) produced renal failure in all rats studied. Mean plasma concentrations of creatinine and urea were substantially increased at both 3 and 7 days after injection of drug (Table 3). Comparison of urine from cisplatin-injected rats and normal animals indicated a significant ($P < 0.001$) fall in 24 h excretion of Na^+ (0.25 ± 0.03; $n = 15$ vs. 0.76 ± 0.06 mmol 100 g^{-1} 24 h^{-1}; $n = 8$), K^+ (0.44 ± 0.05; $n = 15$ vs. 1.1 ± 0.1 mmol 100 g^{-1} 24 h^{-1}; $n = 8$) and Cl^- (0.30 ± 0.03; $n = 15$ vs. 0.99 ± 0.07 mmol 100 g^{-1} 24 h^{-1}; $n = 8$). Reduced inorganic ion excretion, 3–4 days after injection of cisplatin, was accompanied by a substantial increase in urine output (14.4 ± 1.7; $n = 15$ vs. 3.3 ± 0.3 ml 100 g^{-1} 24 h^{-1}; $n = 8$). In addition, Figure 4 shows that on day 7 C_{IN} and C_{PAH} were both reduced by about 60% ($P < 0.001$) relative to their values in normal animals. Cisplatin increased total wet kidney weight by about 35% ($P < 0.001$) and caused extensive tubule necrosis when compared to data for normal rats (Table 2).

Effect of treatment with either CPX or its vehicle on cisplatin-induced acute renal failure

There were no significant differences between rats given cisplatin alone and those injected with cisplatin but treated with vehicle in any of the indices of renal function, other than a small reduction ($P < 0.01$) in tubular damage in vehicle-treated animals (Table 2).

When compared to cisplatin-injected vehicle treated rats, treatment with CPX, at a dose of 0.03 mg kg^{-1} every 12 h for 2 days, did not significantly reduce plasma levels of the nitrogenous compounds creatinine and urea (Table 3). By contrast, cisplatin-injected rats treated with either 0.1 or 0.3 mg kg^{-1} CPX had lower ($P < 0.01$) plasma levels of creatinine and urea on days 3 and 7 than vehicle-treated animals (Table 3). Moreover, treatment with either 0.1 or 0.3 mg kg^{-1} CPX resulted in a significant reduction of creatinine levels on both days 3 and 7 compared to treatment with 0.03 mg kg^{-1} (Table 3). Table 3 shows that although mean urea levels on days 3 and 7 were lower in rats given 0.1 mg kg^{-1} compared to animals treated with 0.03 mg kg^{-1}, these differences were not statistically significant. However, rats dosed with 0.3 mg kg^{-1} of CPX had lower ($P < 0.05$) urea concentrations than animals given 0.03 mg kg^{-1}. Creatinine and urea concentrations were not significantly different in animals treated with either 0.1 or 0.3 mg kg^{-1} CPX (Table 3).

Figure 4 illustrates that treatment with CPX at a dose of 0.03 mg kg^{-1} did not result in a statistically significant increase in C_{IN} or C_{PAH} when compared to cisplatin-injected vehicle-treated rats. At 0.1 mg kg^{-1}, CPX significantly improved both C_{IN} and C_{PAH}; but at a dose of 0.3 mg kg^{-1} only C_{PAH} was significantly greater than the corresponding mean value for cisplatin-injected vehicle-treated rats (Figure 4). C_{IN} at 0.1 mg kg^{-1} and C_{PAH} at 0.3 mg kg^{-1} were significantly greater than in rats treated with 0.03 mg kg^{-1} CPX. Polyuria was not attenuated by any of the doses used (Table 4). In fact, Table 4 shows that there was a tendency for increased polyuria in all groups of cisplatin-injected rats treated with CPX, although these increases were not statistically significant. Inorganic ion excretion was largely unaffected by CPX treatment (Table 4). However, the 0.3 mg kg^{-1} dose did increase ($P < 0.05$) excretion of Na^+ and K^+ com-

Table 2 Total wet kidney weight and histological damage score 7 days after injection with saline or cisplatin

Group	n	*Total kidney weight* (g)	*Damage score*
Saline-injected, saline treated	8	2.16 ± 0.06	0.3 ± 0.2
Saline-injected, CPX treated (0.1 mg kg^{-1})	8	2.35 ± 0.09	0.1 ± 0.1
Saline-injected, CPX-treated (0.3 mg kg^{-1})	8	2.36 ± 0.06	0.7 ± 0.5
Cisplatin-injected, no treatment	15	2.92 ± 0.07***	4.0 ± 0.0
Cisplatin-injected, vehicle treated	15	2.61 ± 0.10***	3.3 ± 0.2#
Cisplatin-injected, CPX-treated (0.03 mg kg^{-1})	15	2.86 ± 0.10***	2.1 ± 0.2†
Cisplatin-injected, CPX-treated (0.1 mg kg^{-1})	15	2.75 ± 0.07***	2.5 ± 0.1†
Cisplatin-injected, CPX-treated (0.3 mg kg^{-1})	25	2.73 ± 0.08***	2.3 ± 0.2‡

Mean ± s.e.mean.
*** $P < 0.001$ relative to saline-injected saline treated (*t* test).
# $P < 0.01$ relative to cisplatin-injected no treatment (Mann-Whitney test).
† $P < 0.05$; ‡ $P < 0.001$ relative to cisplatin-injected vehicle treatment (Mann-Whitney test).
CPX, 8-cyclopentyl-1,3-dipropylxanthine.

Table 3 Plasma creatinine and urea concentrations in cisplatin-injected rats treated by i.v. injection with either vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.03, 0.1 and 0.3 mg kg^{-1}) twice daily for 2 days

Day	*No treatment* ($n = 15$)	*Vehicle* ($n = 15$)	*CPX* 0.03 mg kg^{-1} ($n = 15$)	*CPX* 0.1 mg kg^{-1} ($n = 15$)	*CPX* 0.3 mg kg^{-1} ($n = 25$)
(a) Creatinine (mg 100 ml^{-1})					
0	0.60 ± 0.02	0.61 ± 0.06	0.54 ± 0.3	0.60 ± 0.04	0.66 ± 0.02
3	2.0 ± 0.2	2.7 ± 0.3	2.1 ± 0.2	1.1 ± 0.1**#	1.2 ± 0.1**#
7	1.8 ± 0.3	2.3 ± 0.4	2.6 ± 0.3	1.3 ± 0.2**#	1.3 ± 0.1**#
(b) Urea (mg 100 ml^{-1})					
0	33 ± 2	43 ± 3	35 ± 1	35 ± 2	35 ± 1
3	126 ± 12	152 ± 10	114 ± 13	83 ± 9***	70 ± 5***#
7	148 ± 20	217 ± 25	153 ± 19	98 ± 11***	72 ± 6***#

Mean ± s.e.mean and number of rats in parentheses.
** $P < 0.01$; *** $P < 0.001$ relative to cisplatin-injected vehicle treated group (ANOVA).
# $P < 0.05$ relative to cisplatin-injected CPX (0.03 mg kg^{-1})-treated group (ANOVA).

pared to vehicle-treated animals (Table 4). All three doses of CPX significantly reduced the renal damage score relative to vehicle-treated rats, but the reduction in tubule necrosis was not dose-dependent and CPX treatment did not reverse the increase in kidney weight caused by cisplatin (Table 2).

Discussion

The results show that a single 6 mg kg^{-1} i.v. injection of cisplatin produced acute renal failure in the rat, and the changes in renal function were characterized by increases in plasma creatinine and urea concentration; reduced C_{IN} and C_{PAH} as well as polyuria. Cisplatin also caused an increase in kidney weight, necrosis to the S_3 segment of the proximal tubule and some cast formation. Treatment of cisplatin-injected rats with the vehicle for CPX (alkaline dimethyl sulphoxide) resulted in a small improvement in kidney morphology, but overall the vehicle did not reduce the severity of renal failure and this finding agrees with previous work using CPX vehicle in the glycerol model of acute renal failure (Kellett *et al.*, 1989). By comparison to vehicle-treated rats, animals given all three doses of CPX had reduced tubule necrosis, but with the 0.03 mg kg^{-1} dose, there were no other beneficial effects of therapy. However, increasing the dose to 0.1 mg kg^{-1} resulted in significant reductions in plasma creatinine and urea levels as well as marked improvements in C_{IN} and C_{PAH}. The highest dose of CPX, 0.3 mg kg^{-1}, also ameliorated renal dysfunction (including increases in Na^+ and K^+ excretion) but the increase in C_{IN}, about 75% relative to vehicle-treated group, was not statistically significant. There was some evidence in saline-injected rats that the 0.3 mg kg^{-1} dose of CPX depressed C_{IN}, which might account for the lack of a significant improvement of this variable in cisplatin-injected CPX-treated rats.

In the rat the polyuric effect of cisplatin is biphasic. The first phase occurs 1 to 2 days after exposure to the drug and is associated with normal GFR (Safirstein *et al.*, 1986). This phase usually abates spontaneously and seems to be due to cisplatin-induced inhibition of anti-diuretic hormone release or synthesis (Clifton *et al.*, 1982; Gordon *et al.*, 1982). The second phase, however, occurs 3 to 4 days after administration of drug and develops despite a persistent reduction of GFR. This polyuria results from a direct effect of cisplatin on the kidney, probably as a consequence of reduced medullary hypertonicity brought about by inhibition of urea recycling in the renal medulla (Safirstein *et al.*, 1981; Gordon *et al.*, 1982). In the present study, urine was collected 3 to 4 days after injection of cisplatin, so the polyuria detected was likely to be the second phase. There was no attentuation by CPX of the polyuria, in fact urine output was increased by about 4 ml 100 g^{-1} 24 h^{-1} in the three groups of CPX-treated rats. Although this increase was not statistically significant, it may be an indication of improved renal function. By antagonizing adenosine-induced vasoconstriction, CPX might elevate GFR and so increase the flow of filtrate entering the proximal tubule. As a consequence of the urine concentrating defect (Safirstein *et al.*, 1981; Gordon *et al.*, 1982) the kidney is unable to conserve this fluid, resulting in enhanced polyuria.

Neither inhibition of angiotensin II formation with captopril (Safirstein *et al.*, 1986) nor blockade of α-adrenoceptors (Daugaard & Abildgaard, 1989) improves cisplatin-induced nephrotoxicity. These findings suggest that activation of the renin-angiotensin system and increased sympathetic activity are not major pathogenic factors in the development of this type of renal damage. However, the observations that CPX as well as theophylline (Heidemann *et al.*, 1989) ameliorate nephrotoxicity imply that adenosine mediates, at least in part, the increase in renal vascular resistance seen during the development of cisplatin-induced renal failure. The effects of exogenous adenosine on the renal vasculature involve both

Table 4 Urine output and inorganic ion excretion 3 to 4 days after cisplatin injection in rats treated by i.v. injection with either vehicle (1.0 ml kg^{-1}) or 8-cyclopentyl-1,3-dipropylxanthine (CPX, 0.03, 0.1 and 0.3 mg kg^{-1}) twice daily for 2 days

	No treatment ($n = 15$)	*Vehicle* ($n = 15$)	*CPX* 0.03 mg kg^{-1} ($n = 15$)	*CPX* 0.1 mg kg^{-1} ($n = 15$)	*CPX* 0.3 mg kg^{-1} ($n = 25$)
Urine volume (ml 100 g^{-1} 24 h^{-1})	14.4 ± 1.7	13.3 ± 1.5	16.9 ± 2.1	18.9 ± 2.3	15.8 ± 1.6
Na^+ (mmol 100 g^{-1} 24 h^{-1})	0.25 ± 0.03	0.23 ± 0.02	0.28 ± 0.03	0.31 ± 0.04	0.39 ± 0.04**
K^+ (mmol 100 g^{-1} 24 h^{-1})	0.44 ± 0.05	0.36 ± 0.03	0.42 ± 0.04	0.46 ± 0.04	0.60 ± 0.05*
Cl^- (mmol 100 g^{-1} 24 h^{-1})	0.30 ± 0.03	0.31 ± 0.03	0.37 ± 0.04	0.34 ± 0.04	0.35 ± 0.03

Mean ± s.e.mean and number of rats in parentheses.
* $P < 0.05$; ** $P < 0.01$ relative to cisplatin-injected vehicle-treated group (ANOVA).

constriction of vessels in the outer cortex and vasodilatation in the inner cortex/medulla (Osswald, 1984). Vasoconstriction is likely to be mediated via A_1-adenosine receptors, whereas activation of A_2-receptors evokes vasodilatation (Murray & Churchill, 1984; Holz & Steinhausen, 1987). A_1-selective adenosine antagonists may be more beneficial than their non-selective counterparts, such as theophylline, since they will help to sustain GFR yet maintain vasodilatation and, therefore, provide better perfusion to the tubules. This selective effect could explain why treatment with CPX not only increased GFR but also reduced damage to the S_3 segment of the tubule.

The beneficial effects of CPX treatment were dose-dependent in as much as there was a clear differentiation between the improvements in renal function noted with the $0.03\,mg\,kg^{-1}$ dose and those obtained with the 0.1 and $0.3\,mg\,kg^{-1}$ doses. Increasing the dose from 0.1 to $0.3\,mg\,kg^{-1}$ did not lead to increased protection, so it is probable that the maximum beneficial effect had been reached with the $0.1\,mg\,kg^{-1}$ dose. Unfortunately it was not possible to confirm this by testing doses greater than $0.3\,mg\,kg^{-1}$ because of the poor aqueous solubility of CPX. All three doses of CPX antagonized adenosine-induced bradycardia in control rats, which results from A_1-receptor stimulation (Kellett *et al.*, 1989). These doses also blocked bradycardia in cisplatin-injected animals and there appeared to be no difference in the magnitudes of blockade seen in the two groups of rats. On increasing the dose of CPX, the displacement of the dose-response curve was not proportional in both groups of rats. The reason for this is not clear, but it could be explained by non-equilibrium conditions existing at the receptor sites. Such conditions might be produced by dose-dependent clearance of CPX and/or adenosine. In the latter case it is interesting that Vidrio *et al.* (1987) found that repeated administration of adenosine resulted in an enhancement of its cardiovascular effects. These authors concluded that this self-potentiation might be due to saturation or inhibition of the cellular uptake of adenosine.

Treatment of cisplatin-injected rats with $0.03\,mg\,kg^{-1}$ dose of CPX had little beneficial effect, yet this dose attenuated adenosine-induced bradycardia. This raises the possibility that another pharmacological property of CPX might be responsibile for its protective effects in renal failure. Alkylxanthines inhibit phosphodiesterases to different degrees (Amer & Kreighbaum, 1975) and there is evidence that CPX can inhibit these enzymes in cultured opossum kidney cells (Coulson & Scheinman, 1989). However, we have recently shown that in rats with glycerol-induced acute renal failure that neither 0.1 or $0.3\,mg\,kg^{-1}$ CPX reduced total renal phosphodiesterase activity for up to 6 h after i.v. administration (Panjehshahin *et al.*, 1992). Furthermore, in the same study we showed that repeated administration of CPX, twice daily for two days, at either 0.1 or $0.3\,mg\,kg^{-1}$ was also without effect on renal phosphodiesterase activity. This evidence and the finding by Heidemann *et al.* (1989) that enprofylline, an alkylxanthine which inhibits phosphodiesterase (Fredholm, 1985) but has a low affinity for adenosine receptors (Collis *et al.*, 1984), does not ameliorate cisplatin-induced renal failure, suggests that inhibition of renal phosphodiesterases does not account for the protective effects of CPX. The lack of a beneficial effect of the $0.03\,mg\,kg^{-1}$ dose, despite evidence of adenosine blockade, might be due to inadequate blockade of adenosine receptors within the kidney or to a shorter duration of adenosine blockade with this dose. This latter possibility is supported by the finding that the duration of blockade of adenosine-induced bradycardia with $0.03\,mg\,kg^{-1}$ was considerably shorter than that produced by either 0.1 or $0.3\,mg\,kg^{-1}$ CPX.

In conclusion, the results of this study demonstrate that the A_1-selective adenosine receptor antagonist CPX at doses of 0.1 and $0.3\,mg\,kg^{-1}$, i.v., is able to reduce significantly the severity of cisplatin-induced nephrotoxicity. These renal protective effects occur at doses which significantly antagonize adenosine-induced bradycardia. Our results support the work of Heidemann *et al.* (1989) and imply that adenosine plays a significant role in the pathogenesis of cisplatin-induced acute renal failure. The administration to patients of an adenosine antagonist with cisplatin should reduce the subsequent risk of nephrotoxicity and may allow higher doses of cisplatin to be employed in cancer chemotherapy.

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