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Sumatriptan (GR43175) inhibits cyclic-AMP accumulation in dog isolated saphenous vein

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Sumatriptan (GR43175) contracts rings of dog isolated saphenous vein by an action at 5-HT_1-like receptors. We have now examined the effects of sumatriptan on prostaglandin E_2 (PGE_2)-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in this tissue. Sumatriptan and 5-hydroxytryptamine (5-HT) produced a concentration-dependent inhibition of PGE_2-stimulated cyclic AMP accumulation (EC_{50} values of 250 nM and 80 nM respectively), responses that were mimicked by 5-carboxamidotryptamine but not by U-46619 or methoxamine. The response to sumatriptan (1 μM) was antagonised by methiothepin (1 μM), but not by metergoline (0.1 μM), spiperone (1 μM) or ondansetron (GR38032, 1 μM). These results suggest that 5-HT_1-like receptors which mediate contraction of the dog isolated saphenous vein are negatively coupled to adenylate cyclase in this preparation.

Introduction Sumatriptan (GR43175) is a novel 5-HT_1-like receptor agonist which shows a high degree of selectivity for the 5-HT_1-like receptor mediating smooth muscle contraction in dog isolated saphenous vein (Humphrey *et al.*, 1988). It has been shown that sumatriptan has modest affinity for 5-HT_1 binding sites, with greatest affinity for the 5-HT_{1D} subtype which seems similar, but not identical to the 5-HT_1-like receptor in dog saphenous vein (Humphrey *et al.*, 1988; Peroutka & McCarthy, 1989; Sumner & Humphrey, 1989). Interestingly, 5-HT_{1D} binding sites appear to be negatively coupled to adenylate cyclase (Schoeffter *et al.*, 1988). We therefore sought to explore the receptor coupling mechanism for the 5-HT_1-like receptor in dog isolated saphenous vein. We now report that this receptor appears to be negatively coupled to adenylate cyclase in this preparation.

Methods Lengths of saphenous vein (4–7 cm long) were removed from beagle dogs (Glaxo, 7–10 kg of either sex) either during anaesthesia or after being killed by an overdose of sodium pentobarbitone. These tissues were used immediately or after storage overnight at 4°C in gassed (95% O_2, 5% CO_2), modified Krebs solution (Apperley *et al.*, 1976). Rings of 2–3 mm in length were pretreated for 30 min with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100 μM) and used for measurements of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation and protein content as previously described (Sumner *et al.*, 1989). Results are expressed as arithmetic means ± s.e.mean of '*n*' observations, which is also the number of veins used. Student's unpaired *t* test was used to assess the significance of differences between mean values.

Results Sumatriptan (10 μM) reduced both basal and prostaglandin E_2 (PGE_2, 5 μM)-stimulated cyclic AMP accumulation in rings of dog isolated saphenous vein (basal: control 6.35 ± 1.23, sumatriptan 3.12 ± 0.61 pmol mg^{-1} protein, $n = 6$; PGE_2-stimulated: control 11.47 ± 1.48, sumatriptan 6.16 ± 1.34 pmol mg^{-1} protein, $n = 6$). The stimulation of cyclic AMP accumulation by PGE_2 (5 μM) and its inhibition by sumatriptan (10 μM) followed similar time courses, both effects being maximal after 1–2 min at 37°C (results not shown). Accordingly an incubation time of 2 min was used throughout the study. Sumatriptan and 5-hydroxytryptamine (5-HT) produced a concentration-dependent inhibition of PGE_2-stimulated cyclic AMP accumulation (Figure 1a), with both agonists producing a maximum of about 50% inhibition. Based upon this level of effect, the EC_{50} values for sumatriptan and 5-HT were 250 nM and 80 nM respectively ($n = 7$). The archetypal 5-HT_1-like receptor agonist, 5-carboxamidotryptamine (5-CT), also inhibited PGE_2-stimulated cyclic

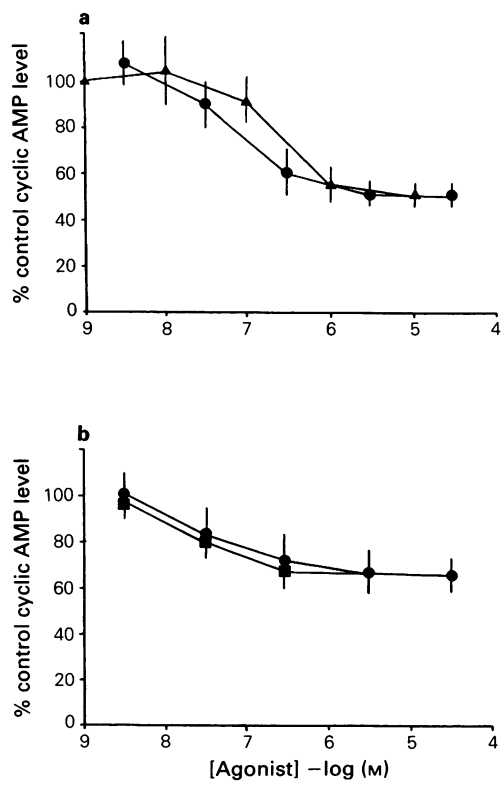


Figure 1 Inhibition of prostaglandin E_2 (PGE_2, 5 μM)-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in rings of dog isolated saphenous vein by 5-hydroxytryptamine (5-HT, ●) and sumatriptan (▲), (a) or by 5-HT (●) and 5-carboxamidotryptamine (5-CT, ■) (b). Calculated pEC_{50} values were 7.10, 6.60, 7.47 and 7.85 respectively, control levels being 9.43 ± 1.07 (a) and 14.02 ± 1.99 (b) pmol cyclic AMP mg^{-1} protein. Results are means of 7 determinations (14 rings) with vertical lines showing s.e.mean.

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AMP accumulation (Figure 1b) with an EC_{50} value of 14 nM compared to 34 nM for 5-HT ($n = 7$). The contractile agonists U-46619 (11,9 epoxymethano-PGH_2) (1 μM) and methoxamine (10 μM) however, did not significantly inhibit PGE_2-stimulated cyclic AMP accumulation (levels of 16.54 ± 1.86 pmol mg^{-1} protein and 10.88 ± 1.54 pmol mg^{-1} protein, respectively, compared to a value for sumatriptan (10 μM) of 6.77 ± 0.43 pmol mg^{-1} protein, and a common control of 13.50 ± 0.64 pmol mg^{-1} protein, $n = 6$ for each). The inhibition of PGE_2-stimulated cyclic AMP accumulation (control 11.39 ± 0.92 pmol mg^{-1} protein ($n = 6$)) by a submaximal concentration (1 μM) of sumatriptan (7.96 ± 0.88 pmol mg^{-1} protein) was attenuated by methiothepin (1 μM; 12.29 ± 1.18 pmol mg^{-1} protein) but not by spiperone (1 μM), ondansetron (GR38032, 1 μM) or metergoline (0.1 μM) (levels of 8.21 ± 0.82, 7.18 ± 0.83 and 7.30 ± 0.88 pmol mg^{-1} protein respectively, $n = 6$ for each). None of these antagonists had any appreciable effect on PGE_2-stimulated cyclic AMP accumulation in their own right at these concentrations.

Discussion The inhibition of cyclic AMP accumulation (either basal or PGE_2-stimulated) by sumatriptan in rings of dog isolated saphenous vein provides the first indication that receptors for this agonist in smooth muscle may be negatively coupled to adenylate cyclase. Thus the receptors mediating both smooth muscle contraction (Humphrey *et al.*, 1988) and inhibition of cyclic AMP accumulation (this study) in this tissue appear identical and represent a sub-type of the 5-HT_1-like receptor class. In keeping with this, 5-HT was some 3–4 fold more potent than sumatriptan, with 5-CT being approximately twice as potent as 5-HT in this study. These relative potencies are almost the same as those for producing contraction in this preparation (Humphrey *et al.*, 1988). In addition, both the contraction of smooth muscle and the inhibition of cyclic AMP accumulation evoked by sumatriptan can be antagonized by methiothepin, but not by metergoline, spiperone, or ondansetron (Humphrey *et al.*, 1988; this study). Inhibition of adenylate cyclase has been implicated as the coupling mechanism for 5-HT_{1A} (Schoeffter & Hoyer, 1988), 5-HT_{1B} (Bouhelal *et al.*, 1988) and 5-HT_{1D} (Schoeffter *et al.*, 1988) binding sites in brain homogenates. Furthermore, sumatriptan shows greatest affinity for 5-HT_{1B} and 5-HT_{1D} binding sites (Peroutka & McCarthy, 1989; Hoyer & Middlemiss, 1989; Sumner & Humphrey, 1989). The 5-HT_1-like receptor in dog saphenous vein shows many similarities to 5-HT_{1D} binding sites (and 5-HT_{1B} sites in rats). There is, however, one obvious exception, namely the lack of effect of metergoline (0.1 μM) in the dog saphenous vein as already described, yet this antagonist shows a high affinity for 5-HT_{1D} binding sites in both radioligand binding and adenylate cyclase assays (Hoyer & Middlemiss, 1989). Despite this exception, there is a lot of data to suggest that the subtype of 5-HT_1-like receptor which subserves the responses to sumatriptan in the dog saphenous vein has its counterpart in 5-HT_{1D} (or 5-HT_{1B} in rats) binding sites, although the apparent heterogeneity of 5-HT_{1D} binding sites may confuse interpretation (Sumner & Humphrey 1989). Finally, although further experiments are required to reach definitive conclusions, it is interesting to note that smooth muscle contraction evoked by U-46619 and methoxamine in the dog saphenous vein was not accompanied by changes in cyclic AMP levels. Thus mechanistically, there does not appear to be an obligatory control of smooth muscle contraction by reduction of cyclic AMP levels in this vessel.

In summary, this study suggests that the subtype of 5-HT_1-like receptor which mediates sumatriptan-induced vascular smooth muscle contraction in dog saphenous vein is coupled to the inhibition of adenylate cyclase in this vessel. This further reinforces the similarity between this 5-HT_1-like receptor and the 5-HT_{1D} binding site.

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Effects of a selective 5-HT_2 agonist, DOI, on 5-HT neuronal firing in the dorsal raphe nucleus and 5-HT release and metabolism in the frontal cortex

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Systemic administration of the 5-HT_2 agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (50 and 100 $\mu g kg^{-1}$, i.v.) inhibited dorsal raphe neuronal firing. DOI (100 $\mu g kg^{-1}$, i.v.) also produced a decrease in extracellular 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the frontal cortex measured by microdialysis. However, local administration of DOI into the frontal cortex produced no change in extracellular 5-HT and 5-HIAA at any dose given (1, 10 and 100 ng). The results demonstrate that DOI is a potent inhibitor of 5-HT neuronal firing and terminal release and that the effects on release are not mediated by an action within the terminal region. The site of action and the receptor involved in the inhibition remains to be determined.

Introduction 5-Hydroxytryptamine (5-HT) receptors can be divided into number of types of which the 5-HT_{1A} and 5-HT_2 subclasses are most thoroughly characterized (Glennon, 1987). The 5-HT_{1A} selective agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OHDPAT) (Hjorth *et al.*, 1982) is a potent inhibitor of dorsal raphe nucleus (DRN) neuronal firing via action on autoreceptors located on the cell bodies and/or dendrites (but not presynaptic terminals) of ascending 5-HT neurones in the DRN (Verge *et al.*, 1985). Systemic administration of the 5-HT_2 agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Glennon, 1987) has been found to inhibit DRN neuronal firing (Garratt & Marsden, 1989). Changes in DRN neuronal function normally lead to corresponding changes in 5-HT release and metabolism in the regions containing 5-HT terminals (Marsden *et al.*, 1989).

This study investigated the effect of DOI on DRN 5-HT neuronal firing and release and metabolism in the frontal cortex to determine whether this 5-HT_2 agonist altered 5-HT release in the frontal cortex *in vivo* and whether effects observed were due to a direct effect of DOI in the frontal cortex or secondary to changes in DRN neuronal firing.

Methods In all experiments male Wistar rats (250–300 g) were housed in groups of five under a 12 h light-dark cycle and allowed food and water *ad libitum*. They were anaesthetized with halothane (1.5–2%)/O_2/N_2O, the jugular vein was cannulated to allow intravenous administration of drugs.

Electrophysiology Single barrelled glass electrodes with an *in vitro* impedance of 4–6 MΩ were used. These were filled with 2 M NaCl containing 2% pontamine sky blue. The animal was placed in a stereotaxic frame and a hole was drilled 7.8 mm posterior of bregma on the midline. The dorsal raphe nucleus is located 5.0–6.5 mm below the dura. 5-HT cells were physiologically identified by the criteria of Aghajanian *et al.* (1968), and pharmacologically by the intravenous administration of 8-OHDPAT which temporarily reduces DRN 5-HT cell firing (Figure 1a). DOI (50 or 100 $\mu g kg^{-1}$, i.v.) or control injection was then administered. After a recording, the location of the cell was marked with a spot of dye permitting the confirmation of the electrode position histologically.

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Dialysis Dialysis probes were constructed and perfused with artificial CSF (pH 7.4) at a rate of 1 $\mu l min^{-1}$. The dialysis probe was implanted into the frontal cortex (rostal-caudal +3.6 mm from bregma, saggital ±1.5 mm from bregma, vertical 2.5 mm from the dura). For local injections a guide cannula was implanted alongside the dialysis probe. From 2 h after implantation, dialysis samples were collected every 20 min.

H.p.l.c. assay of 5-HT and 5-HIAA 5-HT and 5-HIAA were separated by ion-pair, reverse phase chromatography, by use of column packed with 3 μm Hypersil. Mobile phase was pumped through the column at a flow rate of 0.25 $ml min^{-1}$. The amines eluted from the column were measured with a dual glassy carbon working electrode maintained at a potential of +0.85 V. Basal levels were measured for 80 min after which one group of rats received either systemic DOI (100 $\mu g kg^{-1}$, i.v.) or saline (i.v.) while the other group was given either DOI (1, 10 and 100 ng in 0.5 μl CSF) or CSF (0.5 μl) into the frontal cortex and dialysis samples collected for a further 180 min.

Results DOI (50 $\mu g kg^{-1}$, i.v.) produced a 60% inhibition of the firing rate, the higher dose (100 $\mu g kg^{-1}$, i.v.) caused complete cessation of DRN 5-HT neuronal firing for over an hour (Figure 1a). In the frontal cortex the higher dose of DOI produced a decrease in both extracellular 5-HT (44 ± 10.2% of saline control) and 5-HIAA (32 ± 7.4% of saline control) (Figure 1b). DOI (1, 10, and 100 ng) administered locally in the frontal cortex produced no change in either extracellular 5-HT or 5-HIAA at any dose given (Figure 1c).

Discussion The results demonstrate that the 5-HT_2 agonist DOI is a potent inhibitor of DRN 5-HT neuronal firing. Furthermore, the effects of DOI appear to be dose-related. Although DOI has produced a similar effect to 8-OHDPAT it is unlikely that the inhibition seen with DOI is due to an action at the 5-HT_{1A} site since the affinity of DOI for this site is very low (Glennon, 1987) and it would require large doses of DOI for any binding to 5-HT_{1A} sites to occur. Autoradiographic studies have shown a high density of postsynaptic 5-HT_2 sites in the cortex (Hoyer *et al.*, 1986) and more recent studies using [^{125}I]-DOI revealed highest binding in the

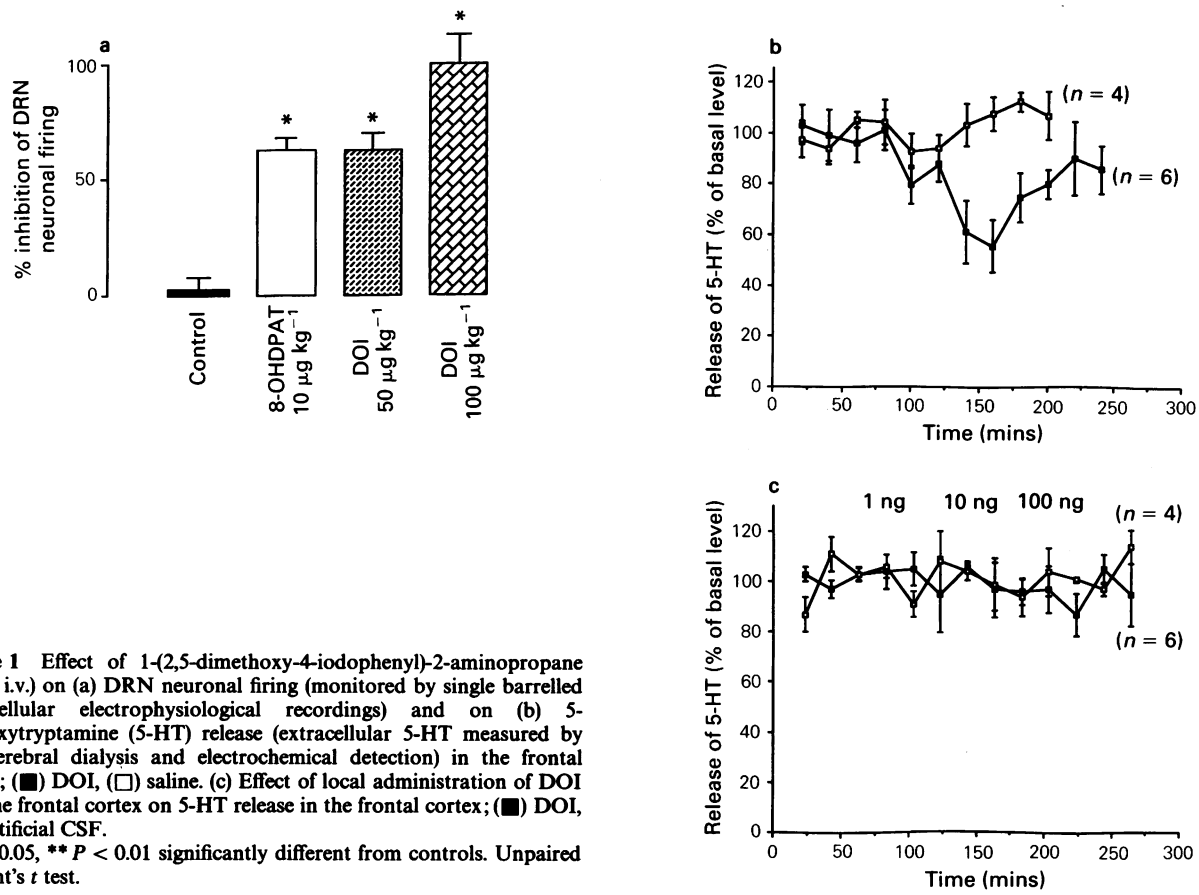


Figure 1 Effect of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, i.v.) on (a) DRN neuronal firing (monitored by single barrelled extracellular electrophysiological recordings) and on (b) 5-hydroxytryptamine (5-HT) release (extracellular 5-HT measured by intracerebral dialysis and electrochemical detection) in the frontal cortex; (■) DOI, (□) saline. (c) Effect of local administration of DOI into the frontal cortex on 5-HT release in the frontal cortex; (■) DOI, (□) artificial CSF.
* $P < 0.05$, ** $P < 0.01$ significantly different from controls. Unpaired Student's t test.

choroid plexus with high binding in the frontoparietal cortex and nucleus accumbens (McKenna *et al.*, 1989). As systemic but not local injection (frontal cortex) of DOI produced a significant decrease in both 5-HT release and metabolism in the frontal cortex it would appear that the decrease in release is not a result of a direct action of DOI on these [^{125}I]-DOI binding sites within the terminal region and indirectly supports evidence that the 5-HT$_2$ binding sites in the frontal cortex are located postsynaptically.

It is possible that the effects of DOI described in this paper may not be due to an action on the classical 5-HT$_2$ site since initial binding data suggested that [^{125}I]-DOI labelled a high-affinity state of the 5-HT$_2$ recognition site (Glennon *et al.*, 1988). McKenna & Peroutka (1989) have shown that structural congeners of R-(−)-DOI display a higher affinity for the [^{125}I]-R-(−)-DOI binding site than the [^{3}H]-ketanserin labelled binding site, which may suggest that there are two subtypes of the 5-HT$_2$ site.

The method(s) of action and receptor type(s) through which DOI produces inhibition of DRN 5-HT neuronal firing and decreases 5-HT release and metabolism warrant further investigation.

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Characterization of presynaptic vascular muscarinic receptors inhibiting endogenous noradrenaline overflow in the portal vein of the freely moving rat

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1 In the portal vein of permanently cannulated, freely moving, unanaesthetized rats, methacholine (MCh) is able to inhibit the electrically-evoked endogenous noradrenaline (NA) overflow. This inhibition is mediated by presynaptic inhibitory muscarinic heteroreceptors.

2 By use of pirenzepine, 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP) and AF-DX 116 as M_1-, M_3-, and M_2-selective antagonists respectively, the MCh (0.1 μM)-induced inhibition of the electrically-evoked NA overflow could be reversed to the control stimulation value dose-dependently.

3 The potency order of the antagonists was: 4-DAMP > AF-DX 116 > pirenzepine, pIC_{50} values being 8.50, 7.96 and 7.01, respectively.

4 From these results it was concluded that the inhibitory presynaptic heteroreceptors in the portal vein of concious unrestrained rats are of the cardiac M_2-subtype.

Introduction

More than twenty years ago, Lindmar *et al.* (1968) demonstrated the presence of muscarinic receptors on terminal sympathetic nerves of the rabbit heart. Agonists for these receptors were able to inhibit noradrenaline (NA) overflow from those terminals (Lindmar *et al.*, 1968; Löffelholz & Muscholl, 1969). Since then, modulation of the noradrenaline (NA) overflow by prejunctional cholinoceptors has been demonstrated in a number of tissues (see Vanhoutte & Levy, 1980; Langer, 1981; Shepherd & Vanhoutte, 1985, for reviews). The nature of the prejunctional cholinoceptors appeared to be muscarinic, because atropine and not hexamethonium fully antagonized the acetylcholine-induced reduction of stimulus-evoked NA overflow (Löffelholz & Muscholl, 1969; Rorie *et al.*, 1981).

Subtypes of muscarinic receptors were first proposed by Birdsall *et al.* (1978) and they are currently divided into at least three subclasses: M_1, found mainly on cerebral cortex, sympathetic ganglia and in the digestive tract, M_2 or M_{2A}, found on the heart and in certain parts of the central and peripheral nervous system, and M_3 or M_{2B}, found on exocrine glands, smooth muscle and in parts of the central nervous system. This subclassification has been derived from results obtained with a number of selective antagonists, mainly in *in vitro* studies (see Eglen & Whiting, 1986, for a review).

Recently, we showed the existence of presynaptic muscarinic receptors that inhibited NA release from the nervous plexus of the portal vein in freely moving, unanaesthetized rats, with methacholine (MCh) as an agonist and atropine as a muscarinic antagonist (Remie *et al.*, 1989).

In the present study, we investigated the subtype of these receptors by antagonizing the MCh-induced inhibition of electrically-evoked endogenous NA overflow in the rat portal vein, by use of pirenzepine as an M_1-selective antagonist, 11[[2-[(diethylamino)methyl]-1-piperidyl]acetyl]-5,11-dihydro-6H-pyrido [2,3-b][1,4]benzodiazepine-6-one (AF-DX 116) as an M_2-selective antagonist and 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP) as an M_3-selective antagonist.

Methods

Male normotensive Wistar rats, weighing 270–350 g, were used. All animals were housed in individual cages and were permitted tap water and standard laboratory chow *ad libitum*. The lights were on from 7 h 00 min–19 h 00 min. The rats were prepared surgically according to the method described recently (Remie & Zaagsma, 1986; Remie *et al.*, 1988). Briefly, under ether anaesthesia a five fine-stitch purse-string suture was placed in the wall of the portal vein at the side opposite the gastroduodenal vein and a silicon cannula was inserted downstream into the portal vein. Similarly, a second cannula, used for the infusion of drugs was inserted into the portal vein, at the side opposite the superior pancreatico-duodenal vein. A bipolar stimulation electrode was placed around the portal vein, between the two cannulae. Both cannulae and the electrode wires were run subcutaneously and emerged at the crown of the head, where they were affixed to the skull.

With this technique, we were able to stimulate the local nervous plexus of the portal vein, to apply drugs locally and to take blood samples from the portal vein without disturbing the animal. The blood samples (200 μl) were collected according to the technique described previously (Remie & Zaagsma, 1986), into small ice-cooled cups containing some EDTA crystals, and red blood cells were spun down immediately (Beckmann Microfuge).

Catecholamines were determined by high performance liquid chromatography with electrochemical detection, in 100 μl plasma which was extracted according to the method described recently (Remie *et al.*, 1988). The analytical column was an Alltech Rosil C18 5μ cartridge (150 × 4.6 mm) and the mobile phase was composed of ultra pure water/methanol (94.0:6.0, v/v), citric acid 1.35%, NaH_2PO_4 1.3%, heptanesulphonic acid 0.06%, and 0.01% of both EDTA and NaCl. The pH was adjusted to 4.1 with citric acid. Before use, the solution was filtered (Schleicher and Schull, 0.2 μm) and degassed by sonication at low pressure.

An ESA Coulochem 5100 A electrochemical detector with a high sensitivity analytical cell (5011) was used for the detection of the catecholamines. The guard potential was set to

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+450 mV, the first detector potential to −350 mV and the second high sensitivity detector to +350 mV. The chromatograms were recorded on a Kipp BD 41 dual channel flatbed recorder. Corrections for incomplete recovery were performed, with 3,4-dihydroxybenzylamine as the internal standard.

Experiments were started approximately ten days after surgery. The nervous plexus of the portal vein was stimulated by square wave constant current (5 mA) biphasic pulses with a frequency of 2 Hz and a duration of 3 ms. Electrical stimulation started at t = 0 and lasted 2 min. Blood samples of 200 μl were taken at the following times: t = −20, −15, −10, −5, 0, 2, 3 min and during the electrical stimulation period every 15 s. For the control experiments, intravenous infusion of MCh was on from t = −10 to t = 2 min. Electrical stimulation started at t = 0 and lasted 2 min. During electrical stimulation a blood sample was taken every 15 s. Intravenous infusion of the antagonist alone started after the t = −20 sample and lasted 10 min, followed by an infusion of the antagonist together with MCh from t = −10 until t = 2 min. The amount of NA overflow during electrical stimulation was calculated from the area under the curve as illustrated in Figure 1. The ratio between the two areas, obtained in the presence and in the absence of MCh, respectively, reflects the change in NA overflow. The increase in the amount of NA overflow during electrical stimulation in the absence of drugs was set to 100%.

Agonist and antagonists were given via the infusion cannula at a flow rate of 6 ml h^{-1}. Drug concentrations in the portal vein were calculated, based on a portal blood flow of 15 ml min^{-1}, measured by Daemen *et al.* (1989), with an electromagnetic flow probe and by Tsuchiya *et al.* (1978), who used microspheres. These calculated concentrations are used throughout the paper (see also Discussion).

The pIC_{50} values of the antagonists for muscarinic receptors in the portal vein were calculated as the −log of the portal vein concentration (molar) of the antagonist causing 50% inhibition of the MCh-induced decrease of electrically-evoked NA overflow. Atropine (0.6 mg kg^{-1}), administered 15 min before stimulation, was used in separate experiments to determine the maximal amount of electrically-evoked NA overflow in the presence of an antagonist; this was 122 ± 8% of the control stimulation value which indicates a minor inhibition of NA overflow by endogeneous acetylcholine, released during co-stimulation of cholinergic neurones (Remie *et al.*, 1989). To allow calculation of the pIC_{50} values this maximal effect was used for all the selective antagonists and the least square regression line analysis was used to determine the concentration needed to obtain 61% (pIC_{50}) of the maximal effect.

Statistical analysis

The results are expressed as the means ± s.e.mean, the number of experiments is indicated by *n*. The statistical significance of the results was analysed by Student's *t* test for unpaired data and a *P* value of less than 0.05 was regarded as being significant.

Drugs used

Methacholine chloride (MCh, Aldrich Chemical Company Inc., Milwaukee, WIS, U.S.A.); AF-DX 116 and pirenzepine were gifts from Dr Karl Thomae GmbH (Biberach an der Riss, F.R.G.); 4-DAMP was a gift from Dr R.B. Barlow (Bristol, U.K.). All drugs were dissolved in sterile saline and were given via the infusion cannula.

Results

Figure 1 represents a typical experiment showing the effect of electrical stimulation of the portal vein nervous plexus on NA overflow in the absence and presence of MCh (0.1 μM). The total amount of NA released during stimulation in the presence of MCh is reduced to 4 ± 12% of the control stimulation value (Figures 1 and 2).

Blockade with the M_1-selective antagonist pirenzepine at concentrations of 70, 400 and 1000 nM, resulted in a dose-dependent reversion of the MCh-induced inhibition of NA overflow during electrical stimulation to 54 ± 18%, 88 ± 13% and 110 ± 6% of the control stimulation value respectively (Figure 2).

Much lower concentrations of the M_3-selective antagonist 4-DAMP (1, 10 and 100 nM) were already capable of blocking presynaptic muscarinic receptors, resulting in a dose-dependent reversal of the MCh-induced inhibition of electrically-evoked NA overflow to 40 ± 11%, 79 ± 9% and 102 ± 20% of the control stimulation value, respectively (Figure 2).

The M_2-selective antagonist AF-DX 116 showed a similar high potency towards the MCh-induced inhibition of evoked NA overflow. At concentrations of 5, 30 and 50 nM AF-DX 116, a reversal to 42 ± 15%, 67 ± 7% and 121 ± 9% respectively of the control stimulation value was seen, (Figure 2).

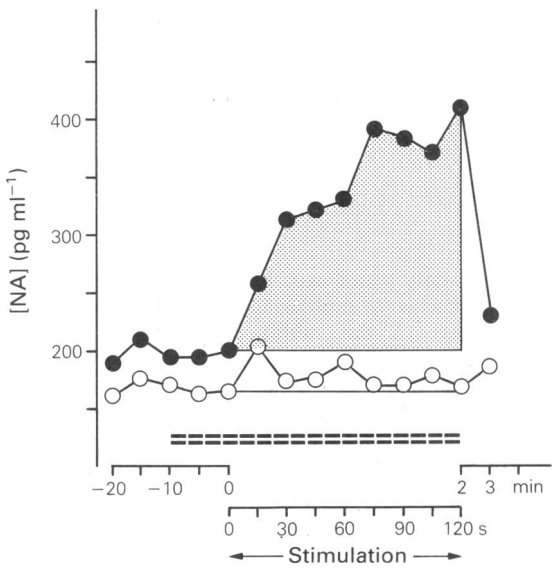


Figure 1 Typical experiment showing the effect of portal methacholine (MCh) infusion (0.3 μg min^{-1}, indicated by ::::) on basal and electrically-evoked (5 mA, 2 Hz and 3 ms) noradrenaline (NA) overflow in the portal vein of the freely moving, unanaesthetized rat (○). The stippled area under the curve (●) represents the total amount of NA overflow during 120 s of electrical stimulation in the absence of MCh (control stimulation).

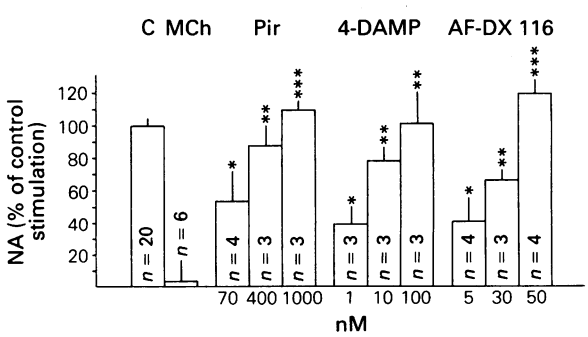


Figure 2 The effect of portal administration of increasing concentrations (nM) of the muscarinic antagonists pirenzepine (Pir), 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP) and AF-DX 116 on the methacholine (MCh, 0.1 μM)-induced inhibition of electrically-evoked endogenous noradrenaline (NA) overflow in the portal vein of freely moving unanaesthetized rats. The amount of NA overflow in the absence of agonist and antagonist was set as 100 ± 6% (C). Asterisks denote values that differ significantly from the corresponding control value (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

From these data the following potency order was calculated for the three antagonists: 4-DAMP > AF-DX 116 > pirenzepine, with pIC_{50} values of 8.50, 7.96 and 7.01, respectively.

Discussion

Much evidence has accumulated that, in both cholinergic and adrenergic nerve terminals, acetylcholine is able to inhibit stimulus-evoked transmitter release mediated via muscarinic receptors (see Vanhoutte & Levy, 1980; Shepherd & Vanhoutte, 1985, for reviews).

Recently we showed, with freely moving, unanaesthetized, permanently cannulated rats (Remie & Zaagsma, 1986; Remie *et al.*, 1988), a MCh-induced inhibition of NA overflow during electrical stimulation of the portal vein nervous plexus. This inhibition could be reversed to control values by using the non-selective muscarinic antagonist atropine, indicating the muscarinic nature of these inhibitory presynaptic receptors (Remie *et al.*, 1989).

Based on binding studies with agonists to brain membranes, Birdsall *et al.* (1978) were the first to propose heterogeneity of muscarinic receptors. After the discovery of antagonists like pirenzepine, 4-DAMP and AF-DX 116, this concept was strengthened significantly and muscarinic receptors were subclassified into M_1, M_2 or M_{2A} and M_3 or M_{2B} receptors (see Eglen & Whiting, 1986, for a review). Most of these results were obtained from *in vitro* studies. Some of these *in vitro* selectivities have been confirmed in *in vivo* experiments. For example, the M_1-selective antagonist pirenzepine has been shown to antagonize the rise in blood pressure induced by the M_1-selective agonist, McN-A-343, in the pithed rat with 16 to 40 fold lower dosages than the MCh-induced fall in blood pressure or vagally-induced bradycardia, being M_3 and M_2 responses, respectively (Hammer & Giachetti, 1982; Wess *et al.*, 1984).

The M_2-selective antagonist AF-DX 116 has been shown to possess the opposite selectivity in antagonizing vagally-induced bradycardia with a 7 fold lower dose than the McN-A-343-induced pressor response; moreover, it antagonized glandular and smooth muscle responses only at high dosages (Giachetti *et al.*, 1986). Interestingly, in *in vivo* radioligand binding experiments, AF-DX 116 was also shown to exhibit the above mentioned selectivity pattern (Hammer *et al.*, 1986).

Recently the results of Giachetti *et al.* (1986) and Hammer *et al.* (1986) were corroborated and extended with the M_3-selective antagonist 4-DAMP (Doods *et al.*, 1987). In this study 4-DAMP showed a 2,000 fold higher affinity *in vivo* for muscarinic receptors on exocrine gland (M_3-subtype) compared to AF-DX 116. However, by measuring antagonism of oxotremorine-induced reduction of the heart rate, 4-DAMP was found to possess almost the same affinity, when compared to AF-DX 116, for the cardiac M_2-subtype.

In the present study we investigated the antagonism by 4-DAMP, AF-DX 116 and pirenzepine of the MCh-induced inhibition of endogenous NA overflow in the rat portal vein. Because all drugs were applied by local infusion, molar drug concentrations in the portal vein were calculated on the basis of a portal blood flow of $15\,ml\,min^{-1}$ (Tsuchiya *et al.*, 1978; Daemen *et al.*, 1989). Naturally, the administration of MCh could change the flow, either by vaso-constriction or, possibly, by (EDRF-mediated) vasodilatation. However, such changes of the vascular diameter will be diminished dose-dependently by the antagonists as well, so that the measured IC_{50} values would differ from the 'real' IC_{50} values by approximately the same factor with all antagonists.

The pIC_{50} values for the reversion of the MCh-induced inhibition of NA overflow by pirenzepine, AF-DX 116 and 4-DAMP were 7.01, 7.96 and 8.50, respectively. These data show that 4-DAMP was 31 times more potent than pirenzepine and 3.5 times more potent than AF-DX 116. These potency-ratios have also been observed in radioligand binding experiments on bovine cardiac left ventricular membranes and rat atrial membranes: the pK_i values revealed 4-DAMP to have a 38 to 41 fold higher affinity than pirenzepine and a 6 fold higher affinity than AF-DX 116. However, they are in sharp contrast to the affinity (pA_2) ratios obtained on bovine tracheal smooth muscle contraction (4-DAMP being 129 and 537 times more potent than pirenzepine and AF-DX 116, respectively) and pK_i ratios (126 and 1514, respectively) found in binding experiments with rat glandular membranes (Doods *et al.*, 1987; Roffel *et al.*, 1988). The excellent agreement between the relative potencies of the three antagonists to reverse the MCh-induced inhibition of evoked NA overflow *in vivo* and the affinity ratios obtained with cardiac muscarinic receptors *in vitro*, confirms the M_2-nature of the inhibitory presynaptic muscarinic heteroreceptor.

One of the reasons for the striking similarity between the *in vivo* and *in vitro* potency-ratios might be our experimental set-up in which permanently cannulated unanaesthetized, freely moving rats were used. The continuous infusion of the antagonist and MCh, which had already started 20 and 10 min, respectively, before the electrical stimulation of the nervous plexus, results in stable local drug concentrations in the portal vein, creating optimal conditions to study locally the nature of the presynaptic receptors *in vivo*.

Our results are in agreement with recent *in vitro* data showing an M_2-like potency of AF-DX 116 in antagonizing the MCh-induced inhibition of [^{3}H]-NA overflow, both in rat heart and guinea-pig iris (Dammann *et al.*, 1989).

In conclusion, the relative potencies of pirenzepine, AF-DX 116 and 4-DAMP, to antagonize the MCh-induced decrease of stimulus-evoked portal NA overflow, revealed the presynaptic muscarinic heteroreceptor involved to have a typical cardiac M_2-nature.

We are grateful to Dr Karl Thomae GmbH (Biberach an der Riss, F.R.G.) for the gifts of AF-DX 116 and pirenzepine, and to Dr R.B. Barlow (Bristol, U.K.) for the gift of 4-DAMP.

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Effect of 5-HT_3 receptor antagonists on responses to selective activation of mesolimbic dopaminergic pathways in the rat

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1 The effects of 5-hydroxytryptamine$_3$ (5-HT_3) receptor antagonists on the behavioural hyperactivity response which results from injection of the neurokinin receptor agonist [pGlu^5, MePhe^8, Sar^9]-substance P (5–11) (DiMe-C7) into the ventral tegmental area (VTA) of the rat midbrain have been determined.

2 Subcutaneous administration of ondansetron (GR38032) (0.001–0.3 mg kg^{-1}), GR65630 (0.01 mg kg^{-1}), ICS 205–930 (0.1 mg kg^{-1}) and MDL 72222 (0.1 mg kg^{-1}), inhibited the DiMe-C7-induced hyperactivity response.

3 The effects of ondansetron on DiMe-C7-induced changes in dopamine and 5-HT metabolism in discrete areas of rat forebrain were studied in order to investigate further the possible mechanism of action of 5-HT_3 antagonists in modifying mesolimbic dopaminergic systems.

4 Intra-VTA administration of DiMe-C7 increased levels of dihydroxyphenylacetic acid (DOPAC) in the nucleus accumbens, olfactory tubercules and right amygdala, indicating increased mesolimbic dopamine metabolism. DOPAC levels were not significantly increased in the frontal cortex, left amygdala or striatum. Dopamine levels were not altered in any of these brain areas. DiMe-C7 also increased 5-hydroxyindoleacetic acid (5-HIAA) levels in the amygdala but this was only statistically significant in the right amygdala. 5-HT levels were not changed significantly by DiMe-C7 treatment.

5 In control rats, pretreatment with ondansetron (0.1 mg kg^{-1}) had no effect on the levels of dopamine, 5-HT or their metabolites, but in rats given DiMe-C7, ondansetron significantly inhibited the increase in DOPAC levels in the nucleus accumbens.

6 These results are in agreement with the proposed facilitatory role of 5-HT_3 receptor activation on mesolimbic dopaminergic transmission, and suggest that 5-HT_3 antagonists may have important therapeutic indications for the treatment of CNS disorders in which mesolimbic dopamine systems are perturbed.

Introduction

The 5-hydroxytryptamine$_3$ (5-HT_3) receptor antagonists which include ondansetron (GR38032) (Butler *et al.*, 1988), ICS 205-930 (Richardson *et al.*, 1985), MDL 72222 (Fozard, 1984), zacopride (Smith *et al.*, 1988) and BRL43694 (Fake *et al.*, 1987) can reduce the behavioural consequences of raised mesolimbic dopamine activity in the rat and marmoset (Hagan *et al.*, 1987; Costall *et al.*, 1987a, b). The mechanism(s) underlying this action are unclear. Using radioligand binding with the 5-HT_3 receptor antagonist [^{3}H]-GR65630, Kilpatrick *et al.* (1987) have recently demonstrated that binding sites with the characteristics of 5-HT_3 receptors are present predominantly in limbic and cortical areas of the forebrain of the rat such as the entorhinal cortex, frontal cortex, amygdala and nucleus accumbens/olfactory tubercules. In contrast, few binding sites are found in striatum and lower brain areas, with the exception of the area postrema, where the highest density of 5-HT_3 receptors exist. 5-HT_3 receptors in the area postrema may mediate the anti-emetic properties of 5-HT_3 antagonists in other species (Higgins *et al.*, 1989).

It is possible, therefore, that the effect of 5-HT_3 receptor antagonists on dopamine-induced hyperactivity responses may be mediated through limbic or cortical 5-HT_3 receptors. Indeed, Costall *et al.* (1987a,b) have shown that 5-HT_3 receptor antagonists inhibit raised mesolimbic dopaminergic activity through an action on limbic 5-HT_3 receptors. Since 5-HT_3 receptor antagonists have no overt effect on normal rat behaviour, it appears necessary to excite mesolimbic dopaminergic pathways to demonstrate their behavioural effects.

We have previously shown (Hagan *et al.*, 1987) the effects of ondansetron on mesolimbic dopaminergic systems, utilizing a model of dopamine-mediated behavioural hyperactivity in the rat, first described by Eison *et al.* (1982a). In this model, the injection of the metabolically stable neurokinin receptor agonist, [pGlu^5, MePhe^8, Sar^9]-substance P(5–11) (DiMe-C7), into the ventral tegmental area (VTA) causes a complex behavioural hyperactivity response which is thought to arise, at least in part, from activation of the mesolimbic dopaminergic pathway. The increased locomotor activity induced by DiMe-C7 can be blocked by haloperidol (Eison *et al.*, 1982b) and is accompanied by increases in dopamine metabolism in the mesolimbic and mesocortical areas of the forebrain (Elliott *et al.*, 1986). We found that ondansetron reduced both the hyperactivity response and the increase in dopamine metabolism produced by the DiMe-C7 treatment in the nucleus accumbens. We have now extended these observations to include other 5-HT_3 receptor antagonists and have further investigated the effects of ondansetron on DiMe-C7-induced changes in dopamine metabolism in other areas of rat forebrain, including the amygdala. By use of discrete injection techniques, other workers have shown that some of the behavioural effects of 5-HT_3 receptor antagonists may be mediated by the amygdala (see Costall *et al.*, 1987a, 1989). Interestingly, ondansetron was found to be more effective at inhibiting the hyperactivity response to unilateral infusion of dopamine into the left amygdala when it was injected into the non-infused right amygdala (Costall *et al.*, 1987a). Therefore, in the present experiments, possible neurochemical effects were investigated in both left and right amygdala. In order to investigate the involvement of 5-HT neurones in the hyperactivity response we have also looked at 5-HT metabolism in DiMe-C7-treated animals.

Methods

Animals

Male Random-Hooded strain rats (Rodent Breeding Unit, Glaxo Group Research, Ware) weighing 250–320 g were used.

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The rats were housed in groups of 5 at a temperature of 20°C on a 12 h light-dark cycle of lights on between 06 h 30 min and 18 h 30 min. Rats were fed Rat and Mouse No. 1 expanded maintenance diet (Special Diet Services) and allowed water *ad libitum*.

Stereotaxic surgery

Rats were anaesthetized with chloral hydrate (550 mg kg^{-1} s.c.) and placed in a Kopf stereotaxic frame. Guide cannulae, consisting of stainless steel tubing (23 gauge) in a perspex holder, were implanted bilaterally, positioned stereotaxically 3.0 mm above the VTA (Ant. +3.0, Vert. −2.5, Lat. ±1.0) with the incisor bar 5.0 mm above the inter-aural line. Stainless steel stylets (30 gauge) were used to keep the guide cannulae patent during the two week recovery period. These extended 0.5 mm beyond the guide tips.

Intracerebral injections

Ten days after surgery the stylets were removed and replaced, to facilitate intracerebral injections. Four days later, the rats were briefly restrained, their stylets removed and intracerebral injection needles (30 gauge) inserted bilaterally into the guide cannulae such that they terminated 3.0 mm below the guide tips. Peptide solutions or vehicle were infused manually over a 30 s period with a micrometer screw gauge, calibrated to deliver a volume of 1 μl by means of Bolab V3 polythene tubing attached to an Agla glass syringe. The injection needles were left in place for a further 60 s, then removed and the stylets replaced. Rats were used on a maximum of two separate occasions, with a 7 day period between testing.

Behavioural testing

Testing was conducted between 08 h 00 min and 14 h 00 min. Rats were allowed to adapt to the test room for at least 1 h before experimentation began. In experiments where rats were to be pretreated with the drug under test or vehicle, this was given subcutaneously into the back of the neck and the animals were then placed immediately into individual perspex observation boxes (25 × 20 × 20 cm high). Locomotor activity was assessed by means of a single infra-red beam photocell-unit placed along the longer axis of the box and located off-centre 4 cm above the floor of the box and 8 cm from the side. Infra-red beam crossings were counted by a microcomputer in 10 min epochs. After 40 min, the rats were removed from the boxes whilst intra-cerebral injections were made and then immediately replaced in the same box for a further period of locomotor counting (typically 90 min). In some experiments, wet dog shakes and rearing were also counted by an observer. In experiments designed to show the neurochemical changes produced by the treatments, the above procedures were adhered to, except that 50 min after intra-cerebral injection the rats were removed from the boxes and taken into a separate room.

Neurochemical experiments

Following removal from the observation room the rats were immediately killed, their brains rapidly removed and several brain areas dissected out from coronal sections with the aid of a stainless-steel punch. With the exception of the amygdala, tissues taken from the left and right hemisphere were pooled. These tissues were then immediately homogenised in 250 μl 0.1 M perchloric acid (containing 0.1 mM disodium EDTA, 0.26 mM sodium metabisulphite and 50 ng ml^{-1} dihydroxybenzylamine (DHBA)) by means of an ultrasonic probe (MSE Soniprep). Homogenised samples were then frozen in liquid nitrogen until centrifugation. Homogenates were centrifuged at 4°C for 3 min at 18,000 r.p.m. in a Burkard Coolspin bench centrifuge. The resulting supernatants were frozen in liquid nitrogen and stored at −70°C until analysed. The pellets were resuspended in 0.1 M NaOH and protein content was measured by the method of Lowry *et al.* (1951).

High performance liquid chromatography (h.p.l.c.) with electrochemical detection

Baseline separation of dopamine, dihydroxyphenylacetic acid (DOPAC), 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and DHBA in standard mixtures and the supernatants was accomplished by h.p.l.c. separation on a Waters Nova-Pak C_{18} reverse-phase radial compression column (mobile phase: 0.1 M disodium hydrogen phosphate buffer, pH 2.85, containing 4 mM heptane sulphonic acid, 0.2 mM disodium EDTA, 9% v/v acetonitrile and 3.5% v/v methanol, flow rate 1.5 ml min^{-1}). The eluate was analysed by oxidative electrochemical detection with an ESA model 5100A detector with model 5011 electrodes, the first set at +0.1 V to act as a filter and the second set at +0.45 V for chromatographic peak detection. Detected peaks were analysed by a Trivector Trio computing integrator, which calculated the area under peaks of interest. The amounts of dopamine, DOPAC, 5-HT and 5-HIAA appearing in the samples were quantitated with reference to external standard calibration curves for each amine and metabolite (5–250 pg, repeated every 24 samples) with correction for the known amount of the internal standard DHBA which was present in each sample. The minimum detectable level of the amines and their metabolites which could be reliably quantitated by this procedure was 5 pg. Tissue levels were expressed as ng mg^{-1} of tissue protein.

Histological examination

Following the described experimental procedures all animals were killed and their brains removed. Sections of brain containing the VTA were preserved in buffered formalin solution for subsequent histological examination by section with a freezing microtome. The injection sites were located from the tracks of the injection needles. Rats in which cannulae were sited outside the VTA were excluded from the study.

Statistical analyses

Results were analysed by analysis of variance followed by Student's *t* test or Dunnett's *t* test for multiple comparisons.

Drugs and solutions

All reagents used were of analytical grade (Analar, BDH). Methanol and acetonitrile for h.p.l.c. were of h.p.l.c. grade (Rathburn). Heptane sulphonic acid was obtained from Kodak. Except where indicated, pure (h.p.l.c. grade) water was used throughout.

DiMe-C7 (Bachem (U.K.) Ltd.) was dissolved to a final concentration of 10 mM in nitrogen de-gassed water, which had been filtered through a 0.2 μm filter (Millipore). Aliquots of this solution were stored at −70°C until required. A sample of each aliquoted batch of DiMe-C7 was subjected to analysis for amino acid composition and peptide content by the Medicinal Chemistry Department of Glaxo Group Research, Greenford. Correction was then made for peptide content in the aliquots.

Ondansetron (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)-methyl]-4*H*-carbazol-4-one); GR65630 (3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-1*H*-indol-3-yl)-1-propanone) and MDL 72222 (1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate) were all synthesised in the Department of Chemical Research, Glaxo Group Research, Ware). ICS 205 930 ((3α-tropanyl)-1*H*-indole-3-carboxylic acid ester) was a gift from Dr G. Engel, Sandoz, Basle.

All drugs for subcutaneous injection were dissolved in sterile water for injection and diluted in sterile 0.9% w/v saline solution. Drug solutions were injected as base equivalents in dose volumes of 0.1 ml per 100 g body weight.

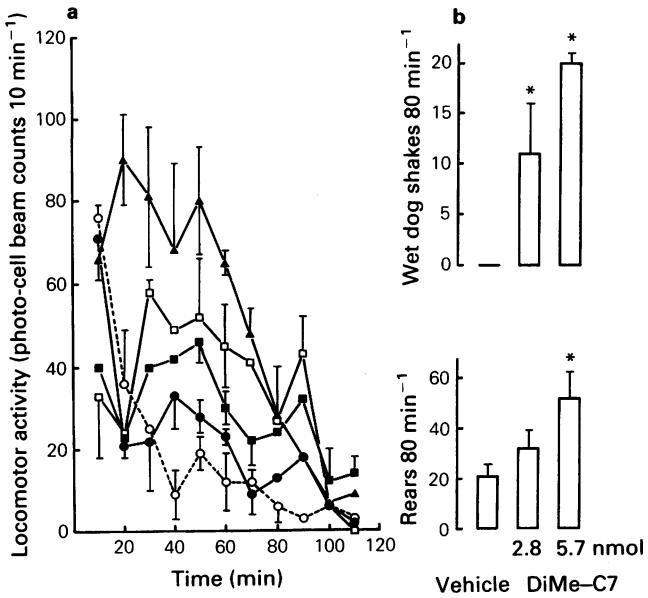


Figure 1 Responses to bilateral injection of DiMe-C7 into the ventral tegmental area (VTA). (a) Locomotor activity response (in counts 10 min^{-1}) to injection of vehicle (○) or DiMe-C7 (●) 1.4, (□) 2.8, (□) 5.7 and (△) 11.5 nmol intra-VTA. Each point represents the mean of 4–6 animals; vertical lines show s.e.mean. (b) Wet dog shake and rearing responses, expressed as total number observed in the 80 min period following intra-VTA injection for the doses of DiMe-C7 indicated. Each column represents the mean of 4–6 animals; vertical bars show s.e.mean. Statistically significant differences from vehicle-treated control animals are shown. * $P < 0.05$.

Standard compounds for the h.p.l.c. analyses were obtained from Sigma. These were dissolved to a final concentration of 50 ng ml^{-1} of base in 0.1 M perchloric acid (containing 0.1 mM disodium EDTA and 0.26 mM sodium metabisulphite).

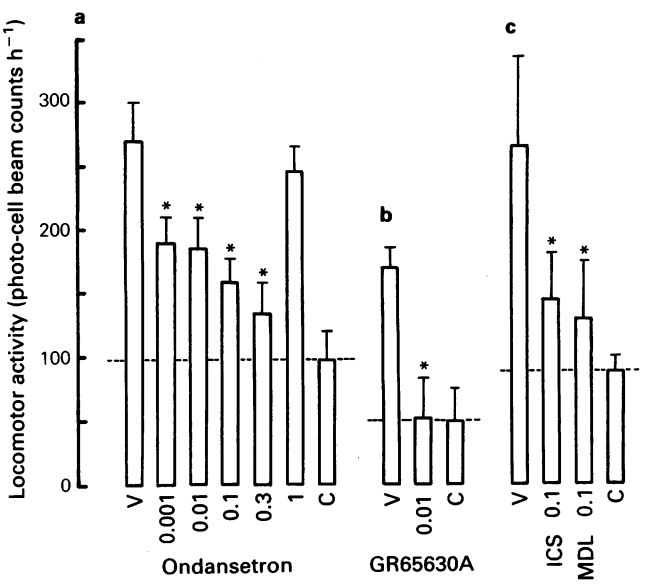


Figure 2 Effect of subcutaneous administration of (a) ondansetron, (b) GR65630A, (c) ICS 205 930 and MDL 72222 on the locomotor hyperactivity induced by injection of DiMe-C7 into the ventral tegmental area (VTA). The response of those animals receiving subcutaneous vehicle pretreatment before DiMe-C7 are indicated as V, those receiving subcutaneous vehicle followed by vehicle into the VTA as C. Pretreatments were administered 40 min before intracerebral injections. In each case the dose of DiMe-C7 was 2.8 nmol, except in the experiments with ondansetron, where a dose of 5.7 nmol was used. Each column shows the mean for 4–12 animals; vertical bars show s.e.mean. By use of Dunnett's t test, the statistically significant differences from animals receiving vehicle followed by DiMe-C7 are shown. * $P < 0.05$.

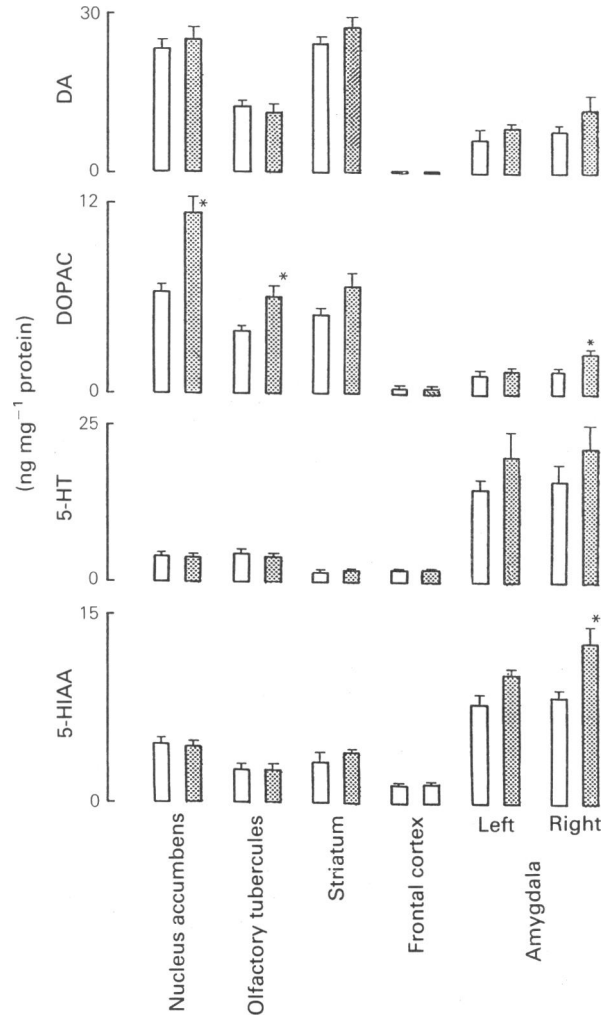


Figure 3 Effect of DiMe-C7 (5.7 nmol, injected into the ventral tegmental area (VTA), stippled columns) on the levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the nucleus accumbens, olfactory tubercules, striatum, frontal cortex, left and right amygdala. Each column shows the mean for 4–8 animals; vertical bars indicate s.e.mean. Statistically significant differences from animals receiving vehicle intra-VTA are shown. * $P < 0.05$.

Results

Effect of intra-VTA DiMe-C7 on locomotor activity

Bilateral intra-VTA injection of DiMe-C7 (1.4–11.4 nmol) caused a behavioural hyperactivity syndrome which included increased locomotor activity, increased rearing and wet-dog shakes (Figure 1). Both the time-course and magnitude of the locomotor hyperactivity response were dose-dependent. At the lower doses of DiMe-C7 (1.4, 2.8 and 5.7 nmol) the increase in locomotor activity in some animals was preceded by a period of reduced locomotor activity with respect to vehicle controls. Marked lachrimation was often noted during this period. At these doses the peak hyperactivity response occurred at 30–50 min. In contrast, at the highest dose of DiMe-C7 (11.4 nmol) the onset of the hyperactivity was more immediate and the peak response occurred after 20 min. However, there was also a second peak of hyperactivity which occurred after 50 min. At all doses of DiMe-C7, locomotor activity returned to control levels by 100 min.

In further studies where the rats received subcutaneous pretreatments, doses of DiMe-C7 were used which produced a submaximal hyperactivity. In these experiments, the rats were

acclimatized to the test environment and the initial hypoactivity when compared to control animals was reduced or absent. The locomotor hyperactivity response of the rats was expressed as the mean total counts (± s.e.mean) in the period 20–80 min after intra-VTA injection.

Effect of 5-HT$_3$ antagonists on the locomotor hyperactivity-induced by DiMe-C7

Pretreatment (40 min s.c.) with ondansetron (0.001–0.3 mg kg^{-1}), GR65630A (0.01 mg kg^{-1}), ICS 205 930 (0.1 mg kg^{-1}) or MDL 72222 (0.1 mg kg^{-1}) inhibited, in a statistically significant manner, the hyperactivity response to DiMe-C7 (2.8 or 5.7 nmol) (Figure 2). A high dose of ondansetron (1 mg kg^{-1}), was without effect on the hyperactivity response. None of these compounds reduced spontaneous locomotion in the rats, measured in the 30 min period prior to DiMe-C7 injection and wet dog shakes induced by DiMe-C7 were not affected by pretreatment with ondansetron (data not shown).

Effect of ondansetron on DiMe-C7-induced changes in dopamine and 5-HT metabolism

Intra-VTA injection of DiMe-C7 (5.7 nmol) increased, in a statistically significant manner ($P < 0.05$), the levels of DOPAC in the nucleus accumbens (+59%), olfactory tubercules (+41%) and right amygdala (+78%) but not the left amygdala (+25%), frontal cortex (+2%) or striatum (+31%) (Figure 3). DiMe-C7 had no significant effect ($P < 0.05$) on the levels of dopamine in any of these brain areas. DiMe-C7 increased 5-HIAA levels in the amygdala but these changes only reached statistical significance in the right amygdala, where 5-HIAA levels were increased by 54% ($P < 0.05$, Figure 3). 5-HT levels were also increased in the amygdala, but these changes were not significant in either hemisphere ($P > 0.05$).

In control rats, pretreatment with ondansetron (0.1 mg kg^{-1}) had no significant effect ($P > 0.05$) on DOPAC or 5-HIAA levels, but in rats treated with DiMe-C7, ondansetron inhibited the increase in DOPAC levels in the nucleus accumbens and right amygdala (Figure 4), but this effect was only significant in the nucleus accumbens ($P < 0.05$). The increase in 5-HIAA levels in the right amygdala also appeared to be reduced by ondansetron, but this reduction was not significant ($P > 0.05$) when compared to animals treated with DiMe-C7 and was still significantly different ($P < 0.05$) from animals receiving ondansetron alone.

Histology

Injection sites were confirmed by subsequent histological examination. In general, injection needle tracks were found to terminate within antero-medial aspects of the VTA. Data from those animals with injection tracks terminating within the VTA (approximately 80% of animals tested) were included in the analyses of results. Representative data from 25 animals with injection tracks terminating in the VTA are shown in Figure 5.

Discussion

Administration of certain neurokinin agonists into the VTA of rat brain enhances locomotor activity (Elliott & Iversen, 1986). This effect can be produced by substance P (Stinus *et al.*, 1978), but is short-lived. Administration into the VTA of the metabolically more stable analogue of substance P, DiMe-C7, mimics the locomotor stimulant effects of substance P, but the duration of effect is much increased (Eison *et al.* 1982a). In the present experiments, intra-VTA administration of DiMe-C7 produced a dose-related, complex behavioural hyperactivity syndrome, which included increased locomotion, rearing and wet dog shakes. These effects are similar to those described by Eison *et al.* (1982b), although the time course of

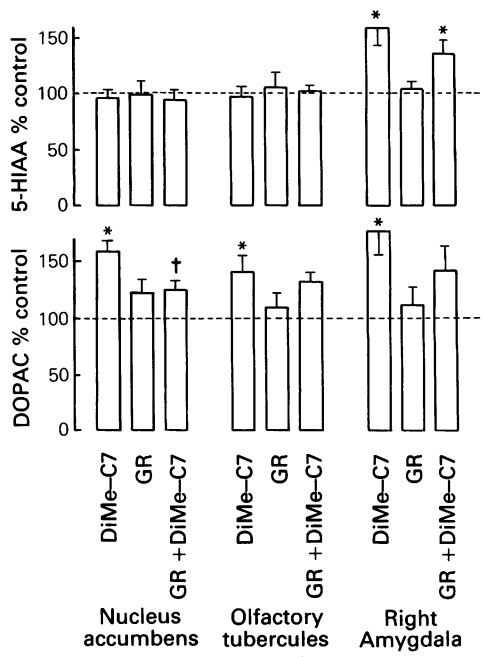


Figure 4 Effect of ondansetron (0.1 mg kg^{-1}, GR) on the changes in dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), induced by an injection of DiMe-C7 (5.7 nmol) into the ventral tegmental area (VTA), in the nucleus accumbens, olfactory tubercules and right amygdala. Columns represent the mean percentage of the metabolite level in control animals. Vertical bars indicate s.e.mean. Statistically significant differences are shown. * $P < 0.05$ versus vehicle controls; † $P < 0.05$ versus DiMe-C7-treated animals.

the locomotor hyperactivity which we observed was somewhat different, as described in detail in the results section.

The reason for this difference in profile between the present results and those of Eison *et al.* (1982b) is unclear, but may reflect rat strain differences or small differences in cannula placement. We are currently investigating the possibility that the relative lack of selectivity of DiMe-C7 between neurokinin receptors may also be important. DiMe-C7 has agonist activity at NK_1 and NK_3 receptors in peripheral tissues *in vitro* (Regoli *et al.*, 1987). It is therefore possible that a combination of actions at more than one neurokinin receptor subtype in

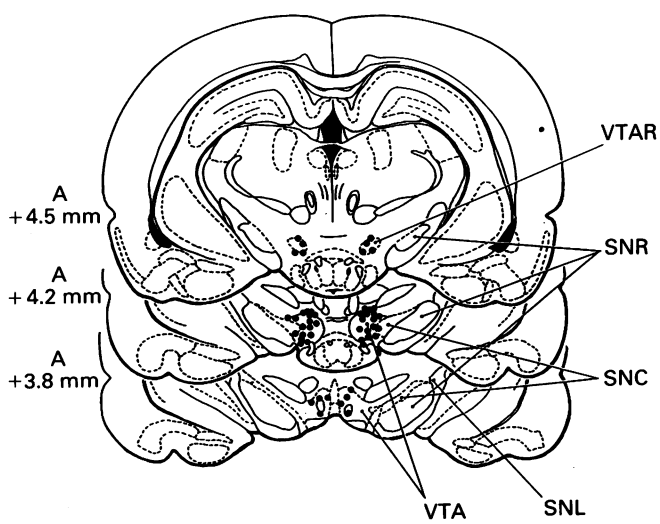


Figure 5 Diagrammatic representation of the sites of DiMe-C7 injection (●). Representative data from 25 animals with injection tracks terminating in the ventral tegmental area (VTA) are shown. The coordinates are according to Paxinos & Watson (1986). SNR, substantial nigra, pars compacta; SNL, substantia nigra, pars lateralis; SNR, substantia nigra, pars reticulata; VTA, ventral tegmental area; VTAR, ventral tegmental area pars rostralis.

the VTA may mediate the effects of DiMe-C7. The relative contribution from each action may determine the overall activity profile. For example, the ability of DiMe-C7 to produce wet dog shakes after central administration to rats is not mimicked by substance P (Eison *et al.*, 1982b) but is mimicked by the selective NK_3 agonist senktide (Stoessl *et al.*, 1988). This effect of senktide presumably involves activation of 5-hydroxytryptaminergic mechanisms since it can be blocked by the selective 5-HT$_2$ receptor antagonist ketanserin. In our experiments, ondansetron was found to have no effect on DiMe-C7-induced wet dog shakes. This is in agreement with the lack of effect of low doses of the 5-HT$_3$ receptor antagonist ICS 205-930 on wet dog shakes induced by the 5-HT precursor, 5-hydroxytryptophan (Shearman & Toksvai, 1987).

The hyperactivity-induced by DiMe-C7 is almost completely abolished by a low dose of haloperidol (Hagan *et al.*, 1987; Eison *et al.*, 1982b), supporting the hypothesis that activation of dopamine systems is involved in the hyperactivity induced by the intra-VTA injection of DiMe-C7. In the present study, we have demonstrated that the 5-HT$_3$ antagonists ondansetron, GR65630 (Kilpatrick *et al.*, 1987), ICS 205-930 and MDL 72222 also reduce this hyperactivity response. This effect would appear to be independent of any effect of the 5-HT$_3$ receptor antagonists on spontaneous locomotor activity, which was not affected in the 30 min period before DiMe-C7 administration. It could be argued that an intrinsic effect on locomotor activity with a longer latency could account for the observed effects. Whilst we cannot rule out this possibility, it seems unlikely, since we have shown that, at a dose (0.1 mg kg^{-1}) which was effective in the present study, ondansetron has no effect on spontaneous locomotion in rats over an 8 h period (unpublished observations). Our results support previous observations (Costall *et al.*, 1987a; Hagan *et al.*, 1987) and suggest that activation of 5-HT$_3$ receptors may facilitate overactivity in mesolimbic dopaminergic systems. Although ondansetron was active at doses as low as 1 μg kg^{-1}, its effect was not maintained at the highest dose of antagonist tested (1 mg kg^{-1}). This decline in effect at supra-effective doses has also been demonstrated for several 5-HT$_3$ receptor antagonists in the continuous dopamine infusion model of hyperactivity in the rat (Costall *et al.*, 1987b). However, it should be noted that at 1 mg kg^{-1} ondansetron was effective at inhibiting intra-accumbens amphetamine-induced hyperactivity (Costall *et al.*, 1987a). In our experiments, the lack of effect of the highest dose of ondansetron was not accompanied by any overt changes in behaviour in the rats and spontaneous locomotor activity was not affected. The mechanism underlying this loss of effect is unknown but may indicate a secondary opposing action of 5-HT$_3$ receptor antagonists which is only observed at high doses.

In order to further our understanding of the mechanisms by which 5-HT$_3$ antagonists reduce mesolimbic hyperactivity, we investigated the effect of ondansetron on DiMe-C7-induced changes in dopamine and 5-HT metabolism in several areas of rat forebrain. DiMe-C7 significantly increased dopamine metabolism in the nucleus accumbens and olfactory tubercules. In the amygdala, DOPAC and 5-HIAA levels were increased in both hemispheres, but these changes were more pronounced in the right amygdala, where both DOPAC and 5-HIAA levels were significantly increased. In control animals, there was no significant difference between left and right amygdala in the levels of either metabolite. This apparent asymmetry in the effect of bilateral injections of DiMe-C7 is puzzling. One possibility is that there is laterality in the response to DiMe-C7. Alternatively, these changes could arise out of small differences in cannula placement between the left and right VTA. The levels of dopamine and DOPAC in the frontal cortex supernatants were low, approximately 3–5 times the reliable detection limits of the h.p.l.c. with electrochemical detection assay. Hence, the data must be interpreted with some caution. However, the complete lack of effect of DiMe-C7 on frontal cortex dopamine metabolism is surprising, given that this structure is innervated by dopaminergic A10 neurones and that Elliott *et al.* (1986) found that a similar treatment with DiMe-C7 caused marked increases (+120%) in DOPAC levels in pre-frontal cortex. Possibly, the dopaminergic neurones which project to the frontal cortex may not respond in the same way to DiMe-C7 as those projecting to the pre-frontal cortex. The localization of the major component of the DiMe-C7 response to mesolimbic dopaminergic systems is supported by the observation that DiMe-C7 had a smaller, not statistically significant effect on striatal dopamine metabolism (31% increase in DOPAC levels, compared to 59% in the nucleus accumbens). DiMe-C7 also produces locomotor hyperactivity after injection into the substantia nigra (SN) of rats (Eison *et al.*, 1982b). Thus, the smaller effect of intra-VTA injection of DiMe-C7 on striatal DOPAC levels suggests that the contribution of striatal dopaminergic neurones to the locomotor hyperactivity induced by intra-VTA DiMe-C7 in the behavioural experiments is not major; although, it cannot be ruled out, given the likely spread by diffusion of the injected peptide to the substantia nigra (see Eison *et al.*, 1982a) and the existence of a population of VTA neurones which project to the ventral striatum (Fallon & Moore, 1978).

In common with its lack of effect on overt normal rat behaviour, ondansetron had no effect on either dopamine or 5-HT metabolism in control animals, supporting previous observations that it has no effect on normal levels of activity of dopamine and 5-HT neurones in cortical and limbic brain areas (Hagan *et al.*, 1987; 1988). In contrast, when mesolimbic dopaminergic activity was stimulated by DiMe-C7, ondansetron reduced both the resulting behavioural hyperactivity and the increase in nucleus accumbens DOPAC levels. Ondansetron has no significant antagonist activity at neurokinin receptors *in vivo* (unpublished data), nor does it inhibit neuronal dopamine uptake (A. Bradbury, personal communication) or monoamine oxidase A or B *in vitro* (M. Dixon, personal communication). Hence, the present results may indicate that ondansetron antagonises the peptide-induced release of mesolimbic dopamine by an action on 5-HT$_3$ receptors. This would imply that DiMe-C7 can increase the release of 5-HT, which then acts on 5-HT$_3$ receptors to amplify the mesolimbic dopaminergic activation. Indeed, the present experiments show that DiMe-C7 increases 5-HIAA levels in the amygdala, suggesting that 5-HT release may be increased here and, thus, that this region may be involved in the observed effects. In support of this hypothesis, it is known that the amygdala contains significant numbers of 5-HT$_3$ receptor binding sites (Kilpatrick *et al.*, 1987). Furthermore, administration of ondansetron into the central nucleus of either left or right amygdala inhibits the hyperactivity response induced by dopamine infusion into the left amygdala (Costall *et al.*, 1987a). Ondansetron had no statistically significant effect on DiMe-C7-induced increase in 5-HIAA levels in the amygdala, suggesting that it does not work by reducing the release of 5-HT. To date, no direct evidence exists that 5-HT, acting at 5-HT$_3$ receptors in the amygdala can facilitate mesolimbic dopamine release. However, Blandina *et al.* (1988) have recently shown that the 5-HT$_3$ receptor antagonist ICS 205–930 can reduce the 5-HT-induced increase in spontaneous overflow of endogenous dopamine from superfused slices of rat brain striatum, supporting the concept that 5-HT$_3$ receptor antagonists can modulate dopamine release. Further studies are required to demonstrate whether this effect can be observed in limbic areas, in particular, the amygdala.

These results may have implications for the use of 5-HT$_3$ receptor antagonists in man. Circumstantial evidence suggests that a disturbance of mesolimbic dopaminergic activity may underlie the symptoms of acute psychoses. Thus, by controlling the effects of excessive dopamine, 5-HT$_3$ receptor antagonists may be useful in the treatment of schizophrenia. In addition, activation of the VTA, leading to enhanced mesocortical and mesolimbic dopaminergic activity is thought to be important in the rewarding action of a number of drugs of abuse, including narcotic analgesics, alcohol, nicotine and

cocaine (see Wise & Bozarth, 1984; Bozarth & Wise, 1986). Therefore, 5-HT_3 receptor antagonists may be of use in the treatment of drug dependence and to alleviate the craving which occurs during drug withdrawal. Recent evidence suggests that ondansetron attenuates the symptoms of withdrawal from chronic alcohol intake in marmosets (Oakley *et al.*, 1988; Costall *et al.*, 1988) and inhibits the decrease in social interaction seen in rats following withdrawal from chronic diazepam treatment given peripherally or directly into the amygdala (Costall *et al.*, 1989). Moreover, ICS 205-930 and MDL 72222 can reduce place preference in rats induced by morphine or nicotine (Carboni *et al.*, 1989).

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Evaluation of the effect of azapropazone on neutrophil migration in anaesthetized swine using a multichamber blister suction technique

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1 The purpose of this study was to determine the *in vivo* inhibitory efficacy of azopropazone on neutrophil migration. The effects of azapropazone given at a dose of 100 mg kg^{-1} i.v. every 2 h on the neutrophil migration into skin inflammation sites (blister fluid) as well as into an autologous serum (± chemoattractant) placed above the blister surface (2nd chamber) were determined.

2 Azapropazone treatment schedule maintained blood levels at 70–100 μg ml^{-1} throughout the time course (360 min) of the experiment.

3 Azapropazone significantly inhibited (48 ± 6%) neutrophil migration into the blister fluid (as evident from the decrease in myeloperoxidase activity).

4 Azapropazone significantly inhibited the neutrophil migration into the autologous serum either with (65 ± 5%) or (35 ± 6%) without the chemoattractant, formyl-methionyl-leucyl-phenylalanine (FMLP). The chemoattractant, FMLP, markedly increased neutrophil migration into the autologous serum by approximately 1.5 to 2 times the non-FMLP treated group. Azapropazone was more efficacious in inhibiting the neutrophil migration in the presence of FMLP than in its absence.

5 We conclude that azapropazone is an effective inhibitor of neutrophil migration into topically inflamed sites in anaesthetized swine.

Introduction

Azapropazone is a nonsteroidal anti-inflammatory agent with a dual inhibitory efficacy on both xanthine oxidase and neutrophil functions (Rakich & Mackin, 1988; Jahn & Thiel 1988). *In vitro* studies have demonstrated that azapropazone is a xanthine oxidase inhibitor with an IC_{50} of 70–140 μg ml^{-1} and an inhibitor of a variety of neutrophil functions (migration, aggregation, superoxide production) at 40–400 μg ml^{-1} (Rakich & Mackin, 1988). Numerous reports have implicated xanthine oxidase and neutrophil derived oxygen free radicals in reperfusion-induced myocardial damage (Rowe *et al.*, 1984; Granger *et al.*, 1986). For these reasons, it has been postulated that azapropazone should exert a beneficial effect in myocardial infarction due to ischaemia and reperfusion (Montor *et al.*, 1987). Subsequent studies in rats and dogs have supported these suggestions (Montor *et al.*, 1987; Knabb *et al.*, 1988).

Neutrophil migration into injured tissue is a fundamental host defence mechanism. Many methods of studying neutrophil migration *in vitro* (Belsheim, 1981; Wilkinson, 1982; Rakich & Mackin, 1988) and *in vivo* have been described (Hellum & Solberg, 1977; Zimmerli & Gallin, 1987). The *in vivo* methods include the injection of an irritant under the skin as well as a variety of tissue chamber techniques (Zimmerli & Gallin, 1987). The most reproducible method used in man for the measurement of neutrophil migration is the suction blister technique as described by Hellum & Solberg (1977).

This study was undertaken to determine the *in vivo* inhibitory efficacy of azapropazone on neutrophil migration by use of a suction multiblister chamber technique (Rowe *et al.*, 1984; Granger *et al.*, 1986).

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Methods

Experimental preparation

Twelve male Hampshire/Yorkshire swine (SPF, 10–15 kg) obtained from Bio Medical Alternatives International, Inc., Raleigh, NC, U.S.A., were used in the study. Swine were sedated with an intramuscular injection of ketamine (10 mg kg^{-1}) and anaesthetized by intravenous administration of sodium pentobarbitone (30 mg kg^{-1}). Animals were placed in a respiratory ventilator with a positive pressure pump (Harvard Instrument). The common carotid artery and the left femoral vein were exposed and cannulated for monitoring heart rate/arterial blood pressure and blood samplings, respectively. Azapropazone was administered intravenously via the marginal ear vein.

Skin chamber device

The skin suction and chamber units were obtained from Neuro probe (Neuro probe, Cabin John, MD, U.S.A.). The suction unit has two rows of four wells in each row. Each well has a diameter of 7 mm, allowing blisters to form with a maximal area of 0.38 cm^2. Each row can be connected separately or together (with a Y-tube) to a vacuum pump. The second chamber unit which covers the blister lesions is very similar to the suction unit, but has bigger wells with a diameter of 12 mm and a volume of 1 ml. A 2 mm wide and 1 mm deep trough filled with a plastic ring surrounds the wells in order to prevent the leakage of the serum. A hole in the top of each well allows the introduction of a 20 gauge subcutaneous needle for the injection and the withdrawal of fluid.

Experimental protocol

The flat surface of the swine's thigh muscle was shaved and disinfected with ethanol. The suction unit was secured in place

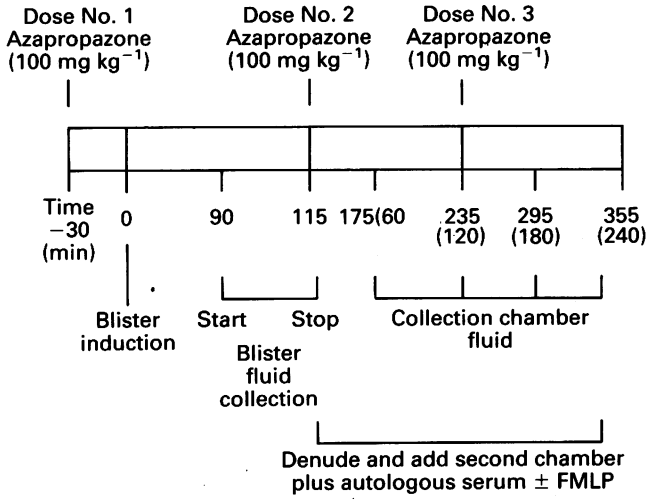


Figure 1 Diagram showing the protocol used in the study. Azapropazone was given at a dose of 100 mg kg^{-1} i.v., 30 min before the induction of the blister. Eight blisters were induced over the swine's thigh muscle (time for induction = 90 min). Blister fluid from each blister was removed and frozen for the analysis of neutrophil migration (myeloperoxidase (MPO) activity). A second chamber was placed above the lesion site after the removal of the epidermal surface of the lesion. An autologous serum ± chemoattractant (formyl-methionyl-leucyl-phenylalanine, FMLP) was added to each well of the chamber. At different time intervals, the autologous serum and blood samples were withdrawn for MPO and azapropazone analysis.

with a velcro bandage. A constant suction of 300 mmHg was applied using a vacuum pump. The suction field was warmed up in order to increase the rate of the blister formation. After 1.5 h, the blisters were complete. The blister fluid was removed and frozen for further analysis of myeloperoxidase (MPO) activity. The blister roofs were then gently removed with scissors. The eight lesions (0.38 cm^2 area) were covered with the skin chamber unit and filled with 0.5 ml autologous serum with or without the chemoattractant, FMLP (1 μg ml^{-1}). At different time intervals the fluids from one chamber with FMLP and from another chamber without FMLP were withdrawn (Figure 1) and frozen at −70°C for further analysis of neutrophil migration (MPO activity). The study was carried out in an azapropazone-treated group ($n = 5$) as well as in a control group ($n = 5$) given only saline (Figure 1). Azapropazone was given at a dose of 100 mg kg^{-1} i.v. 30 min before the induction of the multiblister and every 2 h throughout the study (Figure 1).

Table 1 Effect of azapropazone on the neutrophil migration (MPO) into the blister fluid in anaesthetized swine

Myeloperoxidase activity (MPO)[a]			*Azapropazone plasma level* (μg ml^{-1})
Control	*Azapropazone*	%I[b]	
0.598 ± 0.08	0.312 ± 0.05[c]	48 ± 6	115 ± 22

[a] All values are the mean ± s.e. ($n = 5$).
MPO activity (ΔA 460 nm min^{-1} 100 μl^{-1}).
[b] %I = percentage inhibition of neutrophil migration (MPO activity) as compared to control.
[c] Significantly different from the control group, $P < 0.01$.

Myeloperoxidase assay

Myeloperoxidase (MPO) is a neutrophil-specific enzyme (Mullane *et al.*, 1985). MPO was assayed in the blister fluid and in the autologous serum at different intervals (Figure 1) for the determination of the *in vivo* migration or infiltration of PMN into topically inflamed sites. The assay is as described by Mullane *et al.* (1985). to a 100 μl of centrifuged blister fluid or autologous serum samples, 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing hexadecyltrimethyl ammonium bromide 0.05 mg ml^{-1} (to solubilize MPO) plus O-dianisidine HCl 0.167 mg ml^{-1} plus 0.003% H_2O_2 was added. This reaction mixture was mixed in a spectophotometric cuvette and the changes in absorbance at 460 nm at room temperature were recorded. The slope (changes in absorbance per time) was used to determine the MPO activity. Data were expressed either as the changes in absorbance per min per 100 μl sample or as a percentage of basal MPO activity.

Determination of azapropazone blood levels

Blood samples were withdrawn from the femoral vein of anaesthetized and heparinized swine at different intervals (ranging from 1 to 120 min) post administration of azapropazone (100 mg kg^{-1}, i.v.). Azapropazone blood levels were determined by a previously described high performance liquid chromatographic (h.p.l.c.-u.v.) method (Kline *et al.*, 1983).

Chemicals

Azapropazone (Du Pont, Wilmington, DE, U.S.A.), 100 mg ml^{-1} was dissolved in 0.9% NaCl saline, bubbled with N_2 and the pH adjusted to 9.0–9.5. Monastral blue dye, hexadecyltrimenthyl ammonium bromide, O-diansidine HCl, 30% hydrogen peroxide and formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis

The experimental results are given as the mean ± s.e. The statistical significance of the difference between control and

Table 2 Effect of azapropazone on the neutrophil migration (MPO) into autologous serum (± FMLP) in anaesthetized swine

Autologous serum sampling time (min)	*Myeloperoxidase activity* (MPO)[a] (% of basal)				*Azapropazone plasma level* (μg ml^{-1})
	Control group		*Azapropazone group*		
	(−)FMLP[b]	(+)FMLP[c]	(−)FMLP(%I)	(+)FMLP(%I)	
60	183 ± 20	373 ± 124	145 ± 57	92 ± 36[e]	83.2 ± 2.0
120	280 ± 106	477 ± 137	163 ± 21[d]	129 ± 23[e]	67.1 ± 12.0
180	263 ± 98	417 ± 134	139 ± 19[d]	133 ± 60[e]	103.9 ± 19.1
240	227 ± 71	468 ± 184	160 ± 38[d]	80 ± 36[e]	90.4 ± 13.6
60–240 (% inhibition)			35 ± 6	69 ± 5	

[a] All values are the mean ± s.e. ($n = 5$).
[b] (−)FMLP = autologous serum without formyl-methionyl-leucyl-phenylalanine.
[c] (+)FMLP = autologous serum with FMLP (1 μg ml^{-1}).
[d] $P < 0.05$.
[e] $P < 0.01$.

azapropazone-treated groups was determined by Student's t test. Comparisons (changes with time) were made by use of a one-way analysis of variance. A Newman-Keults test was used for multiple comparisons. Differences were considered statistically significant if $P < 0.05$.

A NONLIN programme (Lexington Consultant, Lexington, KY, U.S.A.) was used for the analysis of the pharmacokinetics of azapropazone in swine blood.

Results

Azapropazone given at a dose of $100\,mg\,kg^{-1}$ i.v. 30 min before the induction of the blisters produced a $48 \pm 6\%$ inhibition of the neutrophil migration into the blister fluid as evident by a decrease in MPO activity (Table 1). The blood levels of azapropazone at the time of the removal of the blister fluid were $115 \pm 22\,\mu g\,ml^{-1}$.

After the removal of the blister, the neutrophil migration (MPO activity) into the autologous serum chamber was examined. An increase of neutrophil migration (2–3 times above the basal value) with a peak value at 2 h post-treatment was observed. Addition of FMLP produced a much greater increase in the neutrophil migration (4–5 times above the basal value) into the serum chamber (Table 2). The neutrophil migration into the skin chambers was significantly inhibited in the azapropazone-treated animals by $35 \pm 6\%$ in the serum without FMLP and by $69 \pm 5\%$ in the serum with the FMLP (Table 2). The blood levels of azapropazone ranged from 70–$100\,\mu g\,ml^{-1}$ throughout the study (Table 2).

Pharmacokinetic analysis of blood vessels following a single i.v. dose in the swine demonstrated that azapropazone had a relatively short elimination half-life $t_{\frac{1}{2}} = 161.4$ min (Table 3).

Discussion

Oxygen derived free radicals have been identified as important mediators of tissue injury (McCord, 1985; Lucchesi & Mullane, 1986). Increased production of oxygen free radicals can cause direct tissue injury via lipid peroxidation of cellular and subcellular membranes leading to irreversible injury (Freeman & Crapo, 1982). Additionally, free radicals can play an important role in releasing chemotactic factors for neutrophil migration into damaged tissue with the subsequent liberation of cytotoxic free radical species (O_2^-, $\cdot OH$, HOCl). In that regard, activation of neutrophils could result in the release of lysosomal enzymes and the obstruction of capillaries and small arterioles (Lucchesi & Mullane, 1986). Activated xanthine oxidase and neutrophils upon ischaemia/reperfusion have been shown to be involved in myocardial injury as evident from the different pharmacological intervention studies at either the xanthine oxidase or neutrophil migration and function levels (McCord, 1985; Lucchesi & Mullane, 1986). It has been demonstrated that infarct size should be reduced in regional myocardial ischaemia/reperfused animal models when animals were made leukopenic before ischaemia (Lucchesi & Mullane, 1986). A decreased infarct size was accompanied by a less dense leukocyte infiltration in the risk region and the adjacent myocardium (Lucchesi & Mullane, 1986). These studies clearly show that the inflammatory response can be accepted as one determinant for ischaemic cell death. Because leukocyte depletion before ischaemia is not a reasonable treatment for patients with acute myocardial infarction, compounds that inhibit neutrophil infiltration may be particularly effective against myocardial ischaemia/reperfusion injury.

A role for neutrophils has been implicated in different tissue destructive events including rheumatoid arthritis, myocardial reperfusion injury, adult respiratory distress syndromes (ARDs), blistering skin disorders and ulcerative colitis (Malech & Gallin, 1987). The neutrophil releases toxins normally to defend the host against invading microbes but the neutrophil has little intrinsic ability to control the release of these toxins under different sets of situations. Therefore, an agent which inhibits neutrophil function may be therapeutically important. This study was undertaken to determine the *in vivo* inhibitory effects of azapropazone on neutrophil migration in a topical inflammation model.

The present results demonstrated an *in vivo* inhibitory efficacy of azapropazone on neutrophil migration ($48 \pm 6\%$ inhibition) into topical sites of injured skin sites at systemic blood levels ranging from $70–100\,\mu g\,ml^{-1}$ ($2–3 \times 10^{-4}$ M). This is in agreement with earlier *in vitro* studies with azapropazone which reported an IC_{50} of 10^{-4} M for the inhibition of neutrophil migration in an *in vitro* chemotaxis assay (Rakich & Mackin, 1988). Furthermore, azapropazone produced a very distinct inhibition of neutrophil migration into the inflamed site either with or without the chemoattractant FMLP. In addition, azapropazone was shown to be more efficacious in the presence of the chemoattractant. This *in vivo* inhibitory effect of azapropazone on neutrophil migration is in agreement with recent studies with azapropazone in a regional ischaemic/reperfused rabbit heart model. In that study, azapropazone was shown to inhibit neutrophil migration into ischaemic/reperfused rabbit myocardium either with or without *in vivo* phorbol myristate acetate ester activation (Mousa *et al.*, 1989). The broad spectrum of the actions of azapropazone on neutrophils suggests that azapropazone may inhibit an early process of neutrophil activation, which precedes mediator release (Rakich & Mackin, 1988). It is concluded that azapropazone is an inhibitor of neutrophil migration and the multiblister suction technique is a potentially useful method for the evaluation of the *in vivo* effects of drugs on neutrophil migration.

Table 3 Pharmacokinetic parameters of azapropazone after i.v. bolus administration of azapropazone ($100\,mg\,kg^{-1}$) to swine

AUC ($\mu g\,ml^{-1}$, min)	66090
$t_{\frac{1}{2}\alpha}$ (min)	2.9
$t_{\frac{1}{2}\beta}$ (min)	161.4
V_d ($ml\,kg^{-1}$)	352.2
Cl ($ml\,min^{-1}\,kg^{-1}$)	1.51
C_{max} ($\mu g\,ml^{-1}$)	647.5

AUC = area under curve; $t_{1/2\alpha}$ = half-life of the α-phase; $t_{1/2\beta}$ = half-life of the β-phase; Cl = Clearance rate; C_{max} = maximum concentration

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Phorbol esters inhibit smooth muscle contractions through activation of Na^+-K^+-ATPase

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1 The role of protein kinase C (PKC) in agonist-induced contractions of guinea-pig ileum longitudinal smooth muscle has been investigated.

2 The phorbol esters, phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-diacetate (PDA) and phorbol 12-myristate 13-acetate (PMA), relaxed tissues precontracted by submaximal concentrations of carbachol, histamine or substance P.

3 This inhibitory action of the phorbol esters was reversed following the application of ouabain, a specific inhibitor of Na^+-K^+-ATPase. Similarly, pretreatment with ouabain inhibited the ability of phorbol esters to relax tissues precontracted by the above agonists.

4 The slow relaxation of the tonic component of contraction induced by submaximal concentrations of carbachol and histamine, and all concentrations of substance P, was abolished in the presence of ouabain.

5 In Na^+-loaded tissues, PDBu and carbachol caused a concentration-dependent increase of Na^+-K^+-ATPase activity, assessed by ouabain-sensitive $^{86}Rb^+$-uptake. Extrusion of Na^+, assessed by the cellular content of the ion, was also stimulated by PDBu (the effect of carbachol was not investigated).

6 We conclude that phorbol esters inhibit the tonic component of contractions induced by submaximal concentrations of these agonists through activation of Na^+-K^+-ATPase. We suggest that PKC may exert feedback control over the tonic component of agonist contractions through stimulation of the pump.

Introduction

Ca^{2+}-mobilizing hormones or neurotransmitters stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate generating two second messengers inositol 1,4,5-trisphosphate ($InsP_3$), which releases Ca^{2+} from non-mitochondrial intracellular stores, and 1,2-diacylglycerol (DAG), which stimulates a Ca^{2+}-activated, phospholipid-dependent enzyme, protein kinase C (PKC; for review see Nishizuka, 1984; Berridge, 1987). In smooth muscles such as porcine coronary artery and guinea-pig ileum, Ca^{2+} released by $InsP_3$ has been proposed to contribute to the formation of agonist-induced contractions (Hashimoto *et al.*, 1986; Watson *et al.*, 1988). In contrast, the role of the DAG/PKC system in the regulation of contraction is unclear in spite of a growing number of investigations (Watson & Godfrey, 1988).

PKC is activated by tumour promoting phorbol esters (Castagna *et al.*, 1982). In our recent study on the guinea-pig ileum longitudinal smooth muscle (Sasaguri & Watson, 1989a), we found that phorbol esters enhance the phasic component of carbachol-induced contractions when added before the agonist, but quickly inhibit the tonic component of contraction when added after the phasic component. Further, the long-term exposure of tissue to a high-concentration of phorbol 12-myristate 13-acetate (PMA) which leads to a down-regulation (or loss) of PKC, has little effect on the phasic component of the carbachol contraction but markedly enhances the tonic component. Taken together, we concluded that PKC may have an important feedback role in limiting the tonic component of contraction but that it plays little part during the phasic component, since this was unchanged following the down-regulation of PKC. The mechanism of inhibition of the tonic component of contraction by PKC was unrelated to an effect on phosphoinositide hydrolysis (Sasaguri & Watson, 1989a).

Na^+-K^+-ATPase is crucial for the maintenance of ionic gradients across biological membranes (for review, see Glynn & Karlish, 1975; Jorgensen, 1982; Kaplan, 1985) and its activation causes hyperpolarization of the membrane (Bolton, 1973a). This action leads to the relaxation of excitable smooth muscles such as the guinea-pig ileum. Since Na^+-K^+-ATPase is believed to be a substrate for PKC (Greene & Lattimer, 1986; Nishizuka, 1986; Yingst, 1988) and is stimulated by phorbol esters in rat hepatocytes (Lynch *et al.*, 1986), guinea-pig pancreatic acinar cells (Hootman *et al.*, 1987) and rat aortic smooth muscle (Moisey & Cox, 1987), we have investigated the possibility that relaxation of ileal smooth muscle by phorbol esters results from stimulation of this enzyme. We conclude that phorbol esters inhibit the tonic component of contractions induced by submaximal concentrations of these agonists through activation of Na^+-K^+-ATPase. We suggest that PKC may exert a feedback control over the tonic component of agonist contractions through the stimulation of this pump. Part of this work has been published in a preliminary form (Sasaguri & Watson, 1989b).

Methods

Hartley-strain guinea-pigs of either sex (300–500 g) were killed by a blow on the head. The whole length of small intestine (except for the duodenum and the ileocaecal region) was quickly dissected and put into Krebs-Henseleit solution gassed with 95% O_2/5% CO_2 at room temperature. Longitudinal smooth muscle was carefully removed as described previously (Sasaguri & Watson, 1988).

Measurement of contractions

A longitudinal muscle strip (approximately 10 mm length without tension) was set up in a 3 or 5 ml glass organ bath to record isotonic contractions under a tension of 1.5 g. Experiments were carried out following 1 h equilibration in HEPES-buffered solution gassed with 100% O_2 at 37°C. When substance P-induced contractions were recorded, atropine (3 μM) was added 5 min before substance P to eliminate the influence of muscarinic neuromuscular transmission (Holzer & Lembeck, 1980). All contraction records, if not otherwise indicated, are shown as percentage response to a maximally effective concentration (1 μM) of carbachol, which was routinely measured at the beginning of each experiment.

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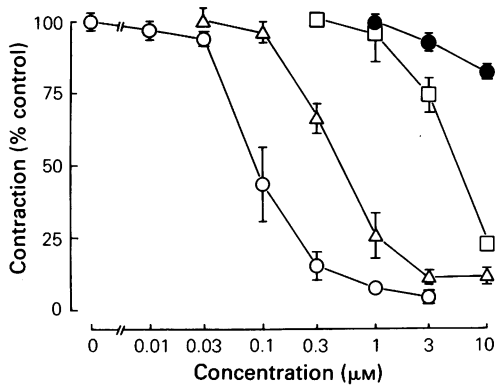


Figure 1 Relaxation by phorbol esters of carbachol-induced contractions. Phorbol 12,13-dibutyrate (○), phorbol 12,13-diacetate (△), phorbol 12-myristate 13-acetate (□) or 4-α-phorbol (●) was added 2 min after the application of 100 nM carbachol (i.e. during the tonic component). Contractions were measured 3 min after the addition of carbachol (1 min after phorbol esters or phorbol) and are demonstrated as percentages of the control (vehicle-treated) contraction induced by 100 nM carbachol. Data represent mean of four separate experiments; vertical lines show s.e.mean.

Measurement of $^{86}Rb^+$-uptake

These studies were carried out essentially as described by Brading & Widdicombe (1974). After 1 h equilibration in normal HEPES-buffered solution, muscle strips were incubated for 3 h in K^+-free solution bubbled with 100% O_2 at 37°C, to increase intracellular Na^+ concentration through inhibition of the Na^+-K^+-pump. Tissues were then placed in low-K^+ solution (K^+ = 1 mM) containing $^{86}Rb^+$ (approximately 5 μCi ml^{-1}) with other reagents as indicated. Four minutes later, they were removed and washed for 5 min in ice-cold low-K^+ solution to remove extracellular radioactivity. Tissues were then blotted, weighed and placed into tubes containing 2 ml of distilled water and counted for gamma radioactivity. When ouabain-insensitive uptake was determined, tissues were pre-incubated with 0.1 mM ouabain for the last 10 min of the incubation in K^+-free solution and the same concentration of ouabain was added to the reaction medium. Ouabain-sensitive $^{86}Rb^+$-uptake was determined by subtracting ouabain-insensitive uptake from total uptake.

Determination of intracellular Na^+ and K^+ content

This assay was based on the method described by Brading (1975). The content of Na^+ and K^+ in smooth muscles was measured by atomic absorption flame photometry. Muscle pieces (approximately 20 mg), treated as indicated in the legend to Table 1, were extracted for 24 h in tubes containing 3 ml of 'diluting fluid' which consisted of 1 N HNO_3 containing 18 mM La^{3+} (La_2O_3) and 10 mM Li^+ (Li_2CO_3) to minimize ionic interactions during the flame photometry and to avoid precipitation of Ca^{2+}. Standard solutions were prepared in the same diluting fluid.

Solutions

Krebs-Henseleit solution (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. The solution was bubbled with 95% O_2 and 5% CO_2; the pH was 7.4 at room temperature.

HEPES-buffered solution (mM): HEPES/Tris buffer 5, NaCl 135, KCl 4, $MgCl_2$ 2, $CaCl_2$ 2, KH_2PO_4 1 and glucose 12. The solution was bubbled with 100% O_2; the pH was adjusted to 7.4 at 37°C. HEPES/Tris buffer was made by the method described previously (Sasaguri & Watson, 1989a). For K^+-free and low-K^+ solutions NaCl substituted for KCl and KH_2PO_4 was replaced with NaH_2PO_4.

Chemicals

Phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-diacetate (PDA), PMA, 4-α-phorbol, ouabain (G-strophanthin), atropine, tetrodotoxin, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), hydroxymethylaminomethane (Tris), histamine and nifedipine were all purchased from Sigma. Carbamylcholine chloride (carbachol) was from BDH. Substance

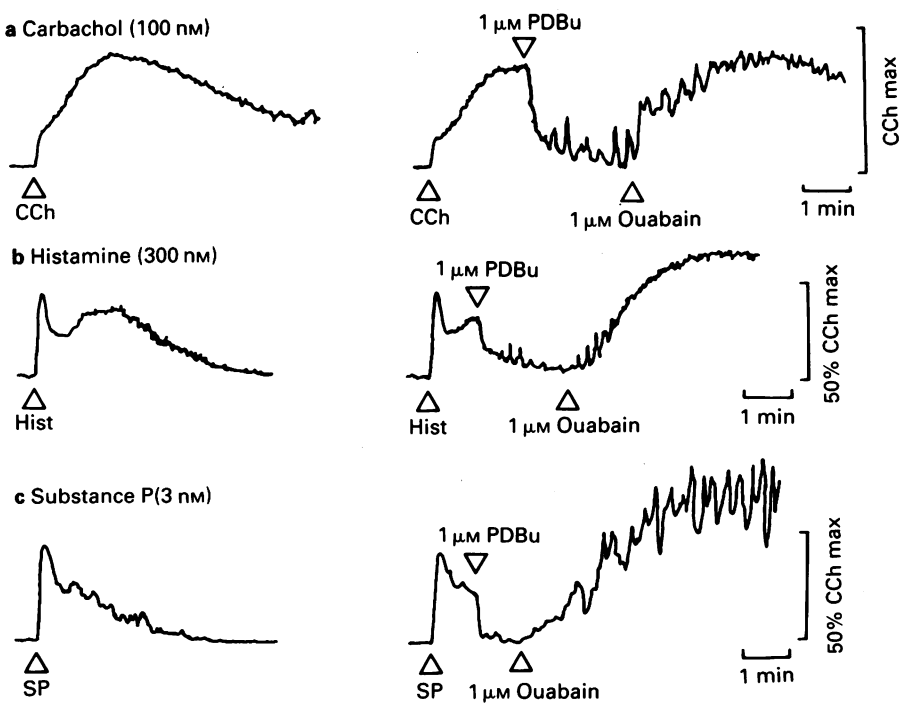


Figure 2 Effect of ouabain on phorbol 12,13-dibutyrate (PDBu)-induced relaxation. Right-hand contractions were recorded 30 min after the control (left). PDBu (1 μM) was applied when the contraction reached a plateau and ouabain (1 μM) was added after maximal relaxation was achieved. Substance (SP)-induced contractions were recorded in the presence of 3 μM atropine. Traces are representative of four independent experiments. CCh = carbachol; Hist = histamine.

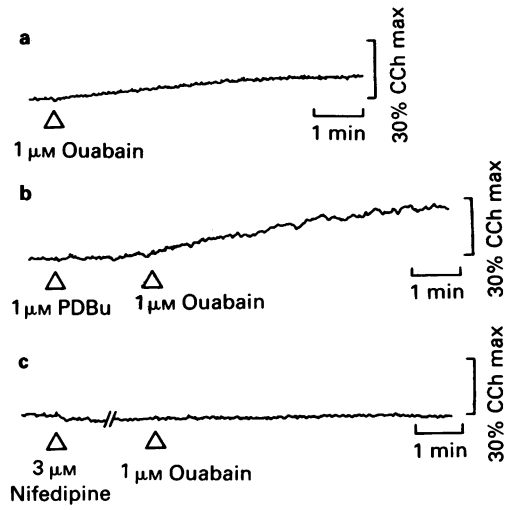


Figure 3 Ouabain-induced contractions. (a) Contraction induced by ouabain (1 μM) on its own. (b) Effect of phorbol 12,13-dibutyrate (PDBu, 1 μM) added 2 min before ouabain. (c) Effect of nifedipine (3 μM) added 5 min before ouabain. These are representative traces from three separate experiments. CCh = carbachol.

P was purchased from Peninsula Laboratories. Rubidium chloride ($^{86}Rb^+$; 0.171 Ci mmol^{-1}) was from Amersham International.

Statistics

The results are shown as the mean ± s.e.mean of the number of observations (n). The statistical significance was assessed by use of Student's t test for paired or unpaired values. P values less than 0.05, were considered to be significant.

Results

Phorbol esters inhibit the tonic component of contractions induced by carbachol, histamine and substance P

We have shown that PDBu quickly inhibits the tonic component of contractions induced by submaximal concentrations of carbachol (30–300 nM), although oscillatory contractions are enhanced (Sasaguri & Watson, 1989a). In the present study we observed similar effects with two further phorbol esters, PDA and PMA. All three compounds relaxed tissues precontracted by 100 nM carbachol in a concentration-dependent manner and exhibited parallel concentration-response curves (Figure 1). The EC_{50} values for PDBu, PDA and PMA were 0.086 μM, 0.53 μM and 3.5 μM, respectively. 4-α-Phorbol, which is unable to activate PKC, produced little relaxation (Figure 1).

The three phorbol esters also induced a similar relaxation of the tonic component of contraction induced by submaximal concentrations of histamine and substance P. Example traces of the effect of PDBu on contractions induced by these agonists in comparison with carbachol are shown in Figure 2.

Ouabain reverses inhibition by phorbol esters

Contractions induced by carbachol, histamine and substance P consisted of a rapid phasic component followed by a slower tonic component which could be inhibited by PDBu (see Figure 2 for example). The addition of ouabain (1 μM) after PDBu caused a rapid reversal of this inhibition and tension quickly rose to a level exceeding that in the control tissues (Figure 2). Higher concentrations of ouabain (3–10 μM) produced a more rapid reversal of PDBu inhibition (not shown).

Ouabain (1 μM) produced a small, slowly-developing contraction which levelled after 5–7 min at 13 ± 3% (n = 10) of the maximal tension generated by carbachol (Figure 3a).

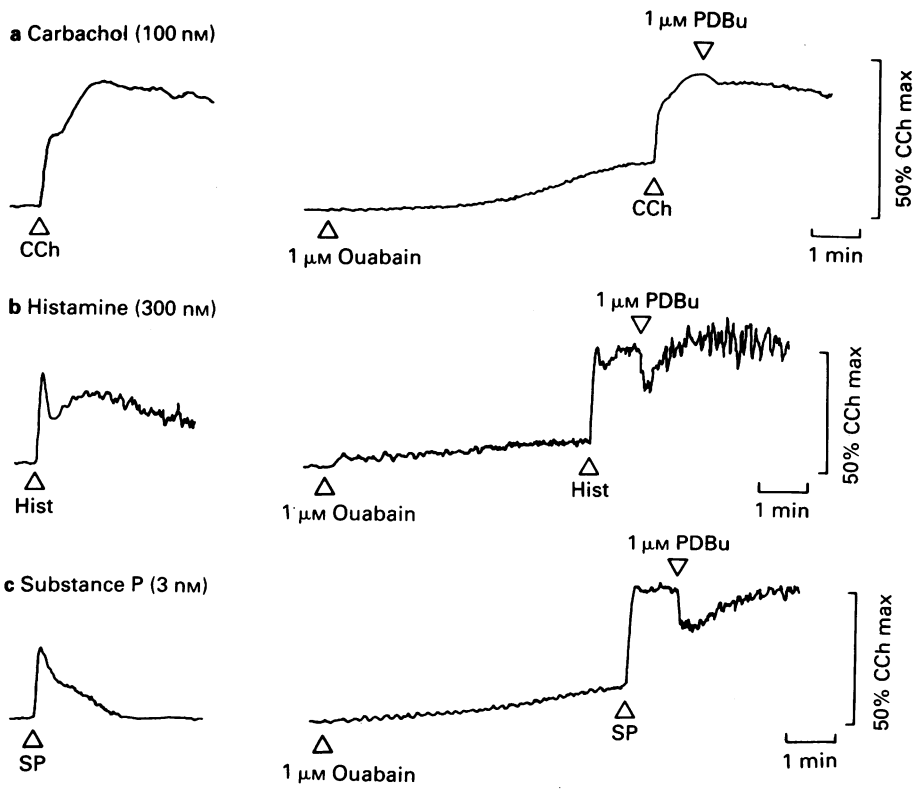


Figure 4 Effect of pretreatment with ouabain on the relaxation induced by phorbol 12,13-dibutyrate (PDBu). Right-hand traces were recorded 30 min after the control (left). Agonist was applied after ouabain-induced contraction reached a plateau and PDBu (1 μM) was added during the tonic component of contractions. Atropine (3 μM) was present during experiment. Traces are representative from three separate experiments. CCh = carbachol and SP = substance P.

Pretreatment of the tissue with PDBu (1 μM) for 2 min caused a small potentiation of the ouabain-induced contraction to 21 ± 4% ($n = 3$, $P < 0.05$) (Figure 3b). However, the size of the contraction generated by ouabain in the presence of PDBu was considerably less than the reversal of the PDBu-induced inhibition of agonist-induced contractions by ouabain, which, in some cases, amounted to almost 75% of the maximal contractile force (e.g. see Figure 2). Contraction to ouabain was inhibited completely in the presence of 3 μM nifedipine (Figure 3c), indicating that it involves the entry of Ca^{2+} through voltage-operated channels consistent with the observation that ouabain induces depolarization in this tissue (Bolton, 1973a).

To look at the effect of pretreatment with ouabain on the phorbol ester-induced relaxation, tissues were incubated with ouabain (1 μM) until the contractions reached a plateau (5 to 7 min) before application of the agonist. Under these conditions, the tonic component of the contractile response to these agonists was sustained and PDBu (1 μM) induced only a small and transient relaxation of the tonic component of contractions (Figure 4). Similarly, if ouabain was given during the tonic component of contraction induced by submaximal concentrations of these agonists (Figure 5) and also maximal concentrations of substance P (up to 1 μM; not shown), the relaxation of the tonic component was inhibited. However, ouabain had little effect on the decay of the tonic component induced by maximally effective concentrations of carbachol (1 μM) and histamine (3 μM). Essentially the same results to those described above were obtained in the presence of tetrodotoxin (300 nM; not shown).

PDBu stimulates the Na^+-K^+-pump

The above data suggest the involvement of the Na^+-K^+-ATPase pump in the inhibition of the tonic component of contractions by phorbol esters. As a test of this, we investigated the action of PDBu on Na^+-K^+-ATPase activity by analysing ouabain-sensitive $^{86}Rb^+$ uptake and the cellular content of Na^+ and K^+ (it should be borne in mind though that these changes may occur partially within neuronal tissue in the preparation). $^{86}Rb^+$ behaves as an isotope of K^+ and so its movement across the membrane is believed to reflect that of K^+.

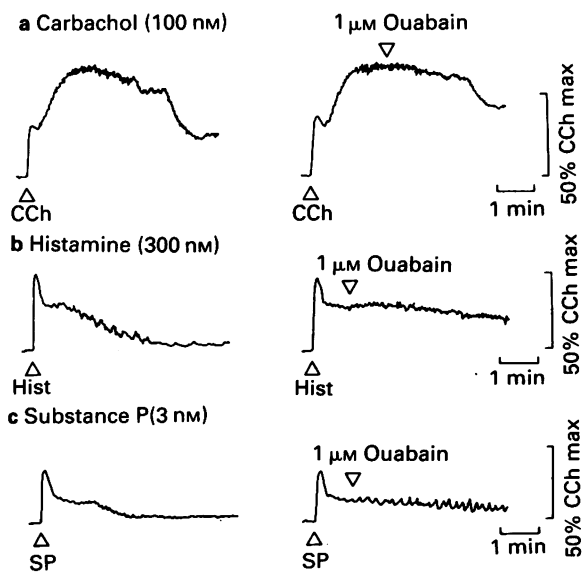


Figure 5 Effect of ouabain on the tonic component of contraction. Ouabain was added before decay of the tonic component began, 2 min after carbachol (CCh) or 1 min after histamine (Hist) and substance P (SP). In (c), atropine (3 μM) was added 5 min before substance P. These are representative traces from three independent experiments.

Table 1 Effect of phorbol 12,13-dibutyrate (PDBu) on the cellular content of Na^+ and K^+

	Na^+ (nmol mg^{-1} wet weight)	K^+ (nmol mg^{-1} wet weight)
Control	70.0 ± 1.8	90.1 ± 4.5
K^+-free	139.9 ± 1.6	1.0 ± 0.1
1 mM K^+	137.4 ± 1.3	4.3 ± 0.3**
1 mM K^+ + 3 μM PDBu	129.4 ± 2.5*	5.0 ± 0.3**

Longitudinal muscle strips were incubated in normal HEPES-buffered solution (control) or K^+-free solution bubbled with 100% O_2 for 3 h at 37°C. Some of the strips treated with K^+-free solution were further incubated for 4 min in the medium containing 1 mM K^+ in the presence or absence of 3 μM PDBu. After being blotted and weighed, the tissues were placed in tubes containing 'diluting fluid' (see Methods). After 24 h, concentrations of Na^+ and K^+ in the solution were measured with an atomic absorption spectrophotometer. Data are expressed as mean ± s.e.mean of 6–12 observations from two or three independent experiments. * $P < 0.02$, ** $P < 0.01$ (vs K^+-free; unpaired t test).

However, preliminary experiments revealed that more than 90% of the basal $^{86}Rb^+$ uptake was insensitive to ouabain. In order to increase the size of the ouabain-sensitive component, therefore, the intracellular Na^+ concentration was elevated by pre-incubation of the tissue in K^+-free solution for 3 h. Confirmation of this increase in cellular Na^+ content is shown in Table 1. The extracellular K^+ concentration during the $^{86}Rb^+$-uptake was maintained at 1 mM to avoid maximal pump activation (Brading & Widdicombe, 1974).

Under these conditions, the ouabain-sensitive portion of the uptake of $^{86}Rb^+$ was between 70–80% of total uptake. A maximally effective concentration of PDBu (3 μM) increased the pump activity by 24 ± 4% above basal (Figure 6). The EC_{50} for this action of PDBu was 225 nM, which is of the same order as the value for inhibition of the tonic component of contraction (86 nM). In contrast, 4-α-phorbol did not stimulate $^{86}Rb^+$-uptake (Figure 6). Carbachol (100 μM) also increased the pump activity by 16 ± 4% above basal, although lower concentrations had no significant effect.

As shown in Table 1, pre-incubation of tissues for 3 h in K^+-free medium almost totally depleted the tissues of K^+, and loaded them with Na^+. A subsequent 4 min incubation in the presence of 1 mM K^+ resulted in a significant cellular

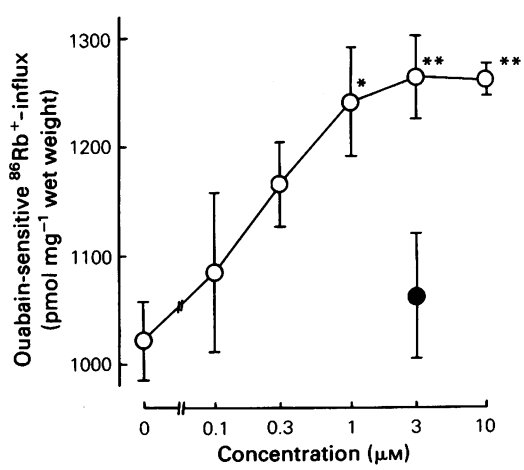


Figure 6 Effect of phorbol 12,13-dibutyrate (PDBu) on ouabain-sensitive $^{86}Rb^+$-uptake. After being loaded with Na^+ by exposure to K^+-free soltuion for 3 h, the tissues were incubated with $^{86}Rb^+$ and the indicated concentration of PDBu (○) or 4-α-phorbol (●) in HEPES-buffered solution containing 1 mM K^+ for 4 min with and without 0.1 mM ouabain. Vertical bars show s.e.mean of 6–12 observations from two or three independent experiments. * $P < 0.05$, ** $P < 0.01$.

uptake of K^+, but the reduction in Na^+ content was not significant. When PDBu was given during incubation with 1 mM K^+, the uptake of K^+ was slightly enhanced, and there was a larger, and significant decrease in cellular Na^+. These results are consistent with the suggestion that there was a greater stimulation of Na^+-K^+-ATPase in the presence of PDBu.

Discussion

Phorbol esters inhibit the tonic component of agonist-induced contractions

The present study provides several lines of evidence to suggest that the activation of PKC leads to an inhibition of the tonic component of agonist-induced contractions of the longitudinal smooth muscle of guinea-pig ileum through stimulation of Na^+-K^+-ATPase.

A number of phorbol esters inhibit the tonic component of contraction induced by a range of agonists. In contrast, 4-α-phorbol, which is unable to activate PKC, has little effect on contraction. The ability of the active phorbol esters to relax precontracted muscle seems to be more closely related to their hydrophilicity than to their potency against PKC. PMA, the most lipophilic of the esters, is the most potent activator of PKC (Nishizuka, 1984) yet is the weakest in inducing relaxation. It seems likely, therefore, that the inhibitory effect of the phorbol esters in influenced by their ability to diffuse through the smooth muscle cell layers, a process which will favour the more hydrophilic agents. A relaxing effect of phorbol esters on the resting tension or on agonist-induced contractions has also been observed in other smooth muscles, including the guinea-pig trachea and rat uterus (Baraban *et al.*, 1985; Menkes *et al.*, 1986; Jackson *et al.*, 1988).

The ability of the phorbol esters to suppress the tonic component of contraction is inhibited by the presence, or reversed by the addition, of the Na^+-K^+-ATPase inhibitor ouabain. Since ouabain induces a weak contraction on its own, mediated through depolarization which leads to the opening of voltage-operated Ca^{2+} channels, it is possible that this effect could account for the reversal of the phorbol ester-induced relaxation. However, this seems unlikely, since the onset of contraction by ouabain is very slow (several minutes) and also small. In contrast, the ability of ouabain to reverse the relaxation of the tonic component induced by phorbol esters is both rapid (significant recovery occurs with 60 s) and powerful, with the final contractile force being greater than in controls (see next section). The possibility that the release of neurotransmitters by ouabain accounts for its ability to reverse the inhibitory action of phorbol esters also appears unlikely. The major transmitter innervating the longitudinal muscle is acetylcholine, but ouabain causes a similar reversal of the responses to substance P, histamine and carbachol despite the fact that the experiments with substance P were carried out in the presence of atropine. Further, similar results were also observed in the presence of tetrodotoxin. Jackson *et al.* (1988) suggested that phorbol esters may relax guinea-pig airways through an increase in cyclic AMP, but this mechanism offers no explanation as to why contraction recovers in the presence of ouabain. However, it is possible that relaxation results from a stimulation of the Na^+-Ca^{2+} exchanger by protein kinase C and that this is reversed by ouabain through an alteration of the Na^+ gradient across the membrane. Nevertheless this does not account for the ability of phorbol esters to increase ouabain-sensitive K^+ uptake into the tissue under conditions of high Na^+ loading.

As discussed in the Introduction, there are a number of studies which have described the activation of Na^+-K^+-ATPase by protein kinase C (Greene & Lattimer, 1986; Nishizuka, 1986; Yingst, 1988). Stimulation of this pump by protein kinase C in guinea-pig ileum would lead to a recovery of membrane potential and relaxation of agonist-induced contractions. Indeed, direct measurements of Na^+-K^+-ATPase activity support this suggestion. The phorbol ester, PDBu, was demonstrated to stimulate the pump over a similar concentration range to that at which it inhibits the tonic component of contraction. Moreover, the observation that PDBu stimulated extrusion of Na^+ confirms this observation. Therefore, taken together, the results obtained provide preliminary evidence that the activation of protein kinase C by phorbol esters plays a role in inhibiting the tonic component of agonist-induced contraction through the stimulation of Na^+-K^+-ATPase. Other actions of protein kinase C (as discussed above may also contribute to this relaxation.

Phorbol esters also enhance contraction

Phorbol esters enhance both the phasic and tonic components of contractions induced by K^+, but only potentiate the phasic component of contractions induced by carbachol and inhibit the tonic component (Sasaguri & Watson, 1989a). When ouabain was added in the presence of phorbol ester (Figure 2, right), the tonic component of the agonist induced contractions exceeded that in control tissues (Figure 2, left) or the tonic component produced in the presence of ouabain (Figure 5, right). This suggests that it is the phorbol ester and not ouabain which is potentiating the tonic component of contraction under these conditions. Thus it appears that, as with K^+, phorbol esters stimulate a pathway which is able to enhance both the phasic and tonic components of agonist-induced contraction, but that the stimulation of Na^+-K^+-ATPase masks this potentiation. The inability of phorbol esters to inhibit the tonic component of contractions induced by high-K^+ may have been due to the large concentrations of K^+ rendering the Na^+-K^+-pump less able to hyperpolarize the cells. The mechanism of enhancement of contraction by phorbol esters is not known.

Physiological significance?

The normal time course of contraction to the above agonists includes a slowly decaying tonic phase. One explanation for this decline therefore is the stimulation of Na^+-K^+-ATPase through the activation of protein kinase C. The observation that ouabain prevents the decay of the tonic component of contraction to submaximal concentrations of these agonists supports this possibility. Under normal conditions an increase in intracellular Na^+, resulting from Na^+-influx following muscarinic receptor stimulation, has been suggested to contribute to activation of Na^+-K^+-ATPase (Bolton, 1973b). However, carbachol stimulated Na^+-K^+-ATPase in Na^+-loaded tissues consistent with the suggestion that carbachol also stimulates pump activity through the activation of PKC.

In contrast with its effects on the tonic component of contraction to submaximal concentrations of the agonists, ouabain had little effect on the decline of the tonic component induced by maximally effective concentrations of carbachol and histamine. It appears therefore that other mechanisms, e.g. receptor desensitization, are responsible for this decay.

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Vasopressin and stress-induced antinociception in the mouse

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1 Arginine vasopressin produced antinociception in the hot-plate test after intracerebroventricular injection (0.5 μg) and in the acetic acid abdominal constriction test after intraperitoneal injection (0.1 mg kg^{-1}).

2 The antinociception produced by arginine vasopressin was sensitive to deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin (0.5 μg i.c.v.; 0.1 mg kg^{-1} i.p.) but not to naloxone (5 μg i.c.v.; 2 mg kg^{-1} i.p.)

3 Arginine vasopressin when administered by the intracerebroventricular route, but not by the intraperitoneal route, produced characteristic behaviour which was sensitive to deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin (0.5 μg, i.c.v.).

4 A 3 min swim at 20°C produced antinociception on the hot-plate which was sensitive to naloxone (0.4 mg kg^{-1}, i.p.) but not to deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin (0.5 μg, i.c.v.).

5 The reduction in the number of acetic acid-induced abdominal constrictions produced by a 30 s swim at 30°C was not sensitive to either naloxone (2 mg kg^{-1}, i.p.) or deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin (0.1 mg kg^{-1}, i.p.).

6 Arginine vasopressin, at high doses, is antinociceptive in the mouse but does not appear to mediate stress-induced antinociception in this species.

Introduction

Stress-induced antinociception (SIA) is a complex phenomenon and the characteristics of a particular model of SIA depend not only on the animal species but also on the nature of the stress and the intensity of noxious stimulus used to assess the antinociception.

Several neurotransmitters, including acetylcholine, noradrenaline, histamine and 5-hydroxytryptamine are capable of producing antinociception and these have all been implicated in various models of SIA (Hutson *et al.*, 1984; Hart & Yadav, 1985; Hart & Oluyomi, 1986; 1987). Much attention has been focused on the involvement of endogenous opioid peptides in SIA (Lewis *et al.*, 1984) and, in addition, it has been suggested that another peptide, namely vasopressin, may also be involved (Bodnar *et al.*, 1980; Hamamura & Onaka, 1987).

The antinociceptive activity of lysine vasopressin, after intraventricular or subcutaneous administration, was demonstrated by Berntson & Berson (1980) in the rat, using radiant heat on the tail as the noxious stimulus; the antinociception was not naloxone-sensitive. Support for a physiological role for vasopressin in nociception was provided by the report that Brattleboro rats have a lowered threshold to foot-shock and a reduced level of antinociception after a cold water swim (Bodnar *et al.*, 1980). Further evidence for the involvement of vasopressin in SIA was provided by Hamamura & Onaka (1987) who demonstrated that the vasopressin antagonist, deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin, inhibited a naloxone insensitive model of SIA in the rat at a dose which also reduced the antinociception produced by exogenous arginine vasopressin. This latter observation was in contrast to the results of Caldwell *et al.* (1987) who found that deamino(CH$_2$)$_5$Tyr(Me) arginine vasopressin did not antagonize the antinociceptive effects of arginine-vasopressin in the mouse.

In the present study we have demonstrated the antinociceptive activity of arginine vasopressin in the mouse, using two different noxious stimuli. The characteristics of this antinociception have been examined by determining its sensitivity to naloxone and to the selective vasopressin antagonist deamino(CH$_2$)$_5$Tyr(Me)AVP (Kruszynski *et al.*, 1980). This antagonist has been employed to investigate the involvement of arginine vasopressin in SIA in the mouse by use of both a naloxone-sensitive and naloxone-insensitive model of SIA.

Methods

Male LACA mice (25–35 g), housed at 22 ± 2°C with illumination from 07 h 00 min to 21 h 00 min and with food and water *ad libitum*, were allowed to acclimatize for at least 90 min in the laboratory prior to experimentation between 11 h 00 min and 17 h 00 min.

Nociception

Two methods were used to assess the degree of antinociception produced by arginine vasopressin or by stress. In the hot-plate test the mouse was placed on a surface maintained at 56°C for 45 s or until lifting or shaking of a hind paw was observed; movement of mice on the hot plate was limited by a plexiglass cylinder (26 cm high and 20 cm diameter). An initial reaction time was obtained for each mouse and only those responding within 10 s were used. Potential antinociceptive agents were injected by the intracerebroventricular (i.c.v.) route 30 min after the screen reaction time had been measured and the effect of the agent was determined 10 min later. When antagonists were studied they were administered (i.c.v.) 30 min after the screen reaction time and 10 min before the antinociceptive agent. The abdominal constriction test involved the intraperitoneal (i.p.) injection of acetic acid (0.6% w/v, 0.01 ml g^{-1}) with the abdominal constrictions counted between 10 and 20 min after the injection of the acetic acid. Potential antinociceptive agents were administered 15 min before the acetic acid and antagonists 10 min before the antinociceptive agent, both by the i.p. route.

In the investigation of arginine vasopressin-induced antinociception, i.c.v. administration was used in the hot-plate test as this method involves a supraspinal response whilst the i.p. route was used for the abdominal constriction test which is sensitive to antinociceptive agents which act peripherally (Smith *et al.*, 1982). When investigating the possible involvement of opioids, naloxone was administered by the same route as the arginine vasopressin.

Stress-induced antinociception

Two models of SIA were investigated which differed in the intensity of the stress, the method of assessment of antinociception and in their sensitivity to naloxone. These have

been described previously (Hart *et al.*, 1983) and are summarised as follows:

(1) Mice were stressed by being placed in a water bath (57 cm × 30 cm, depth of 19 cm) at 20°C for 3 min. Hot-plate reaction times were determined three times: 30 min before the administration of antagonist or saline; 10 min later, which was immediately before the stress, and 2 min after the stress. The 2 min delay between the end of the stress and the measurement of a reaction time was occupied by the drying of the mouse. SIA was expressed as the difference between the post and pre-stress values for reaction times. Mice receiving saline were treated in the same manner apart from the swim. Naloxone was administered by the i.p. route and deamino(CH_2)$_5$Tyr(Me)AVP was injected by the i.c.v. route.
(2) Mice were placed in the water bath for 30 s at a water temperature of 30°C with antagonists or saline being injected (i.p.) 15 min before the stress. On removal from the water the mice were injected with acetic acid (as described above) and abdominal constrictions counted for 10 min after a delay of 10 min.

Drugs

Morphine hydrochloride, arginine vasopressin and the vasopressin antagonist [1-(β-mercapto-β,β-cyclopentomethylene propionic acid), 2-(O-methyl) tyrosine] arginine vasopressin (deamino(CH_2)$_5$Tyr(Me)AVP) were obtained from Sigma Ltd., naloxone hydrochloride was a gift from Endo Laboratories. In most experiments using the hot-plate, drugs or saline were administered by the i.c.v. route by a modification of the method described by Haley & McCormick (1957) with the injection volume not exceeding 5 μl. Coded solutions were used throughout the investigation with at least six mice in each experimental group. The investigator was not allowed access to the code until after the completion of the experiment.

Analysis

Results from both the hot-plate test (change in reaction times) and from the abdominal constriction test (number of constrictions in 10 min period) are expressed as medians and comparisons between groups has been assessed by the appropriate test; either the Kruskal-Wallis test or the Mann-Whitney test.

Results

Vasopressin-induced antinociception

Arginine vasopressin (0.5, 1 μg) injected i.c.v. produced a small but significant increase in reaction times of mice on the hot-plate (Table 1). The antinociception produced by 0.5 μg arginine vasopressin was abolished completely by the vasopressin antagonist deamino(CH_2)$_5$Tyr(Me)AVP (0.5 μg, i.c.v.) but unaffected by a dose of naloxone (5 μg, i.c.v.) which produced significant inhibition (approximately 90%) of morphine (5 μg, i.c.v.)-induced antinociception. In contrast, the vasopressin antagonist did not reduce antinociception due to morphine.

A similar pattern of results were obtained in the abdominal constriction test (Table 2). In mice treated with saline, the administration of acetic acid caused 26.5 (IQR 25–29) constrictions which was significantly reduced by both arginine vasopressin (0.1 mg kg^{-1}, i.p.) and morphine (2.5 mg kg^{-1}, i.p.). The effect of arginine vasopressin was inhibited by deamino(CH_2)$_5$Tyr(Me)AVP (0.1 mg kg^{-1}, i.p.) ($P < 0.001$) but not by naloxone (2 mg kg^{-1}, i.p.) and the reduction in abdominal constrictions produced by morphine was sensitive to naloxone but not to the vasopressin antagonist.

Stress-induced antinociception

In mice receiving saline (5 μl i.c.v.) a 3 min swim at 20°C increased the reaction time on the hot-plate by a median of 12.25 s (Table 3). This SIA was unaffected by deamino(CH_2)$_5$Tyr(Me)AVP at a dose (0.5 μg i.c.v.) which had

Table 1 Change in reaction time of mice on the hot-plate 10 min after administration of morphine or arginine vasopressin alone, or in presence of antagonist

Drug	*Dose* (μg)	*Median* (s)	*IQR*
Saline		0.5	0–0.5
Morphine	5	32.5*	29–39
Naloxone	5	0	0–0.5
Vasopressin	0.5	4*	4–5
	1	5*	4–6
d(CH_2)$_5$Tyr(Me)AVP	0.5	0	0–0.5
Saline +			
Vasopressin	0.5	3.75	3–4.5
d(CH_2)$_5$Tyr(Me)AVP +	0.5		
Vasopressin	0.5	0*	0–0.5
Naloxone +	5		
Vasopressin	0.5	4	4–4.5
d(CH_2)$_5$Tyr(Me)AVP +	0.5		
Morphine	5	31	30–37
Naloxone +	5		
Morphine	5	3*	2.5–4

All injections were by the i.c.v. route. IQR = interquartile range d(CH_2)$_5$Tyr(Me)AVP = deamino(CH_2)$_5$Tyr(Me) arginine vasopressin.
*$P < 0.001$ against appropriate control by Kruskal-Wallis test.

Table 2 Median number of abdominal constrictions recorded 10–20 min after i.p. administration of acetic acid

Treatment	*Dose* (mg kg^{-1})	*Median*	*IQR*
Saline		26.5	25–29
Vasopressin	0.1	12.5*	12–14
Morphine	2.5	8*	6–9
d(CH_2)$_5$Tyr(Me)AVP	0.1	27	24–29
Naloxone	2	28.5	27–30
Saline +			
Vasopressin	0.1	13.5	12–16
d(CH_2)$_5$Tyr(Me)AVP +	0.1		
Vasopressin	0.1	25.5#	24–26
d(CH_2)$_5$Tyr(Me)AVP +	0.1		
Morphine	2.5	7	7–8
Naloxone +	2		
Vasopressin	0.1	11.5	9–14
Naloxone +	2		
Morphine	2.5	22*	20–23
Saline + swim		8.5*	5–9
d(CH_2)$_5$Tyr(Me)AVP + swim	0.1	8.5	7–9
Naloxone + swim	2	9.5	8–10

Swim was for 30 s at 30°C. Morphine and vasopressin were administered 15 min before the acetic acid and antagonists 10 min before the agonist. All injections were by the i.p. route. IQR = interquartile range. d(CH_2)$_5$Tyr(Me)AVP = deamino(CH_2)$_5$Tyr(Me) arginine vasopressin.
*$P < 0.01$ against appropriate control by Kruskal-Wallis test. #$P < 0.001$ against saline + vasopressin control by Mann-Whitney test.

Table 3 Difference between post-swim and pre-swim reaction times on the hot-plate (i.e. SIA) in mice treated with saline, deamino(CH_2)$_5$Tyr(Me) arginine vasopressin (d(CH_2)$_5$Tyr(Me)AVP) or naloxone

Treatment	*Dose*	*Median* (s)	*IQR*
Saline	5 μl i.c.v.	12.25	10–13
d(CH_2)$_5$Tyr(Me)AVP	0.5 μg i.c.v.	12	12–13
Saline	10 ml kg^{-1} i.p.	11	8–12
Naloxone	0.4 mg kg^{-1} i.p.	6*	3–8.5
	4 mg kg^{-1} i.p.	1**	−2–3

Post-swim reaction time was measured 2 min after the 3 min swim at 20°C. In mice treated with saline but not swum the median increase in reaction time was 0.5 s. IQR = interquartile range.
*$P < 0.05$; **$P < 0.01$ by Kruskal-Wallis test.

inhibited the antinociception produced by arginine vasopressin on the hot-plate. The involvement of endogenous opioids in this model of SIA was confirmed by the reduced antinociception observed in the mice treated with naloxone.

Stressed mice (30 s swim at 30°C) responded to acetic acid with a reduced number of abdominal constrictions i.e. 8.5 compared to 26.5 in unstressed animals ($P < 0.01$). In this case the SIA was unaffected by prior treatment of the mice with either deamino$(CH_2)_5$Tyr(Me)AVP or naloxone at doses that reduced the effects of arginine vasopressin and morphine respectively in the abdominal constriction test (Table 2).

Behaviour

Although not estimated quantitatively in this study, animals injected with arginine vasopressin were observed carefully for any gross changes in behaviour. The i.c.v. administration of arginine vasopressin elicited an immediate and consistent pattern of behaviour involving excessive grooming and alternate hind limb scratching directed at the head. Between 4 min and 6 min after injection of vasopressin, vocalisation occurred which continued for more than an hour whilst the characteristic grooming behaviour lasted for 10 min. Both the grooming behaviour and vocalisation responses to arginine vasopressin were inhibited by deamino$(CH_2)_5$Tyr(Me)AVP (0.5 μg, i.c.v.) which alone produced no marked changes in behaviour. The behaviour of mice given arginine vasopressin by the intraperitoneal route did not differ from that of animals treated with saline.

Discussion

The present study confirms that arginine vasopressin is antinociceptive in the mouse and, significantly, demonstrates antinociception occurring after both i.c.v. and i.p. administrations of this peptide. Previous workers have failed to show antinociception by the i.c.v. route. Tulunay *et al.* (1982) used doses of between 0.01 and 0.1 μg per mouse and did not observe antinociception using a radiant heat tail-flick method whilst Berkowitz & Sherman (1982) found that 1–10 $\mu g\,kg^{-1}$ was effective intravenously but not intracerebroventricularly in the abdominal constriction test. The positive results obtained in the present study may reflect the higher doses of arginine vasopressin, 0.5 μg and 1 μg per mouse, which were employed. Caldwell *et al.* (1987) showed that arginine vasopressin was antinociceptive in mice on the hot-plate with doses of 1 μg and 4 μg administered intracisternally. In contrast to the results of Berkowitz & Sherman (1982), studies in the rat (Kordower *et al.*, 1982) showed lysine vasopressin to be active by the i.c.v. route (500 ng) but not after subcutaneous injection (1500 ng).

Berkowitz & Sherman (1982) suggested that arginine vasopressin may act peripherally in producing antinociception and this would appear to be supported by the present finding that intraperitoneal arginine vasopressin (0.1 $mg\,kg^{-1}$) reduced the number of abdominal constrictions produced by acetic acid. However, no firm conclusions can be drawn about the antinociceptive site of action of arginine vasopressin from these experiments; the i.c.v. dose was high and may have entered the peripheral circulation whilst it is possible, but unlikely, that the intraperitoneally administered peptide would have penetrated the brain. Banks & Kastin (1987), in a review of peptide movement across the blood:brain barrier, concluded that peripherally administered arginine vasopressin is unlikely to enter the brain whilst the peptide is cleared rapidly from the cerebrospinal fluid after central administration. The spinal cord does not appear to be the site of action of arginine vasopressin in the rat for intrathecal vasopressin was not antinociceptive and lesions of the paraventricular nucleus, which lowered levels of vasopressin in the cord, did not affect tail-flick latencies (Millan *et al.*, 1984).

Antinociception induced by arginine vasopressin was attenuated by the selective antagonist deamino$(CH_2)_5$Tyr(Me)AVP administered 10 min prior to the peptide whereas Caldwell *et al.* (1987) reported no inhibition of antinociception in the mouse when the antagonist was administered at the same time as arginine vasopressin by the intracisternal route. Hamamura & Onaka (1987) found that the increase in the tail-flick latency in the rat induced by the intravenous administration of arginine vasopressin was sensitive to deamino$(CH_2)_5$Tyr(Me)AVP given intravenously 1 h before the vasopressin. It would seem that the lack of activity observed by Caldwell *et al.* (1987) may be explained in the timing of the administration of the antagonist. Kruszynski *et al.* (1980) reported that the pA_2 for deamino$(CH_2)_5$Tyr(Me)AVP against the pressor activity of arginine vasopressin was 8.62 whilst it had weak antidiuretic activity and it may thus be considered a selective antagonist of V_1 vasopressin receptors (Manning & Sawyer, 1984). The inhibition by deamino$(CH_2)_5$Tyr(Me)AVP of both the behavioural activities induced by arginine vasopressin and the antinociception suggests that in the mouse the receptors involved are probably of the same V_1 class. In the present study the vasopressin antagonist had no effect on morphine induced antinociception nor did naloxone affect antinociception due to arginine vasopressin.

This finding that arginine vasopressin-induced antinociception does not involve opioid pathways confirms the results of others in mice (Berkowitz & Sherman, 1982) and rats (Berntson & Berson, 1980; Berson *et al.*, 1983; Watkins *et al.*, 1986). The lack of interaction between opioid- and vasopressin-induced antinociception is surprising in view of the reported co-existence of vasopressin and dynorphin in neurones in the brain (Cox *et al.*, 1982) and the potentiation of morphine antinociception observed with 10–100 ng arginine vasopressin administered intracerebroventricularly to the mouse (Tulunay *et al.*, 1982). This could be studied further by investigating whether there is any interaction between arginine vasopressin and a κ-opioid agonist.

The model of SIA involving a 3 min swim at 20°C with antinociception assessed on the hot-plate is sensitive to low doses of naloxone and to a selective δ-opioid receptor antagonist (Hart *et al.*, 1983; 1985). Endogenous opioids are thus important in this model and there is good evidence for the additional involvement of cholinergic and adrenergic pathways (Hart & Yadav, 1985) but the present investigation shows that arginine vasopressin is unlikely to have a significant role. When the duration of the swim is reduced to 30 s and water temperature increased to 30°C the resulting antinociception is too mild to be measured on the hot-plate but can be assessed as a reduction in the number of constrictions induced by acetic acid. This SIA is not attenuated by high doses of naloxone (Hart *et al.*, 1983), as confirmed in the present study, and was unaffected by the vasopressin antagonist at a dose which produced significant inhibition of the antinociceptive action of vasopressin. The demonstration that arginine vasopressin is not involved in SIA in the mouse, in contrast to its well documented role in the rat (Bodnar *et al.*, 1980; Hamamura & Onaka, 1987), further emphasizes that the characteristics of SIA depend upon the species and the method of assessment of antinociception (Kelly, 1982).

In conclusion, the results of this study show that arginine vasopressin is antinociceptive in the mouse in both the hot-plate and abdominal constriction tests but that there is no evidence for its involvement in either naloxone-sensitive or naloxone-insensitive models of SIA.

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Changes in preganglionic sympathetic nerve function following chronic morphine treatment in rats

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1 The effects of acute or chronic morphine treatment on the changes in blood pressure and pulse rate in response to ganglionic stimulation or blockade and to vagal stimulation, and of isolated atria to field stimulation or noradrenaline, were studied.

2 In pithed rats, intravenously injected hexamethonium significantly depressed the blood pressure responses to sympathetic nerve stimulation. The ganglionic blocking effects of hexamethonium were significantly greater in chronically morphine-treated rats, but were not significantly affected by acute morphine administration in naive animals.

3 Intravenous administration of nicotine dose-dependently increased blood pressure and pulse rate. The magnitudes of these changes were not significantly affected by acute or chronic morphine pretreatment.

4 Studies with rat isolated atrial preparations revealed that the changes in atrial contractile rate and force in response to noradrenaline or field stimulation were not influenced by either acute or chronic morphine treatment.

5 Cervical vagal stimulation produced voltage- or frequency-dependent decreases in pulse rate and blood pressure. The responses were not significantly affected by chronic morphine treatment.

6 These findings suggest that the site of the changes in sympathetic function following prolonged exposure to the opiate appears to be on the preganglionic nerve fibres.

Introduction

Previous work has shown that chronic opiate treatment produces not only physical dependence and tolerance but also other physiological changes which are different from those observed after acute opiate administration. These include altered sensitivity to the actions of catecholamines, acetylcholine, 5-hydroxytryptamine and arachidonic acid (Schulz & Goldstein, 1973; Kromer & Steigeman, 1982; Rae & De Moraes, 1983; Dai *et al.*, 1985; Martin & Takemori, 1986) and altered responses to certain pathophysiological conditions such as acute myocardial ischaemia (Chan *et al.*, 1987; Ko *et al.*, 1988). The mechanisms behind such effects are not clear. Although it has been suggested that prolonged exposure to morphine induces body adaptation which involves the development of postsynaptic supersensitivity to the appropriate neurotransmitters (Schulz & Goldstein, 1973; Rae *et al.*, 1977; Llorens *et al.*, 1978; Collier, 1980), the possibility that chronic morphine treatment may cause changes in the function of the peripheral nervous system at a presynaptic level cannot yet be excluded.

Recently, it has been observed that chronic morphine treatment significantly depresses the cardiovascular changes and the elevation in plasma noradrenaline concentration induced by sympathetic nerve stimulations in pithed rats (Leung *et al.*, 1986a; 1989). Although the data suggest that the function of the postsynaptic adrenoceptors is unchanged and that activity changes may occur between the sympathetic preganglionic fibres and the postganglionic nerve terminals (Leung *et al.*, 1986a), the exact location and nature of the alterations have not yet been defined.

The present study was undertaken to examine further the possible sites of functional changes along the sympathetic neuronal pathway after chronic treatment with morphine. For comparative purposes, similar experiments were carried out in acutely morphine-treated animals. In addition, in order to evaluate the possible effects of chronic morphine treatment on the function of the cholinergic nerves, cardiovascular responses to electrical vagal stimulation were also observed.

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Methods

Animals

Female Sprague-Dawley rats, weighing 140–160 g, were used. They were given a standard rat pellet diet (Ralston Purina Co., U.S.A.) and tap water to drink *ad libitum*, and were housed in a temperature (23 ± 1°C)- and humidity (65 ± 5%)-controlled room.

Measurements of cardiovascular parameters in intact rats

In experiments which measured the responses to nicotine injection or vagal stimulation, anaesthesia was induced by diethyl ether (B.D.H.) and was maintained with chloralose (B.D.H.) 100 mg kg^{-1} i.v. Rats subjected to sympathetic nerve stimulation were pithed under ether anaesthesia. The trachea, jugular vein and right common carotid artery were cannulated. Arterial blood pressure was measured with a Statham P23ID pressure transducer. Integrated pulse rate was recorded by triggering the pressure wave using a biotachometer (Narco Bio-Systems). These variables were displayed on a physiograph (MK-IV, Narco Bio-Systems). The anaesthetized animals were kept warm with a heating lamp throughout the whole experimental period.

Sympathetic nerve stimulation after ganglionic blockade

Sympathetic nerve stimulation was carried out as previously described (Leung *et al.*, 1986a; 1989). Rats were pithed under ether anaesthesia and then artificially ventilated with room air (1 ml 100 g^{-1}, 82 strokes min^{-1}). The autonomic preganglionic fibres between T1–T12 in the spinal canal were stimulated with electrical impulses (10 Hz, 0.5 ms pulse width) of various voltages for 5 s at 5 min intervals. Gallamine triethiodide (Sigma) 5 mg kg^{-1} i.v. and atropine sulphate (E. Merck) 1 mg kg^{-1} i.p. were given to minimize skeletal muscle contraction and the effects of parasympathetic nerve stimulation, respectively. The rats subjected to autonomic ganglion blockade were given hexamethonium bromide (Koch-Light) 6 mg kg^{-1} i.v. The controls received similar volumes

(1 ml kg^{-1}) of 0.9% w/v NaCl (saline) by the same route. All drugs were injected 15 min before the start of sympathetic nerve stimulation; the doses are calculated as the salts.

Responses to nicotine

In order to avoid the complications due to the respiratory effects of nicotine (Silvette *et al.*, 1962), the rats were artificially ventilated with room air (1 ml $100 g^{-1}$, 82 strokes min^{-1}) throughout the experimental period. Doses of nicotine tartrate (BDH) 0.125, 0.25, 0.5 and 1 mg kg^{-1}, calculated as the salt, were injected i.v. at 10 min intervals.

Vagal stimulation

The method of vagal stimulation, as described by Ho *et al.* (1987), was employed in a group of chronic morphine-treated rats. A sleeve electrode was positioned under the cervical section of the left vagus nerve. The nerve was stimulated for 5 s at 54 min intervals with electrical square-wave impulses of 5 ms pulse width, 10 Hz frequency and various voltages, or of 5 ms pulse width, 70 V and various frequencies.

Rat isolated atrial preparations

The rat atria were isolated by the technique described by Blinks (1966) and mounted in an organ bath filled with Krebs–Henseleit solution containing atropine. The force and rate of contraction were measured with a strain-gauge transducer (E & M Instrument Co. Inc., Houston, A-1082) and biotachometer (Narco Bio-Systems), respectively, and were displayed on a physiograph (MK-IV, Narco Bio-Systems). The temperature of the organ bath was kept at 37°C and the physiological solution was gassed with 95% O_2 and 5% CO_2. The composition of the Krebs-Henseleit solution containing atropine was (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-glucose 11.1 and atropine sulphate 0.005.

Experiments were started after a 20 min period of equilibration. Contraction of the atrial muscle was enhanced by either field stimulation or the addition of noradrenaline bitartrate (Sigma) into the bathing medium. The electrical field pulses were monophasic square waves of 0.5 ms pulse width, 15 V and of various frequencies (2, 4, 6, 8, 10, and 12 Hz), or 8 Hz and various voltages (5, 10, 20, 25, and 30 V). The preparation was stimulated for 5 s at 5 min intervals. Various amounts of noradrenaline bitartrate, to achieve concentrations of 10^{-6}, $10^{-5.5}$, 10^{-5} and $10^{-4.5}$ M were added.

Morphine treatment

The acute effects of morphine sulphate (Macfarlan Smith, Middlesex) were observed in naive rats. Intact animals were given 16 mg kg^{-1} i.p. 15 min before the start of the experiments. In isolated atrial preparations, graded doses of morphine sulphate were added sequentially to the bathing solution to achieve concentrations of 10^{-7}, $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, 10^{-5} and 10^{-4} M immediately before stimulation of the preparations with field stimulation (15 V, 8 Hz, 0.5 ms) or noradrenaline (10^{-6} M). The doses are calculated as the salt. Similar volumes of vehicle (saline) were given to the controls by the same route and at the same times.

A previously described method for inducing opiate tolerance and dependence was adopted in the chronic opiate studies (Dai *et al.*, 1984). Morphine sulphate was administered in a drinking solution of 5% w/v sucrose in ordinary tap water. It was given in increasing concentrations (48 h apart) of 0.1, 0.2, 0.3 and finally 0.4 mg ml^{-1}; weights are calculated as the salt. The final concentration was continued until the end of the 3 week treatment period. Control animals were given a 5% sucrose solution to drink. All drinking solutions were provided *ad libitum*. These animals were used for observing the effects of prolonged morphine treatment. In the studies with isolated atrial preparations, the atria isolated from the chronic morphine-treated rats were also used but were bathed in a medium containing morphine sulphate 1 μg ml^{-1} in order to avoid the possible occurrence of withdrawal effects during the experiment. This concentration of morphine was similar to plasma morphine levels found in the dependent rats (Leung *et al.*, 1986b). Control preparations, isolated from sucrose-drinking controls, did not have morphine in their bathing solution.

Statistical analysis

Data were expressed as means ± s.e.mean and were analysed by Student's unpaired *t* test. The upper limit for statistical significance was set at $P < 0.05$.

Results

Effects of ganglionic blockade

In naive rats, sympathetic nerve stimulation at 0.5 ms and 10 Hz produced voltage-dependent increases in blood pressure in the saline-treated controls (Figure 1a). The pulse rate also tended to rise but the changes were less marked and exhibited greater variation (Figure 1d). The blood pressure and pulse rate quickly returned to their pre-stimulation values within 20 s after cessation of electrical stimulation. Similar observations were made in control (sucrose drinking) (Figures 1b and 1e) or chronically morphine-treated rats (Figures 1c and 1f).

In both naive and sucrose-drinking rats treated with hexamethonium bromide 6 mg kg^{-1} i.v., the changes in blood pressure induced by low voltages sympathetic nerve stimulation (10 and 30 V or 10, 30 and 50 V, respectively) were significantly less than those of their vehicle-treated controls. However, their responses to higher voltage stimulation (50, 70 and 90 V in naive animals, 70 and 90 V in sucrose-drinking rats) were not significantly different from those of their vehicle-treated controls (Figures 1a and 1b). In rats treated chronically with morphine for 21 days, hexamethonium administration caused significant decreases in the percentage blood pressure changes in response to both low and high voltage sympathetic nerve stimulation (Figure 1c). The pulse rate responses to sympathetic nerve stimulation in all pretreatment groups showed no significant difference between the saline- and hexamethonium-treated rats (Figures 1d, e and f).

In acutely morphine-treated rats, hexamethonium 6 mg kg^{-1} i.v. also caused significant depression of the blood pressure rises induced by low voltage (30 and 50 V) sympathetic nerve stimulation (Figure 2a). The effects of hexamethonium on the blood pressure changes induced by high voltage (70 and 90 V) stimulation (Figure 2a), or on the pulse rate increases due to sympathetic nerve stimulation at all voltages (Figure 2b), were not statistically significant.

Responses to nicotine

In naive rats and in sucrose-drinking controls, i.v. administered nicotine increased the mean blood pressure (Figures 3a and b) and pulse rate (Figures 3c and d) in a dose-dependent manner. The changes were immediate but transient, and lasted no longer than 30 s. Similar patterns and intensities of responses to nicotine injection were observed in animals treated either acutely (Figures 3a and c) or chronically (Figures 3b and d) with morphine; no statistically significant differences were found when compared with their acute saline-treated or chronic sucrose-drinking controls.

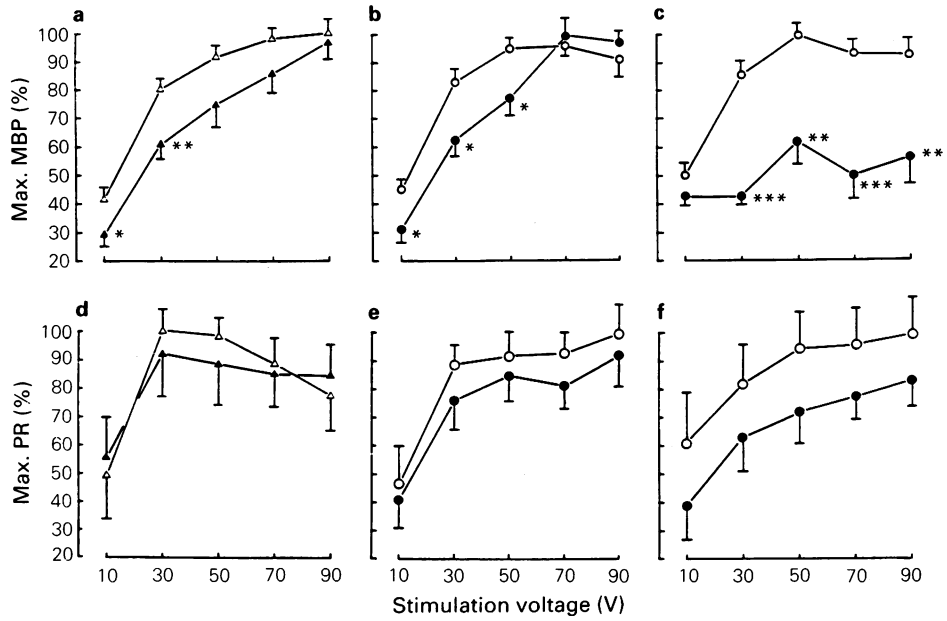


Figure 1 Effects of hexamethonium on the percentage change in maximum mean blood pressure (Max. MBP) (a, b and c) and maximum pulse rate (Max. PR) (d, e and f) responses of pithed rats subjected to sympathetic nerve stimulation (10–90 V, 10 Hz, 0.5 ms). (a) and (d) Naive rats injected with saline (△, 6 ml kg^{-1}, i.v.) ($n = 8$) or hexamethonium bromide (▲, 6 mg kg^{-1}, i.v.) ($n = 8$). (b) and (e) Sucrose-drinking rats on day 21 injected with saline (○, 1 ml kg^{-1}, i.v.) ($n = 8$) or hexamethonium bromide (●, 6 mg kg^{-1}, i.v.) ($n = 8$). (c) and (f) Morphine/sucrose-treated rats on day 21 injected with saline (○, 1 ml kg^{-1}, i.v.) ($n = 8$) or hexamethonium bromide (●, 6 mg kg^{-1}, i.v.) ($n = 9$). The values plotted are the means with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared with the corresponding values in the saline-injected groups.

Responses of rat isolated atria to field stimulation and noradrenaline

The baseline contraction rate and force of the atria isolated from naive, or from chronically sucrose- or morphine-treated rats, before field stimulation or noradrenaline addition were not significantly different from each other.

Table 1 shows that the basal contraction force and rate in atria isolated from naive rats were increased by 10^{-6} M noradrenaline or field stimulation at 0.5 ms, 8 Hz and 15 V, and such increases were not significantly altered by the addition of morphine to the bathing medium even at a concentration of 10^{-4} M.

In atria isolated from chronically sucrose- or morphine/sucrose-drinking rats, noradrenaline dose-dependently increased the force and rate of contraction (Figures 4a and b). Field stimulation of these preparations also displayed frequency- (Figures 4c and d) or voltage- (Figures 4e and f)

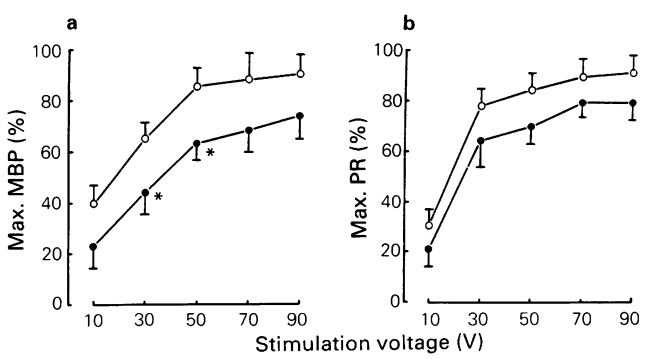


Figure 2 Effects of hexamethonium on the percentage change in (a) maximum mean blood pressure (Max. MBP) and (b) maximum pulse rate (Max. PR) responses in acutely morphine-treated rats subjected to sympathetic nerve stimulation. Saline-injected (○, 1 ml kg^{-1}, i.v.) ($n = 8$) and hexamethonium-injected (●, 6 mg kg^{-1}, i.v.) ($n = 8$) rats. The values plotted are the means with s.e.mean shown by vertical bars. $*P < 0.05$ when compared with the corresponding values in the saline-injected groups.

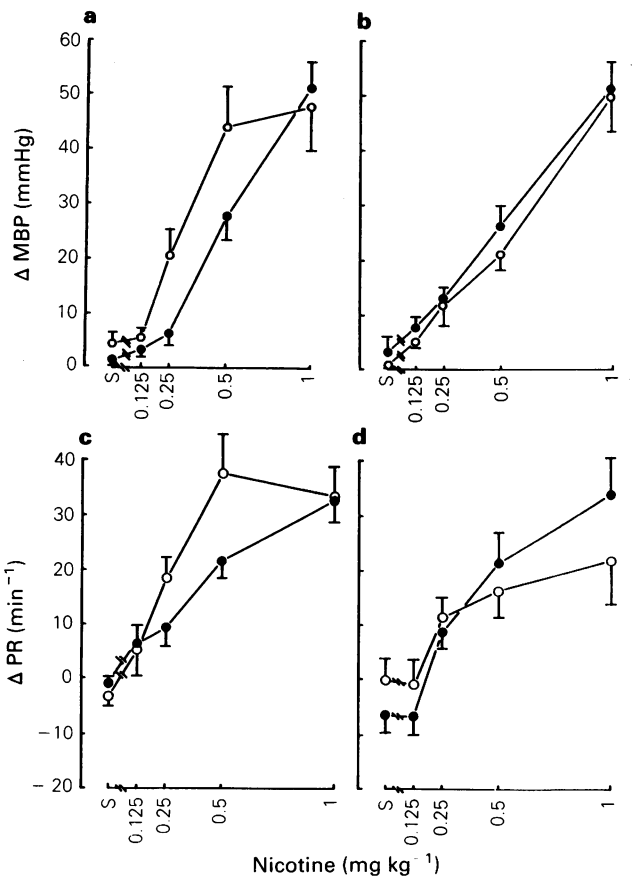


Figure 3 Changes in mean blood pressure (Δ MBP) (a and b) and pulse rate (Δ PR) (c and d) in the response to nicotine i.v. in acute or chronic morphine-treated rats. S = saline. (a) and (c) Naive rats injected with saline (○, 1 ml kg^{-1}, i.p.) ($n = 8$) or morphine sulphate (●, 16 mg kg^{-1}) ($n = 8$). (b) and (d) Sucrose-drinking (○) ($n = 8$) or morphine/sucrose-treated (●) ($n = 8$) rats on day 21. The values plotted are the means with s.e.mean shown by vertical bars. No significant differences were found when the values were compared with their corresponding controls.

Table 1 Acute effects of morphine on the responses of atria isolated from naive rats to 10^{-6} M noradrenaline and field stimulation (15 V, 8 Hz, 0.5 ms)

Increases in contraction parameters	*Saline*	−log [*Morphine* (M)] 8	7	6.5	6	5.5	5	4
(A) *Noradrenaline*								
Δ Force (%)	86.5 ± 3.6	86.3 ± 0.7	91.2 ± 4.9	81.5 ± 4.8	89·2 ± 4.0	91·6 ± 3.2	86·7 ± 1.7	82·3 ± 6.8
Δ Rate (min^{-1})	79.7 ± 5.4	87.6 ± 7.6	85.1 ± 3.4	86.6 ± 3.6	90.6 ± 6.2	89.5 ± 4.9	91.2 ± 7.2	82.5 ± 4.5
(B) *Field stimulation*								
Δ Force (%)	89.7 ± 5.6	79.0 ± 2.1	89.3 ± 7.3	79.7 ± 6.8	88.8 ± 6.2	81.5 ± 5.8	75.8 ± 8.8	83.0 ± 4.7
Δ Rate (min^{-1})	75.3 ± 9.1	79.0 ± 3.5	66.2 ± 5.2	90.1 ± 4.4	82.0 ± 10.3	86.0 ± 2.7	83.2 ± 9.7	74.0 ± 5.8

The values are the means ± s.e.means. Each value is the mean of 8 measurements.
No significant differences were found when compared with values of those in saline controls.

dependent increases in both contractile force and rate. There were no significant differences between the values of the contraction parameters of these two pretreatment groups.

Vagal stimulation

Electrical vagal stimulation at 0.5 ms produced voltage- (Figures 5a and c) and frequency- (Figures 5b and d) dependent decreases in pulse rate and mean blood pressure. The responses were immediate and transient. Both parameters quickly returned to the pre-stimulation levels within 15 s after cessation of electrincal stimulation. The intensities of the responses to vagal stimulation in the chronically morphine-treated rats were not significantly different from those in the sucrose-drinking controls.

Discussion

It was found in the present study that, in rats chronically treated with morphine, the blocking effects of hexamethonium on blood pressure responses to sympathetic nerve stimulation were significantly greater when compared with those in naive

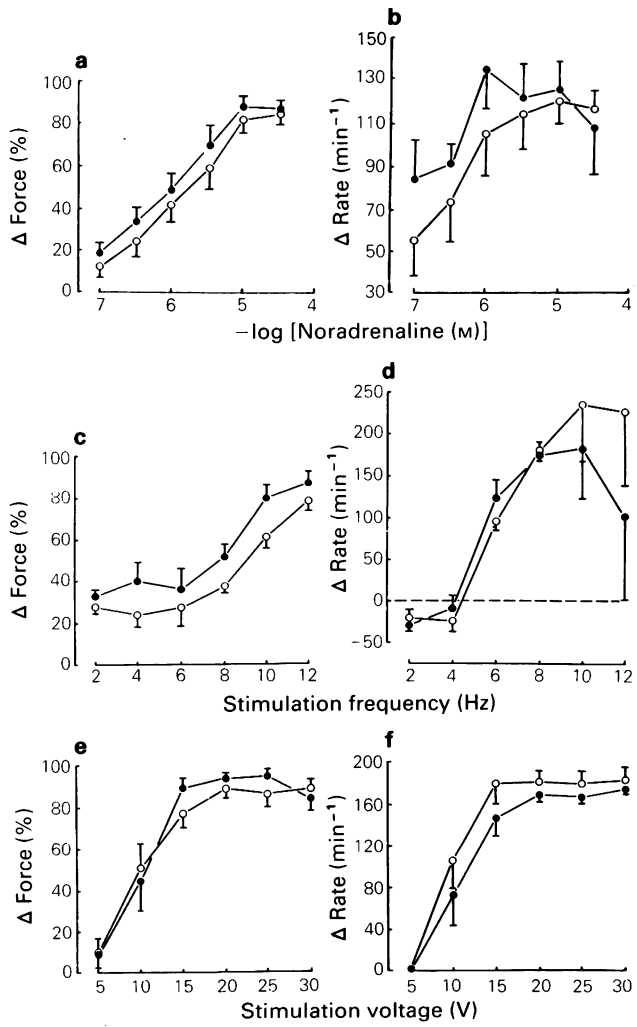


Figure 4 Effects of noradrenaline (a and b) and field stimulation of 2–12 Hz, 15 V, 0.5 ms (c and d) or of 5–30 V, 8 Hz, 0.5 ms (e and f) on force and rate of contraction in rat isolated atrial preparations obtained from (○) sucrose-drinking ($n = 9$) and (●) morphine/sucrose-treated ($n = 8$) rats. The values plotted are the means with s.e.mean shown by vertical bars. No significant differences were found when compared the corresponding value in two groups.

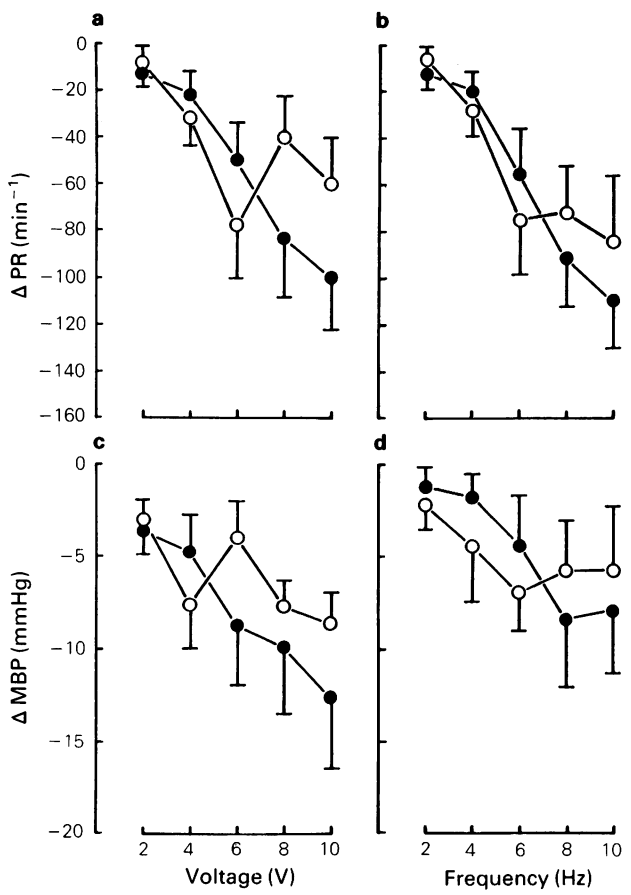


Figure 5 Effects of chronic morphine administration on the changes in pulse rate (Δ PR) and mean blood pressure (Δ MBP) induced by electrical vagal stimulation at 10 Hz, 0.5 ms with variable voltages (a and c) or at 10 V, 0.5 ms with variable frequencies (b and d): (○) sucrose-drinking controls ($n = 9$), (●) chronic morphine-treated rats ($n = 10$). The values plotted are the means with s.e.mean shown by vertical bars. No significant differences were found when compared with the corresponding values in the two groups.

and sucrose-drinking animals. This depressant effect, unlike those seen in the sucrose-drinking controls and in the acutely morphine-treated rats, could not be restored even by increasing the stimulation voltage to 90 V. It is, therefore, suggested that chronically morphine-treated rats are more susceptible to the ganglionic blocking actions of hexamethonium than their controls. The increased susceptibility is not likely to be related directly to the plasma morphine concentrations because this phenomenon was not observed in naive animals treated acutely with a high dose of morphine sulphate.

There are several possible explanations of the inability of high voltage sympathetic nerve stimulation to reverse the ganglionic blocking actions of hexamethonium in chronically morphine-treated rats. (1) An overdose of hexamethonium had been administered and, therefore, even at the highest sympathetic stimulation voltage, the amount of acetylcholine released from the preganglionic fibres was still not sufficient to overcome the blockade. (2) The sympathetic pathways distal to the ganglionic region may have been functionally impaired and, consequently, a relatively smaller amount of hexamethonium would have been sufficient to block sympathetic function. (3) The sympathetic pathways proximal to the ganglionic region could have been functionally altered, hence, the amount of acetylcholine released during sympathetic stimulation was smaller and, therefore, could not displace hexamethonium from the binding sites.

In the current investigation, a relatively small dose of hexamethonium was selected so as not to depress the blood pressure responses to high voltage sympathetic nerve stimulation in naive and sucrose-drinking rats. This would enable the possible enhancement or decrease, if any, of the ganglionic blocking actions due to acute or chronic morphine treatment to be observed. Therefore, the first possibility can be excluded.

The cardiovascular responses of the acutely or chronically morphine-treated rats to i.v. injection of nicotine were not significantly different from those of their naive or sucrose-drinking controls. This indicates that the number, or sensitivity, of ganglion nicotinic receptors and the functions of the sympathetic postganglionic fibres are not significantly changed by chronic morphine treatment. Thus, the second possibility is unlikely to be the cause of the apparently irreversible blocking effects of hexamethonium in chronically morphine-treated animals.

It was also found in the present study that the responses to noradrenaline and field stimulation of the atria isolated from the chronic morphine-treated rats were not significantly different from those of atria isolated from sucrose-drinking controls. These results suggest that chronic morphine treatment in rats does not significantly alter the functions of the postganglionic sympathetic nerve fibres or the postsynaptic adrenoceptors. These findings support the observations on the effects of i.v. administered nicotine in intact rats and also previous findings on the cardiovascular responses to i.v. injected noradrenaline in pithed animals (Leung *et al.*, 1986a). They also reinforce the idea that enhanced susceptibility to the ganglionic blocking effects of hexamethonium in chronically morphine-treated animals is not due to functional impairment of the sympathetic pathway distal to the ganglion. These results are, however, at variance with those of Rae *et al.* (1977, 1983); these workers showed that the vas deferens of chronically morphine-treated mice developed supersensitivity to noradrenaline. However, it may not be feasible to compare their results with the present findings because entirely different species of animals, tissues, doses and routes of chronic administration of morphine and experimental designs were used.

Elimination of the first and second possibilities mentioned above means that the data favour the third alternative. It is therefore, suggested that the reduced cardiovascular responses and plasma noradrenaline increase during sympathetic nerve stimulation in rats chronically treated with morphine (Leung *et al.*, 1986a; 1989) may have resulted from dysfunction of the preganglionic sympathetic nerve fibres, leading to diminished release of acetylcholine in response to sympathetic nerve stimulation. However, this idea is not supported by results of the present studies on vagal stimulation which show that chronic opiate administration does not significantly affect the cardiovascular responses to electrical vagal stimulation. It is difficult to explain the discrepant findings in different cholinergic nerves. It is possible that different neuronal fibres may have different types or numbers of opiate receptors. Thus, different nerve fibres may have dissimilar sensitivities to the chronic actions of the opiates. Further studies are required before a definitive conclusion can be reached.

Evidence has shown the existence of presynaptic and postsynaptic opiate receptors (Harder & Madden, 1984; Illes *et al.*, 1985; Berzetei *et al.*, 1988). It has also been suggested that opioid peptides are co-stored with noradrenaline in postganglionic sympathetic nerves (Lang *et al.*, 1983). However, the physiological role of endogenous opioids in the control of cardiovascular functions has not been fully elucidated, and various tissues from different or even the same species of experimental animals differ in response to opiates; this is probably because of the presence of different types of opioid receptors. The present investigation in rats shows that acute morphine administration did not significantly alter the blood pressure and pulse rate responses to i.v. injected nicotine, the ganglionic blocking effect of hexamethonium, or the responses of the isolated atria to field stimulation or noradrenaline. This may be due to the doses of morphine (which is generally regarded as a prototype μ-agonist) used in the present study not being sufficiently high to activate other opiate receptors such as the inhibitory presynaptic δ- and κ-receptors. In the nicotine tests, the responses of the intact animals may also be complicated by cardiovascular reflexes. Nevertheless, these findings agree with previous observations that acute morphine treatment in naive rats does not alter the cardiovascular responses to sympathetic nerve stimulation or to i.v. injected noradrenaline (Leung *et al.*, 1986a), nor does such treatment influence the elevation of plasma catecholamine level in response to sympathetic nerve stimulation (Leung *et al.*, 1989).

In summary, the findings of the present study suggest that functional impairment of the sympathetic nervous system following prolonged morphine administration involves mainly the preganglionic nerve fibres.

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An examination of the sources of calcium for contractions mediated by postjunctional α_1- and α_2-adrenoceptors in several blood vessels isolated from the rabbit

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1 The roles of intracellular and extracellular-derived Ca^{2+} in α-adrenoceptor-mediated contractions to noradrenaline (NA) have been investigated in several isolated blood vessels from the rabbit by examining responses in the presence of a modified Krebs-Henseleit saline with 2.5 mM Ca^{2+} and a Ca^{2+}-buffered saline with 0.1 μM free Ca^{2+}.

2 NA was tested in preparations of the abdominal aorta, distal saphenous artery, renal vein, lateral saphenous vein, plantaris vein and ear vein exposed to a Ca^{2+}-buffered saline with 0.1 μM [Ca^{2+}]. A concentration of NA which was maximally effective in modified Krebs-Henseleit saline, produced an initial transient contraction (ITC) followed by a relaxation towards baseline. This is evidence that α-adrenoceptor-mediated responses in all these blood vessels depend upon calcium from both sources.

3 The ITC was particularly pronounced in the arteries and was associated more closely with the α_1-receptor subtype. In the abdominal aorta, distal saphenous artery and renal vein the ITC can almost exclusively be attributed to an α_1-adrenoceptor (prazosin-sensitive, rauwolscine-resistant). In the ear vein, and to a lesser extent the plantaris vein, the ITC was mediated in part by an α_2-adrenoceptor (prazosin-resistant, rauwolscine-sensitive).

4 α_2-Adrenoceptors in the lateral saphenous vein largely account for the response to NA in modified Krebs-Henseleit saline, but α_1-adrenoceptors mediate the ITC in Ca^{2+}-buffered saline. After selective inactivation of α_1-adrenoceptors with a combination of phenoxybenzamine and rauwolscine, responses to NA in modified Krebs-Henseleit saline are slow in onset and there is no ITC in Ca^{2+}-buffered saline.

5 The possible significance of the coupling of postjunctional α_2-adrenoceptors to dual sources of Ca^{2+} is discussed in relation to the interaction between α-adrenoceptor subtypes and the ease of demonstrating functional α_2-adrenoceptors in isolated blood vessels.

Introduction

Postjunctional α_1- and α_2-adrenoceptors both mediate contraction of vascular smooth muscle (McGrath, 1982; Starke, 1987). Pressor responses mediated by each subtype exhibit differential sensitivity to calcium channel blockers. It has been suggested that α_1-adrenoceptors are coupled to both the release of cellular-bound calcium and the opening of calcium channels, while α_2-adrenoceptors are linked only to the latter (van Zwieten & Timmermans, 1987). There are, however, several reports not in accord with this view. For example, calcium entry blockers failed to exhibit any major differential sensitivity when examined against responses arising from the infusion of various selective agonists in the spinal dog (Morita *et al.*, 1985), pithed rat (O'Brien & McGrath, 1987) and conscious unrestrained rat (Lefevre-Borg *et al.*, 1988). In view of these conflicting observations and the difficulty in relating pressor responses to changes in peripheral resistance (Hiley & Thomas, 1987), we have now sought clarification by examining isolated blood vessels.

In contrast to the necessary reliance on drugs in *in vivo* experiments, the relative importance of extracellular Ca^{2+} for α_1-adrenoceptor-mediated responses has been assessed by the omission of Ca^{2+} and the addition of calcium-chelating agents. In general this has provided support for the view that contractions elicited via this subtype are linked to two sources of Ca^{2+} (van Breemen *et al.*, 1986; McGrath & Wilson, 1988). Thus, in low Ca^{2+} salines containing EGTA, noradrenaline (NA) produced transient contractile responses via α_1-adrenoceptors in the rabbit aorta, rat aorta and rat anococcygeus (Deth & van Breemen, 1974; McGrath *et al.*, 1984; McGrath 1985; Wilson *et al.*, 1987). In contrast, responses in the rat isolated portal vein were completely abolished by a similar procedure (Sigurdsson *et al.*, 1975). However, the difficulty associated with demonstrating functional α_2-adrenoceptors in isolated vascular smooth muscle (McGrath, 1982; Hieble *et al.*, 1986) has prevented a rigorous study of the Ca^{2+}-dependence of contractile responses mediated by this subtype. In two tissues examined to date, canine isolated saphenous vein (Jim & Matthews, 1985) and rat isolated femoral vein (Stubbs *et al.*, 1988), interventions known to prevent Ca^{2+} entry were found to abolish α_2-adrenoceptor-mediated contractile responses.

In the present study we have examined the ability of NA to elicit contractions in the presence of a low concentration of extracellular Ca^{2+} in several isolated blood vessels taken from the rabbit. These include the saphenous and plantaris veins (where the response mediated by α_2-adrenoceptors exceeds that produced by α_1-adrenoceptors), ear vein ($\alpha_2 \gg \alpha_1$), left renal vein and abdominal aorta (α_1) (see: Daly *et al.*, 1988a,b) and saphenous artery ($\alpha_1 > \alpha_2$; Dunn *et al.*, 1989). Our findings provide further support for the view that α_1-adrenoceptors are generally linked to two Ca^{2+} coupling mechanisms (release and entry), but we have identified two isolated veins in which postjunctional α_2-adrenoceptors appear to be similarly coupled. The possible relationship between postjunctional α_2-adrenoceptor-mediated release of cellular-bound Ca^{2+} and prazosin-sensitive, rauwolscine-sensitive contractile responses mediated by this receptor subtype in isolated blood vessels is also discussed.

Methods

White albino New Zealand rabbits of either sex weighing 2.3–3.0 kg were killed by stunning followed by exsanguination. Segments of the abdominal aorta, distal saphenous artery, the left renal vein, the ear vein, the lateral saphenous vein and the plantaris vein were cleaned of fat and connective tissue *in situ* and then placed in ice-cold modified Krebs-Henseleit physiological salt solution (Krebs-Henseleit saline, for composition

see below). The ear vein was defined as the 10 mm region either side of the first bifurcation of the vein running parallel to the ear artery. The plantaris vein was represented as the 15 mm distal segment of the continuation of the lateral saphenous vein measured from the ankle. The distal saphenous artery was taken as a 20 mm segment of the saphenous artery (medial side) between the knee and ankle. Segments 3 mm in length were taken from each vein (5 mm for the ear vein) or artery and suspended between two 0.2 mm thick stainless steel supports as previously described (Daly et al., 1988a). The upper support was connected by surgical silk to a Grass FT03 isometric transducer while the lower support was connected to a rigid glass tissue holder. Preparations were then mounted in 30 ml isolated organ baths under an initial resting tension of 4 g wt. (aorta), 1.5 g wt. (distal saphenous artery), 0.5 g wt. (renal vein), 0.3 g wt. (ear vein), 1.5 g wt. (lateral plantaris vein), 2 g wt. (lateral saphenous vein) and allowed to relax. Each segment was bathed in modified Krebs-Henseleit saline maintained at 37°C and gassed with 5% CO_2 in O_2. No attempt was made to remove the endothelium in any preparation.

In one group of experiments postjunctional α_2-adrenoceptors in the isolated lateral saphenous vein and the plantaris vein were isolated by the receptor protection procedure described previously (Daly et al., 1988b). Briefly, preparations were exposed to 1 μM rauwolscine 10 min before the addition of 0.3 μM phenoxybenzamine. Both antagonists were applied to the tissue for 30 min and the irreversible antagonist was then washed out by replacing the bathing solution twice with modified Krebs-Henseleit saline containing 1 μM rauwolscine only. Finally, 1 μM rauwolscine was removed by replacing the bathing solution 5 times over 60 min.

After a 90 min equilibration period, during which a steady resting tension was achieved, each preparation was exposed to 3 μM NA and allowed to contract for 10 min. After a complete washout, an additional one hour equilibration period was allowed before the experiment was commenced. This procedure was found to minimize changes in the sensitivity of the preparation to subsequent additions of the agonist. Basal tension after the sighting response remained stable for the rest of the experiment; 1.5–2.0 g wt. (aorta); 0.5–0.6 g wt. (distal saphenous artery); 0.15–0.2 g wt. (renal vein); 0.1–0.15 g wt. (ear vein); 0.2–0.3 g wt. (lateral plantaris vein, lateral saphenous vein). Isometric contractions were recorded by a Grass FT03 transducer connected to a Linseis 2065 pen recorder.

Preparations were then exposed to either 55 mM KCl (without equimolar replacement of NaCl) or NA and the responses allowed to proceed to equilibrium. The concentration of NA employed was approximately 30–100 fold greater than the EC_{50} determined from the equilibrium concentration-response curve (Daly et al., 1988a,b); 3 μM NA (aorta, distal saphenous artery, ear vein, saphenous vein and plantaris vein), 10 μM NA (post-phenoxybenzamine/rauwolscine plantaris vein) and 30 μM NA (renal vein and post-phenoxybenzamine/rauwolscine saphenous vein). In general high concentrations of NA are required for the release of cell-derived Ca^{2+} (see: McGrath et al., 1984; McGrath 1985; Wilson et al., 1987). The agonist was removed by repeated washing with modified Krebs-Henseleit saline. Preparations were then allowed to re-equilibrate for a further 60 min. The bathing solution was then replaced twice, separated by 1 min, with a modified Krebs-Henseleit saline incorporating 0.1 μm free Ca^{2+}, buffered by the combination of 0.5 mM Na_2EGTA and 0.25 mM $CaCl_2$ (0.1 μM Ca^{2+} buffered saline), and 9 min later the response to NA or KCl tested.

Three different protocols were then performed. (i) *Abdominal aorta, distal saphenous artery, saphenous vein and plantaris vein:* preparations were repeatedly washed with 0.1 μM Ca^{2+}-buffered saline over 40 min to effect complete removal of NA, *i.e.* the preparations were not re-exposed to 2.5 mM Ca^{2+}-modified Krebs-Henseleit saline. The effect of NA was then re-examined. (ii) *Abdominal aorta, distal saphenous artery, saphenous vein and plantaris vein:* preparations were then washed repeatedly with 2.5 mM Ca^{2+}-modified Krebs-Henseleit saline to remove NA and to allow 're-filling' of cellular Ca^{2+} stores. The effect of NA was re-examined 40 min later after a 10 min exposure to either 0.01 μM Ca^{2+}-buffered saline (0.025 mM $CaCl_2$ and 0.5 mM Na_2EGTA) or 1 μM Ca^{2+}-buffered saline (0.475 mM Ca^{2+} and 0.5 mM Na_2EGTA). (iii) *All preparations:* NA was again removed by repeated washing with modified Krebs-Henseleit saline and the preparations exposed to 0.1 μM prazosin, 1 μM rauwolscine or a combination of both antagonists for a minimum of 40 min. These concentrations of the antagonists have previously been shown to be selective for α_1-(prazosin-sensitive) and α_2-adrenoceptor (rauwolscine-sensitive) subtypes and are 30–100 fold greater than the antilog of the negative pA_2 value (Daly et al., 1988a,b). At the end of this period, the response to NA was tested in the 0.1 μM Ca^{2+}-buffered saline incorporating the appropriate concentration of the antagonist(s) using the previous cycle.

In experiments with 55 mM KCl, responses in modified Krebs-Henseleit saline and then 0.1 μM Ca^{2+}-buffered saline were evoked after the initial reference response to 3 μM NA in modified Krebs-Henseleit saline. Unless stated otherwise, the first response in 0.1 μM Ca^{2+}-buffered saline was expressed as a percentage of the maximum response to NA in modified Krebs-Henseleit saline (mean ± s.e.mean). Subsequent responses in 0.1 μM Ca^{2+}-buffered saline (with and without antagonists) were expressed as a percentage of the first response to the particular agonist in 0.1 μM Ca^{2+}-buffered saline. Differences between means were considered statistically different if $P < 0.05$ for either paired or unpaired observations (Student's t test). In all experiments one preparation of each blood vessel was not exposed to an antagonist and served as a time control.

The composition of the modified Krebs-Henseleit saline was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 . 7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA 23 μM was included in all experiments to prevent oxidative degradation of NA and 1 μM propranolol and 10 μM cocaine were also included to inhibit β-adrenoceptors and uptake$_1$, respectively. A 0.1 M stock solution of EGTA in 0.1 M NaOH, prepared fresh each week, was used to produce 0.5 mM Na_2EGTA in the Ca^{2+}-buffered saline. The final concentration of 'free' Ca^{2+} in each of the solutions was calculated by the method outlined by Miller & Smith (1984).

The following drugs were used: (−)-noradrenaline bitartrate (Sigma), prazosin HCl (Pfizer), rauwolscine HCl (Roth), phenoxybenzamine HCl (SK&F) (±)-propranolol HCl (Sigma) and cocaine HCl (Macarthys). With the exception of NA, which was dissolved in 23 μM Na_2EDTA, all drugs were dissolved in distilled water and added to the organ baths in 0.1 ml volumes.

Results

The effect of Ca^{2+}-buffered saline on responses to 55 mM KCl and NA

Contractile responses to the addition of 55 mM KCl to modified Krebs-Henseleit saline in three venous preparations were 85.5 ± 7.5% (lateral saphenous vein, $n = 5$), 70.6 ± 6.9% (plantaris vein, $n = 5$) and 63.4 ± 6.1%, (ear vein, $n = 4$) of the maximum response to NA. These responses to 55 mM KCl were abolished in 0.1 μM Ca^{2+}-buffered saline. Responses of the abdominal aorta (66.6 ± 4.1%, $n = 4$) and distal saphenous artery (89.7 ± 3.2%, $n = 5$) to 55 mM KCl in modified Krebs-Henseleit saline were markedly reduced to 4.2 ± 1.2% and 4.2 ± 1.3%, respectively, in 0.1 μM Ca^{2+}-buffered saline. In these two vessels the residual responses consisted of a slowly developing contraction.

Figure 1 shows representative trace recordings of responses in all vessels examined of the maximal responses to NA in modified Krebs-Henseleit saline and 0.1 μM Ca^{2+}-buffered saline. In arterial preparations an equilibrium response in modified Krebs-Henseleit saline was achieved 5–8 min after

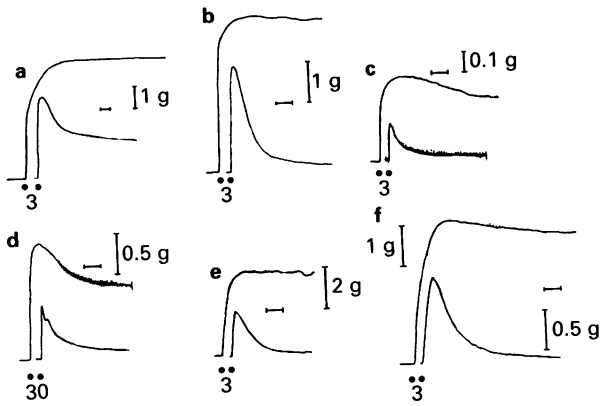


Figure 1 Representative trace recordings of (−)-noradrenaline-induced contractions in several isolated blood vessels from the rabbit. Responses were elicited in physiological salt solution (modified Krebs-Henseleit saline; upper responses) and then repeated after a 10 min exposure to a 'buffered' low ionized calcium (0.1 μM) physiological salt solution (Ca^{2+}-buffered saline; lower response). The blood vessels shown are as follows: (a) the abdominal aorta; (b) the distal saphenous artery; (c) the left renal vein; (d) the ear vein; (e) the lateral plantaris vein; (f) the lateral saphenous vein. Note the change in response calibration for (f) in the presence modified Krebs-Henseleit salinea and Ca^{2+}-buffered saline, and the change in time base for (a). Values indicating the concentration of noradrenaline employed in each preparation are in μM units. Calibrations represent g wt.

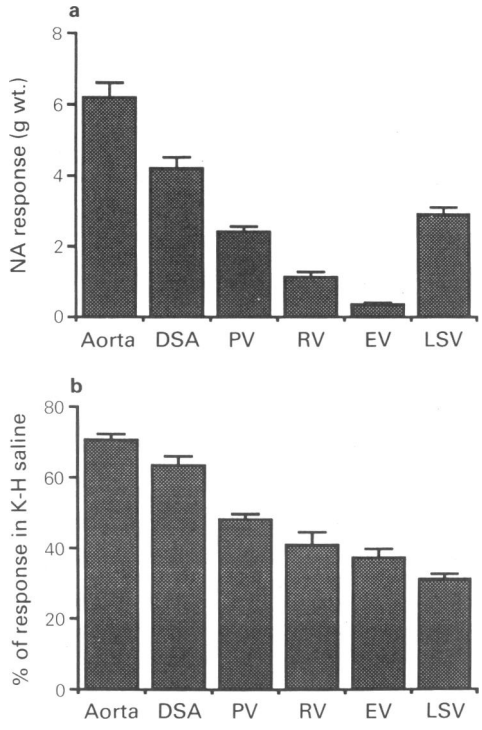


Figure 2 A comparison of the responses of several isolated blood vessels from the rabbit to a maximal concentration (30–100 fold log EC_{50}) of (−)-noradrenaline (NA) in (a) physiological salt solution (modified Krebs-Henseleit saline) and (b) 'buffered' low ionized calcium (0.1 μM) physiological salt solution (Ca-buffered saline). The blood vessels shown are as follows: the abdominal aorta (aorta); distal saphenous artery (DSA); plantaris vein (PV); left renal vein (RV); ear vein (EV); lateral saphenous vein (LSV). Responses in (a) are the maximum equilibrium response (g wt.) and in (b) the peak response of the initial transient contraction in Ca^{2+}-buffered saline (relative to maximum responses to NA in modified Krebs-Henseleit (KH) saline). Values shown are the mean of 5–10 observations from different animals and the vertical bars represent the s.e.mean.

addition of NA, while in the venous preparations the peak response was attained after 3–4 min, after which the contraction waned to 70–80% of the maximum. Responses to NA in all tissues were reduced in the presence of Ca^{2+}-buffered saline and consisted of an initial transient contraction (ITC), which peaked within 60 s of the initiation of the response, followed by relaxation to a residual response.

Both the equilibrium response (modified Krebs-Henseleit saline) and the ITC (Ca^{2+}-buffered saline) to NA in the two arterial preparations were significantly greater than those elicited in the venous preparations, but there was no obvious correlation between the magnitude of the responses in modified Krebs-Henseleit saline and the relative size of the ITC (Figure 2). For example, in the lateral saphenous vein NA produced the third largest equilibrium response (modified Krebs-Henseleit saline) but the smallest ITC. The ITC elicited by NA in Ca^{2+}-buffered saline varied from 30–70% of the maximum response in modified Krebs-Henseleit saline (Figure 2). In the ear vein, the ITCs to NA in 3 of 32 preparations (10 animals) were less than 10% of the maximum response in modified Krebs-Henseleit saline and were excluded from the analysis. Contractile responses of the aorta to NA were more resistant to Ca-buffered saline; time to peak of the ITC was 90–120 s and the residual response was 32.8 ± 3.9% ($n = 10$) of the maximum in modified Krebs-Henseleit saline. In all other

Table 1 The effect of Ca^{2+}-buffered saline on responses to 3 μM noradrenaline in four isolated vessels from the rabbit

		A *0.01 μM Ca^{2+} buffered saline*	B *0.1 μM Ca^{2+} buffered saline*	C *1 μM Ca^{2+} buffered saline*	D *0.1 μM Ca^{2+} buffered saline (no prior exposure to modified Krebs-Henseleit saline)*
Abdominal aorta	ITC	3.47 ± 0.34 *	3.3 ± 0.16 *	3.3 ± 0.39 *	—
	Residual response	1.2 ± 0.1 *	1.55 ± 0.19 *	1.83 ± 0.16 *	0.75 ± 0.17
Distal saphenous artery	ITC	3.3 ± 0.14 *	3.45 ± 0.21 *	3.9 ± 0.2 *	—
	Residual response	0.15 ± 0.05	0.25 ± 0.08	0.4 ± 0.1 *	0.16 ± 0.02
Plantaris vein	ITC	1.06 ± 0.06 *	1.05 ± 0.06 *	1.37 ± 0.21 *	—
	Residual response	0.05 ± 0.02	0.07 ± 0.03	0.3 ± 0.12 *	0.02 ± 0.02
Saphenous vein	ITC	0.61 ± 0.17 *	0.61 ± 0.12 *	0.81 ± 0.24 *	—
	Residual response	0.07 ± 0.02	0.11 ± 0.03	0.3 ± 0.19 *	0.07 ± 0.02

After 30 min in modified Krebs-Henseleit saline, preparations were exposed to the Ca-buffered saline for 10 min before the addition of noradrenaline (columns A, B and C). Noradrenaline was removed by repeated washing with modified Krebs-Henseleit saline. In some experiments (column D), removal of noradrenaline between consecutive responses was effected by repeated washing with 0.1 μM Ca^{2+} buffered saline, thereby ensuring that the calcium stores were not refilled before the next exposure to noradrenaline. The values (g wt.) shown are the mean ± s.e.mean of four observations. The initial transient contraction (ITC) is defined as the peak response of the contraction in Ca-buffered saline which attains a peak (usually within 40 s of agonist addition) followed by a relaxation (> 50%) back towards the baseline. The residual response is the contraction in Ca-buffered saline remaining after relaxation.

* Responses significantly different from column D (Student's paired t test $P < 0.05$).

preparations, the residual responses in Ca^{2+}-buffered saline were <5% of the maximum response in modified Krebs-Henseleit saline.

Table 1 shows the effect of Ca^{2+}-buffered saline with different $[Ca^{2+}]$ on responses to 3 μM NA in several blood vessels. In all preparations examined, the magnitude of the ITC was independent of the concentration of buffered $[Ca^{2+}]$ (0.01 μM–1 μM). However, an ITC to NA could not be repeated unless preparations were re-equilibrated in modified Krebs-Henseleit saline after exposure to Ca^{2+}-buffered saline and NA and removal of the agonist (column D).

The effects of single 'selective' concentrations of prazosin and rauwolscine on responses to NA in 0.1 μM Ca^{2+}-buffered saline

Figure 3 shows the effects of 0.1 μM prazosin, 1 μM rauwolscine and a combination of both antagonists on the ITC responses to NA in several isolated blood vessels in Ca^{2+}-buffered saline. In the aorta 0.1 μM prazosin practically abolished responses. In the renal vein and distal saphenous artery, prazosin reduced the ITC by more than 60% but the combination of 0.1 μM prazosin and 1 μM rauwolscine was no more effective than 1 μM prazosin alone (Figure 3b and c). In contrast to the aorta and renal vein, 1 μM rauwolscine produced a significant reduction in the ITC elicited by NA in the distal saphenous artery. Based upon the selectivity of these concentrations of the antagonists for α_1- and α_2-adrenoceptors (Daly *et al.*, 1988a,b), these observations indicate that the ITCs to NA in these preparations are mediated exclusively via α_1-adrenoceptors.

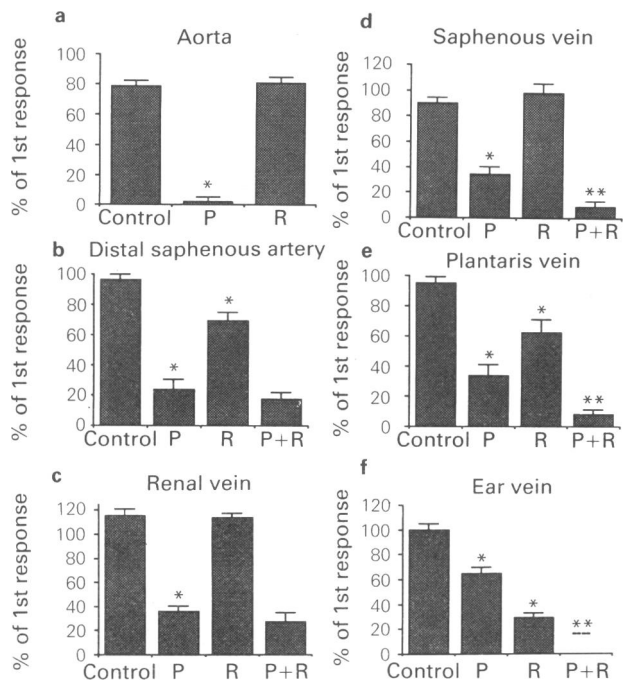


Figure 3 The effect of saline (Control), 0.1 μM prazosin (P), 1 μM rauwolscine (R) and both antagonists in combination (P + R) on the initial transient contraction to (−)-noradrenaline (NA) in several isolated blood vessels from the rabbit exposed to a 0.1 μM Ca^{2+}-buffered saline for 10 min. Responses shown are the mean of 4–6 observations and the vertical bars represents the s.e.mean. Comment: 0.1 μM prazosin produced a significant inhibition of responses to NA in all preparations, but 1 μM rauwolscine produced a significant inhibition only in the distal saphenous artery, ear vein and plantaris vein. * Indicates a significant reduction in the initial transient contraction compared to saline control. ** Indicates a significant difference ($P < 0.05$) between the effect of 0.1 μM prazosin alone and the effect of the combination of 0.1 μM prazosin and 1 μM rauwolscine. In the ear vein only 1 of 5 preparations responded to NA in the presence of both antagonists, the dashed lines indicate the limit of detection for a response in this preparation.

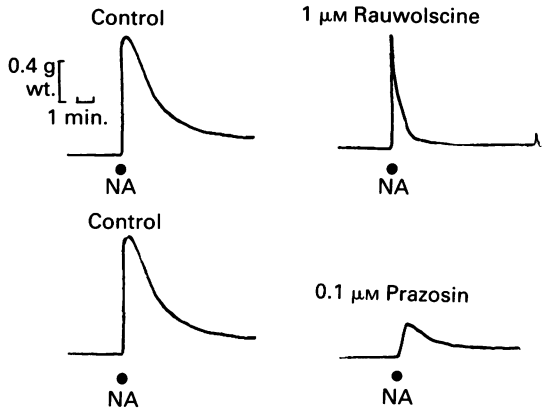


Figure 4 Representative trace recordings of the effects of 1 μM rauwolscine and 0.1 μM prazosin on the contraction to 3 μM noradrenaline (NA) in the lateral saphenous vein after a 10 min exposure to $[0.1\ \mu M\ Ca^{2+}]_{free}$ buffered physiological salt solution. It is noteworthy that although rauwolscine did not affect the size of the initial transient contraction it did reduce the time course of the response.

In the plantaris vein and lateral saphenous vein, 0.1 μM prazosin produced a 60% reduction in the ITC to NA. Rauwolscine 1 μM effected a significant inhibition of the peak response of the ITC to NA in the plantaris vein. In the lateral saphenous vein the peak height of the response was unchanged but the duration of the response was reduced (Figure 4). In both preparations, the combination of the antagonists produced a greater effect than did prazosin alone (Figure 3d and e). In the isolated ear vein, 1 μM rauwolscine produced a greater inhibition of the NA ITC than 0.1 μM prazosin, and the combination of both antagonists abolished responses in 4 out of 5 of preparations (Fig. 3f). Thus, although α_1-adrenoceptors participate in the ITC to NA in these three venous preparations, α_2-adrenoceptors play a dominant and subsidiary role, respectively, in the isolated ear vein and plantaris vein. In the lateral saphenous vein, the precise role of α_2-adrenoceptors in the ITC elicited by NA is unclear.

The effects of 0.1 μM Ca^{2+} buffered saline on isolated α_2-adrenoceptor-mediated responses in the lateral saphenous vein and plantaris vein

After exposure of the lateral saphenous vein and plantaris vein to NA and to 0.3 μM phenoxybenzamine/1 μM rauwolscine to inactivate the α_1-adrenoceptors, the maximum equilibrium responses of the vessels in modified Krebs-Henseleit saline were reduced to 65.3 ± 5.4% ($n = 7$) and 59.1 ± 4.3% ($n = 8$), respectively, of the maximum response in modified Krebs-Henseleit saline. In the lateral saphenous vein, a response to NA in the presence of 0.1 μM Ca^{2+}-buffered saline was observed in just 4 of 7 preparations. Furthermore, these were slow in onset without a well defined 'peak' and less than 10% of the maximum response (Figure 5). The effects of the antagonists were not examined. In contrast, in phenoxybenzamine/rauwolscine-treated preparations of the plantaris vein NA produced a small ITC (11.0 ± 1.2% ($n = 8$) of the response in modified Krebs-Henseleit saline). This response was abolished by 1 μM rauwolscine in 3 out of 4 preparations but was unaffected by 0.1 μM prazosin ($n = 4$).

Discussion

Contractile responses in vascular smooth muscle depend upon an elevation in intracellular $[Ca^{2+}]$ via (i) release of cellular-

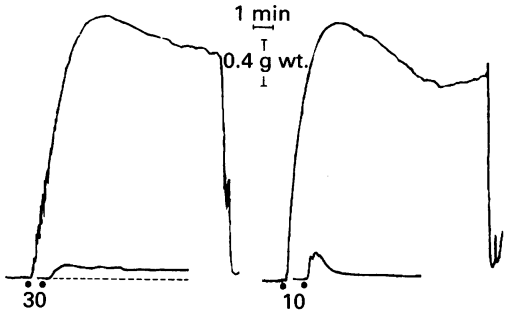


Figure 5 Representative trace recording of noradrenaline (NA)-induced contractions of (a) the lateral saphenous vein and (b) the plantaris vein after 60 min exposure to a combination of 1 μM rauwolscine (35 min) and 0.3 μM phenoxybenzamine (30 min). Responses were elicited in modified Krebs-Henseleit saline (upper responses) and then repeated after a 10 min exposure to 0.1 μM Ca^{2+}-buffered saline (lower response). The values indicating the concentration of noradrenaline employed in each preparation are in μM units. Note that compared with the control responses in Figures 3d and e, the responses in 0.1 μM Ca^{2+}-buffered saline are considerably reduced.

bound or sequestered Ca^{2+}, (ii) entry of Ca^{2+} via voltage-operated calcium channel (VOCs) or (iii) entry of Ca^{2+} via receptor-operated calcium channels (ROCs) (Bolton, 1979). The present study concentrates on the contribution of the release of intracellular Ca^{2+} to responses mediated by post-junctional α_1- and α_2-adrenoceptors in isolated vascular smooth muscle.

Three observations indicate that the ITC to NA in 0.1 μM Ca^{2+}-buffered saline is a consequence of the release of intracellular Ca^{2+} from a store. Firstly, 0.1 μM Ca^{2+}-buffered saline virtually abolished contractions to KCl in all preparations—indicating that VOCs require a higher concentration of 'free' Ca^{2+} to contribute to responses. Secondly, the magnitude of the ITC to NA in four preparations examined was independent of the buffered $[Ca^{2+}]$ in the bathing medium from 1 μM down to 0.01 μM. Thirdly, the ITC to NA in Ca-buffered saline could not be repeated unless the preparation was re-exposed to modified Krebs-Henseleit saline (2.5 mM Ca^{2+}) between successive responses. Furthermore, the choice of 0.1 μM for the $[Ca^{2+}]_{free}$ of the Ca^{2+}-buffered saline was made with the aim of reducing to a minimum the magnitude of the outwardly-directed diffusion gradient for Ca^{2+}, thereby preserving the integrity of the intracellular Ca^{2+} stores. It should be noted, however, that confirmation that an ITC is associated with the liberation of cellular-bound Ca^{2+} requires demonstration of an increase in intracellular $[Ca^{2+}]$ such as increased $^{45}Ca^{2+}$ efflux (e.g. Chui *et al.*, 1986), or direct measurement of changes in intracellular $[Ca^{2+}]_{free}$ with indicators such as fluorescent Ca^{2+}-sensitive dyes (e.g. Reynolds & Dubyak, 1985).

The ability of Ca^{2+}-buffered saline to reduce the maintained contraction to NA in all preparations (with the exception of the aorta) to less than 5% of the modified Krebs-Henseleit saline maximum indicates that responses mediated by both α_1- and α_2-adrenoceptors are highly dependent upon the presence of extracellular Ca^{2+} (>1 μM) to support contractions. Although the relative contributions of VOCs and ROCs to these responses were not examined in the present study, preliminary experiments with 1 μM nifedipine in the lateral saphenous vein revealed that responses to KCl were abolished but those to NA were relatively unaffected (unpublished observations). Similarly, Schümann & Lues (1983) have shown that nitrendipine failed to reduce responses to phenylephrine in the lateral saphenous vein. These observations also highlight the danger inherent in making assumptions about receptor-response coupling based solely upon the effect of Ca^{2+}-entry blockers, which is necessitated *in vivo* (see van Zweiten & Timmermans, 1987). Clearly, responses to NA require extracellular free Ca^{2+}, which presumably moves into the cells, but not via dihydropyridine-sensitive channels: this is similar to the non-vascular smooth muscle in the rat anococcygeus (McGrath, 1985).

The relatively large residual response of the aorta to NA in the presence of Ca^{2+}-buffered saline is similar to that described by Hester (1988) who employed 'zero' calcium, EGTA and the calcium-entry blocker D-600. This indicates that the relative contribution of cell-derived Ca^{2+} to α-adrenoceptor-mediated contractions in the aorta is considerably greater than that for most other blood vessels, and is in accord with data suggesting an inverse relationship between vessel calibre and the significance of extracellular Ca^{2+} in these responses (Cauvin *et al.*, 1985; Owen *et al.*, 1987).

Initial transient contractions, release of cellular-bound Ca^{2+} and postjunctional α_1- and α_2-adrenoceptors

The ITC in each of the different vessels was between 30 and 70% of the maximum response in modified Krebs-Henseleit saline but was consistent within each. Thus, the relative contribution of cell-derived Ca^{2+} to contractile responses varies considerably between anatomically distinct vessels. In general, it is greater in arteries than in veins. It should be emphasized that a comparison based upon the height of the ITC alone probably underestimates the differences between preparations; no attempt has been made to compare the area under the ITC response (cf. measurement of $^{45}Ca^{2+}$ efflux from pre-labelled preparations. Chui *et al.*, 1986).

As judged by the effect of 0.1 μM prazosin on ITC responses to NA, α_1-adrenoceptors contributed to the release of intracellular Ca^{2+} in all preparations even when the α_2-adrenoceptor was the dominant subtype responsible for the equilibrium response (Table 2). In the abdominal aorta, the ITC response was practically abolished by 0.1 μM prazosin, while 1 μM rauwolscine was without effect. This is similar to observations in the renal vein, where the combination of both antagonists produced no greater inhibition of the ITC than that observed with prazosin alone. Since α_1-adrenoceptors mediate the equilibrium response to NA in these two preparations (Daly *et al.*, 1988a), the failure of 1 μM rauwolscine to reduce responses is further evidence that this concentration of the antagonist is devoid of activity at α_1-adrenoceptors. In addition, the pA_2 value for rauwolscine at postjunctional α_1-adrenoceptors in both the rabbit isolated pulmonary artery and ear artery has been found to be less than 6 (Weitzell *et al.*, 1979; Hieble & Woodward, 1984). It should be noted, however, that the

Table 2 A comparison of the relative roles of α-adrenoceptor subtypes in the equilibrium response (modified Krebs-Henseleit saline) and the initial transient contraction (Ca^{2+}-buffered saline) to noradrenaline (NA) in several isolated blood vessels from the rabbit.

Preparation	*Equilibrium response (modified Krebs-Henseleit saline)**	*ITC (Ca^{2+}-buffered saline)*
Abdominal aorta	α_1	α_1
Left renal vein	α_1	α_1
Distal saphenous artery	α_1**	α_1
Lateral saphenous vein	$\alpha_2 > \alpha_1$	$\alpha_1 \gg \alpha_2$
Lateral saphenous vein (SV.RP)	α_2	[NP]
Plantaris vein	$\alpha_2 > \alpha_1$	$\alpha_1 > \alpha_2$
Plantaris vein (PV.RP)	α_2	α_2
Ear vein	$\alpha_2 > \alpha_1$	$\alpha_2 > \alpha_1$

* Data taken from Daly *et al.* (1988a,b,c).
** In the absence of angiotensin II all responses are prazosin-sensitive and the rank order of potency of agonists is NA > phenylephrine > UK-14304. α_2-Adrenoceptors are apparent only in the presence of angiotensin II (Dunn *et al.*, 1989). NP – not possible.

ability of 1 μM rauwolscine to inhibit the ITC to NA in the distal saphenous artery, without significantly enhancing the effect of prazosin, indicates that in this blood vessel the situation is more complex (see: Dunn *et al.*, 1989).

Even in the isolated ear vein, for which there is little evidence for postjunctional α_1-adrenoceptors making a significant contribution to the equilibrium response to NA, 0.1 μM prazosin produced a significant inhibition of the NA ITC (Table 2). Similarly in the lateral plantaris vein and the saphenous vein, despite the apparently greater contribution of postjunctional α_2-adrenoceptors to the equilibrium response (Daly *et al.*, 1988a,b), prazosin-sensitive (and thus α_1) adrenoceptors are chiefly responsible for the ITC to NA (Table 2). These observations agree with the well documented associations between postjunctional α_1-adrenoceptors, liberation of cellular-bound Ca^{2+} and inositol phosphate metabolism (Reynolds & Dubyak, 1985; Abdel-Latif, 1986).

However, the novel finding of this study is that postjunctional α_2-adrenoceptors can mediate ITCs to NA in vascular smooth muscle. In both the ear vein and lateral plantaris vein, the combination of both antagonists produced greater inhibition of the ITC to NA than observed with 0.1 μM prazosin alone (see Figure 3). Indeed, in the ear vein 1 μM rauwolscine produced greater inhibition of the ITC than did 0.1 μM prazosin. Further support for α_2-adrenoceptor-mediated release of cell-derived Ca^{2+} is provided by prazosin-resistant, rauwolscine-sensitive ITCs to NA in preparations of the plantaris vein previously exposed to 1 μM rauwolscine/0.3 μM phenoxybenzamine to effect functional inactivation of the α_1-adrenoceptors.

These observations contrast sharply with those obtained in other isolated veins and some in the pithed rat. In the canine saphenous vein (Jim & Matthews, 1985), the rat femoral vein (Stubbs *et al.*, 1988) and the cat middle cerebral artery (Skärby, 1984), the use of either the inorganic calcium channel blocker, La^{3+}, or exposure to Ca^{2+}-free modified Krebs-Henseleit solution (including EGTA) resulted in abolition of α_2-adrenoceptor-mediated contractions. Moreover, in the present study an ITC to NA was not observed in 3 of 7 preparations of the saphenous vein following irreversible removal of α_1-adrenoceptors. Nevertheless, it is clear that like α_1-adrenoceptors, the α_2-subtype in some preparations can be coupled to two transduction mechanisms (Ca^{2+} release and entry). It would, therefore, be fruitless to base any therapeutic or experimental strategy on an exclusive correlation between α_2-adrenoceptor activation and Ca^{2+} entry to cells.

Of the two putative second messenger systems linked to activation of postjunctional α_2-adrenoceptors, inhibition of adenylate cyclase activity (Fain & Garcia-Sainz, 1980) and acceleration of Na^+/H^+ exchange (Isom *et al.*, 1987), only the former could conceivably directly regulate the content of cellular-bound Ca^{2+} stores (see: Meisheri & van Breemen, 1982). Since there are no major differences in the time course of α_1- and α_2-adrenoceptor-mediated ITCs (compare ear vein and renal vein; Figure 1), the change in [cyclic AMP] (produced by a reduction in adenylate cyclase activity: α_2) would have to proceed at a rate similar to that of the rise of inositol triphosphate production (α_1). An alternative possibility is that α_2-adrenoceptor-mediated release of cellular-bound Ca^{2+} is the product of a novel transduction process or inositol triphosphate.

Demonstration of postjunctional α_2-adrenoceptors in vitro *and possible relationship to calcium-coupling mechanisms*

Negative coupling to a physiological process is a characteristic feature of many α_2-adrenoceptors, e.g., neurotransmitter release (Starke, 1987), insulin secretion (Morantes *et al.*, 1988), lipolysis in adipocytes (Berlan *et al.*, 1982), growth hormone secretion or calcitonin secretion (Brown *et al.*, 1985). Thus, demonstration of the functional consequence of α_2-adrenoceptor stimulation requires either basal or drug-induced elevation of a positively-coupled response. This is analogous to the conditions required for demonstrating β-adrenoceptor-mediated relaxation of smooth muscle, i.e. a pre-existing contractile response. In contrast, α_2-adrenoceptors linked to contraction of smooth muscle and the aggregation of platelets are examples of positively-coupled processes. A comparison of these two functional responses provides further insights into the difficulties associated with demonstrating prazosin-resistant, rauwolscine-sensitive smooth muscle contractions *in vitro*.

In rat and rabbit platelets, demonstration of a pro-aggregatory response to α_2-adrenoceptor stimulation requires the presence of a submaximal concentration of an aggregatory agent, e.g. thrombin or ADP-α-S (Kerry *et al.*, 1984). Although both processes are of the same functional polarity, this is analogous to the negatively-coupled α_2-adrenoceptors described above. However, in human platelets adrenaline can elicit a prazosin-resistant aggregatory response in the absence of a secondary agent (Kerry *et al.*, 1984; Clare *et al.*, 1984). Since the densities of α_2-adrenoceptors in human and rabbit platelets are similar (Kerry *et al.*, 1984), the observed functional differences can only be attributed to qualitative differences in the stimulus coupling mechanism for aggregation in these two species. This could be explained as follows. α_2-Adrenoceptors in human platelets are linked to two activation processes, one of which is responsible for initiation of the aggregatory response, while α_2-adrenoceptors on rabbit platelets are linked to a process dependent upon pre-existing aggregatory response. Thus, although α_1-adrenoceptors are not present on rabbit platelets (Grant & Scrutton, 1980), if they were present and able to initiate aggregation (e.g. like thrombin), it would be predicted that α_2-adrenoceptor mediated pro-aggregatory responses would appear be sensitive to both rauwolscine and prazosin.

We have noted that the α-adrenoceptor population in the isolated lateral saphenous vein is unique among veins from the rabbit (Daly *et al.*, 1988a,b,c). In spite of the overwhelming evidence for postjunctional α_2-adrenoceptors (agonist potency; UK-14304 > NA > phenylephrine, and antagonist potency; rauwolscine > corynanthine), responses to NA and UK-14304 were sensitive to low concentrations of prazosin (Daly *et al.*, 1988c) with no evidence for the 'resistant' component observed *in vivo* (McGrath, 1982). In contrast, α-adrenoceptors in the isolated ear vein and the isolated lateral plantaris vein possess the same agonist and antagonist profile yet contractile responses to NA are resistant to prazosin (Daly *et al.*, 1988a). This basic difference in the α-adrenoceptor-mediated equilibrium contractile response between these preparations is also apparent for the ITC to NA (Table 2). In particular, although rauwolscine reduced the duration of the ITC to NA in the saphenous vein (further evidence for an interaction between subtypes?) no part of this response could be described as resistant to prazosin.

Assuming that α_2-adrenoceptors are not efficiently coupled to the process responsible for the ITC, the greater facility of α_1-adrenoceptors for eliciting an ITC response will result in potentiation of α_2-adrenoceptor-mediated equilibrium responses, thereby rendering them 'sensitive' to prazosin. However, this does not exclude the possibility that in the absence of α_1-adrenoceptors, α_2-adrenoceptors are still able to elicit a contractile response (e.g. saphenous vein after removal of α_1-adrenoceptors). Thus, prazosin-resistant α-adrenoceptor-mediated responses in the ear vein and plantaris vein could arise from either a differential distribution of the two subtypes within the blood vessel wall, or from an α_2-adrenoceptor-mediated ITC. For the preparations examined in the present study, the latter possibility is supported by the observations that in the saphenous vein and distal saphenous artery, preparations for which there is no evidence for an appreciable α_2-adrenoceptor-mediated ITC (see Table 2), α_2-adrenoceptor-mediated equilibrium responses are facilitated and uncovered, respectively, by the presence of angiotensin II (Schümann & Lues, 1983; Daly *et al.*, 1988d; Dunn *et al.*, 1989). Interestingly, angiotensin II produced little facili-

tation of α_2-adrenoceptor-mediated responses to NA in the ear vein (unpublished observations). In the context of an interaction between α_2-adrenoceptors and other receptor systems, it is noteworthy that angiotensin II (like α_1-adrenoceptors) has long been associated with activation of the phosphatidylinositol cycle (Adbel-Latif, 1986).

In conclusion, we have presented evidence that both α_1- and α_2-adrenoceptors that mediate contraction in vascular smooth muscle can utilise Ca^{2+} from cellular and extracellular sources to support contraction. In general, the role for cellular-bound Ca^{2+} in α-adrenoceptor-mediated responses is greater for the α_1-subtype and more pronounced in arteries. However, for the α_2-subtype there appears to be a relationship between the release of intracellular Ca^{2+} and the ease of demonstrating this subtype in isolated veins.

Note added in proof

Michel *et al.* (1989) have recently demonstrated that α_2-adrenoceptors on a human erythroleukaemia cell line can mobilise intracellular calcium, an effect apparently not linked to inhibition of adenylate cyclase, Na^+/H^+ exchange or PI turnover.

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Caffeine acting on pregnant rat myometrium: analysis of its relaxant action and its failure to release Ca^{2+} from intracellular stores

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1 The effect of caffeine on mechanical activity was studied in pregnant rat myometrium.

2 In muscle cells with intact plasmalemmae, caffeine (0.1–50 mM) produced no contraction whatever the experimental conditions.

3 Caffeine (0.1–10 mM) inhibited, in a concentration-dependent manner, contractions induced by electrical stimulation, potassium-rich (60 mM K^+) solution, sodium-free solution or oxytocin (22.5 nM).

4 In Ca^{2+}-free solution, various substances (oxytocin, sodium orthovanadate and prostaglandin E_2) evoked sustained contractions that were suppressed by caffeine (5–10 mM). When caffeine (>5 mM) was applied during Ca^{2+}-loading of the tissue (2.1 mM Ca^{2+}, 5 min) in the presence of a K^+-rich solution, the subsequent transient contraction induced by a short application (10 s) of oxytocin (22.5 nM) in Ca-free solution was reduced (63 ± 3.5% reduction for 20 mM caffeine, $n = 4$).

5 In saponin-skinned strips, application of caffeine (5–10 mM) during loading of the Ca^{2+}-store increased the subsequent contraction induced by myo-inositol 1,4,5 trisphosphate (IP_3, 10 μM). Caffeine (10–30 mM) decreased calcium-activated contractions in skinned fibres lacking a functional internal Ca-store. This effect was reduced by the cyclic AMP-dependent protein kinase inhibitor Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp (8 μM).

6 In conclusion, it is suggested that the inability of caffeine to cause spasm of rat myometrium is due to the absence of a caffeine-sensitive calcium-release channel in the sarcoplasmic reticulum. Relaxant effects of caffeine can be explained by mechanisms leading to a decrease in both the cytoplasmic free Ca^{2+} concentration and the Ca^{2+}-sensitivity of the contractile machinery.

Introduction

In a large variety of muscular tissues (heart: Fabiato & Fabiato, 1977; skeletal muscle: Endo, 1977; vascular muscle: Itoh *et al.*, 1981) caffeine produces contractions by releasing calcium ions from the sarcoplasmic reticulum (SR).

In smooth muscle, caffeine does not always induce a contraction and its effect appears to be dependent on experimental conditions (Bolton, 1979; Saida, 1982). For example, caffeine inhibits contractions induced by structurally-specific agonists and KCl in pulmonary artery (Ito *et al.*, 1977), aorta (Leijten & Van Breemen, 1984) and intestinal muscle (Nasu *et al.*, 1975; Ahn *et al.*, 1988). In uterine smooth muscle, caffeine fails to evoke a contraction (Ashoori *et al.*, 1985) although the existence of an internal calcium-store that is sensitive to several substances has clearly been demonstrated both in intact (Mironneau *et al.*, 1984) and in skinned strips (Savineau *et al.*, 1988). In some smooth muscle cells (e.g., rabbit aorta: Leijten & Van Breemen, 1984) caffeine has two opposing effects on mechanical activity: a spasmogenic effect that involves interaction with the sarcoplasmic reticulum, and a relaxing effect. The relative importance of these two effects may depend on the type of smooth muscle. The nature of the relaxing effect remains unclear and has been attributed to several mechanisms including an inhibition of calcium influx, an increase in calcium-extrusion and in calcium-storage in the sarcoplasmic reticulum (SR) and a decrease in calcium sensitivity of the contractile machinery (Leijten & Van Breemen 1984; Ahn *et al.*, 1988). Some of these mechanisms could stem from an increase in the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content of the cells following inhibition of phosphodiesterases (Polson *et al.*, 1982).

The aim of the present study was to investigate the effect of caffeine on the mechanical activity of pregnant rat myometrium with regard both to its relaxing action and to its possible spasmogenic activity. Experiments were performed on both intact and saponin skinned strips.

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Methods

Preparation

Most of the experiments were performed on rat myometrium obtained at the end of pregnancy (16–18 days). Thin bundles (50–80 μm in diameter, 2–3 mm in length) were isolated from the longitudinal layer, and were free of both endometrium and circular muscle. Experiments were also performed on strips (150–200 μm in diameter, 3–5 mm in length) isolated from the longitudinal muscle of the portal vein and the circular muscle of the colon from the same pregnant rat.

Mechanical recordings

Mechanical activity was recorded in an experimental chamber (Mironneau *et al.*, 1980) by means of a highly sensitive isometric force transducer (Akers 801 AME, Horten, Norway).

Solutions for experiments with unskinned muscle

Physiological solutions had the following composition: (a) reference solution (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.1, $MgCl_2$ 0.5 and glucose 11. The solution was gassed with O_2, buffered with Tris-HCl (8.3 mM) at pH 7.4 and maintained at 35°C. (b) K^+-rich solution was prepared by substituting equimolar amounts of KCl for NaCl. (c) Na^+-free solution was prepared by substituting 130 mM choline chloride (in the presence of 0.1 mM atropine) for 130 mM NaCl. (d) Ca^{2+}-free solution was

prepared by omitting $CaCl_2$ and adding EGTA (0.2 mM) or lanthanum ions (0.1 mM).

Concentration-response curves for caffeine in tissue induced to contract by several agents.

Several stimuli, such as electrical stimulation, potassium chloride, sodium-free solution and oxytocin, were used to contract myometrium in Ca^{2+}-containing solution.

Twitch contractions were elicited by single electrical pulses (50 ms, 4–5 V, 0.033 Hz) delivered through platinum electrodes located on each side of the chamber. Control twitches of constant amplitude were recorded for at least 15 min before the addition of caffeine.

Application of K^+-rich (60 mM K^+) solution for 30 min induced a phasic contraction followed by a sustained plateau (tonic component) which was maintained without significant decrease in amplitude ($2.1 \pm 0.5\%$ reduction after 30 min, $n = 5$, $P > 0.05$). Application of sodium-free solution for 30 min induced a maximal contraction of the strip (Savineau *et al.*, 1987) which was maintained without significant variation in amplitude ($1.8 \pm 0.6\%$ increase after 30 min, $n = 6$, $P > 0.05$). At the beginning of the experiment, K^+-rich solution or sodium-free solution was repeatedly applied in order to obtain at least two contractions which were similar in amplitude and time-course.

Concentrations of caffeine were added in a cumulative manner during electrical stimulation or tonic component of either potassium-induced contraction or sodium-removal-induced contraction. A concentration increment was made once the maximum inhibitory effect of the preceding concentration had been recorded (usually 3–6 min).

The action of caffeine against the contractile response induced by oxytocin (22.5 nM) was studied by measuring the change in the amplitude of contraction at the end of the first minute of a short application (2 min) of oxytocin. This amplitude, which was checked to be constant upon repeated application at the beginning of the experiment, was used as a control value. Each concentration of caffeine was applied for 10 min before its effect on the oxytocin-induced contraction was examined. This experimental procedure was chosen because prolonged application of oxytocin induces a maximal contraction of the strip that is not maintained, and followed by an oscillatory activity after 3–5 min. Thus, it was not possible to examine the effect of caffeine on a plateau response.

Drug-induced contractions in Ca^{2+}-free solution

In a Ca^{2+}-free EGTA-containing solution, brief applications (10–20 s) of a supramaximal concentration of acetylcholine (ACh) or oxytocin (Oxy) evoke only one transient contraction from rat myometrium. The response reappears after the preparation has been bathed with a Ca^{2+}-containing solution (calcium-loading) (Lalanne *et al.*, 1984). The effect of caffeine on the transient drug-induced contraction was studied. Caffeine was added during calcium loading in polarized (5.6 mM K^+) as well as depolarized (60 mM K^+) tissue. The objective of these experiments was to study the effects of caffeine on the filling of the calcium-store through the leak pathway or through voltage-dependent channels, respectively (Figure 2).

For longer applications (> 2 min) of acetylcholine or oxytocin, the transient contraction is followed by a maintained component. When the same concentration of drug is applied at regular intervals in Ca^{2+}-free solution, the maintained component only is observed. Repeated applications of sodium orthovanadate and prostaglandin E_2 also induce maintained contractions that are reproducible for exposure times to Ca^{2+}-free solution as long as 1 h (Mironneau *et al.*, 1984). The effect of caffeine on these maintained contractions was studied by adding caffeine during the plateau of these responses (Figure 3).

Skinned strips

Skinned muscle preparations were obtained by use of saponin (50 $\mu g\,ml^{-1}$) according to the method of Savineau *et al.* (1988). After the skinning procedure, strips were incubated for 10 min in the relaxing solution in the presence or absence of caffeine. Contracting solutions were then applied in a cumulative manner in order to construct the pCa-tension curve. In some experiments Brij 58 (2%) a detergent known to destroy the sarcoplasmic reticulum (Orentlicher *et al.*, 1974) was added to the relaxing solution.

For studying the effect of caffeine on the intracellular store, the EGTA-concentration was reduced from 4 to 0.1 mM, and potent inhibitors of mitochondrial function such as sodium azide (5 mM) and sodium cyanide (5 mM) were added.

Chemicals

Chemicals used in the present work were: acetylcholine chloride (ACh), adenosine 5'-triphosphate (ATP), adenosine 3'-5' monophosphate (cyclic AMP), atropine, ethyleneglycol bis-(β-aminoethylether)N,N,N',N' tetraacetic acid (EGTA), 3-isobutyl 1-methylxanthine (IBMX), isoprenaline, D-myo-inositol 1,4,5 trisphosphate (IP_3), oxytocin, polyoxyethylene ether (Brij 58), propranolol, prostaglandin E_2 (PGE_2), sodium orthovanadate, cyclic AMP-dependent protein kinase inhibitor (PKA-inhibitor) (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp.) all from Sigma (St. Louis, MO, U.S.A.); saponin from ICN (Cleveland, OH, U.S.A.); caffeine from Merk (Darmstadt, F.R.G.), theophylline from Prolabo (Paris, France) and phentolamine from Ciba-Geigi (Paris, France).

Analysis of results

Results are expressed as the mean ± s.e.mean for n number of experiments. Significance was assessed by means of unpaired Student's t test. A difference between means was considered significant when $P < 0.05$.

Results

Effects of caffeine in unskinned smooth muscle

Effects of caffeine both in different preparations and in various experimental conditions In pregnant rat uterine smooth muscle, caffeine (0.1–50 mM) did not induce a contraction either in the presence or in the absence of external calcium ions (20 strips). The same caffeine solutions contracted other smooth muscles dissected from the same pregnant rat (e.g. portal vein—7 strips, and colon—5 strips) either in the presence or in the absence of calcium ions in the bathing solution.

It has been shown in guinea-pig trachealis that caffeine exhibits a spasmogenic action only when the temperature of the bathing solution is lowered below 22°C (Small *et al.*, 1988). However, on cooling rat myometrium, even to 4°C, no contraction was evoked by caffeine (0.1–50 mM) (8 strips). In guinea-pig ventricular muscle it has been shown that caffeine contraction is maximal in the absence of external sodium ions (Kitazawa, 1988). However, in rat uterus, removal of sodium ions itself induces a maximal contraction when external calcium ions are present (Savineau *et al.*, 1987). Thus caffeine (0.1–50 mM) was applied in solutions free of both sodium and calcium: again, no contraction was observed (5 strips).

Effect of caffeine in uterus induced to contract by several agents Caffeine (0.05–10 mM) reduced, in a concentration-dependent manner, the amplitude of uterine contractions elicited by electrical stimulation (twitches— Figure 1a), K^+-rich solution (60 mM K^+, tonic spasm—Figure 1b), sodium-free sol-

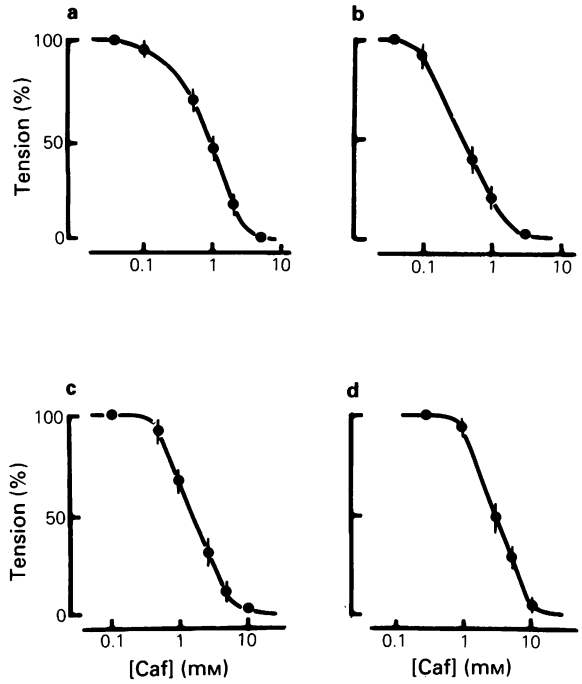


Figure 1 Effect of caffeine on the mechanical activity of pregnant rat myometrium. Concentration-response curves for the effect of caffeine (Caf) on twitches (a), and on contractions induced by K^+-rich (60 mM K^+) solution (tonic spasm) (b), sodium-free solution (c) and oxytocin (22.5 nM) (d). Abscissae: concentration (mM) of caffeine on a log scale. Ordinates: tension as a percentage of the initial value. In each case, the response obtained in the absence of caffeine was considered as 100%. Data points are mean, $n = 5$. Vertical bars show s.e. mean.

ution (Figure 1c) and oxytocin (Figure 1d). The concentrations of caffeine required to produce 50% inhibition of these contractions were (mM): 0.9 ± 0.08, 0.35 ± 0.06, 1.7 ± 0.3 and 3 ± 0.2 ($n = 5$), respectively. The effect of caffeine was unaffected by the presence of propranolol (3 μM) or phentolamine (3 μM).

Effect of caffeine on the transient uterine contraction induced by drugs in Ca^{2+}-free solutions A brief (10 s) application of oxytocin (22.5 nM) induced a transient contraction in Ca^{2+}-free solution (Figure 2Aa). This contraction was increased by $370 \pm 20\%$, $n = 4$, (Figure 2Ab) when K^+-rich (60 mM K^+) solution was applied during the Ca^2-loading period (5 min, 2.1 mM Ca^{2+}), demonstrating that a large amount of calcium ions had been accumulated in the store, as a consequence of activation of voltage-dependent calcium influx. The action of caffeine on activated-calcium influx could thus be studied by comparing the amplitude of the oxytocin response after the store in depolarized tissue had been filled with (test condition) or without (control condition) caffeine. We did check, in preliminary experiments, that in the control condition (i.e. after a calcium loading in the presence of 60 mM K^+) oxytocin induced a consistent contraction in Ca-free solution upon repeated loading protocols (data not shown). Figure 2B shows that 5 mM caffeine had no effect on the oxytocin-induced transient contraction in the latter experimental conditions (i.e. added in K^+-rich solution). However, it should be noted that the potassium contracture in the presence of 2.1 mM Ca^{2+} (calcium loading) was virtually abolished (4 strips). Moreover, higher concentrations of caffeine (10 and 20 mM) diminished the oxytocin-induced contraction by $15.3 \pm 0.6\%$ and $63 \pm 3.5\%$ ($n = 4$, $P < 0.05$) respectively (see for example Figure 2C),

In another set of experiments, it was verified that, when the calcium loading was performed in the reference solution (5.6 mM K^+), caffeine (10–20 mM) had no significant effect on the amplitude of the subsequent contraction induced by oxytocin or acetylcholine in Ca-free solution ($-2.3 \pm 0.4\%$ and $+1.2 \pm 0.2\%$ respectively, $n = 4$, $P > 0.05$).

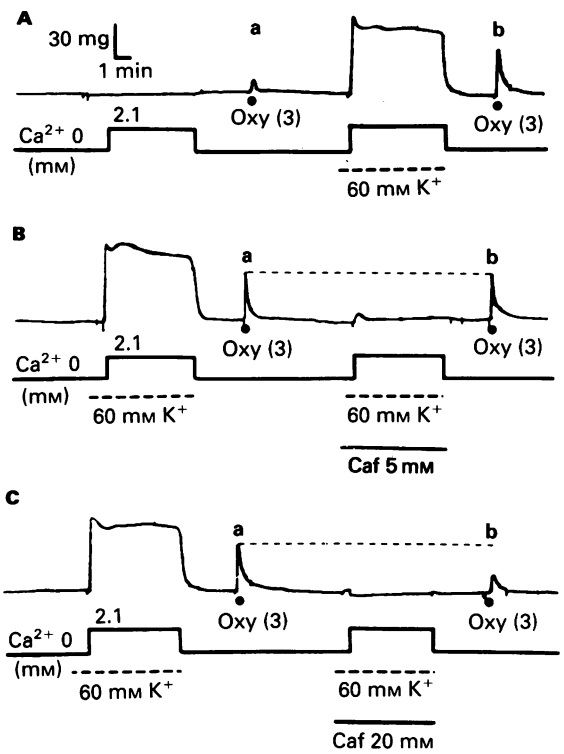


Figure 2 Effect of caffeine (Caf) on the transient uterine contraction induced by a short application (10 s) of 22.5 nM oxytocin (Oxy) in a Ca^{2+}-free EGTA-containing solution. (A) When the Ca-loading (2.1 mM Ca^{2+} for 5 min) was performed in K^+-rich (60 mM K^+) solution, the oxytocin-induced contraction measured after 3 min of perfusion in Ca^{2+}-free solution (b) was increased compared with that obtained when the Ca-loading was performed in reference solution (5.6 mM K^+) (a). In the following experiments, the Ca-loading was always performed in the presence of 60 mM K^+ (control condition). (B) When 5 mM caffeine was added during the Ca-loading of the depolarized tissue, the oxytocin-induced contraction in Ca^{2+}-free solution was not modified (compare Ba and Bb), whereas (C) 20 mM caffeine reduced the oxytocin-induced contraction, (compare Ca and Cb). Figures in parentheses indicate times in Ca-free solution (min).

Effect of caffeine on maintained and repeated contractions induced by drugs in Ca^{2+}-free solutions Figure 3 shows an example of maintained uterine contractions induced by oxytocin (22.5 nM), sodium orthovanadate (0.25 mM) or prostaglandin E_2 (PGE_2, 1 μM) applied for a long period (> 2 min) at different times of perfusion in a Ca-free medium. The amplitudes of these contractions were 2.9 ± 0.2, 13 ± 0.6 and 5.3 ± 0.3 mg ($n = 7$), respectively. When caffeine (5–10 mM) was added to the Ca^{2+}-free solution during the plateau of these contractions, there was no additional increase in tension. In contrast, complete relaxation occurred within 2 ± 0.1, 4.5 ± 0.2 and 8.2 ± 0.5 min ($n = 4$) for oxytocin, vanadate and PGE_2-induced contractions, respectively in the presence of 10 mM caffeine (Figure 3Aa, Ba, Ca). These contractions were also inhibited by isoprenaline (0.5 μM), theophylline (10 mM) and IBMX (1 mM) (Figure 3Ab, Bb, Cb).

Furthermore, when caffeine (5–20 mM) was applied for 3 min between two successive contractions evoked by oxytocin, vanadate or PGE_2, the amplitude of the second agonist-induced contraction was not diminished, provided that a time interval of at least 2 min was allowed between the removal of caffeine and the subsequent addition of agonist (see for example Figure 3D).

Effect of caffeine in skinned uterine strips

Effect on the internal calcium-store We have shown in previous studies that IP_3 but not caffeine induced a contraction

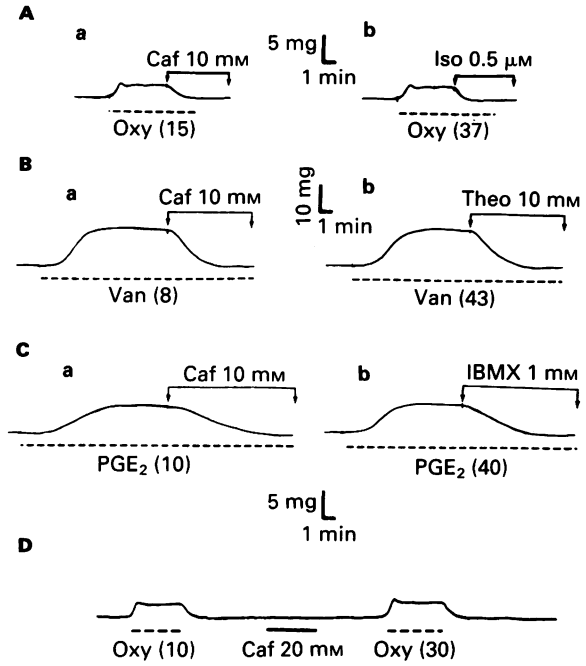


Figure 3 Pregnant rat myometrium bathed in a Ca^{2+}- free medium: the effects of some alkylxanthines and isoprenaline on the maintained contractions evoked by long (>2 min) application of some spasmogens. (A) Caffeine (Caf, a) and isoprenaline (Iso, b) tested against contraction induced by oxytocin (Oxy 22.5 nM). (B) Caffeine (Caf, a) and theophylline (Theo, b) tested against contraction induced by sodium orthovanadate (Van, 0.25 mM). (C) Caffeine (Caf, a) and isobutylmethylxanthine (IBMX, b) tested against contraction induced by prostaglandin E_2 (PGE_2, 1 μM). (D) Effect of the addition of 20 mM caffeine between two successive contractions induced by oxytocin (Oxy, 22.5 nM). Note that caffeine did not reduce the second contraction. Figures in parentheses indicate times in Ca^{2+}-free solution (min).

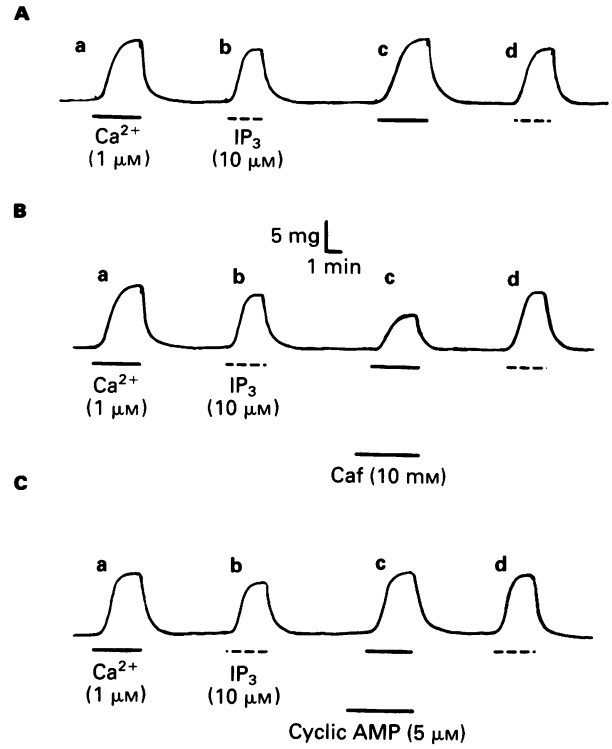


Figure 4 Effects of caffeine (Caf) in saponin-skinned uterine strips. After saponin treatment (50 μg ml^{-1} for 20 min), the EGTA-concentration of the relaxing solution was lowered from 4 to 0.1 mM. (A) Control experiment: the internal calcium-store was loaded by addition of 1 μM Ca^{2+} for 3 min: a contractile response occurred (a). After removal of the Ca^{2+}-solution and complete relaxation of the strip, addition of inositol trisphosphate (IP_3, 10 μM) evoked a contraction (b), which could be obtained again (d), without change in amplitude after replenishment of the store (c). (B, C). When the loading of the Ca^{2+}-store was performed in the presence of 10 mM caffeine (Bc) or 5 μM cyclic AMP (Cc), the subsequent IP_3-induced contraction (Bd, Cd) was increased in comparison with the reference response (Bb, Cb). Experiments were performed in the presence of 5 mM NaN_3 and 5 mM NaCN.

in chemically skinned strips when the Ca^{2+}-store had been loaded with 1 μM Ca^{2+} for 3 min (Savineau *et al.*, 1988; Savineau, 1988). In the following experiments, we tested the effect of caffeine on the loading of the Ca^{2+}-store by measuring the amplitude of the subsequent contraction induced by IP_3 (10 μM). Figure 4B shows that IP_3-induced contraction was increased by 14 ± 0.7% ($n = 4$, $P < 0.05$) in comparison with the control response, when 10 mM caffeine was present during the loading procedure. A similar effect was observed with 5 μM cyclic AMP: +17 ± 1.2% ($n = 4$, $P < 0.05$) (Figure 4C).

Effect on the contractile machinery. The pCa-tension curve was constructed in the presence of 4 mM EGTA on strips which had been treated with saponin as well as with Brij 58 in order to destoy the internal store. In the presence of caffeine (15–30 mM) the pCa-tension relationship was clearly shifted to the right, and the maximum Ca-activated contractions were decreased when compared to those obtained in the absence of caffeine, indicating a decreased sensitivity of the contractile machinery to calcium ions (Figure 5a). These effects were also observed in the presence of cyclic AMP (0.3 mM, Figure 5a).

In the subsequent experiments, we tested the effect of the PKA-inhibitor Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp (Cheng *et al.*, 1986) on the relaxation induced by caffeine. Figure 5b shows that preincubation of strips for 10 min with 8 μM of the protein kinase inhibitor, which had by itself no effect on the contractile response, significantly reduced the inhibitory effect of caffeine (2.5–30 mM) on the calcium-activated contraction (pCa = 6).

Discussion

The present study addresses two major points concerning the action of caffeine on the mechanical activity of pregnant rat myometrium: (i) the absence of a spasmogenic effect; (ii) the suppression by caffeine of contractions induced by various spasmogens i.e. its relaxing effect.

Absence of a spasmogenic action of caffeine

The absence of a spasmogenic action of caffeine in uterus seems to be a peculiarity of this tissue, since caffeine causes contraction of other smooth muscles from the same animal (e.g. portal vein and colon). It is widely accepted that caffeine-induced contraction in striated muscles is due to the release of calcium ions from an internal store: the sarcoplasmic reticulum. It is unlikely that this lack of spasmogenic effect of caffeine in uterus can be related to the absence of a functional calcium store. A store of this kind has been shown to be involved in the contractile activity of this muscle both in intact (Mironneau *et al.*, 1984) and in skinned preparations (Savineau *et al.*, 1988).

An alternative explanation might be that release of stored calcium ions was masked at the contractile level by the activation of opposing mechanisms. However, we failed to find experimental evidence for Ca^{2+}-release in each of the following circumstances: (i) in contrast to ventricular muscle (Kitazawa, 1988), when external sodium ions were removed (in a Ca^{2+}-free solution) caffeine did not induce uterine contraction. This result does not accord with the suggestion that rapid and complete extrusion of released calcium via the Na-Ca exchange may have masked caffeine-induced spasm. (ii) In contrast to airways smooth muscle (Small *et al.*, 1988), cooling the myometrium to 4°C did not reveal a spasmogenic

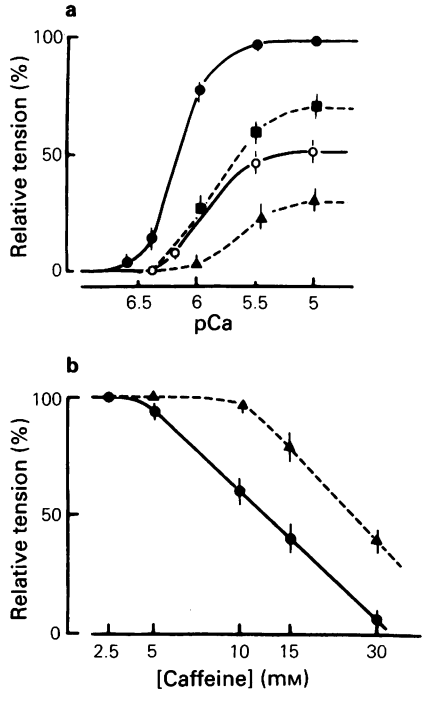


Figure 5 Effect of caffeine on calcium-activated contractions in skinned strips. Experiments were performed in the presence of 4 mM EGTA after Brij 58 treatment. (a) pCa-tension relationship in reference conditions (●), and after preincubation of the strips for 10 min in the presence of 15 mM (■) and 30 mM (▲) caffeine of 0.3 mM cyclic AMP (○). Abscissa scale: concentration of calcium ions (pCa = $-\log[Ca^{2+}]$). Ordinate scale: % of maximum contraction. The tension developed by the unskinned strip in response to 0.1 mM acetylcholine was considered as 100%. Data points are mean, $n = 6$ (●) or 4 (■, ○, ▲). (b) Concentration-response curves for the relaxing effect of caffeine on calcium-activated contractions in the absence (●) and presence (▲) of 8 μM cyclic AMP-dependent protein kinase inhibitor. Calcium-activated contraction was induced by a pCa = 6 with (pretreatment for 10 min) or without the protein kinase inhibitor. Cumulative concentrations of caffeine were then added. Abscissa scale: concentration of caffeine (mM) on a log scale. Ordinate scale: tension as a percentage of the initial value. The tension developed by a pCa = 6 in the absence of caffeine was considered as 100%. Data points are mean, $n = 5$. In (a) and (b) vertical lines show s.e.mean.

response to caffeine. (iii) In unskinned uterine muscle incubated in a Ca-free solution, application of caffeine between two successive agonist-induced contractions did not depress the second one (Figure 3D). A similar result was obtained with the IP_3-induced contraction in skinned strips (Savineau, 1988; Kanmura *et al.*, 1988).

The lack of spasmogenic action of caffeine in rat myometrium could thus be due to the absence of a specific calcium release mechanism activated by caffeine in that preparation, as is the case in the sarcoplasmic reticulum of canine aorta (Ehrlich & Watras, 1988). In this respect, the sarcoplasmic reticulum of longitudinal uterine muscle could be of the 'Sβ' type according to the classification proposed by Iino *et al.* (1988), i.e. with a calcium-release mechanism activated by IP_3 but not by caffeine.

The relaxing effect

Caffeine exhibits a potent inhibitory effect on uterine contractile activity in both intact and skinned strips. Our experiments suggest that this effect can be mediated by various mechanisms which involve: (i) an increase in calcium-storage, (ii) a decrease in calcium-sensitivity of the contractile machinery, (iii) a decrease in stimulated calcium influx.

In different experimental conditions, the relaxing effect of caffeine was well mimicked in skinned strips by cyclic AMP itself (Figure 4 and 5a), and in intact strips by different substances (isoprenaline, theophylline, isobutyl methylxanthine) known to increase the cyclic AMP concentration of myometrial cells (Harbon *et al.*, 1976; Fortier *et al.*, 1983) (Figure 3), thus, suggesting that this effect is mediated at least partially by cyclic AMP-dependent mechanisms. The IP_3-induced contraction was slightly increased when either caffeine (10 mM) or cyclic AMP (5 μM) was applied during the Ca-loading (Figure 4) showing that more calcium ions had been stored. A similar effect of cyclic AMP on the Ca-storage has been observed in vascular smooth muscles (Itoh *et al.*, 1982; Hwang & Van Breemen, 1987). In addition cyclic AMP could act on calcium extrusion. In rat myometrium it is known that: (i) isoprenaline and cyclic AMP increase the extrusion of $^{45}Ca^{2+}$ in cultured cells (Fortier *et al.*, 1983) and stimulate both the calcium-transport and the Ca-ATPase activity of vesicles enriched in plasma membranes (Krall *et al.*, 1976), (ii) the Na/K pump is stimulated by cyclic AMP (Turi & Somogyi, 1988). The subsequent decrease in the cytoplasmic sodium concentration should, in turn, increase the Ca-efflux via the Na/Ca exchange previously described in the myometrium (Grover *et al.*, 1981; Savineau *et al.*, 1987). More detailed experiments are required to investigate a possible action of caffeine on these mechanisms of Ca^{2+}-extrusion.

Caffeine (> 5 mM) decreased the Ca-sensitivity of the contractile machinery of rat myometrium (Figure 5). This effect can be explained, according to the hypothesis of Adelstein & Hataway (1979), by the cyclic AMP-dependent phosphorylation of the myosin light chain kinase (MLCK). When phosphorylated, MLCK becomes less effective in phosphorylating myosin light chains (LC_{20}) resulting in relaxation. In our study, this hypothesis was supported by the observation that a specific cyclic AMP-dependent protein kinase inhibitor reduced the relaxing effect of caffeine on Ca^{2+}-activated contractions in skinned strips (Figure 5b).

Another matter of debate concerns the effect of caffeine on calcium influx. In the present work, the agonist-induced contraction in Ca-free solution was not reduced when caffeine (10–20 mM) was present during the loading period, suggesting that basal calcium influx (leak pathway) was not inhibited by caffeine. As regards voltage-activated calcium influx, the effect of caffeine was dependent on the concentration used. For caffeine concentrations up to 5 mM, the transient contraction induced by oxytocin (in Ca^{2+}-free solution) was not modified, whereas the potassium-contracture was inhibited (Figure 2B). This result seems of importance because it shows that twitch and potassium-induced contraction can be antagonized by caffeine (Figure 1) without decreasing the calcium entry. For higher concentrations (10–20 mM), the oxytocin-induced contraction was depressed indicating an inhibitory effect of caffeine on calcium influx through voltage-sensitive channels. Previous studies have yielded conflicting results as to whether the effect of caffeine on calcium-influx was mediated by cyclic AMP. Dibutyryl cyclic AMP has been shown to inhibit calcium-influx in rabbit aorta (Meisheiri & Van Breemen, 1982), but not in taenia-caecum (Tomiyama *et al.*, 1973). Moreover, intracellularly perfused cyclic AMP had no effect on voltage-dependent calcium current in smooth muscle cells isolated from rabbit intestine (Ohya *et al.*, 1987).

It should be noted that the IC_{50} of the spasmolytic action of caffeine in intact muscles bathed in Ca^{2+}-containing solutions was about 1 mM (Figure 1), whereas in all other conditions this effect occurred at higher concentrations (generally > 5–10 mM). This could be due to the existence of different mechanisms applicable at different caffeine concentrations: (i) inhibition of cyclic AMP-dependent phosphodiesterase requiring high concentrations of caffeine; (ii) another mechanism sensitive to low concentrations of caffeine, could be involved with the integrity of the plasma membrane as suggested in rabbit intestine (Bolton & Lim, 1989). However, this mechanism has not yet been identified.

In conclusion, the present work shows that in the rat myometrium caffeine does not elicit contractile responses and in

addition antagonizes those evoked by depolarization or agonists. The lack of spasmogenic action might be related to the absence of a functional calcium-release channel sensitive to caffeine in this smooth muscle. The inhibitory effect appears to be due to the action of caffeine on various mechanisms leading to a decrease in both the cytoplasmic calcium concentration and the calcium-sensitivity of the contractile machinery.

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Eosinophil accumulation in pulmonary airways of guinea-pigs induced by exposure to an aerosol of platelet-activating factor: effect of anti-asthma drugs

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1 Exposure of guinea-pigs to aerosols of platelet activating factor (PAF) (0.01 to 100 $\mu g\,ml^{-1}$) induced a dose-dependent increased incidence of eosinophils in bronchoalveolar lavage fluid (BAL) at 48 h. Total leucocyte numbers and the percentages of lymphocytes and neutrophils were unchanged in BAL fluid.

2 Increased numbers of eosinophils were detected in BAL 1 h after exposure to PAF but eosinophilia was not maximal until 48 h. One week after exposure to PAF, the percentage of eosinophils in BAL was within the normal range.

3 Depletion of circulating platelets or neutrophils by intravenous injection of specific antisera did not modify accumulation of eosinophils in the airway lumen following inhalation of PAF (10 $\mu g\,ml^{-1}$).

4 PAF-induced pulmonary airway eosinophil accumulation was inhibited by treatment with SDZ 64–412, a selective PAF-antagonist, whether the compound was administered before, or 30 min after, inhalation of PAF.

5 Pulmonary airway eosinophil accumulation due to inhaled PAF (10 $\mu g\,ml^{-1}$) was inhibited by prior treatment with aminophylline, cromoglycate, ketotifen, dexamethasone and AH 21-132.

6 Pulmonary airway eosinophil accumulation due to inhaled PAF (10 $\mu g\,ml^{-1}$) was not inhibited by prior treatment with indomethacin, salbutamol or mepyramine.

Introduction

The presence of an eosinophilic infiltrate in the conducting airways of asthmatics was reported by Huber & Koessler in 1922. Eosinophil numbers in peripheral blood can be used as an index of disease severity in bronchial asthma (Horn *et al.*, 1975; Brown *et al.*, 1977) and the occurrence of eosinophils in bronchoalveolar lavage (BAL) fluid has been reported to precede late-onset responses to allergen in asthma patients (De Monchy *et al.*, 1985). However, only recently has the contribution of the eosinophil to the pathology of asthma been emphasised (Dahl & Venge, 1982; Frigas & Gleich, 1986). Toxic proteins derived from eosinophils have been demonstrated in sputum (Frigas *et al.*, 1981), BAL (De Monchy *et al.*, 1985) and blood (Dahl *et al.*, 1978) from asthma patients. Additionally, it is likely that lipid mediators such as peptidoleukotrienes (Verhagen *et al.*, 1984) and PAF (Lee *et al.*, 1984) may, in large part, be generated by eosinophils.

PAF has been reported to induce a selective accumulation of eosinophils in the airways of guinea-pigs (Aoki *et al.*, 1988a) and primates (Arnoux *et al.*, 1988). Hence, we have studied the dose relationship and time course of PAF-induced bronchial eosinophil accumulation in the guinea-pig and have examined the capacity of a range of anti-asthma drugs to inhibit this response.

Methods

Animals

Male Dunkin-Hartley guinea-pigs (400–600 g) were used for PAF exposure studies and antisera were raised in male New Zealand white rabbits (3–4 kg).

Antisera

Anti-serum to guinea-pig neutrophils Neutrophil-rich peritoneal exudates were induced by intraperitoneal injection of 5 ml of preformed immune complexes (bovine gamma globulin (BGG) + anti-BGG). After 4 h, guinea-pigs were killed (ether inhalation) and the peritoneal cavity was washed successively with four 10 ml aliquots of Hank's balanced salt solution (HBSS) containing EDTA (10 mM), HEPES (10 mM) and BSA (0.3%). Lavage fluid was centrifuged (200 *g* for 10 min) and the cell pellet resuspended in HBSS (1 ml). Approximately 3–5 × 10^7 cells were applied to a discontinuous Percoll gradient (densities: 1.07; 1.08; 1.09; 1.11) and centrifuged at 1000 *g* for 20 min at 20°C. At the interface between 1.09 and 1.11 density bands neutrophils were found in >95% purity. These cells were washed three times and resuspended in HBSS without EDTA or BSA. Neutrophils (2–3 × 10^7) in 1 ml were emulsified with an equal value of Freund's complete adjuvant (FCA) in a high speed blender and the emulsion was injected subcutaneously into the neck and flank of a rabbit. This procedure was repeated on two occasions at three-weekly intervals. Subsequently, blood was collected from anaesthetized (sodium pentothal 30–50 $mg\,kg^{-1}$, i.v.) rabbits by cardiac puncture and after clotting, was stored for 24 h. Serum was centrifuged (2000 *g* for 10 min), heat inactivated (56°C for 30 min) and adsorbed with guinea-pig red blood cells prior to storage as aliquots at −20°C.

Anti-serum to guinea-pig platelets Blood was collected by cardiac puncture of ether-anaesthetized guinea-pigs into sodium citrate (2.8% w/v). Blood was centrifuged (400 *g* for 10 min) at room temperature and platelet-rich plasma was centrifuged (1000 *g* for 10 min). The platelet pellet was resuspended in citrate buffer (1 ml) and approximately 5–10 × 10^8 platelets were emulsified with FCA. Rabbits were immunised according to the procedure described above.

Inhalation procedure

Guinea-pigs were restrained by the neck, with the snout inserted into a nose-cone which was connected to the outlet of

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Table 1 Time course of eosinophil accumulation in pulmonary airways of guinea-pigs exposed to an aerosol of PAF (10 $\mu g\,ml^{-1}$)

Time	n	Total cells ($\times 10^6$)	% Cell populations in BAL			
			Mac	Eos	Lym	Neut
0	15	17.5 ± 2.5	83 ± 1	8 ± 1	7 ± 1	2.1 ± 0.4
1 h	10	19.9 ± 1.4	82 ± 2	11 ± 1†	5 ± 2	1.0 ± 0.1
4 h	10	21.0 ± 2.0	79 ± 2	14 ± 2*	5 ± 1	2.0 ± 0.1
24 h	10	17.9 ± 1.5	74 ± 3*	16 ± 2*	6 ± 1	3.0 ± 0.1
48 h	20	19.9 ± 2.5	70 ± 2*	21 ± 2*	5 ± 1	2.0 ± 0.1
72 h	10	19.6 ± 2.0	80 ± 2	13 ± 2†	6 ± 1	1.4 ± 0.6
1 week	10	18.3 ± 1.6	82 ± 8	9 ± 1	2 ± 1	1.0 ± 0.2
2 week	4	23.3 ± 2.9	85 ± 2	6 ± 1	7 ± 1	1.3 ± 0.5

Results are expressed as mean ± s.e.mean. Student's t test for groups of unequal size was used to assess the statistical significance of observed differences between no exposure (time 0) and various time points after exposure to PAF; †$P < 0.05$, *$P < 0.005$. Mac = macrophages; Eos = eosinophils; Lym = lymphocytes; Neut = neutrophils; BAL = bronchoalveolar lavage fluid.

a Devilbiss nebuliser. Aerosols were generated by use of compressed air (7 litres min^{-1}). Guinea-pigs were exposed for 1 h to aerosols generated from 10 ml of a solution of PAF (0.01 to 100 $\mu g\,ml^{-1}$) in BSA (0.25%) or BSA (0.25%) alone. For kinetic studies, animals were exposed to PAF (10 $\mu g\,ml^{-1}$) for 1 h and lungs were washed 1, 4, 24, 48, 72 h and 1 or 2 weeks after exposure. For dose-effect studies, guinea-pigs were exposed to aerosols of PAF at increasing concentrations (0–100 $\mu g\,ml^{-1}$) and eosinophilia in BAL was determined 48 h after exposure.

Bronchoalveolar lavage

Guinea-pigs were killed with sodium pentobarbitone (100 $mg\,kg^{-1}$, i.p.), the trachea was cannulated to allow the lungs to be washed with six aliquots of modified HBSS (10 ml). Total fluid recovery exceeded 85%. Cell suspensions were concentrated by low speed centrifugation (200 g for 10 min) and the cell pellet resuspended in modified HBSS (1 ml). Total cell counts were made in a haemocytometer. Differential cell counts were made from smears fixed in methanol (1 min) and stained with Leishman's stain (4 min). A total of 500 cells per smear were counted by light microscopy under oil immersion ($\times$ 1000), in order to differentiate cell types.

Drugs

PAF (C_{16}; Novobiochem) was dissolved in ethanol at 1 $mg\,ml^{-1}$ and stored at −18°C. Dilutions of PAF were made in 0.25% bovine serum albumin (BSA, Fluka) in physiological saline.

Ketotifen, aminophylline, cromoglycate, mepyramine, SDZ 64-412, 2,3-dihydro-5-[4-(3,4,5-trimethoxyphenylethyl)phenyl]-imidazo[2,1-α]isoquinolinium hydrochloride, AH 21-132, *cis*-6-(*p*-acetoamidophenyl)-1,2,3,4a,10b-hexahydro-8,9-demethoxy-2-methylbenzo-[c][1,6]-naphthyridine, (Sandoz), salbutamol (Glaxo) and indomethacin (Sigma) were dissolved in physiological saline; dexamethasone (Sigma) was dissolved in a mixture of ethanol and polyethylene glycol (50:50 v/v).

Drug administration

With the exception of SDZ 64-412, drug solutions were loaded into osmotic minipumps (Alzet, 2001 with a delivery rate of 1 $\mu l\,h^{-1}$) and implanted subcutaneously in the nuchal region of guinea-pigs during ether anaesthesia. Five days after implantation of minipumps, animals were exposed to an aerosol of PAF (10 $\mu g\,ml^{-1}$) and 48 h later, BAL was collected. SDZ 64-412, a selective PAF-receptor antagonist, was given orally either 1 h before PAF challenge or 30 min after Paf challenge at a dose of 10 $mg\,kg^{-1}$.

Statistical comparisons

Student's t test (two tailed) was used for comparison between treatments.

Results

Time-course of eosinophil accumulation

In untreated animals, BAL fluid yielded 17.5 ± 2.5 × 10^6 leukocytes (n = 15). These comprised 83 ± 1% macrophages, 8 ± 1% eosinophils, 7 ± 1% lymphocytes and 2 ± 0.4% neutrophils. Occasionally, other cell types such as ciliated and non-ciliated epithelial cells were observed; however, since these cells typically formed strips or clumps, they could not be quantified. In animals exposed to PAF (10 $\mu g\,ml^{-1}$), the leucocyte content of BAL was examined at 1, 4, 24, 48, 72 h, as well as 1 and 2 weeks after exposure. The percentage of eosinophils in BAL 1 h after cessation of aerosol exposure was significantly greater than that in untreated animals ($P < 0.05$) but maximal eosinophilia was not observed until 48 h had elapsed (Table 1, Figure 1). Eosinophil numbers in PAF-treated animals remained significantly greater than in untreated animals at 72 h ($P < 0.05$), but had returned to values within

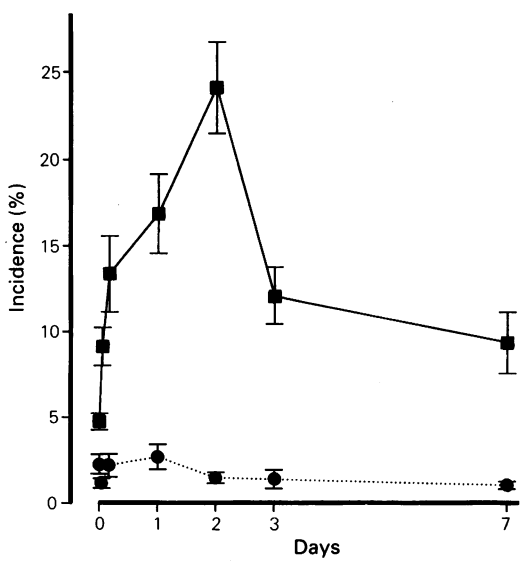


Figure 1 Incidence of eosinophils (■) and neutrophils (●) in bronchoalveolar lavage fluid collected from guinea-pigs at various times after inhalation of an aerosol of PAF (10 $\mu g\,ml^{-1}$) for 1 h. Bars depict s.e.mean and n = 10–20 animals per time point.

Table 2 The effect of exposure of guinea-pigs to increasing concentrations of aerosolised PAF on BAL cells recovered 48 h after exposure

	n	Total cell ($\times 10^6$)	% Cell populations in BAL			
			Mac	Eos	Lym	Neut
No treatment	15	17.5 ± 2.5	83 ± 1	8 ± 1	7 ± 1	2.1 ± 0.4
BSA 0.25%	15	25.1 ± 3.2	80 ± 2	10 ± 2	7 ± 1	2.0 ± 0.5
PAF 0.01	10	20.4 ± 1.9	76 ± 2	12 ± 1	8 ± 1	2.3 ± 0.6
($\mu g ml^{-1}$) 0.1	10	19.3 ± 2.6	72 ± 2*	15 ± 2†	7 ± 1	2.0 ± 0.7
1.0	10	20.8 ± 1.5	71 ± 2*	19 ± 2*	6 ± 1	1.8 ± 0.5
10.0	10	18.9 ± 1.6	69 ± 2*	23 ± 2*	6 ± 1	1.4 ± 0.3
100.0	10	23.3 ± 2.9	62 ± 3*	28 ± 4*	7 ± 1	1.3 ± 0.3

Results are expressed as mean ± s.e.mean. Student's t test for groups of unequal size was used to assess the statistical significance of observed differences between bovine serum albumin (BSA) and PAF-treated groups; † $P < 0.05$; * $P < 0.005$. Mac = macrophages; Eos = eosinophils; Lym = lymphocytes; Neut = neutrophils. BAL = bronchoalveolar lavage fluid.

the normal range after 1 week (Table 1). No significant changes in leucocyte number were observed, other than a decrease in the percentage of macrophages 24 and 48 h after exposure to PAF (Table 1). Exposure of guinea-pigs to an aerosol of BSA did not significantly increase the percentage of eosinophils in BAL fluid at 24 (8.7 ± 1.4%; $n = 7$) or 48 h (10 ± 2%; $n = 15$).

Dose-effect relationship

Since maximal eosinophil accumulation was observed 48 h after exposure to an aerosol of PAF ($10 \mu g ml^{-1}$), this time-point was chosen to examine the effect on leucocyte populations in BAL of aerosols of PAF at concentrations between 0.01 and $100 \mu g ml^{-1}$. Increasing concentrations of Paf induced a dose-related increase in the percentage of eosinophils observed in BAL, with significant eosinophilia being detectable after inhalation of an aerosol containing $0.1 \mu g ml^{-1}$ of PAF (Table 2). There was a concomitant decrease in the percentage of macrophages recovered in BAL, whilst neutorphil or lymphocyte populations remained unaltered (Table 2).

Effect of neutrophil or platelet depletion

To ascertain whether circulating platelets or neutrophils participate in eosinophil recruitment to the lungs after exposure to an aerosol of PAF ($10 \mu g ml^{-1}$), each cell type was depleted selectively by specific antisera.

In vitro specificity of the anti-neutrophil serum (ANS) used for this study has been reported elsewhere (Hutson *et al.*, 1989). Intravenous injection of ANS ($2 ml kg^{-1}$) produced a neutropaenia in blood which was maximal (>96%) at 24 h and persisted for 48 h with recovery being evident at 96 h (control 4490 ± 600 μl^{-1}; ANS 24 h, 260 ± 68 μl^{-1}; ANS 48 h, 993 ± 227 μl^{-1}; ANS 96 h, 3836 ± 558 μl^{-1}; $n = 3$). A marginal reduction of platelet counts (at 24 h) was not significant.

Intravenous injection of anti-platelet serum (APS) caused depletion of 94.5 ± 1.7% of circulating platelets 24 h after injection (control 448 ± 63.1 × $10^3 \mu l^{-1}$; APS 24 h, 23.2 ± 1.9 × $10^3 \mu l^{-1}$; $n = 5$). Recovery from platelet depletion was more rapid than from neutrophil depletion, so that platelet counts were within the normal range by 48 h.

Antisera were injected intravenously 24 h prior to PAF exposure, to induce maximal depletion of each cell type. Neither platelet depletion nor neutrophil depletion affected the basal cell numbers recovered in BAL or the pulmonary airway eosinophil accumulation that occurred 48 h after exposure to an aerosol of PAF ($10 \mu g ml^{-1}$) (Figure 2).

Effect of a PAF-receptor antagonist

Eosinophil accumulation in the pulmonary airways was fully inhibited by administration of a selective PAF-receptor

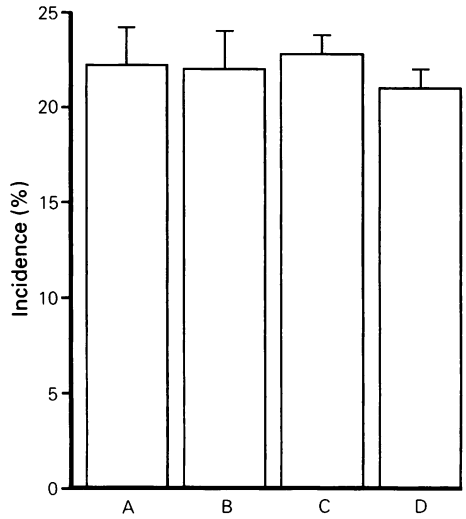


Figure 2 Incidence of eosinophils in bronchoalveolar lavage fluid collected from guinea-pigs 48 h after inhalation of an aerosol of PAF ($10 \mu g ml^{-1}$) following no treatment (A), pretreatment with normal rabbit serum (B), anti-platelet antiserum (C) or anti-neutrophil antiserum (D). Bars depict s.e.mean and $n = 10$ animals per treatment.

Table 3 The effect of anti-asthma drugs on PAF-induced eosinophil accumulation in pulmonary airways of the guinea-pig

Treatment	Dose ($mg kg^{-1}$)	Total cells ($\times 10^6$)	Eosinophils (%)	P^1
Saline	—	20.0 ± 2.1	22.2 ± 1.6	
SDZ 64-412	10.0	24.7 ± 4.4	10.0 ± 2.5	<0.001
Cromoglycate	1.0	17.8 ± 2.3	13.4 ± 2.0	<0.01
Ketotifen	1.0	16.9 ± 0.9	11.1 ± 1.1	<0.001
	0.1	18.0 ± 2.0	14.0 ± 0.9	<0.001
AH 21-132	1.0	18.7 ± 0.8	9.8 ± 0.8	<0.001
	0.1	15.6 ± 1.0	17.7 ± 1.4	<0.05
Indomethacin	1.0	14.9 ± 1.1	18.8 ± 1.2	NS
Salbutamol	1.0	12.8 ± 0.9	19.2 ± 2.0	NS
Mepyramine	1.0	24.7 ± 3.5	20.4 ± 3.1	NS
Dexamethasone	1.0	18.3 ± 2.4	8.8 ± 1.0	<0.001
Aminophylline	10.0	20.6 ± 1.6	10.3 ± 1.6	<0.001

Results are expressed as mean ± s.e.mean. [1] Student's t test was used to assess the statistical significance of observed differences between drug treatment and saline treatment of the eosinophil response. Drugs were given subcutaneously by osmotic minipumps implanted for 7 days, except SDZ 64-412 which was given orally 1 h before aerosol exposure. Each treatment group comprises 10 animals exposed to PAF $10 \mu g ml^{-1}$.

antagonist SDZ 64-412 (Handley et al., 1988) (10 mg kg^{-1}, p.o.), 1 h before exposure to an aerosol of PAF (10 μg ml^{-1}) (Table 3). In a separate study, SDZ 64-412 (10 mg kg^{-1}, p.o.) was administered 30 min after exposure to an aerosol of PAF (10 μg ml^{-1}). Guinea-pigs which were not exposed to PAF had basal eosinophil numbers in BAL of 5.1 $\pm$ 0.9% in the absence of drug treatment; sham-treated guinea-pigs exposed to an aerosol of PAF had a pulmonary airway eosinophilia of 14.9 $\pm$ 1.6% ($P < 0.0005$ compared to no treatment) and animals exposed to PAF and subsequently treated with SDZ 64-412 exhibited a pulmonary airway eosinophilia of 6.5 $\pm$ 3.2%, which was not significantly different from the incidence of eosinophils in untreated animals.

Effect of pretreatment with anti-asthma drugs

Daily pretreatment of guinea-pigs with aminophylline (10 mg kg^{-1}), cromoglycate (1 mg kg^{-1}), ketotifen (0.1–1 mg kg^{-1}), AH 21-132 (0.1–1 mg kg^{-1}) or dexamethasone (1 mg kg^{-1}) caused a significant decrease in the percentage of eosinophils in BAL, 48 h after exposure to PAF (10 μg ml^{-1}), whereas pretreatment with mepyramine (1 mg kg^{-1} day^{-1}), indomethacin (1 mg kg^{-1} day^{-1}) or salbutamol (1 mg kg^{-1} day^{-1}) did not diminish eosinophil accumulation significantly (Table 3). The lack of inhibition observed with the latter three drugs was not related to the administered dose since arachidonic acid-induced bronchoconstriction was reduced (although not fully inhibited) in indomethacin-treated animals and histamine-induced bronchoconstriction was fully inhibited in mepyramine-treated guinea-pigs (data not shown). The effectiveness of salbutamol was assessed by β-adrenoceptor desensitization which was observed after 5 days of treatment.

Discussion

Platelet activating factor (PAF), which was first identified in the supernatant of rabbit basophils challenged with antigen (Benveniste et al., 1972), is now recognised as a potent inflammatory mediator with a wide range of biological activities (Braquet et al., 1987). PAF and lyso-PAF, the precursor and metabolite of PAF, are released into the airway lumen of lungs from sensitized guinea-pigs after exposure to antigen (Fitzgerald et al., 1986) and increased levels of lyso-PAF have been detected in serum of asthma patients in whom there was a late-onset reaction to allergen (Nakamura et al., 1987). PAF is released during allergic reactions, is chemoattractant for both neutrophils and eosinophils *in vitro* (Wardlaw et al., 1986) and induces accumulation of such cells *in vivo* (Dewar et al., 1984; Henocq & Vargaftig, 1986); hence, it is reasonable to anticipate that PAF formation will contribute to the accumulation of such inflammatory cells in the lungs of asthma patients.

In the present experiments, exposure of guinea-pigs to an aerosol of PAF induced a selective, dose-dependent accumulation of eosinophils in the airway lumen which was maximal at 48 h. Microscopically, these eosinophils appeared larger than eosinophils from untreated guinea-pigs, yet there was little evidence of degranulation. An attempt was made to separate eosinophils on the basis of their density by use of discontinuous Percoll gradients (densities 1.07, 1.08, 1.09, 1.10). Eosinophils sedimented at the interface of layers 1.08/1.09 (hypodense) and 1.09/1.10 (normodense) in normal and PAF-treated guinea-pigs. Variability of the ratio between hypodense and normodense eosinophils was too large to allow an assessment of changes in density.

Guinea-pigs were exposed to PAF over a prolonged period (1 h) in order to avoid the severe bronchospasm that can occur upon exposure to an aerosol of PAF (Lefort et al., 1984) and which would limit entry of PAF into the airways. Guinea-pigs are obligatory nose-breathing animals so that only small quantities of aerosols enter the pulmonary airways. This has been shown in studies with radiolabelled BSA (Broder et al., 1978) or ovalbumin (Richerson, 1972) which estimated that between 0.01 and 0.05% of the total dose is deposited within guinea-pig airways and use of radiolabelled materials for imaging has confirmed this conclusion (Gazeley et al., 1986). It may be presumed that nanogram quantities of PAF enter the lung in the present experiments and that the major proportion of inhaled PAF will have been deposited in the nasal mucosa.

Despite having a short half-life *in vivo* (Latrigue-Mattei et al., 1984), PAF can cause chronic inflammatory changes when injected intradermally (Dewar et al., 1984) or instilled into the lungs of experimental animals (Camussi et al., 1983). Results of the present study confirm that the cellular reaction to PAF is protracted, since maximal eosinophilia was observed 48 h after a single exposure to an aerosol of PAF. In the guinea-pig mesentery, local application of PAF induces persisting thrombosis that can be accounted for by a protracted formation of PAF by vascular endothelium (Bourgain et al., 1986). An analogous mechanism may contribute to eosinophil accumulation, since the accumulation of eosinophils within the airways was diminished by SDZ 64-412, even when administered 30 min after exposure to PAF. This observation suggests that either PAF is not destroyed rapidly on the epithelial surface, possibly by binding to glycoproteins in the mucus layer, or that PAF induces sustained generation of PAF. Both alveolar macrophages and eosinophils are capable of generating PAF when activated by a variety of stimuli (Arnoux et al., 1980; 1982; Lee et al., 1984). These cells may be appropriate candidates for sustained PAF generation.

It has been proposed that during eosinophil accumulation in the guinea-pig, platelet activation is an obligatory intermediate step (Lellouch-Tubiana et al., 1988). It is known that intravenous injection of PAF causes entrapment of platelets and neutrophils (Dewar et al., 1984) to be succeeded by emigration of eosinophils (McManus, 1987). Nonetheless, the present experiments show that platelet depletion with lytic anti-serum did not diminish the accumulation of eosinophils in guinea-pig airways exposed to an aerosol of PAF. In the study of Lelluch-Tubiana et al. (1988), PAF was given intravenously, which may account for the differing observations since there are clear differences between the effects upon the guinea-pig lung of PAF given by the intravenous and inhaled routes. Intravenous PAF causes a platelet-dependent bronchoconstriction (Vargaftig et al., 1980) and airway hyperreactivity (Mazzoni et al., 1985), whereas bronchoconstriction due to an aerosol of PAF is not dependent upon circulating platelets (Lefort et al., 1984). In the present experiments, an absence of circulating neutrophils did not influence eosinophil accumulation after inhalation of PAF, in agreement with recent findings that neutrophil depletion did not affect the magnitude of late-onset airway obstruction or eosinophil accumulation in sensitized guinea-pigs challenged with an aerosol of antigen (Aoki et al., 1988b; Hutson et al., 1989). It is of interest to note that at no time after PAF inhalation was an increased neutrophil infiltration observed in BAL fluid. This observation is in marked contrast to the pre-eminent accumulation of neutrophils which is evident after intradermal injection of PAF in guinea-pigs (Dewar et al., 1984) and man (Archer et al., 1985). It is possible that airway and skin vasculature differ in their response to PAF.

Results of the present study reveal that pretreatment of guinea-pigs with ketotifen, aminophylline, cromoglycate, dexamethasone or AH 21-132 inhibited pulmonary eosinophil accumulation that followed inhalation of an aerosol of PAF. On the other hand, mepyramine, indomethacin or salbutmol were without effect. Cyclo-oxygenase inhibitors do not inhibit eosinophil accumulation (Spicer et al., 1985; Fugner, 1989) and anti-histamines are not considered to affect eosinophil accumulation, since most experiments using antigen as the stimulus are conducted under cover of an antagonist of histamine to prevent death from anaphylaxis. The action of β-

adrenoceptor agonists is more controversial with one study reporting an inhibition of antigen-induced eosinophilia by fenoterol (Fugner, 1989) and another confirming our finding of no inhibition with albuterol (Hutson *et al.*, 1988). Whether these differences represent different dose effects, differences in duration of action or other actions of the drugs is not clear at present.

Our observations are consistent with the available clinical observations that a reduction of eosinophils in blood, sputum or lungs occurs in atopic asthmatic patients following treatment with cromoglycate (Diaz *et al.*, 1984) or ketotifen (Gobel, 1978) and in patients with intrinsic bronchial asthma when treated with glucocorticosteroids (Horn *et al.*, 1975). Furthermore, theophylline and glucocorticosteroids, but not cromoglycate or salbutamol, inhibit the production of an eosinophil-activating factor by peripheral blood monocytes obtained from individuals with moderate eosinophilia (Thorne *et al.*, 1988). The question as to whether these drugs share a common site of action or whether they act at different points along the pathway from eosinophilpoiesis to accumulation and degranulation of eosinophils in the bronchial walls and mucus of asthmatics remains to be determined.

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Locomotor activity in rats after administration of nicotinic agonists intracerebrally

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1 Nicotine (0.13 and 0.4 mg kg^{-1}, s.c.) increased the ambulatory component of locomotor activity in rats previously exposed to the drug. Nicotine did not increase repeated movements reliably.

2 An infusion of either nicotine (8 μg) or the potent nicotinic agonist cytisine (4 μg) into the ventral tegmental area of the forebrain increased ambulation but not repeated movements.

3 An infusion of nicotine or cytisine into the nucleus accumbens, striatum, dorsal hippocampal formation or motor thalamus did not increase ambulatory or repeated movements.

4 Mecamylamine (0.1–1.0 mg kg^{-1}, s.c.) blocked increases in locomotor activity produced by an infusion of nicotine or cytisine into the ventral tegmental area.

5 The locomotor activity produced by systemically administered nicotine may be mediated, in part, through nicotinic receptors located in the ventral tegmental area of the mesolimbic dopamine system.

Introduction

Nicotine produces a wide range of behavioural changes, many of which are mediated through the central nervous system. Nicotine can enhance locomotor activity in rats, an effect which is more marked in animals that have been previously exposed to the drug (Morrison & Stephenson, 1972; Clarke & Kumar, 1983a, b). The nicotinic antagonist mecamylamine can block this effect of nicotine. Neuroanatomical studies have identified several brain areas which may be implicated in the behavioural effects of nicotine. Binding sites identified with [^{3}H]-(−)-nicotine or [^{3}H]-acetylcholine in the presence of atropine are concentrated, *inter alia*, in the thalamus, ventral tegmental area (VTA), nucleus accumbens, substantia nigra, striatum and molecular layer of the dentate gyrus of the hippocampal formation, (Schwartz *et al.*, 1984; Clarke *et al.*, 1985). As the hyperkinetic effect of dopamine agonists is mediated by the mesolimbic system (Kelly *et al.*, 1975), it is interesting that recent evidence suggests that at least some of the effects of nicotine involve interaction with central dopamine mechanisms of that system (Imperato *et al.*, 1986; Rowell *et al.*, 1987; Clarke *et al.*, 1988; Lapin *et al.*, 1989).

Apart from one short study (Pert & Chieuh, 1986), the effects on locomotor activity of direct infusions of nicotinic agonists into discrete brain areas have not been determined. The present study investigates the effects on locomotor activity of an infusion of nicotine, or the potent but relatively lipophobic nicotinic agonist cytisine, into several brain regions. Rats that were treated chronically with nicotine (s.c.) were used in all studies because the locomotor stimulant effect of nicotine is clearer in such animals (Morrison & Stephenson, 1972; Clarke & Kumar, 1983a, b). The brain areas for investigation were selected because previous evidence showed that they contained large numbers of binding sites for nicotine, and because they have been implicated in locomotor behaviour.

Methods

Animals

Male hooded Lister rats (Olac) were housed individually with free access to food and water. Lights were on from 7 h 30 min to 19 h 30 min.

Apparatus

Photocell activity cages (30 × 30 × 30 cm) were constructed from transparent Perspex sheet with wire mesh floors. Two parallel beams of infra-red light, modulated at a frequency of 7.5 kHz, were located 3 cm from opposite walls, and 4 cm above the floor of each cage. Interruptions of the beams were recorded by a microcomputer in an adjoining room. During each session, two measures of activity were taken: interruptions of either beam that followed interruptions of the other beam were recorded as 'cage crosses' (ambulation), whereas consecutive interruptions of the same beam were recorded as 'repeated moves'. The activity cages used and the measures taken were modified from Clarke & Kumar (1983a, b). The duration of each test session was 1 h, within which data were recorded separately for 6 consecutive periods of 10 min each.

Behavioural procedures

Rats received daily injections of nicotine (0.4 mg kg^{-1}, s.c.) and were placed in the activity cages for 1 h each day. This procedure continued for 5 days a week for at least 2 weeks, to produce tolerance to locomotor depressant effects of nicotine (Morrison & Stephenson, 1972; Clarke & Kumar, 1983a, b) and to familiarise the rats with the cages. In rats which were to receive intracerebral infusions, guide cannulae were then implanted as described below. One week was allowed for recovery from surgery, and the rats then received at least five more daily injections of nicotine (0.4 mg kg^{-1}, s.c.) before tests began.

Tests of drugs on locomotor activity took place twice weekly (Tuesdays and Fridays), and on the intervening days the rats continued to receive once daily injections of nicotine (0.4 mg kg^{-1}, s.c.). The very few rats in which nicotine increased cage crosses by less than 20% were excluded from these studies. Different doses of test drugs given systemically or intracerebrally were administered in random order and each dose was tested once in each rat. Test sessions began immediately after administrations of drugs and lasted for 1 h. To provide a basis for comparison, each experiment with intracerebral administrations of drugs also included tests with systemic injections of saline (1 ml kg^{-1}, s.c.) or nicotine (0.4 mg kg^{-1}, s.c.).

The effects of nicotine administered systemically were determined first. Saline and three doses of nicotine (0.04 mg kg^{-1}, 0.13 mg kg^{-1} and 0.40 mg kg^{-1}) were tested in random order, in rats into which intracerebral guide cannulae had not been implanted.

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To determine the effects of nicotine or cytisine given intracerebrally, rats were given bilateral infusions of vehicle or a dose of drug into the brain regions detailed below. Normally three doses of each drug were tested. The rats were restrained gently by hand while the drugs were infused through 28 gauge internal cannulae (Plastic Products) connected via polypropylene tubing to 10 μl Hamilton syringes. The solutions were delivered at a rate of 1 $\mu l\,min^{-1}$ for 30 s by a Sage Model 341A infusion pump, and the internal cannulae were left *in situ* for a further 1 min before withdrawal. Normally, doses of only one agonist were infused into each brain region and thus, individual rats received no more than 4–5 intracerebral infusions (two per week).

To study the effects of mecamylamine, rats received either saline or mecamylamine systemically (s.c.) 30 min before tests. Mecamylamine was administered in doses of 0.1 $mg\,kg^{-1}$, 0.32 $mg\,kg^{-1}$ and 1.0 $mg\,kg^{-1}$. These doses of mecamylamine were chosen from previous work demonstrating blockade of locomotor activity produced by nicotine (Clarke & Kumar, 1983a, b). Then, either saline, nicotine or cytisine was infused intracerebrally, through guide cannulae aimed at the ventral tegmental area (VTA), immediately before 1 h tests. A single intracerebral dose of nicotine (8 μg) or cytisine (3.2 μg) was used in these experiments, selected from the results of the earlier experiments.

At the conclusion of each experiment, all rats with implanted cannulae were killed by a humane method and brains were removed for histological examination.

Surgery

Tolerant rats were anaesthetized with halothane and held on a Stoelting stereotaxic frame. A hole was drilled in the skull over each hemisphere to facilitate placement of 22 gauge guide cannulae (Plastic Products). The cannulae were adjusted to an angle of 10° from vertical and they were lowered to just above the desired site. The stereotaxic coordinates for the injection sites were based on the atlas of Pellegrino *et al.* (1979) and were as follows: dorsal hippocampus, A3.0, L2.5, V.3.0; VTA, A3.0, L1.5, V8.8; nucleus accumbens, A9.0, L2.0, V7.0; motor thalamus, A5.2, L2.0, V6.7; striatum, A8.2, L3.0, V4.7. The cannulae were embedded in acrylic dental cement and anchored to the skull with 4 stainless steel screws (10BA, cheesehead).

Histology

Brains were fixed in 40% formaldehyde/glacial acetic acid/methanol (1:1:8), processed, and embedded in paraffin wax. Serial sections (20 μm) were cut on a sledge microtome and stained with Luxol fast blue and Mallory's phosphotungstic acid Haematoxylin or cresyl violet. The extent of the infusion sites was examined microscopically and recorded on composite diagrams taken from the atlas of Konig & Klippel (1963).

Data analysis

Activity scores for the entire hour of each test were examined by one factor or two factor analyses of variance with repeated measures (Winer, 1971). Multiple comparisons with control groups were made with Dunnett's modified *t* test.

Drugs

All doses were calculated as those of the bases. (−)-Nicotine hydrogen-(+)-tartrate (BDH) was dissolved in distilled water and the pH was adjusted to 7.0 with sodium hydroxide. Mecamylamine hydrochloride (Merck, Sharp & Dohme) was dissolved in distilled water. All systemic injections were subcutaneous in volumes of 1.0 $ml\,kg^{-1}$ and the control was 0.9% saline. For intracerebral infusions, aqueous solutions of nicotine or cytisine (Koch-Light) were made isotonic with sodium chloride. Control (vehicle) intracerebral infusions were of isotonic sodium chloride.

Results

Systemic administration of nicotine

Nicotine increased locomotor activity as measured by the number of cage crosses ($F3, 21) = 9.61$, $P < 0.001$). The effect was dose-related and significant after 0.13 $mg\,kg^{-1}$ ($t(21) = 3.38$, $P < 0.05$) and 0.4 $mg\,kg^{-1}$ of nicotine ($t(21) = 4.96$, $P < 0.01$). The lowest dose of nicotine (0.04 $mg\,kg^{-1}$) did not increase the number of cage crosses above baseline (Figure 1a). Figure 2a shows that nicotine (0.4 $mg\,kg^{-1}$) increased cage crosses uniformly throughout the 1 h period of measurement. Although there was a significant overall effect of nicotine on the number of repeated moves ($F(3, 21) = 3.99$, $P < 0.05$), no individual dose of nicotine differed significantly from saline.

Focal infusions of nicotine or cytisine into brain areas

The results with infusions into the VTA are described first. There were clear overall effects of drug treatments (cage crosses, $F(5, 85) = 6.26$, $P < 0.001$; repeated moves, $F(5, 85) = 10.1$, $P < 0.001$). Figure 3a shows that after bilateral infusions of nicotine (8 μg) into the VTA, the number of cage crosses increased to 178 ± 26 as compared with 112 ± 15 after vehicle ($t(85) = 2.87$, $P < 0.02$). Figure 2b shows that this effect was clearest during the 20–40 min after infusion. In the same

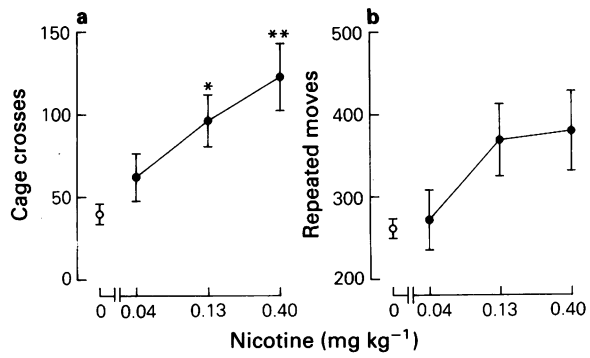


Figure 1 Locomotor activity in rats exposed to nicotine (0.4 $mg\,kg^{-1}$, s.c.) daily for two weeks and then tested twice weekly ($n = 8$). Each dose of nicotine shown was tested once in each rat (●). Activity after an injection of saline is also shown (○). Each point in this and subsequent figures represents the mean of data obtained from 1 h sessions in photocell activity cages. Vertical lines show s.e.mean. (a) Shows results for ambulation; (b) results for repeated moves. Significant differences from saline are marked (*$P < 0.05$; **$P < 0.01$).

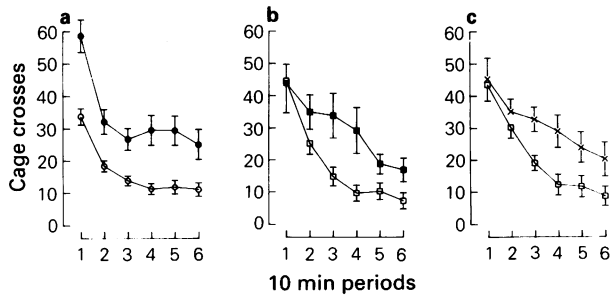


Figure 2 Locomotor activity in three groups of rats exposed to nicotine (0.4 $mg\,kg^{-1}$, s.c.) daily. Ambulation (cage crosses) is shown for 6 successive periods of 10 min each, beginning immediately after administration of drug or vehicle. Results are shown for nicotine administered systemically (0.4 $mg\,kg^{-1}$, s.c., $n = 8$, ●) and intracerebrally (8 μg infused bilaterally into ventral tegmental area (VTA), $n = 18$, ■), for cytisine administered intracerebrally (3.2 μg infused bilaterally into the VTA, $n = 13$, ×), and for saline administered systemically (○) or infused into VTA (□).

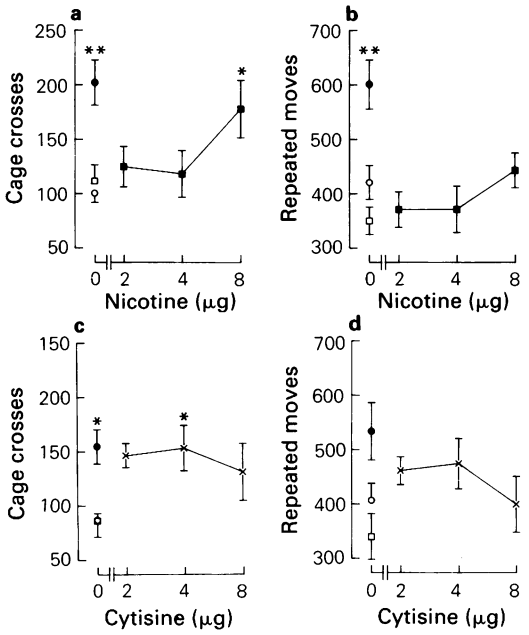


Figure 3 Locomotor activity in rats exposed to nicotine (0.4 mg kg^{-1}, s.c.) daily and tested with different doses of nicotine ($n = 18$, ■) or cytisine ($n = 9$, ×) infused into the ventral tegmental area (VTA). Activity is also shown after an infusion of saline into VTA (□), and after an injection of saline (○) or 0.4 mg kg^{-1} of nicotine (●) subcutaneously. Asterisks show significant differences from vehicles (*$P < 0.05$; **$P < 0.01$).

rats, nicotine (0.4 mg kg^{-1}, s.c.) increased cage crosses to 202 ± 21 as compared with 101 ± 9 after saline (Figure 3a). Nicotine (0.4 mg kg^{-1}, s.c.) also increased the number of repeated moves to 601 ± 45 from 421 ± 31 after saline ($t(85) = 4.38$, $P < 0.01$), but this effect was not present after infusions of nicotine into the VTA (Figure 3b). An infusion of the vehicle into the VTA did not significantly increase either cage crosses or repeated moves in comparison with results after systemically administered saline, either in the preceding experiments with nicotine or those with cytisine that are described next.

The effects of cytisine infused into the VTA were tested in different rats. There was an overall effect of drug treatments ($F(5, 40) = 3.45$, $P < 0.05$). Figure 3c shows that a 4 μg dose of cytisine increased the number of cage crosses to 153 ± 21 as compared with 86 ± 15 after vehicle ($t(40) = 2.75$, $P < 0.05$). Figure 2c shows that this effect was clearest during the 20–40 min after infusion. Cytisine in doses of 2 μg or 8 μg did not increase the number of cage crosses significantly, although inspection of Figure 2 shows that there were no substantial differences between the results after the three doses of cytisine. Systemic administration of nicotine (0.4 mg kg^{-1}, s.c.) increased ambulation to 155 ± 16 as compared with 87 ± 6 crosses after saline (Figure 3c). Neither an infusion of cytisine into the VTA, nor an injection of nicotine (0.4 mg kg^{-1}, s.c.) systemically, increased repeated moves (Figure 3d). In a separate experiment in other rats ($n = 14$), cytisine (3.2 μg) infused into the VTA increased cage crosses to 181 ± 18, as compared with 124 ± 13 after vehicle ($t(13) = 3.04$, $P < 0.01$).

An infusion of nicotine (2–8 μg) into the nucleus accumbens did not increase the number of cage crosses in comparison with infusion of vehicle into the same area. However, analysis of variance showed an overall effect due to drug treatments ($F(5, 55) = 13.2$, $P < 0.001$), and nicotine (0.4 mg kg^{-1}, s.c.) enhanced ambulation (Figure 4a). Similarly, Figure 4b shows that an infusion of cytisine (0.32–3.2 μg) into the nucleus accumbens did not alter locomotor activity in comparison with vehicle, whereas there was an overall effect due to drug treatments ($F(5, 50) = 9.27$, $P < 0.001$), and nicotine (0.4 mg kg^{-1}, s.c.) increased the number of cage crosses. An

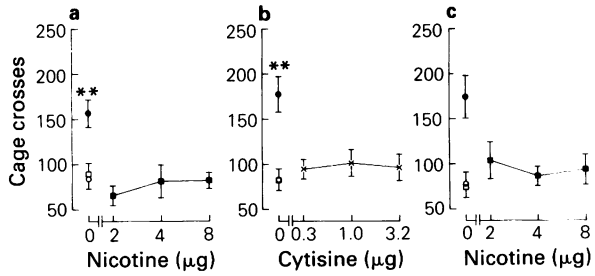


Figure 4 Locomotor activity in rats exposed to nicotine (0.4 mg kg^{-1}, s.c.) daily and tested with different doses of nicotine (■) or cytisine (×). (a) and (b) Show effects of an infusion of nicotine and cytisine into nucleus accumbens ($n = 11$–12). (c) Shows the effect of an infusion of nicotine into striatum ($n = 9$). Activity is also shown after an infusion of saline into striatum or accumbens (□), and after an injection of saline (○) or 0.4 mg kg^{-1} of nicotine (●) subcutaneously. Asterisks show significant differences from saline (**$P < 0.01$).

infusion of the vehicle intracerebrally did not significantly increase either cage crosses or repeated moves in comparison with results after systemically administered saline, either in the preceding experiments with infusions into the nucleus accumbens or with infusions into other regions as described below.

An infusion of nicotine (2–8 μg) into the striatum did not significantly increase the number of cage crosses in comparison with infusions of vehicle ($n = 9$). However, Figure 4c shows that in the same rats, there was an overall effect due to drug treatments ($F(5, 40) = 7.08$, $P < 0.001$), and nicotine (0.4 mg kg^{-1}, s.c.) increased cage crosses significantly.

Nicotine (2–8 μg) infused into the dorsal hippocampal formation did not increase ambulation ($n = 8$). For example, after nicotine (8 μg bilaterally), the number of cage crosses was 103 ± 14, as compared with 121 ± 23 after vehicle. However, there was an overall effect of drug treatments ($F(5, 35) = 7.64$, $P < 0.001$) and nicotine (0.4 mg kg^{-1}, s.c.) increased the number of cage crosses to 192 ± 33, as compared with 96 ± 18 after saline (s.c.). Similarly, cytisine (0.32–3.2 μg) did not increase locomotor activity when infused into the dorsal hippocampal formation ($n = 7$). After cytisine (3.2 μg bilaterally), the number of cage crosses was 112 ± 19, as compared with 103 ± 17 after vehicle. However, there was an overall effect of drug treatments ($F(5, 30) = 16.7$, $P < 0.001$) and nicotine (0.4 mg kg^{-1}, s.c.) increased the number of cage crosses to 231 ± 30, as compared with 109 ± 16 after saline (s.c.).

Nicotine (2–8 μg) infused into the motor thalamus did not increase ambulation ($n = 7$). Nicotine (8 μg bilaterally) produced the largest activity scores (116 ± 31, as compared with 81 ± 23 after vehicle), but the increase was not significant. However, there was an overall effect of drug treatments ($F(5, 30) = 6.87$, $P < 0.001$) and nicotine (0.4 mg kg^{-1}, s.c.) increased the number of cage crosses to 143 ± 24, as compared with 57 ± 16 after saline (s.c.). Similarly, cytisine (0.32–3.2 μg) did not increase locomotor activity when infused into the motor thalamus ($n = 5$). After cytisine (3.2 μg bilaterally), the number of cage crosses was 76 ± 24, as compared with 58 ± 13 after vehicle. However, there was an overall effect of drug treatments ($F(5, 20) = 7.03$, $P < 0.01$) and nicotine (0.4 mg kg^{-1}, s.c.) increased the number of cage crosses to 189 ± 40, as compared with 67 ± 11 after saline (s.c.).

Neither nicotine nor cytisine significantly increased the number of repeated moves when infused into any of the areas under investigation. For this reason, data for repeated moves have been presented only for nicotine administered systemically (Figures 1 and 3), and for infusions of nicotine or cytisine into the VTA (Figure 3).

Effects of mecamylamine

An injection of nicotine (0.4 mg kg^{-1}) systemically markedly increased cage crosses as shown by a two-factor analysis of

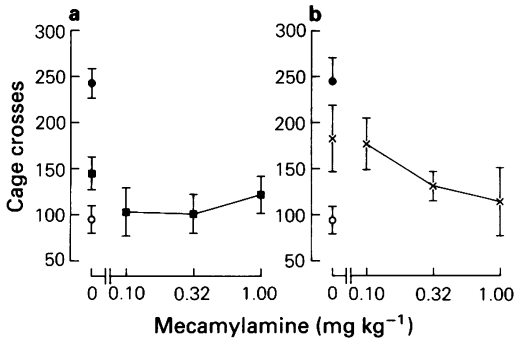


Figure 5 Locomotor activity in rats exposed to nicotine (0.4 mg kg^{-1}, s.c.) daily and tested after intracerebral infusions of nicotinic agonists (n = 6–7). (a) Effects of three doses of mecamylamine (s.c.) 30 min before an infusion of 8 μg nicotine bilaterally into the ventral tegmental area (VTA, ■). (b) Effects of three doses of mecamylamine (s.c.) 30 min before an infusion of 3.2 μg of cytisine bilaterally into VTA (×). In both (a) and (b), points above 0 show effects of subcutaneous saline (○), 0.4 mg kg^{-1} (s.c.) nicotine (●), and 8 μg nicotine (■) or 3.2 μg cytisine (×) infused into the VTA.

variance ($F(1, 77) = 54.3$, $P < 0.01$). There was no overall difference between the activity scores for two subgroups of the rats ($F(1, 11) = 1.19$). Nicotine (8 μg, Figure 5a) or cytisine (3.2 μg, Figure 5b) infused into the VTA of the subgroups slightly increased cage crosses and the overall effect was significant when compared with results after infusion of vehicle ($F(1, 77) = 6.13$, $P < 0.05$). Nicotine and cytisine did not increase locomotor activity when infused into the VTA after administration of any dose of mecamylamine (largest $F(1, 77) = 1.69$). There was no significant difference between the effects of mecamylamine in the two groups of rat (interaction $F(7, 77) = 0.91$).

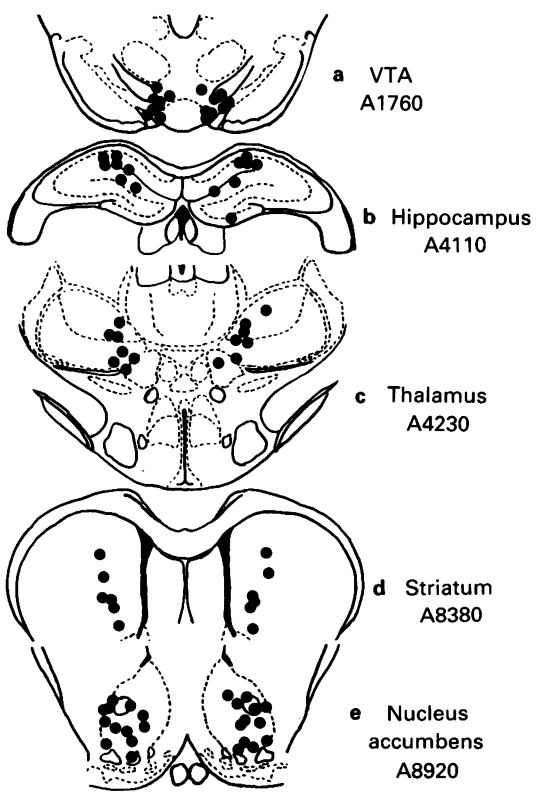


Figure 6 Composite diagrams showing the sites where nicotine was infused intracerebrally into the ventral tegmental area (VTA) (a), dorsal hippocampal formation (b), motor thalamus (c), striatum (d) and nucleus accumbens (e). Anterior coordinates taken from the atlas of Konig & Klippel (1963) are shown. In the case of VTA, only a representative selection of injection sites is shown for clarity. All infusions were bilateral.

Histology

Histological examination revealed that the sites into which nicotine was infused in the VTA were concentrated in an area ventral to the red nucleus and intermediate between substantia nigra and interpeduncular nucleus, with occasional encroachment on these areas. Figure 6 illustrates the location of these infusion sites. However, in the study with mecamylamine, the infusion sites for the agonists were located towards the rostral pole of the VTA. Within the nucleus accumbens, infusion sites were predominantly in the ventral and medial sector with some overlap into the ventral striatum (Figure 6). Striatal nicotine injections were confined to the medial striatum lying dorsal to the nucleus accumbens. Infusions into the hippocampus were confined exclusively to the dorsal hippocampus. Thalamic nicotine injection sites were located primarily in the ventral sector of the motor thalamus comprising the ventrolateral, ventral anterior and ventromedial thalamic nuclei (Figure 6). The sites into which cytisine was infused were similar to those for nicotine and histological details are not presented. Data for the small number of rats in which the infusion sites were not located in the targeted areas have been excluded throughout.

Discussion

Previous studies have established that nicotine can increase locomotor activity in rats and the present experiments investigate brain regions that may be involved. Locomotor activity was separated into two components, cage crosses that reflected ambulatory activity, and repeated moves that probably encompassed a wider range of behavioural changes (such as grooming and rearing onto the hind legs). The results with the two measures were different. Nicotine consistently increased cage crosses both when administered systemically and by intracerebral infusion into the VTA. Nicotine did not increase repeated moves reliably when administered systemically, and it never did so when given intracerebrally. Direct observation also suggested that nicotine (s.c.) did not produce stereotypy of the type seen with dopamine agonists. The findings support previous observations that nicotine can increase ambulatory activity but produces only low levels of stereotyped responses (Clarke & Kumar, 1983a, b; Lapin *et al.*, 1987), and emphasize again the importance of using recording methods that distinguish adequately between different components of locomotor activity (Krsiak *et al.*, 1970; Sahgal *et al.*, 1989). Crude measures of activity that confound ambulation with other behaviours may fail to detect effects of nicotine, especially after intracerebral injections.

The findings support the view that nicotine may increase locomotor activity by acting in the VTA. Alone among the brain areas examined, the VTA was sensitive to nicotine and cytisine, as shown by increases in ambulation after infusions of the drugs. The finding that the nicotinic antagonist mecamylamine blocked the effects of the agonists suggests the involvement of nicotinic receptors. A previous study also suggests that infusion of cytisine into the VTA may increase locomotor activity (Pert & Chiueh, 1986). The effects of nicotine and cytisine were generally similar, although cytisine acted at smaller doses which accords with its greater potency as an inhibitor of the binding of [^{3}H]-nicotine (Clarke *et al.*, 1985; Reavill *et al.*, 1988). The 8 μg dose of nicotine needed to enhance locomotor activity would have amounted to about 0.05 mg kg^{-1} if given systemically, which is close to the threshold for stimulation of locomotor activity (Figure 1). With both nicotine and cytisine, there was a delay between infusion and effect, the basis for which is not known. For these and other reasons, there is a need for more precise mapping of the neuroanatomical site through which nicotine acts; the inter-

peduncular nucleus and substantia nigra are near to the VTA, and both of these regions contain many binding sites for [^{3}H]-nicotine (Clarke *et al.*, 1985). In the experiments with mecamylamine, infusions of the agonists into the rostral pole of the VTA produced less marked increases in ambulation than when the drugs were infused into the central region of the VTA in the preceding experiments.

The neurochemical mechanisms by which nicotine produces locomotor stimulation are still unclear, but evidence has accumulated that some of its effects are mediated preferentially through the dopaminergic mesolimbic system. The importance of the dopaminergic projection from the VTA to the nucleus accumbens in locomotor activity is well established (Pijnenburg & Van Rossum, 1973; Kelly *et al.*, 1975). Degeneration of forebrain dopamine pathways produced by 6-hydroxydopamine reduces the numbers of binding sites for [^{3}H]-(−)-nicotine on dopamine cell bodies and terminals (Clarke & Pert, 1985). Release of dopamine *in vitro* and *in vivo* occurs with lower concentrations of nicotine in the nucleus accumbens than in the striatum (Imperato *et al.*, 1986; Rowell *et al.*, 1987; Rapier *et al.*, 1988). Evidence from studies of the nicotine discriminative stimulus and of rotational behaviour also support the view that nicotine interacts with dopamine mechanisms (Reavill & Stolerman, 1987). An important recent study shows that the locomotor activation produced when nicotine is administered systemically is accompanied by increased dopamine utilisation in some limbic areas, and that it is attenuated by 6-hydroxydopamine lesions of the nucleus accumbens (Clarke *et al.*, 1988). These findings suggest that the locomotor activity produced by nicotinic agonists infused into the VTA may also involve the mesolimbic dopamine system. It was surprising that infusions of the agonists into the nucleus accumbens did not stimulate ambulation. However, although acute systemic injection of nicotine can increase release and metabolism of dopamine in the nucleus accumbens, chronic exposure to nicotine (as in the present study) can attenuate the effect (Lapin *et al.*, 1989).

There appears to be a close relationship between binding sites for [^{3}H]-(−)-nicotine and dopamine nerve terminals in the striatum, but the experiments showed that focal infusion of nicotine into the striatum did not enhance locomotor activity. Striatal dopamine mechanisms are associated more with stereotyped behaviours than with locomotor activity which is mediated mainly through the mesolimbic system (Fog *et al.*, 1967; Kelly *et al.*, 1975).

The autoradiographic study of Clarke *et al.* (1985) showed that large areas of the thalamus, including the motor thalamus, exhibited some of the greatest numbers of binding sites for [^{3}H]-nicotine in rat brain. The motor thalamus, comprising the ventrolateral and ventral anterior nuclei, is the main thalamic relay centre between two major motor areas, the basal ganglia and cerebellum, which project to the motor cortex (Massion, 1976/77). However, the present results do not provide any evidence that nicotine produces locomotor stimulation by an action in the motor thalamus.

Recent evidence has shown that hippocampal projections to the nucleus accumbens play a role in motor stimulation in the rat, as blockade of glutamate mechanisms in the nucleus accumbens attenuates the locomotor activity produced by carbachol injection into the dentate gyrus (Mogenson & Nielsen, 1984). The hippocampus may also contribute to some behavioural effects of nicotine in the rat; an infusion of nicotine into the dorsal hippocampus of rats trained to discriminate nicotine from saline in two-bar operant conditioning procedures can produce effects like those of nicotine injected systemically (Meltzer & Rosecrans, 1981; Reavill & Stolerman, unpublished data). However, the present results indicate that an infusion of nicotine or cytisine into the dorsal hippocampus does not increase locomotor activity and thus, they suggest that different brain areas may mediate different behavioural effects of nicotine. However, for the present, only the VTA and the mesolimbic dopamine system have been linked to the activating effect of nicotine on ambulation.

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Presynaptic α_2-autoinhibition in a vascular neuroeffector junction where ATP and noradrenaline act as co-transmitters

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1 α_2-Autoinhibition of transmitter release was investigated in the largest rami caecales of the rabbit ileocolic artery. Vasoconstriction, elicited by electrical field stimulation or by exogenous agonists, was measured as an increase in perfusion pressure.

2 Short periods of electrical stimulation elicited monophasic vasoconstriction, whereas longer periods (>10 s) produced biphasic vasoconstriction. Prazosin had no significant effect on the first component of the biphasic vasoconstriction elicited by electrical stimulation, but did reduce the second component at higher frequencies. α,β-Methylene ATP significantly attenuated the first component whilst the second component was relatively resistant.

3 The α_2-adrenoceptor antagonist yohimbine did not change responses evoked by very short pulse trains (<2 s) but enhanced responses to longer pulse trains. When vasoconstriction was biphasic, both phases were potentiated by yohimbine.

4 The results indicate that the vasoconstriction elicited by brief trains of sympathetic nerve impulses is mainly or exclusively mediated by ATP, whereas at longer pulse trains a noradrenergic component comes into play. The potentiation produced by yohimbine is due to interruption of presynaptic α_2-adrenoceptor-mediated autoinhibition of transmitter release. The autoinhibition affects both purinergic and adrenergic components of sympathetic neurotransmission.

Introduction

Investigations have now revealed numerous sympathetic neuroeffector systems where adenosine 5'-triphosphate (ATP) and noradrenaline (NA) act as co-transmitters. These systems include the vas deferens (Sneddon *et al.*, 1983; Sneddon & Burnstock, 1984a; Stjärne & Åstrand, 1984; 1985), as well as a variety of blood vessels, such as rat tail artery (Sneddon & Burnstock, 1984b), rabbit and guinea-pig mesenteric arteries (Ishikawa, 1985), guinea-pig saphenous artery (Cheung & Fujioka, 1986), rabbit central ear artery (Kennedy *et al.*, 1986), dog mesenteric artery (Muramatsu, 1986) and rabbit saphenous artery (Burnstock & Warland, 1987). Apart from these *in vitro* observations NA-ATP co-transmission has also been shown in the vasculature of the pithed rat (Grant *et al.*, 1985; Flavahan *et al.*, 1985; Bulloch & McGrath, 1986; 1988a,b).

The role of presynaptic α_2-adrenoceptor-mediated autoinhibition of transmitter release in a system where ATP and NA act as co-transmitters has been studied previously in the rabbit ileocolic artery (von Kügelgen & Starke, 1985) and in the smaller jejunal branches of the mesenteric artery of the rabbit (Ramme *et al.*, 1987). The α_2-adrenoceptor antagonist yohimbine increased the electrically evoked release of previously stored [^{3}H]-NA in these tissues. In jejunal arteries, yohimbine increased also the excitatory junction potential (e.j.p.) amplitudes from the fourth e.j.p. onwards in a series of e.j.ps evoked by 15 pulses at 2 Hz. In the rabbit ileocolic artery, the vasoconstrictor responses produced by electric field stimulation (5 Hz 100 pulses) were significantly increased by yohimbine.

The aim of the present study was to investigate the potentiation of vasoconstriction by yohimbine under a variety of stimulation parameters, in order to verify whether this action was in fact due to interruption of the α_2-autoinhibition (which does not operate at short pulse trains: Story *et al.*, 1981; Auch-Schwelk *et al.*, 1983) and not to some other effect of yohimbine, such as sensitization of vascular smooth muscle to the contractile effect of NA (Auch-Schwelk *et al.*, 1983). Our second question was whether it was possible to investigate the role of α_2-autoinhibition on the release of NA and ATP separately.

A preliminary communication of these results has been published (Bulloch & Starke, 1988).

Methods

Rabbits of either sex (2–3 kg) were decapitated and the proximal section of the largest ramus caecalis arteriae ileocolicae (Barone *et al.*, 1973) was dissected out and cleared of fatty tissue. Side branches were tied up and ileocolic arteries were cannulated at both ends before being mounted vertically in a 4 ml organ bath. They were perfused at a constant rate of flow (2.7 ml min^{-1}) in such a way that the perfusate did not mix with the bathing fluid (von Kügelgen & Starke, 1985).

Vasoconstriction produced by drugs or electrical field stimulation (platinum electrodes; 0.3 ms pulse width; supramaximal current strength of 200 mA; variable frequencies and train lengths) was observed as an increase in perfusion pressure (monitored via a Statham P23 Db pressure transducer). The perfusate and bathing medium contained (mmol l^{-1}) NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, Na_2 EDTA 0.03 and ascorbic acid 0.3. The medium was saturated with 95% O_2–5% CO_2 and maintained at 37°C.

The arteries were allowed to equilibrate for 1 h before electrical stimulation or the addition of exogenous agents.

Drugs used were adenosine 5'-triphosphate dipotassium salt (ATP) (Sigma), α,β-methylene adenosine 5'-triphosphate lithium salt (mATP) (Sigma), (−)-noradrenaline bitartrate (Sigma), prazosin HCl (Pfizer), SK&F 104078 maleate (6-chloro-9-[(3-methyl-2-butenyl)oxyl]-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine) (gift from SK&F, U.S.A.), tetrodotoxin (TTX) (Sigma), yohimbine HCl (Roth). Stock solutions of drugs were prepared with distilled water. NA, ATP and mATP were added only to the organ bath. The antagonists prazosin, SK&F 104078 and yohimbine were added to both the perfusing fluid and the organ bath.

The means and s.e.mean are given throughout. Means were compared by use of paired Student's *t* test. *P* values of 0.05 or

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less were considered to be significant. n is the number of preparations.

Results

The effect of yohimbine on peak responses to electrical field stimulation

Stimulation of perfused ileocolic arteries produced reproducible vasoconstrictions observed as increases in perfusion pressure. These vasoconstrictions were pulse number and frequency-dependent. Yohimbine ($0.3\,\mu mol\,l^{-1}$) (Figure 1) increased by up to 100% vasoconstrictor responses to 100 or 200 pulses at 10 Hz and 100 pulses at 5 Hz. Yohimbine did not change the responses elicited by 5 or 10 pulses at these frequencies or by 6 or 8 pulses at 40 Hz. Hence, the potentiation by yohimbine was only observed when train lengths $\geqslant 10$ s were employed.

Analysis of the components of the nerve-mediated vasoconstrictor response

When the recording apparatus was run at a faster chart speed, the responses to pulse trains of up to 10 s duration appeared to be monophasic, whereas it was possible to distinguish two phases in the response to 20 s pulse trains (Figure 2). In most tissues the two phases were separated by an intervening dip in tension, or at higher frequencies, by a plateau. The first phase of the contraction reached a peak within 5–9 s, with the second phase peaking within 18–23 s after onset of stimulation. The relative proportion of the two phases was frequency-dependent. At lower frequencies (5 Hz) the first phase was nearly always larger than the second phase but at higher frequencies (20 Hz) the latter dominated the response.

We used the P_{2x}-purinoceptor desensitizing agent, mATP and the α_1-adrenoceptor antagonist prazosin, in an attempt to identify the (main) transmitter for each of the two components of the vasoconstriction. Selected responses from individual experiments are shown in Figure 3. mATP itself elicited marked but transient vasoconstrictions when given at five cumulative concentrations of $3\,\mu mol\,l^{-1}$ each (not shown). Subsequently, the first phase of the vasoconstrictor response

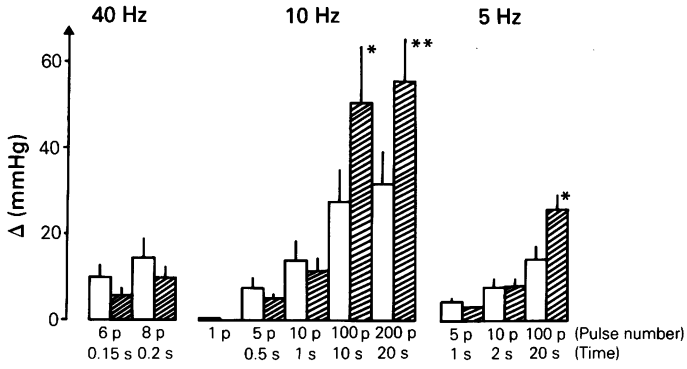


Figure 1 The effect of yohimbine on peak vasoconstrictor responses. Arteries were stimulated with different pulse numbers first at 40 Hz frequency, then at 10 Hz and finally at 5 Hz in the order shown in the graph. Intervals between stimulation periods were 5 min. After an initial sequence in the absence of yohimbine (open columns), the sequence was repeated 10 min after the addition of yohimbine ($0.3\,\mu mol\,l^{-1}$) to the perfusate (hatched columns). Columns represent means of seven experiments; bars show s.e.mean. Significant differences from pre-yohimbine values, for a paired t test: $^*P < 0.05$, $^{**}P < 0.01$. In control tissues (stimulation repeated in the absence of yohimbine) there were no significant differences between the corresponding responses within each tissue. Only two out of seven tissues responded to one pulse. This response was then abolished by yohimbine.

to electrical stimulation was greatly reduced whereas the second phase persisted (Figure 3Bb). Prazosin, in contrast, reduced the second phase with little effect on the first (Figure 3Ab). The combination of prazosin and mATP abolished vasoconstrictor responses almost entirely (Figure 3Ac).

A statistical evaluation of these experiments is presented in Figures 4 and 5. mATP consistently attenuated the first component as well as the monophasic responses to trains of up to 10 s duration but had little effect on the second component (Figure 4). Prazosin tended to reduce the second phase, although this effect was significant only for stimulation at 20 Hz. Prazosin did not change the first phase (except for a reduction of monophasic responses to 12 pulses delivered at 40 Hz) (Figure 5). When mATP was given after prazosin, all responses were abolished (not shown, but see Figure 3Ac).

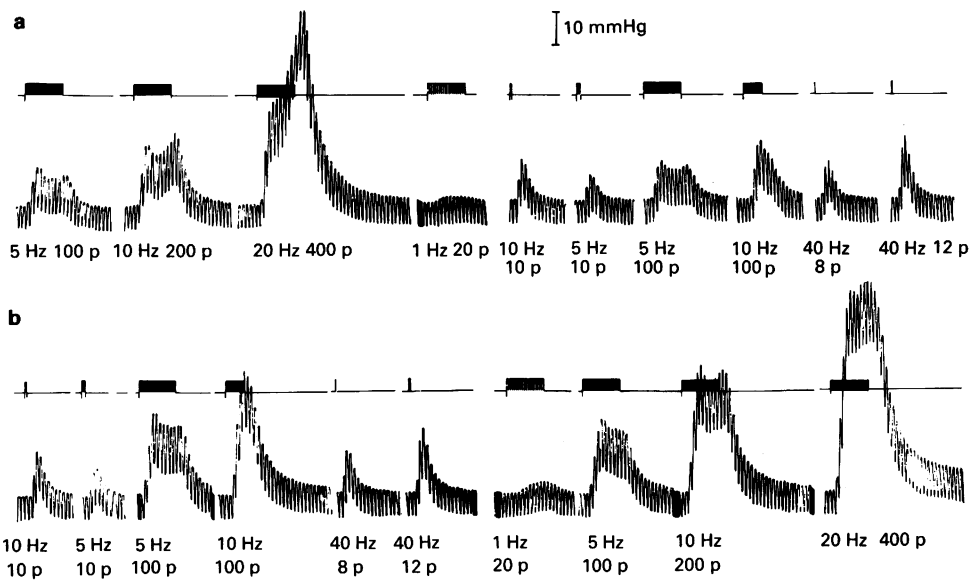


Figure 2 Mono- and biphasic vasoconstrictor responses of the ileocolic artery and the effect of yohimbine. Vessels were stimulated by trains of pulses and in the order defined in the graph. Duration of stimulation is also illustrated above each response. Intervals between stimulation periods were 5 min. The fluctuations in perfusion pressure were due to the pulsatile movements of the pump. (a) Shows the control responses to the first cycle of stimulation, and (b), the responses to the second cycle of stimulation carried out in the presence of yohimbine ($0.3\,\mu mol\,l^{-1}$). Yohimbine was added 10 min before the cycle of stimulations was repeated.

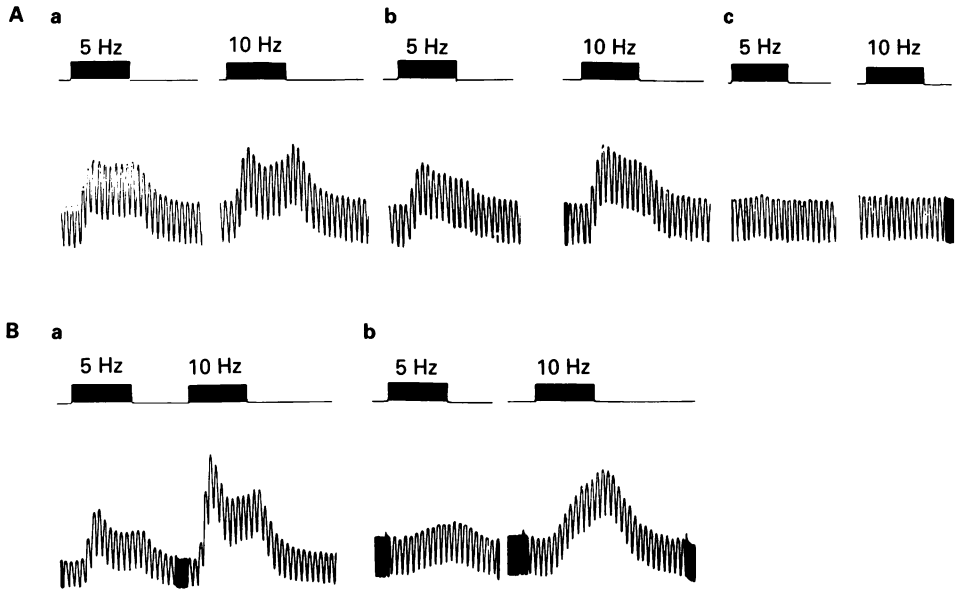


Figure 3 Biphasic vasoconstrictor responses of the ileocolic artery and the effect of prazosin and α,β-methylene ATP (mATP). Vessels were electrically stimulated according to the protocol illustrated in Figure 2, but only the responses to 5 Hz and 10 Hz, 20 s each, are shown here. (A) and (B) represent experiments on two different tissues. (Aa) and (Ba) show control responses to the first cycle of stimulations. (Ab) Shows responses to the second cycle of stimulations, carried out in the presence of prazosin ($0.3\,\mu mol\,l^{-1}$), and (Ac) responses to a third cycle of stimulations, after exposure to mATP ($15\,\mu mol\,l^{-1}$), given as five additions of $3\,\mu mol\,l^{-1}$ over 30 min (see von Kügelgen & Starke, 1985) and then washed out 5 min before electrical stimulation (prazosin still present). (Bb) Shows responses after exposure to mATP ($15\,\mu mol\,l^{-1}$) alone.

The effect of yohimbine on biphasic responses

It was with this biphasic profile in mind that the effect of yohimbine was re-analysed to take into consideration the components of co-transmission. Figures 2 and 6 show that at stimulations of 10 or 20 s duration, yohimbine potentiated both phases of the responses at each frequency, except for the second component at 400 pulses 20 Hz (and for the very small responses to 1 Hz). In contrast, none of the responses to shorter stimulation periods were enhanced. In particular, yohimbine did not change the vasoconstriction elicited by 8 or 12 pulses at 40 Hz even though these responses were of a comparable size to those obtained at 5 Hz 100 pulses and 10 Hz 100 pulses.

The effect of prazosin ($0.3\,\mu mol\,l^{-1}$) in the presence of yohimbine ($0.3\,\mu mol\,l^{-1}$) (Figure 7) on the biphasic response elicited by electrical field stimulation at 5 Hz 100 pulses, was compared to its effect in the absence of yohimbine (Figure 5). In tissues exposed to yohimbine ($0.3\,\mu mol\,l^{-1}$), prazosin ($0.3\,\mu mol\,l^{-1}$) significantly attenuated both components of the response.

The effect of yohimbine on responses to exogenously administered agonists

The introduction of NA ($3\,\mu mol\,l^{-1}$–$30\,\mu mol\,l^{-1}$) to the bathing fluid, produced concentration-dependent vasoconstrictions. These responses were reproducible within any one

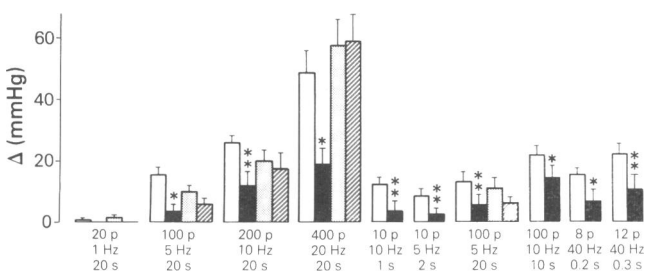


Figure 4 The effect of α,β-methylene ATP (mATP) on the two phases of the vasoconstrictor responses in the ileocolic artery. Arteries were stimulated according to the protocol illustrated in Figure 2, first before the addition of mATP (open columns – first component; stippled columns - second component where present) and then after desensitisation with mATP ($15\,\mu mol\,l^{-1}$) (solid columns – first component; hatched columns – second component where present). The first phase was taken as the peak constrictor height which occurred 5–9 s after the onset of stimulation, and the second phase, as the peak height between 18–23 s. Columns represent mean values of six experiments; bars show s.e.mean. Significant differences from pretreatment values, for paired t test: $^*P < 0.05$, $^{**}P < 0.01$. In control tissues (stimulation repeated in the absence of mATP) there were no significant differences between the corresponding responses within each tissue.

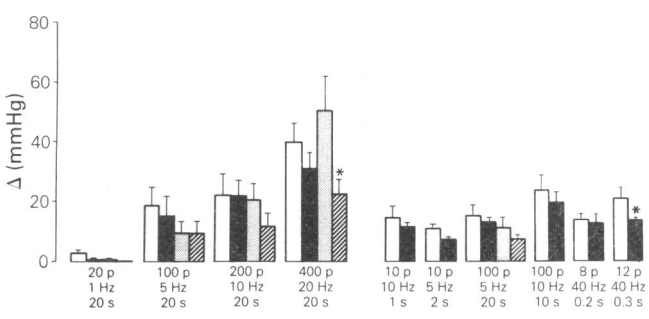


Figure 5 The effect of prazosin on the two phases of the vasoconstrictor response. Arteries were stimulated according to the protocol illustrated in Figure 2, first in the absence of prazosin (open columns – first component; stippled columns – second component where present) and then in the presence of prazosin ($0.3\,\mu mol\,l^{-1}$) (solid columns – first component; hatched columns – second component where present). The subsequent administration of α,β-methylene ATP (mATP, $15\,\mu mol\,l^{-1}$) completely abolished the responses (not shown). The first phase was taken as the peak constrictor height which occurred 5–9 s after the onset of stimulation, and the second phase, as the peak height between 18–23 s. Columns represent mean values of six experiments; bars show s.e.mean. Significant differences from pretreatment values, for paired t test: $^*P < 0.05$, $^{**}P < 0.01$. In control tissues (stimulation repeated in the absence of prazosin) there were no significant differences between the corresponding responses within each tissue.

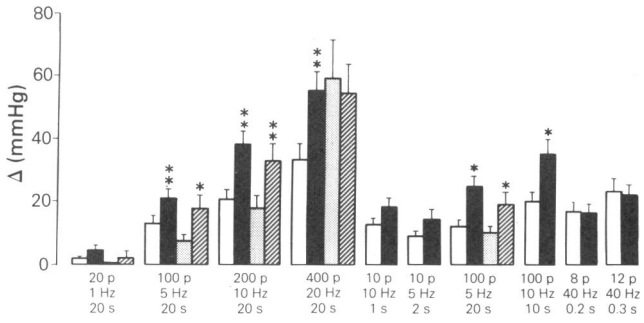


Figure 6 The effect of yohimbine on the two phases of the vasoconstrictor response. Arteries were stimulated according to the protocol illustrated in Figure 2, first in the absence (open columns – first component; stippled columns – second component where present) and then in the presence of yohimbine (0.3 μmol l^{-1}) (solid columns – first component; hatched columns – second component where present). The first phase was taken as the peak constrictor height which occurred 5–9 s after the onset of stimulation, and the second phase as the peak height between 18–23 s. Columns represent values of nine experiments; vertical bars show s.e.mean. Significant differences from pretreatment values, for paired t test: * $P < 0.05$, ** $P < 0.01$. In control tissues (stimulation repeated in the absence of yohimbine) there was no significant difference between the corresponding responses within each tissue.

preparation and were not affected by yohimbine (0.3 μmol l^{-1}) (Table 1).

ATP (300 μmol l^{-1}) administered to the bathing fluid at intervals of 30 min, to avoid problems of tachyphylaxis, produced transient vasoconstrictions which were unaffected by yohimbine (0.3 μmol l^{-1}) (Table 1).

The selectivity of mATP was also tested against exogenously administered NA and ATP. The vasoconstrictor responses produced by NA (3 μmol l^{-1}–30 μmol l^{-1}) were unaffected by mATP (15 μmol l^{-1}) (Figure 8), but those produced by ATP (300 μmol l^{-1}) were abolished (not illustrated).

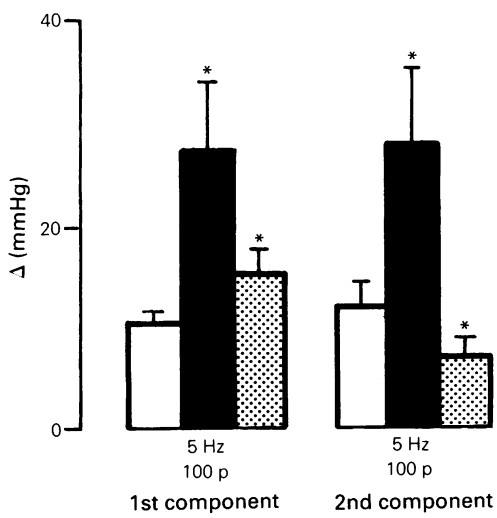


Figure 7 The effect of prazosin administered in the presence of yohimbine on the two phases of the vasoconstrictor response elicited by 5 Hz 100 pulses. The protocol followed here was different from that adopted in previous figures. A frequency-response curve was obtained first (5, 10, and 20 Hz, all for 20 s) and then stimulation by 100 pulses at 5 Hz (20 s) was repeated every 5 min for the remainder of the experiment. Shown are the last responses to 100 pulses, 5 Hz, before drug addition (open columns), responses 10 min after addition of yohimbine (0.3 μmol l^{-1}) (solid columns), and responses 10 min after addition of prazosin (0.3 μmol l^{-1}), still in the presence of yohimbine (stippled columns). Columns represent mean values of five experiments. Significant difference from preceding values, for paired t test: * $P < 0.05$, ** $P < 0.01$. In control tissues (stimulations repeated in the absence of yohimbine and prazosin) there was no significant difference between the corresponding responses within each tissue.

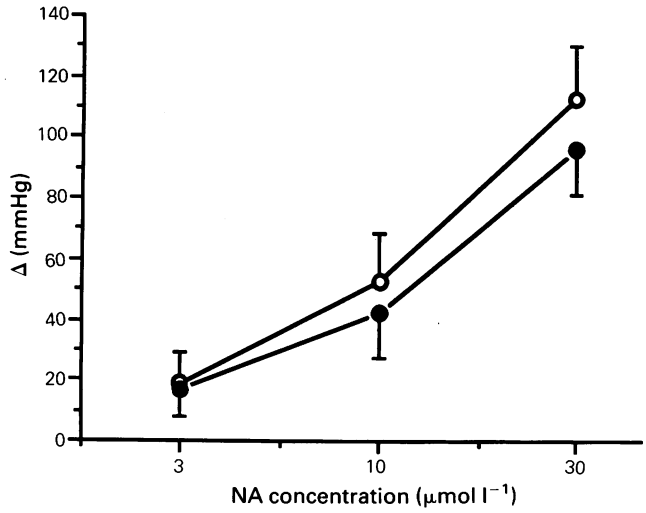


Figure 8 The effect of α,β-methylene ATP (mATP) on vasoconstrictor responses to exogenous noradrenaline (NA). A concentration-response curve was constructed before (○) and after treatment (●) with mATP (15 μmol l^{-1}; given as five additions of 3 μmol l^{-1} over 30 min and then washed out 5 min before the addition of NA). Values are means of five experiments; vertical lines show s.e.mean. In mATP-treated tissues and in control tissues (no treatment with mATP) there was no significant difference (paired t test) between the corresponding responses within each tissue.

The effect of other agents on vasoconstrictor responses to nerve stimulation

The addition of tetrodotoxin (TTX) (0.5 μmol l^{-1}) abolished nerve-mediated vasoconstrictions at all frequencies studied (results not shown).

The effect of the 'selective' postjunctional α_2-adrenoceptor antagonist SK&F 104078 (Ruffolo *et al.*, 1987) was also tested against nerve-mediated vasoconstrictions, as well as those elicited by exogenous NA. SK&F 104078 (3 μmol l^{-1}) potentiated the first component of the electrically-induced vasoconstrictor response, but an attenuation was observed for the second component at 20 Hz (Figure 9). SK&F 104078 (3 μmol l^{-1}) attenuated the response to exogenous NA (10–30 μmol l^{-1}) (Table 1).

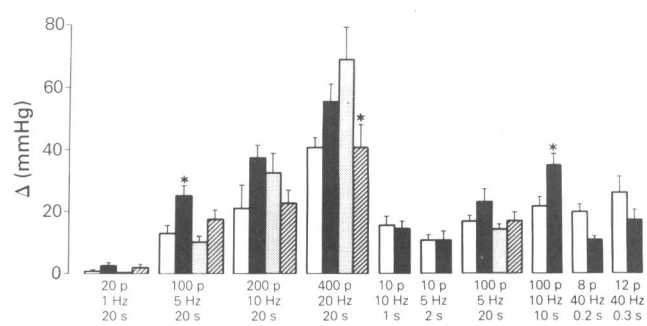


Figure 9 The effect of SK&F 104078 on the two phases of the vasoconstrictor response. Arteries were stimulated according to the protocol illustrated in Figure 2, first in the absence (open columns – first component; stippled columns – second component where present) and then in the presence of SK&F 104078 (3 μmol l^{-1}) (solid columns – first component; hatched columns – second component where present). The first phase was taken as the peak constrictor height which occurred 5–9 s after the onset of stimulation, and the second phase as the peak height between 18–23 s. Columns represent mean values of six experiments; vertical bars show s.e.mean. Significant difference from pretreatment values, for paired t test: * $P < 0.05$, ** $P < 0.01$. In control tissues (stimulation repeated in the absence of SK&F 104078) there was no significant difference between the corresponding responses within each tissue.

Table 1 The effects of antagonists on agonist-induced vasoconstrictor responses of the ileocolic artery

	Increase in perfusion pressure (Δ mmHg) *elicited by*			
	NA (μmol l^{-1}			ATP (μmol l^{-1})
	3	*10*	*30*	*300*
Before yohimbine	8.4 ± 3.5	23.8 ± 10.4	56.7 ± 15.3	18.3 ± 6.3
Yohimbine (0.3 μmol l^{-1}) present	8.9 ± 3.6	20.3 ± 7.1	54.6 ± 16.5	19.6 ± 7.6
Before SKF	16.2 ± 6.3	48.1 ± 14.1	85.9 ± 13.6	—
SKF (3 μmol l^{-1}) present	7.4 ± 4.0	20.1 ± 12.1*	60.9 ± 12.7*	—

Noradrenaline (NA) and ATP were added at 30 min intervals. In any one tissue the same concentration of agonist was used throughout. Agonist additions were made until consistent responses were obtained and the responses to the final 3 control additions of agonist were averaged. The antagonists yohimbine or SK&F 104078 were introduced both to the perfusing fluid and to the organ bath, after these control additions and 10 min before the final addition of agonist. Values are means ± s.e.mean of five experiments. Significant differences from pretreatment values (paired t test): * $P < 0.05$, ** $P < 0.01$. In control tissues (repeated agonist additions in the absence of antagonists) there was no significant difference between the corresponding responses within each tissue.

Discussion

The rabbit ileocolic artery is one blood vessel in which NA-ATP co-transmission has been demonstrated (von Kügelgen & Starke, 1985). Our experiments show that, for longer pulse trains (train lengths of 20 s), two phases of the contraction can be differentiated; the first one (and the only one detected at short pulse trains) was greatly reduced by mATP, and hence mainly mediated by ATP, the second one was mATP-resistant, but at least partly sensitive to prazosin, and hence had a considerable noradrenergic component (Figures 3, 4 and 5). When interpreting the data, it should be noted that the measured height of at least the second of the two phases comprises both purinergic and adrenergic components, with the second component being superimposed upon the first one. This overlap (with the purinergic component dominating the first phase and the adrenergic component dominating the second phase) is probably responsible for the fact that prazosin reduced significantly only the second phase of the response to 20 Hz, at least in the absence of other drugs; in the presence of yohimbine, it significantly reduced both phases of the response to 5 Hz stimulation as well (Figure 7).

Biphasic vasoconstrictor responses have also been obtained in other vascular neuroeffector sites where NA and ATP act as co-transmitters, with each of the two phases being attributed to a different co-transmitter (Kennedy *et al.*, 1986; Burnstock & Warland, 1987; Machaly *et al.*, 1988).

Another example of a neuroeffector system where ATP and NA act as co-transmitters is the vas deferens (Sneddon *et al.*, 1983; Sneddon & Burnstock, 1984a; Stjärne & Astrand, 1984; 1985). The mechanical response of the vas deferens to sympathetic nerve stimulation is also biphasic; a rapid twitch contraction is followed by a slow tonic contraction. Each of the two components seems to be due to both NA and ATP with only quantitative differences in the contribution of either (Stjärne & Åstrand, 1985). This biphasic profile of the mechanical response is therefore comparable to that identified in the vasculature.

Yohimbine has been shown to potentiate electrically-elicited vasoconstrictor responses (von Kügelgen & Starke, 1985). Although the increase in neurogenic vasoconstriction may be due to the interruption of presynaptic α_2-adrenoceptor-mediated autoinhibition, a postsynaptic potentiation by yohimbine has also been observed (Auch-Schwelk *et al.*, 1983). If a postsynaptic mechanism such as the sensitization of the vascular smooth muscle were responsible for the enhancement of neurogenic vasoconstriction, then the effect of yohimbine should be observed at all parameters of stimulation studied. However, the present results indicate an exclusively presynaptic action of yohimbine, since potentiation was only observed when long trains of stimulation were employed but not when trains were too short for autoinhibition (for example 5 Hz 10 pulses, 10 Hz 10 pulses, 40 Hz 8 pulses and 40 Hz 12 pulses; see Story *et al.*, 1981; Auch-Schwelk *et al.*, 1983). In addition, yohimbine had no effect on contractile response to exogenous NA or ATP (Table 1). An ongoing autoinhibition is, in fact, a prerequisite for the effect of yohimbine, and this effect reveals marked postsynaptic sequelae of a primary presynaptic control mechanism.

The blockade of α_2-autoinhibition by yohimbine potentiated both phases of the responses in the ileocolic artery (except for the second component at 20 Hz (Figure 6)). This could indicate that the release of both ATP and noradrenaline is subject to α_2-autoinhibition, in agreement with recent findings in the dog mesenteric artery (Muramatsu *et al.*, 1989).

However, yohimbine changed the vasoconstrictor response not only in size but, apparently, qualitatively as well. In the presence of yohimbine, prazosin attenuated both phases of the response to 5 Hz 100 pulses (Figure 7) and this contrasts to its lack of any significant effect on the same response in the absence of yohimbine (Figure 5). In other words, yohimbine changed a response which was nearly all purinergic (Figure 5) to one which was predominantly adrenergic (Figure 7). This observation opens up the interesting possibility that yohimbine preferentially increased the release of NA. Hence, the storage vesicles in the sympathetic nerve terminals may differ in their ATP/NA ratio, and presynaptic α_2-autoinhibition may decrease (and its interruption enhance) mainly transmitter release from NA-rich vesicles. Another recent observation in our laboratory supports this possibility. (Bulloch & Starke, unpublished observations), where vasoconstriction in the rabbit ileocolic artery was elicited by nicotine or 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) which act by releasing transmitter from sympathetic nerve terminals through presynaptic nicotinic receptors (see Starke, 1977). The responses to these drugs, although comparable in size to those produced by electrical stimulation at 5 Hz 100 pulses (in the absence of yohimbine) were almost entirely blocked by prazosin (0.3 μmol l^{-1}), again indicating differential storage and release of NA and ATP. Recent work (Trachte, 1985; 1988; Ellis & Burnstock, 1989) has also raised questions on methods of storage and release of NA and ATP.

This study also highlighted the difficulties encountered when analysing prejunctional effects of an antagonist like yohimbine, which is also active at postjunctional α_2-adrenoceptors. It has been proposed that postjunctional α_2-adrenoceptors occur in the vicinity of the postganglionic sympathetic varicosities in the vasculature in the pithed rabbit (McGrath *et al.*, 1982; Bulloch *et al.*, 1987) and the pithed rat (Flavahan *et al.*, 1985). Vasoconstrictor responses of the rabbit ileocolic artery to exogenous NA are mediated exclusively by α_1-adrenoceptors, since they are abolished by relatively low concentrations of prazosin but not attenuated by yohimbine (von Kügelgen & Starke, 1985; and present study). However, released endogenous NA might still exert some of its vasoconstrictor effect by acting on smooth muscle α_2-adrenoceptors located close to the sites of release. If so, the adrenergic component of co-transmission would be greater than revealed by prazosin, and the degree of α_2-autoinhibition also would be greater than revealed by yohimbine, because the latter would block the α_2-adrenoceptor-mediated part of the vasoconstrictor response.

It was for this reason that the 'selective' postjunctional α_2-adrenoceptor antagonist SK&F 104078 (Ruffolo *et al.*, 1987) was employed to study any postjunctional α_2-adrenoceptor component in neurogenic vasoconstriction. The concentration of SK&F 104078 used was $3\,\mu mol\,l^{-1}$ and was therefore under the $10\,\mu mol\,l^{-1}$ concentration at which the antagonist has been postulated to cease to be selective for postjunctional α_2-adrenoceptors (Ruffolo *et al.*, 1987). However, at $3\,\mu mol\,l^{-1}$, SK&F 104078 potentiated the first component of the electrically-mediated response indicating possibly blockade of prejunctional α_2-adrenoceptors (Figure 9). The second component of the response to 20 Hz 400 pulses was, in contrast, attenuated. Interestingly, this was the one set of stimulation parameters, in stimulation periods of sufficient length, where the second component was not potentiated by yohimbine. Hence, there is indeed some evidence that postjunctional α_2-adrenoceptors may come into play when longer pulse trains are delivered at higher frequencies. However, non-selective (α_1-adrenoceptor) antagonism by SK&F 104078 cannot be ruled out. Although yohimbine had no effect on responses to exogenous NA (Table 1), responses to exogenous NA ($10–30\,\mu mol\,l^{-1}$) were significantly reduced by SK&F 104078 (Table 1). There is no evidence from the present study to support the proposal that SK&F 104078 is selective for postjunctional α_2-adrenoceptors. This lack of selectivity is in agreement with recent findings (Connaughton & Docherty, 1988).

In conclusion, NA and ATP are co-transmitters of neurogenic vasoconstriction in the rabbit ileocolic artery. When the sympathetic nerves of the artery are stimulated by relatively long pulse trains (more than 10 s), the vasoconstrictor response becomes biphasic. The initial phase is predominantly or exclusively purinergic whereas the second phase contains a considerable adrenergic component. Both the release of NA and the release of ATP are subject to presynaptic α_2-adrenoceptor-mediated autoinhibition. However, the composition of the co-transmitter mixture may change when autoinhibition is interrupted.

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Modulation of seizure susceptibility in the mouse by the strychnine-insensitive glycine recognition site of the NMDA receptor/ion channel complex

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1 In order to determine whether the strychnine-insensitive glycine modulatory site on the N-methyl-D-aspartate (NMDA) receptor/ion channel complex is fully activated *in vivo*, the ability of the selective glycine receptor agonist, D-serine, to modulate seizure susceptibility in the mouse has been examined.

2 D-Serine (10–200 μg per mouse, i.c.v.) dose-dependently increased the potency of NMDLA in inducing seizures in Swiss Webster mice by approximately 3 fold. L-Serine was without significant effect.

3 The potency of pentylenetetrazol in inducing seizures was also enhanced by D-, but not L-serine, although the magnitude of the shift (1.6 fold) was considerably less than for NMDLA.

4 Similar doses of D-serine were also able to block the anticonvulsant effect of the non-selective glycine receptor antagonist, kynurenic acid, against seizures induced by NMDLA, but were without effect on the anticonvulsant effect of the competitive NMDA receptor antagonist, 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP).

5 D-Serine completely antagonized the protective effect of the selective glycine receptor antagonist, 7-chlorokynurenic acid, against sound-induced seizures in DBA/2 mice, but was less effective in this model against the less selective antagonist, kynurenic acid.

6 The results indicate that *in vivo*, NMDA receptors are not maximally potentiated by endogenous glycine and suggest an important involvement of the glycine modulatory site on the NMDA receptor/ion channel complex in the pathophysiology of epilepsy.

Introduction

Electrophysiological studies indicate that glycine is able to potentiate the response evoked by N-methyl-D-aspartic acid (NMDA) in neurones cultured from rat cerebral cortex (Johnson & Ascher, 1987). Glycine is also able to enhance, by a strychnine-insensitive mechanism, the specific binding of radioligands interacting in a non-competitive manner with the ion channel of the NMDA receptor complex (Wong *et al.*, 1987; Reynolds *et al.*, 1987; Bonhaus *et al.*, 1987). Taken with the nearly identical distribution of NMDA-sensitive, [^{3}H]-glutamate binding sites and of strychnine-insensitive, [^{3}H]-glycine binding sites (Monaghan & Cotman, 1985; Bristow *et al.*, 1986; Hudson & Woodruff, 1988), these results suggest the presence of an allosteric glycine recognition site located in the NMDA receptor/ion channel complex (Bowery, 1987). As only low concentrations of glycine are required to occupy this site, and the free amino acid is present in appreciable amounts in body fluids (Ferraro & Hare, 1985), it might seem unlikely that glycine would have any significant influence on NMDA-mediated neurotransmission *in vivo* (Johnson & Ascher, 1987). However, in the present paper we provide evidence that this modulatory system is not normally fully activated since D-serine, an agonist at the glycine modulatory site (Wong *et al.*, 1987; Reynolds *et al.*, 1987; Kemp *et al.*, 1988) is able to potentiate seizures induced in the mouse by NMDLA at doses similar to those antagonizing the anticonvulsant effects of the selective glycine receptor antagonist, 7-chlorokynurenic acid (Kemp *et al.*, 1987; 1988).

Methods

Animals

Male Swiss Webster mice (20–30 g) and audiogenic-seizure-prone DBA/2 mice (21–23 days of age, 5–9 g) were obtained from Bantin and Kingman, Hull. Animals were housed in groups of five under a 12 h light/dark cycle (lights on at 07 h 00 min) with food and water available *ad libitum*.

Intracerebroventricular injections

For intracerebroventricular (i.c.v.) injections, mice were anaesthetized with ether and the skull exposed. Injections (5 μl) were made at lambda with a 3.5 mm long, 27 gauge needle attached to a 10 μl Hamilton syringe and the wound sealed with epoxy resin. Injections of dye by this procedure led to a uniform distribution of injectate throughout the ventricular system within 10 min.

Seizures induced by N-methyl-DL-aspartic acid and pentylenetetrazol

Mice were injected subcutaneously with N-methyl-DL-aspartic acid (NMDLA, 500 mg kg^{-1}) or pentylenetetrazol (PTZ, 120 mg kg^{-1}) and latency to the tonic extension of the forepaws noted. Animals not convulsing within 30 min of injection were considered protected and given a maximum latency score of 30 min. Putative antagonists of seizures were given i.c.v. 15 min before the convulsant and the ED_{50} dose (dose protecting 50% of animals) was determined by probit analysis.

Audiogenic seizures

Tonic seizures were induced in male DBA/2 mice at 23 days of age by exposure to an electric bell (125 dB, 1.4 kHz) for 30 s. Protection was defined as the absence of a tonic seizure during the exposure to sound. Vehicle-treated animals convulsed within 10 s of sound onset.

Drugs

Kynurenic acid, N-methyl-DL-aspartic acid, pentylenetetrazol and D- and L-serine were obtained from Sigma (Poole, Dorset). 3-((+)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) was purchased from Tocris Neuramin, Buckhurst Hill, 7-chlorokynurenic acid was synthesized by the Department of Medicinal Chemistry, Merck Sharp & Dohme

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Research Laboratories, Terlings Park, Harlow, Essex. All compounds were dissolved in 0.9% NaCl with the aid of HCl or NaOH and the pH adjusted to 6.5–7.5 with acid or base as appropriate.

Results

Effect of D- *and* L-*serine on seizures induced by NMDLA and PTZ*

The administration of NMDLA to mice induces wild running, barrell rolling and, at high doses (>200 mg kg^{-1}, s.c.) tonic seizures and death. NMDLA (50–500 mg kg^{-1}) dose-dependently increased the incidence of tonic seizures and decreased the latency to convulse (Figure 1a). When D-serine (50–200 μg per mouse) was injected i.c.v., no seizure activity was seen over the following 45 min, but when given 15 min before NMDLA, the dose-response relationship to NMDLA was shifted to the left. After a 100 μg dose of D-serine, the ED_{50} for the induction of convulsions by NMDLA decreased by more than 3 fold from 245 mg kg^{-1} to 80 mg kg^{-1} (Figure 1a). Increasing the dose of D-serine to 200 μg per mouse had no greater effect than 100 μg D-serine (results not shown). In contrast, L-serine (100 μg per mouse) was without significant effect on either the latency to convulse or the number of animals convulsing in response to NMDLA (Figure 1a).

Pentylenetetrazol (30–120 mg kg^{-1}) also dose-dependently induced tonic seizures in the mouse and the dose-response relationship was again shifted to the left by administration of D-, but not L-serine (Figure 1b). However, the magnitude of the shift was considerably less than that seen when NMDLA was the convulsant, the ED_{50} dose of PTZ decreasing from 93.7 mg kg^{-1} to 60.4 mg kg^{-1} following administration of 200 μg of D-serine.

Effects of D-*serine on the blockade of NMDLA-induced seizures by kynurenic acid and CPP*

In addition to blockade of non-NMDA excitatory amino acid receptors, kynurenic acid can act as an antagonist of the potentiating effects of glycine on NMDA-mediated neurotransmission (Kemp *et al.*, 1987; Watson *et al.*, 1988). When given i.c.v., 15 min beforehand, kynurenic acid dose-dependently increased the latency to convulse to a supramaximal dose of NMDLA (500 mg kg^{-1}, s.c.; Figure 2a). In order to investigate the involvement of the glycine/NMDA receptor in the anticonvulsant effect of kynurenic acid, D-serine (30–100 μg per mouse) was administered i.c.v. concomitantly with a submaximal anticonvulsant dose of kynurenic acid (25 μg per mouse): the increase in the latency to convulse induced by kynurenic acid was antagonized dose-dependently by D-serine (Figure 3a). The competitive NMDA receptor antagonist, CPP (3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, Davies *et al.*, 1986) also dose-dependently antagonized NMDLA-induced seizures over the dose range of 0.015–0.15 μg per mouse, i.c.v. (Figure 2a). However, D-serine (3–300 μg per mouse) failed to antagonize the protective effect of a submaximal dose of CPP (0.075 μg per mouse, Figure 3b).

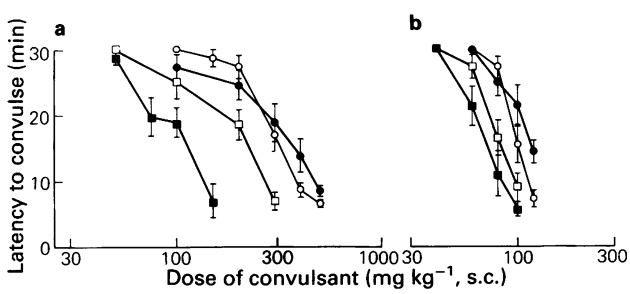


Figure 1 Effect of D- and L-serine on (a) N-methyl-DL-aspartic acid (NMDLA) and (b) pentylenetetrazol (PTZ)-induced tonic seizures. D- or L-serine (50–200 μg per mouse) were injected i.c.v. in Swiss Webster mice. Fifteen min later NMDLA (50–500 mg kg^{-1}) or PTZ (60–120 mg kg^{-1}) were injected s.c. and the latency to the tonic extension of the forepaws noted. Animals not showing a tonic seizure within 30 min were given a maximum latency score of 30 min. Values shown are the mean latency scores (s.e.mean shown by vertical bars) of at least 8 animals per group. (a): (○) Vehicle; (□) D-serine 50 μg; (■) D-serine 100 μg; (●) L-serine 100 μg. (b) (○) Vehicle; (□) D-serine 100 μg; (■) D-serine 200 μg; (●) L-serine 100 μg. The ED_{50} dose (dose inducing seizures in 50% of the animals tested) was calculated by probit analysis, animals not convulsing within 30 min of injection of the convulsant being considered protected. The ED_{50} dose of NMDLA was decreased from 245 mg kg^{-1} to 80 mg kg^{-1} by pretreatment with 100 μg D-serine ($P < 0.05$, F test) whilst L-serine was without significant effect. For PTZ, D-serine significantly reduced the ED_{50} dose from 93.7 mg kg^{-1} to 60.4 mg kg^{-1} ($P < 0.05$, F test) and again L-serine was without significant effect.

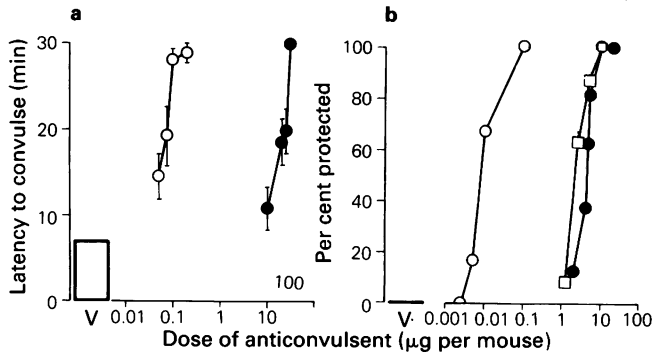


Figure 2 The anticonvulsant activity of compounds against (a) N-methyl-DL-aspartic acid (NMDLA)-induced seizures in Swiss Webster mice and (b) sound-induced seizures in DBA/2 mice. In (a) 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (○) or kynuernic acid (●) were given i.c.v. 15 min before NMDLA (500 mg kg^{-1}, s.c.) and the latency to the tonic extension of the forepaws noted. Animals not convulsing within 30 min of injection of NMDLA were considered protected. Values shown are the mean latencies (s.e.mean shown by vertical bars) of at least 8 animals per dose. The ED_{50} dose (dose protecting 50% of the animals tested) for CPP and kynurenic acid was 0.08 μg per mouse and 25 μg per mouse respectively. In (b), CPP (○), kynurenic acid (●) or 7-chlorokynurenic acid (□) were given i.c.v. 15 min before exposure to an electric bell. Protection was defined as the absence of a tonic seizure during 30 s of sound exposure. Results are shown as the percentage of animals protected ($n = 8$ or more per group). The ED_{50} doses for CPP, kynurenic acid and 7-chlorokynurenic acid were 0.008, 3.75 and 2.32 μg per mouse respectively.

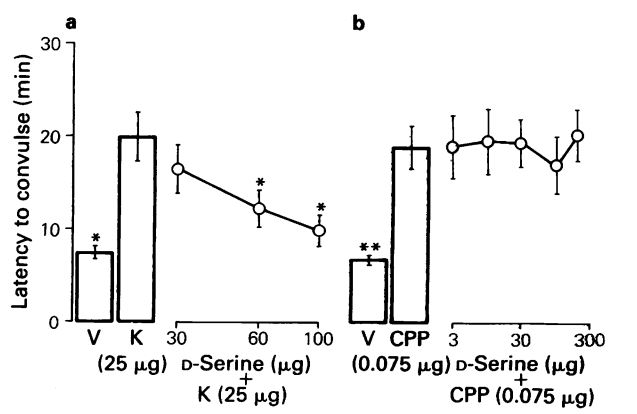


Figure 3 The antagonism of the protective effects of (a) kynurenic acid (K) and (b) 3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) against N-methyl-DL-aspartic acid (NMDLA)-induced seizures. D-Serine was co-administered i.c.v. with either kynurenic acid (25 μg per mouse) or CPP (0.075 μg per mouse) 15 min before the injection of NMDLA (500 mg kg^{-1}, s.c.) and the latency to convulse determined. Values shown are the mean latencies (s.e.mean shown by vertical bars) of at least 8 animals per group. *Significantly different from animals given only kynurenic acid, $P < 0.05$ (Student Newman Keuls test). **Significantly different from animals given only CPP, $P < 0.05$.

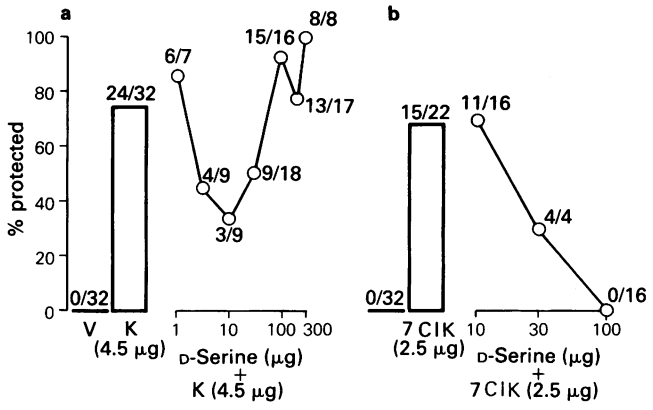


Figure 4 The antagonism of the protective effects of (a) kynurenic acid (K) and (b) 7-chlorokynurenic acid (7ClK) against audiogenic seizures. D-Serine was co-administered i.c.v. with kynurenic acid (4.5 μg per mouse) or 7-chlorokynurenic acid (2.5 μg per mouse) 15 min before exposure to sound (for further details see legend to Figure 2). The number of animals not convulsing was noted. Results are shown as the percentage of animals protected. The numbers at each point on the graph represent the number of mice protected/number tested.

Effects of D-*serine on the blockade of audiogenic seizures by kynurenic acid, 7-chlorokynurenic acid and diazepam*

Kynurenic acid was more than 6 fold as potent as an antagonist of sound-induced seizures in DBA/2 mice than of seizures induced by NMDLA (ED_{50} = 3.8 and 25 μg per mouse respectively, Figure 2a,b). The anticonvulsant effect of a submaximal dose of kynurenic acid (4.5 μg per mouse) was antagonized only partially by D-serine (Figure 4a) and at doses somewhat lower (1–10 μg per mouse) than those required to block the action of kynurenic acid against NMDLA (10–100 μg per mouse). Furthermore, the effect of D-serine was biphasic, 100 μg D-serine giving less antagonism than a 10 μg dose. The possibility that D-serine might even potentiate the anticonvulsant effect of kynurenic acid was examined by co-administration of D-serine (100 μg per mouse) with a lower dose of kynurenic acid (3 μg per mouse): the proportion of mice convulsing was not altered (number of animals convulsing/number tested: controls = 0/8; kynurenic acid, 3 μg = 1/7; kynurenic acid, 3 μg + D-serine, 100 μg = 1/8).

Unlike kynurenic acid, its 7-substituted chloro analogue has appreciably higher affinity for the strychnine-insensitive glycine recognition site than for NMDA, quisqualate or kainate receptors and potently antagonizes the actions of glycine at the NMDA receptor ((Kemp *et al.*, 1988). 7-Chlorokynurenic acid at doses up to 10 μg per mouse was without effect on seizures induced by NMDLA (doses in excess of this induced marked sedation, ataxia and respiratory depression), but dose-dependently blocked sound-induced seizures in DBA/2 mice with an approximate ED_{50} of 2 μg per mouse. As for the anticonvulsant effect of kynurenic acid against NMDLA-induced seizures, concomitant administration of D-serine (10–100 μg per mouse) antagonized the protective effect of a submaximal dose of 7-chlorokynurenic acid (2.5 μg per mouse, Figure 4b). In contrast, D-serine (10–300 μg per mouse) was unable to antagonize an equivalent submaximal protective effect of the benzodiazepine receptor agonist, diazepam (1 mg kg^{-1}, i.p., results not shown).

Discussion

Neuronal responses to the excitatory amino acid, N-methyl-D-aspartate, can be potentiated by glycine acting at a strychnine-insensitive binding site on the NMDA receptor/ion channel complex (Johnson & Ascher, 1987; Wong *et al.*, 1987; Bonhaus *et al.*, 1987; Reynolds *et al.*, 1987; Bowery, 1987). In patch clamp experiments with cultured mouse neurones, glycine potentiates responses to NMDA at high perfusion rates, when leakage from the cells contributes little to the concentration of glycine in the external milieu: concentrations greater than 1 μM are saturating (Johnson & Ascher, 1987). Given the high concentrations of glycine in body fluids (i.e. > 1 μM in CSF, Ferraro & Hare, 1985) it has been argued that the glycine modulatory site on the NMDA receptor may be maximally activated *in vivo* and thus of little relevance to the phasic control of NMDA-mediated neurotransmission (Johnson & Ascher, 1987). However, the concentration of glycine in the synaptic cleft *in vivo* may not always exceed 1 μM. Indeed, when the perfusion rate of rat neocortical slices is kept at a level permitting the ebb and flow of neurotransmitters and neuromodulators to be controlled by natural release and degradation processes, exogenously applied glycine enhanced responses to NMDA (Thomson *et al.*, 1989). Furthermore, there is evidence that the intraventricular injection of glycine can potentiate the rat cerebellar cyclic GMP response to co-administered NMDA (Danysz *et al.*, 1989).

Seizures induced in the mouse by NMDLA are thought to reflect the activation of central NMDA receptors (Leander *et al.*, 1988; Price *et al.*, 1988) and thus present a model with which to probe the functional relevance of the glycine modulatory site on the NMDA receptor *in vivo*. Because of its lack of selectivity between strychnine-sensitive and insensitive receptors, glycine itself is not a suitable tool with which to work. However, this problem can be overcome by use of the glycine receptor agonist D-serine (Bristow *et al.*, 1986; Wong *et al.*, 1987; Kemp *et al.*, 1988) that is devoid of significant affinity for strychnine-sensitive glycine receptors (A. Foster, personal communication). When given directly into the cerebral ventricles, D-serine was not itself convulsant, but clearly potentiated the convulsant action of NMDLA. In contrast, L-serine, which *in vitro* is about 100 fold less potent than the D-enantiomer, was without significant effect. Thus, serine stereoselectively potentiated the convulsant activity of NMDLA, consistent with the glycine modulatory site on the NMDA receptor being unsaturated with respect to substrate *in vivo*.

Since NMDA receptor antagonists also block seizures induced by pentylenetetrazol (Croucher *et al.*, 1982; Hayes & Balster, 1985) it was of interest to determine whether D-serine was also able to potentiate the convulsant action of PTZ. D-, but not L-serine also dose-dependently increased the potency of PTZ in inducing tonic seizures over the same dose range that was effective in the NMDLA seizure model. The magnitude of the effect was, nevertheless, considerably smaller than that seen with NMDLA, possibly reflecting the pharmacological specificity of the D-serine/NMDA receptor interaction. On the other hand, the relative insensitivity of PTZ-induced seizures could also be explained by a differential penetration of D-serine to disparate epileptogenic foci.

Modulation of NMDA receptor activation by the D-serine-sensitive glycine receptor was also illustrated by the ability of D-serine to antagonize the anticonvulsant action of the glycine receptor antagonists, kynurenic acid and 7-chlorokynurenic acid. The blockade of NMDLA-induced seizures by kynurenic acid was reversed by D-serine at doses similar to those potentiating the actions of NMDLA alone. The specificity of this effect is shown by the inability of D-serine to antagonize a similar anticonvulsant effect of the competitive NMDA receptor antagonist, CPP. This result is entirely consistent with electrophysiological studies in which both D-serine and glycine were unable to reverse the competitive antagonist action of CPP and D-2-aminophosphonovaleric acid against NMDA in the rat cortical slice (Kemp *et al.*, 1988; Fletcher & Lodge, 1988).

Kynurenic acid was also an effective anticonvulsant in the audiogenic seizure prone DBA/2 mouse. However, D-serine was less efficacious (although somewhat more potent) in blocking the protective effect of kynurenic acid in DBA/2 mice than in the NMDLA seizure model. Furthermore, the antagonism of kynurenic acid by D-serine in DBA/2 mice was clearly

biphasic. Interestingly, glycine is reported to reverse only partially the kynurenate-induced block of responses to NMDA in rat cortical wedges (Fletcher *et al.*, 1988). These paradoxical results could be explained by the lack of selectivity of kynurenic acid for the glycine modulatory site: in addition to antagonism of the potentiating effects of glycine at the NMDA receptor, kynurenate also antagonizes the actions of glutamate at quisqualate and kainate receptors (Kemp *et al.*, 1987). Blockade of these 'glycine-insensitive' receptors (Johnson & Ascher, 1987) is also likely to have anticonvulsant consequences (Turski *et al.*, 1987). Indeed, when the selective glycine receptor antagonist, 7-chlorokynurenic acid, was used as the anticonvulsant in the DBA/2 mouse, D-serine was able to block totally its protective effect, but was without effect on a similar degree of protection afforded by diazepam.

Thus, the selective potentiation of the convulsant action of NMDLA by D-serine suggests that NMDA receptors are not maximally potentiated by endogenous glycine. A similar conclusion was also recently reached by Larson & Beitz (1988) who showed that the intrathecal injection of glycine in animals pretreated with a subconvulsant dose of strychnine (to block strychnine-sensitive glycine receptors) enhanced the convulsant action of co-administered NMDA. Consequently, it is probable that variations in the availability of glycine occurring in the vicinity of the receptor *in vivo* are sufficient to influence significantly the tone of NMDA-mediated neurotransmission. Given the high concentrations of glycine in epileptogenic foci (Van Gelder *et al.*, 1972) and the anticonvulsant properties of the selective glycine receptor antagonist, 7-chlorokynurenic acid, these results strongly suggest an important involvement of the glycine/NMDA receptor/ion channel complex in the pathophysiology of epilepsy.

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Effect of ethanol on eicosanoid synthesis by human gastric and colonic mucosal pieces

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1 Human gastric and colonic mucosa obtained at operation was cut into small pieces and incubated with different concentrations of ethanol.

2 Ethanol (5–40%) caused a concentration-dependent increase in the amounts of prostaglandin E (PGE), thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and (up to 20% ethanol) leukotriene C_4/D_4 (LTC_4/D_4) in incubates of mucosal pieces from either region.

3 Higher concentrations of ethanol usually caused small increases or reductions of eicosanoid levels; gastric mucosal PGE and 6-keto-$PGF_{1\alpha}$ were still increased by 100% ethanol, whereas TXB_2 was unaltered, and LTC_4/D_4 was reduced. With the colonic mucosa, 100% ethanol increased PGE but reduced the other eicosanoids.

4 Gastric mucosal pieces incubated in water or phosphate buffer yielded generally similar amounts of eicosanoids. However, colonic mucosa yielded more when incubated in water, possibly indicating a greater sensitivity to osmotic damage. This difference between the two regions is consistent with the ability of the gastric mucosa to resist damage by water on its epithelial surface.

Introduction

Some natural cyclo-oxygenase products such as prostaglandin E_2 (PGE_2) and PGI_2, and synthetic analogues such as 16,16-dimethyl PGE_2 and misoprostol, protect the gastrointestinal mucosa against damage (Scratcherd, 1987; Watkinson & Akbar, 1987; Boughton-Smith & Whittle, 1988a). In contrast, 5-lipoxygenase products, particularly leukotriene C_4 (LTC_4), may mediate damage and inflammation in the gastric mucosa (Wallace & Whittle, 1985; Boughton-Smith & Whittle, 1988a) and colonic mucosa (Rampton & Hawkey, 1984; Sharon & Stenson, 1984; Boughton-Smith *et al.*, 1988; Wallace *et al.*, 1989).

Ethanol caused a concentration-dependent increase in the *ex vivo* or *in vitro* release of LTC_4 from the rat gastric mucosa (Peskar *et al.*, 1986; 1988; Boughton-Smith & Whittle, 1988a,b); prostaglandin formation was not significantly affected (Boughton-Smith & Whittle, 1988a; Peskar *et al.*, 1988). Inhibition of gastric LTC_4 formation by compounds such as sodium salicylate (Peskar *et al.*, 1988), 16,16-dimethyl PGE_2 (Boughton-Smith & Whittle, 1988a), cysteamine or diethyl maleate (Lange *et al.*, 1987), carbenoxolone or nordihydroguaiaretic acid, protected the rat gastric mucosa against ethanol-induced damage (Peskar *et al.*, 1986); again prostaglandin release was not significantly affected (Boughton-Smith & Whittle, 1988a; Peskar *et al.*, 1988).

In another recent study (Boughton-Smith & Whittle, 1988b), the selective 5-lipoxygenase inhibitors BW A4C and BW A137C, at doses that almost completely inhibited rat mucosal synthesis of LTB_4 or LTC_4, did not reduce the gastric mucosal damage by ethanol. In contrast, pretreatment with the less specific lipoxygenase/cyclo-oxygenase inhibitor BW 755C did reduce rat gastric mucosal damage by ethanol (Wallace & Whittle, 1985). Thus the relationship between ethanol-induced gastric mucosal damage and eicosanoid formation is unclear. Furthermore, these studies were mainly in the rat, and their relevance to man has not been determined. We now describe the effect of different ethanol concentrations on the eicosanoid accumulation in incubates of human gastric and colonic mucosal pieces.

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Methods

Human gastric and colonic mucosa

Gastric and colonic tissue were taken at least 5 cm from any macroscopically detected lesions in surgical specimens removed for benign or malignant disease. It was transported to the laboratory in 154 mM NaCl at ambient temperature within 30 min of removal, and on arrival it was transferred to ice-cold phosphate buffered saline pH 7.4 (PBS). The layer of mucosa/submucosa was carefully cut off from the underlying muscle while the tissue was bathed in PBS. As far as we know, the patients had not recently consumed drugs that affect eicosanoid synthesis.

Mucosal incubation

Each sample of mucosa/submucosa was rinsed in PBS, cut into pieces 3–5 mm^2, and washed again with PBS which was then drained off. Carefully weighed samples of 100 ± 5 mg were pre-incubated in PBS (1 ml, 0°C, 15–30 min) which was then drained off, and the tissues were transferred to tubes containing 1 ml of either PBS, distilled water or different concentrations of ethanol (5, 10, 20, 40, 80 and 100% in distilled water) for 5 min. The tissues were then taken out, rinsed well in PBS, blotted gently on filter paper and then transferred to tubes containing 1 ml of fresh ice-cold PBS for another 5–10 min.

This pre-incubation fluid, containing any released prostanoids, was discarded and the fluid was again replaced by 1 ml of fresh PBS alone. After further incubation at 37°C for 30 min, the PBS containing the released eicosanoids was removed and stored at −20°C until assayed.

Radioimmunoassays

The method used is based on that of Jaffe & Behrman (1974) and suitable dilutions of antisera and tritiated standards were

Table 1 Effect of different concentrations of ethanol on the accumulation of endogenous eicosanoids in incubates of human gastric mucosal pieces (30 min incubation)

	% difference from the water control			
Incubation fluid	*PGE*	*TXB_2*	*6-keto-$PGF_{1\alpha}$*	*LTC_4/D_4*
Buffer (PBS)	4.4 ± 27.7	−21.4 ± 11.0	0.9 ± 11.7	−5.2 ± 17.2
Ethanol (%)				
5	21.7 ± 9.9	42.3 ± 26.1	46.9 ± 34.5	18.6 ± 26.7
10	57.0 ± 41.1	110.4 ± 50.1	74.8 ± 29.5[a]	149.3 ± 58.5
20	129.7 ± 43.2[c]	117.7 ± 22.7[c]	83.7 ± 40.9	242.2 ± 60.2[c]
40	355.5 ± 114.7[c]	319.3 ± 90.3[c]	159.2 ± 59.4[b]	57.0 ± 18.8[a]
80	471.5 ± 152.5[c]	66.4 ± 55.5	137.7 ± 30.2[c]	−24.6 ± 14.7
100	293.0 ± 61.7[c]	0.3 ± 33.0	111.3 ± 37.7[a]	−66.7 ± 9.8[a]

The results are mean ± s.e.mean expressed as the % difference from the amounts in incubates with water ($n = 6$), and analysed by Student's t test for paired data. [a]$P < 0.05$, [b]$P < 0.02$, [c]$P < 0.01$, compared with the water controls.
Amounts of eicosanoids (ng g^{-1} wet weight 30 min^{-1}) in the water control: PGE 120.0 ± 17.7, TXB_2 57.7 ± 14.3, 6-keto-$PGF_{1\alpha}$ 107.8 ± 16.8 and LTC_4 7.0 ± 2.0

used. Assay sensitivities were 20 pg for prostanoids and 50 pg for LTC_4. The intra- and inter-assay coefficients of variation were 5–9% and 10–11% depending on the substance measured. All assays were in duplicate.

Tritiated prostanoids were purchased from Amersham Radiochemical Centre. The % cross-reactions of the antisera used were: PGE antibody (ICN): PGE_2 100; PGE_1 240; PGA_1 10; PGA_2 8; $PGF_{1\alpha}$ 3.2; $PGF_{2\alpha}$ 4.2; PGB_1 0.7; PGB_2 0.9. 6-keto-$PGF_{1\alpha}$ antibody (Wellcome): $PGF_{2\alpha}$ 0.84; PGE_2 0.1; TXB_2 0.02. TXB_2 antibody (Wellcome): $PGF_{2\alpha}$ 0.11; PGE_2 0.008; 6-keto-$PGF_{1\alpha}$ 0.01.

Tritiated leukotriene C_4 was purchased from New England Nuclear. The % cross-reactions of the LTC_4/D_4 antiserum, kindly provided by Dr J. Zakrzewski, were: LTC_4 100; LTD_4 29.4; LTE_4 0.7; LTB_4 0.05; PGE <0.05; arachidonic acid <0.05.

As the PGE antiserum does not distinguish between PGE_1 and PGE_2, the measurements are expressed as PGE. Since the LTC antiserum distinguishes poorly between LTC_4 and LTD_4, the measurements are expressed as LTC_4/D_4. Because the results reflect only the interaction of released material with antisera, the measurements should really be expressed as, for example, PGE-like. However, for simplicity we have referred to them as the authentic substances. Evidence that these released products are prostanoids and leukotrienes is provided by experiments using the same antisera, where indomethacin inhibited prostanoid formation in human gastric mucosa/submucosa (Tavares *et al.*, 1987), and the cyclo-oxygenase/lipoxygenase inhibitor BW755C blocked eicosanoid formation in human colon mucosa/submucosa (unpublished).

Most of the values are given to 3 significant figures; they are shown in the text as the means, and the s.e.mean are given in the Tables. The results were analysed statistically by Student's t test for paired data (2-tailed), using the groups treated with distilled water (the solvent for ethanol) as the controls unless otherwise stated.

Results

Gastric mucosal prostanoids

Amounts of gastric mucosal prostanoids in the water incubates of gastric mucosa/submucosa (control group, ng g^{-1} 30 min^{-1}) were: PGE 120; TXB_2 57.7; 6-keto-$PGF_{1\alpha}$ 108 ($n = 6$). Incubation in PBS instead of water made little or no difference to the prostanoid yield (PGE, +4% of the amount in the water incubates, $P < 0.4$; TXB_2 −21%, $P < 0.2$; 6-keto-$PGF_{1\alpha}$, +3%, $P < 0.6$).

As shown in Table 1, ethanol (5–40%) induced a concentration-related increase in the prostanoid contents of the incubates. The PGE yield rose further with 80% ethanol, but the increase in the other prostanoids tended to be less than when 40% ethanol was used. With 100% ethanol the amounts of both PGE and 6-keto-$PGF_{1\alpha}$ were still higher than with water, whereas TXB_2 was almost identical to the control.

Gastric mucosal leukotrienes

Incubates of gastric mucosa/submucosa in water contained 7.0 ng g^{-1} wet weight 30 min^{-1} of LTC_4/D_4, compared with

Table 2 Effect of different concentrations of ethanol on the accumulation of endogenous eicosanoids in incubates of human colonic mucosal pieces (30 min incubation)

	% difference from the water control			
Incubation fluid	*PGE*	*TXB_2*	*6-keto-$PGF_{1\alpha}$*	*LTC_4/D_4*
Buffer (PBS)	−33.0 ± 13.8	−27.3 ± 3.9[c]	−42.3 ± 7.1[c]	−29.6 ± 8.9[a]
Ethanol (%)				
5	21.2 ± 15.4	22.8 ± 18.3	−10.0 ± 8.8	41.9 ± 18.2
10	31.0 ± 11.9[a]	14.8 ± 11.9	−4.0 ± 13.0	55.7 ± 19.4[a]
20	95.8 ± 16.4[c]	85.2 ± 24.0[a]	30.7 ± 13.6	160.0 ± 59.7[b]
40	215.7 ± 63.3[c]	140.5 ± 29.0[a]	75.8 ± 31.0[c]	66.6 ± 44.8
80	295.7 ± 68.7[c]	14.0 ± 13.1	16.0 ± 18.6	−29.1 ± 17.1
100	187.8 ± 56.1[b]	−44.2 ± 13.2[a]	−48.5 ± 15.6[a]	−40.3 ± 13.7

The results are shown as means ± s.e.mean, and expressed as % difference from the water control ($n = 6$). P values (Student's t test for paired data): [a]$P < 0.05$, [b]$P < 0.02$, [c]$P < 0.01$, compared with the water incubates.
Amounts of eicosanoids (ng g^{-1} wet weight 30 min^{-1}) in the water incubates were: PGE 89.1 ± 15.1, TXB_2 51.4 ± 11.7, 6-keto-$PGF_{1\alpha}$ 137.4 ± 35.6 and LTC_4/D_4 6.3 ± 1.1

6.6 ng g^{-1} in PBS ($P < 0.8$). Ethanol had a biphasic effect. With 5–20% there was a concentration-related increase in the mean amounts of LTC_4/D_4, whereas 40% ethanol caused a smaller increase than 10 or 20%, and 100% ethanol was inhibitory (Table 1).

Colonic mucosal prostanoids

The mean amounts of prostanoids in the aqueous incubates of colonic mucosa/submucosa were (ng g^{-1} 30 min^{-1}): PGE 89; TXB_2 51; 6-keto-$PGF_{1\alpha}$ 137 ($n = 6$). Amounts in the PBS incubates were lower (PGE by 33%, $P < 0.1$; TXB_2 27%, $P < 0.01$; 6-keto-$PGF_{1\alpha}$ by 42%, $P < 0.01$).

With ethanol (5–40%) there was a concentration-related increase in the prostanoid accumulation (Table 2). At 80% ethanol the PGE accumulation increased further, whereas the mean amounts of TXB_2 and 6-keto-$PGF_{1\alpha}$ were little different from the controls. With 100% ethanol PGE was still increased, whereas the amounts of TXB_2 and 6-keto-$PGF_{1\alpha}$ were less than controls, showing a concentration-dependent biphasic effect of ethanol.

Colonic mucosal leukotriene formation

Aqueous incubates of colonic mucosa/submucosa contained a mean amount of 6.3 ng g^{-1} wet weight 30 min^{-1} of LTC_4/D_4, whereas the PBS incubates contained about 30% less (4.4 ng, $P < 0.05$). With 5–20% ethanol there was a concentration-related stimulation of the LTC_4/D_4 yield, but 80 and 100% ethanol tended to be inhibitory (Table 2).

Discussion

The amounts of eicosanoids in incubates of human gastric mucosa were similar when water or phosphate buffer were used. With colonic mucosa, in contrast, the water resulted in higher mean amounts of eicosanoids compared to buffer (30–40% increase, $P < 0.1$ to < 0.01). Perhaps this smaller effect of water on the gastric mucosa, compared to the colonic mucosa, may reflect a smaller osmotic perturbation of the gastric mucosal cell membranes. The luminal surface of the gastric mucosa is designed to withstand the osmotic effect of intraluminal water, whereas normally the colon is exposed only to iso-osmotic solutions.

In rats, ethanol (40–100%) intraluminally caused a concentration-dependent increase in LTC_4 release with no change in 6-keto-$PGF_{1\alpha}$ (Peskar *et al.*, 1986; Boughton-Smith & Whittle 1988a). By contrast, we found that 100% ethanol increased the amounts of PGE and 6-keto-$PGF_{1\alpha}$, but had no effect on TXB_2, and actually reduced the amount of LTC_4/D_4. At the lower concentrations of 5–40%, ethanol concentration-dependently increased the amounts of all the eicosanoids from both types of human mucosa/submucosa. However, in the *ex vivo* rat experiments (Peskar *et al.*, 1986) and the studies using the whole stomach rather than just the mucosa (Boughton-Smith & Whittle 1988a), the ethanol reached the tissue mainly via the epithelial surface. Furthermore, the mucus adhering to intact mucosa presumably diluted the ethanol before it reached the epithelial cells. By contrast, in our experiments the ethanol penetrated directly into the mucosal pieces and may therefore have reached higher concentrations than in the rat studies. This, rather than a species difference, may explain why we obtained effects at lower ethanol concentrations, and it may be more appropriate to compare our findings at lower ethanol concentrations with those at higher concentrations in the rat studies.

Increased amounts of eicosanoids due to the ethanol may have resulted from perturbation of the cell membranes. The maximum response in our experiments varied with both the ethanol concentration and the individual substances (20% ethanol for maximum LTC_4/D_4 accumulation, 40% for maximum TXB_2 and 6-keto-$PGF_{1\alpha}$, and 80% for PGE in both the gastric and colonic tissues). With the gastric mucosa, 80–100% ethanol increased both the PGE and 6-keto-$PGF_{1\alpha}$ accumulation, but with 100% ethanol the mean amount of TXB_2 was unchanged, and LTC_4/D_4 production was inhibited. With the colonic mucosa, 100% ethanol increased PGE accumulation but considerably decreased the amounts of TXB_2, 6-keto-$PGF_{1\alpha}$ and LTC_4/D_4. The LTC_4/D_4 yield seems to be the most sensitive to inhibition by a high ethanol concentration. This might be because lipoxygenase is a cytosolic enzyme, and therefore more accessible to the ethanol, whereas cyclo-oxygenase is membrane-bound.

We cannot explain the differences between the two types of human mucosa/submucosa, or between different eicosanoids, in the effects of the higher concentrations of ethanol, or why increased prostanoid formation can occur with concentrations of ethanol that might be expected to denature the enzymes involved. Perhaps the 5 min incubation with the alcohol, and its dilution on diffusion through the tissue, did not allow denaturation of the cyclo-oxygenase, particularly at the centre of mucosal pieces.

At concentrations reaching the human stomach on drinking strong alcoholic beverages, gastric mucosal leukotriene synthesis might increase. If leukotriene formation contributes to gastric mucosal damage by ethanol, its effects may be antagonised by the increased amounts of prostaglandins formed, and increased by the greater thromboxane production. It would be of interest to see whether selective 5-lipoxygenase inhibitors reduce gastic mucosal damage by ethanol in man.

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Characterization of the muscarinic receptor subtype involved in phosphoinositide metabolism in bovine tracheal smooth muscle

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1 The muscarinic receptor subtype involved in the methacholine-induced enhancement of phosphoinositide metabolism in bovine tracheal smooth muscle was identified by using the M_2-selective antagonist AF-DX 116 and the M_3-selective antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide, in addition to the M_1-selective antagonist pirenzepine, in a classical Schild analysis.

2 All the antagonists shifted the methacholine dose-response curve to the right in a parallel and concentration-dependent fashion, yielding Schild plots with slopes not significantly different from unity. The pA_2 values (6.94, 6.32 and 8.54 for pirenzepine, AF-DX 116 and 4-DAMP methobromide respectively) indicate that it is the M_3 (smooth muscle/glandular), but not the M_2 (cardiac) muscarinic receptor subtype, present in this tissue, that mediates phosphoinositide turnover, in accordance with our previous contractile studies.

3 The results provide additional evidence for the involvement of phosphoinositide turnover in the pharmacomechanical coupling between muscarinic receptor stimulation and contraction in (bovine tracheal) smooth muscle.

Introduction

Enhancement of phosphoinositide (PI) turnover has been proposed as the transduction mechanism in airway (and other) smooth muscle cells involved in the coupling of muscarinic receptor stimulation and contraction (Baron *et al.*, 1984; Abdel-Latif, 1986). Thus, it has been demonstrated that muscarinic receptor stimulation results in the breakdown of membrane polyphosphoinositides and/or the formation of inositol phosphates in canine (Baron *et al.*, 1984), bovine (Grandordy *et al.*, 1986) and guinea-pig (Robertson *et al.*, 1988) tracheal smooth muscle, and very recently in human bronchial smooth muscle (Meurs *et al.*, 1989). Furthermore, it has been suggested that in canine tracheal smooth muscle inositol 1,4,5-trisphosphate is able to release Ca^{2+} from intracellular stores (Hashimoto *et al.*, 1985), which is thought to be involved in the smooth muscle contraction process (see e.g. Yousufzai *et al.*, 1987), and that the formation of this putative second messenger in bovine tracheal smooth muscle actually precedes contraction (Chilvers *et al.*, 1989). Finally, recent work from our laboratory provided evidence for a direct relationship between PI metabolism and bovine tracheal smooth muscle contraction; the abilities of three muscarinic agonists to elicit inositol phosphates accumulation and contraction were correlated (Meurs *et al.*, 1988). This study also revealed the presence of an agonist-dependent receptor (actually transduction) reserve.

Important additional evidence for the involvement of PI turnover in muscarinic receptor-mediated smooth muscle contraction might come from the pharmacological characterization of these processes with receptor subtype-selective antagonists. Indeed, both contraction in a wide variety of smooth muscle preparations (Eglen *et al.*, 1987; Konno & Takayanagi, 1986; O'Rourke *et al.*, 1987), including bovine trachea (Grandordy *et al.*, 1986; Moore *et al.*, 1988; Roffel *et al.*, 1988), and PI metabolism in a number of these preparations (Grandordy *et al.*, 1986; Akhtar *et al.*, 1987; Noronha-Blob *et al.*, 1987; Gardner *et al.*, 1988) have been shown to be mediated by muscarinic receptors with low affinity for the M_1-selective antagonist pirenzepine.

Since the further subdivision of muscarinic receptors with low affinity towards pirenzepine into M_2 (cardiac) and M_3 (smooth muscle/glandular) subtypes, by use of selective antagonists like AF-DX 116, methoctramine, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide and hexahydrosiladifenidol, it has been established that bovine tracheal smooth muscle contraction is mediated by the M_3 receptor subtype (Roffel *et al.*, 1988; Moore *et al.*, 1988). Interestingly, equilibrium (Giraldo *et al.*, 1988; Lazareno & Roberts, 1988b; Michel & Whiting, 1987; Moore *et al.*, 1988; Roffel *et al.*, 1988) and dissociation (Giraldo *et al.*, 1988; Roffel *et al.*, 1989) radioligand binding experiments, as well as molecular biology techniques (Maeda *et al.*, 1988), have demonstrated the presence of a large population of cardiac but only a small population of smooth muscle/glandular type binding sites in (among others bovine tracheal) smooth muscle membranes. Which of these muscarinic receptor subtypes mediates PI metabolism in smooth muscle is, at present, unknown. Until now, only in one study were M_2/M_3 subtype-selective muscarinic antagonists used. It was found that carbachol-induced PI breakdown in guinea-pig bladder was antagonized with high potency by AF-DX 116 ($pA_2 = 7.3$) and with low potency by hexahydrosiladifenidol ($pA_2 = 6.8$), suggesting the involvement of the M_2 (cardiac) subtype (Noronha-Blob *et al.*, 1987). However, it should be mentioned that atropine showed an anomalous low potency ($pA_2 = 7.7$).

The aim of the present study was to establish the pharmacological subtype of muscarinic receptor that mediates PI turnover in bovine tracheal smooth muscle, following the characterization of these receptors in contraction and binding studies (Roffel *et al.*, 1987; 1988), by using, in addition to pirenzepine, the M_2-selective antagonist AF-DX 116 and the M_3-selective antagonist 4-DAMP methobromide in a classical Schild analysis.

Methods

Tissue prepararation

Fresh bovine tracheae were obtained from the local slaughterhouse and transported to the laboratory within 30 min in Krebs-Henseleit (KH) buffer, at room temperature, pregassed with 95% O_2/5% CO_2; pH 7.4. Trachealis muscle was carefully dissected and smooth muscle strips (15 × 5 mm) were prepared free of mucosa and connective tissue in KH solution gassed with 95% O_2/5% CO_2 at room temperature. The strips

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were kept overnight at 24°C in a system in which they were continuously superfused (flow 3.9 ml min^{-1}) with gassed KH solution.

Inositol phosphates determination

Total inositol phosphates accumulation was determined essentially as described previously (Meurs *et al.*, 1988; 1989). Tracheal smooth muscle strips weighing a total of 6 g were chopped with a McIlwain tissue chopper, twice at a setting of 500 μm followed by three times at 100 μm. The tissue particles were washed four times with 45 ml KH containing 5 mM LiCl (KH/LiCl) and were then loaded with 75 μCi of [^{3}H]-inositol in 30 ml KH/LiCl for 60 min at 37°C, with gentle shaking and continuous gassing with 95% O_2/5% CO_2. After this incubation, the tissue was washed twice with KH/LiCl and was finally resuspended in 32 or 37 ml of this medium, depending on the number of dose-response curves (4–5) in the experiment. Aliquots of 450 μl of tissue suspension were then incubated with 25 μl of antagonist solutions or vehicle for 30 min at 37°C in capped tubes pregassed with 95% O_2/5% CO_2. After this preincubation, 25 μl of muscarinic agonist solutions were added, the tubes were gassed and capped again, and were incubated for another 25 min. Incubations were terminated by the addition of 500 μl ice-cold 10% (w/v) trichloroacetic acid. After the tubes had been on ice for 30 min, the precipitated protein was removed by centrifugation and 800 μl of the supernatants were extracted three times with four volumes of water-saturated diethyl ether. The extracted supernatants were then diluted with 8 ml of water and were applied to columns containing approximately 1 ml of Dowex AG 1X8 anion exchange resin (formate form). The columns were washed with 10 ml of water, followed by the sequential elution of glycerophosphoinositol with 15 ml of 5 mM disodium tetraborate/30 mM sodium formate buffer and of total inositol phosphates with four times 2 ml of 0.1 M formic acid/1.0 M ammonium formate buffer. These inositol phosphate samples were mixed with Plasmasol scintillation cocktail, 15 ml per sample, and counted for radioactivity in a Beckman LS 1800 liquid scintillation counter (35% efficiency).

Tissue protein was measured by the method of Lowry *et al.* (1951), after solubilization with 5 ml 1 N NaOH (10 min in a boiling waterbath) and subsequent neutralization with 5 ml 1 N HCl. Bovine serum albumin was used as the standard.

Data analysis

In each experiment, methacholine dose-response curves in the presence of muscarinic antagonists were related to the control dose-response curve, of which the maximum response was taken as 100%. In most experiments three or four concentrations of a muscarinic antagonist were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. When the slope did not differ significantly from unity (two-tailed Student's t test, $\alpha = 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log$ ([antagonist]/(dose ratio − 1)) (Mackay, 1978).

Materials

[^{3}H]-inositol (L-myo-[1,2-^{3}H(N)]) (40.8–60.8 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA, U.S.A.) and methacholine from Sigmal Chemical Co. (St. Louis, MO, U.S.A.). Plasmasol was purchased from Packard Instrument B.V. (Groningen, The Netherlands). AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) and pirenzepine were gifts from Dr Karl Thomae GmbH (Biberach an der Riss, F.R.G.); 4-DAMP (4-diphenylacetoxy-N-methylpiperidine) methobromide was a gift from Dr R.B. Barlow (Bristol, U.K.). All other chemicals were of reagent grade.

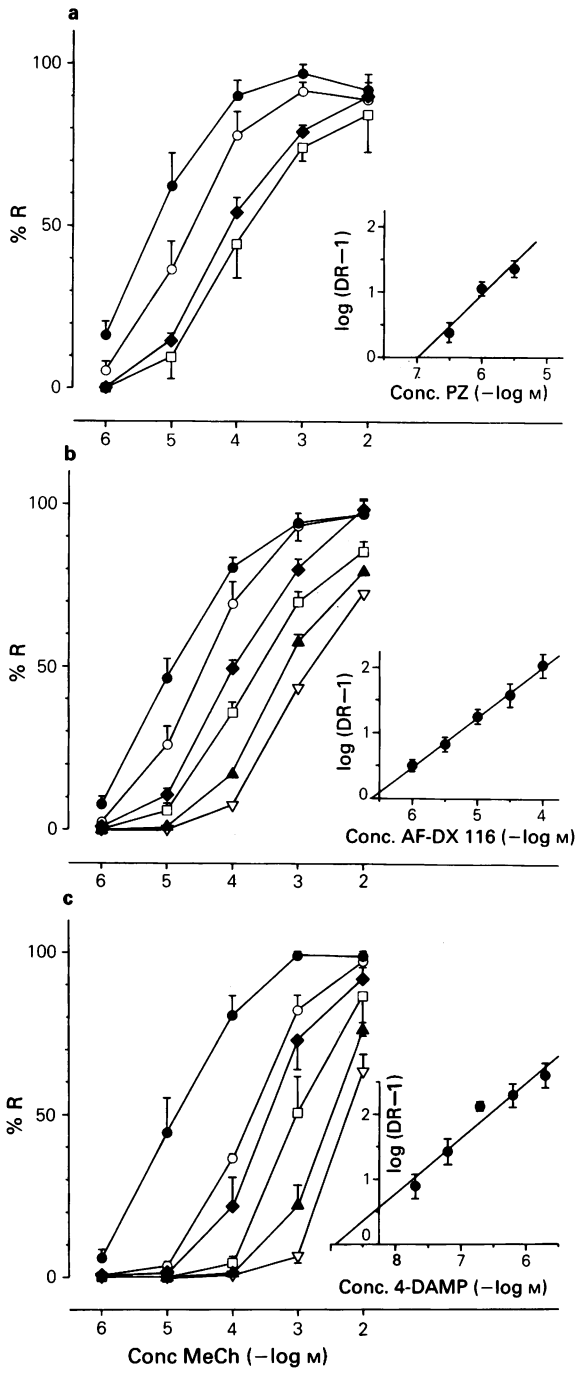


Figure 1 Dose-response curves for methacholine (MeCh)-induced phosphoinositide metabolism in bovine tracheal smooth muscle in the absence and presence of different concentrations of muscarinic antagonists, (a) pirenzepine (PZ) 0 (●) (3), 0.3 (○) (3), 1 (◆) (3) and 3 (□) (3) μM; (b) AF-DX 116 0 (●) (7), 1 (○) (4), 3 (◆) (6), 10 (□) (6), 30 (▲) (3) and 100 (▽) (3) μM; and (c) 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide 0 (●) (5), 0.02 (○) (3), 0.06 (◆) (3), 0.2 (□) (3), 0.6 (▲) (4) and 2 (▽) (4) μM. The number of determinations is given in parentheses. The insets show the corresponding Schild plots. Data points represent means of 3–7 experiments (as indicated in parentheses) each performed in duplicate. Vertical lines show s.e.mean. % R indicates responses relative to the control dose-response curves.

Results

In the absence of muscarinic antagonists, methacholine caused an 8.5 ± 0.9 fold stimulation of inositol phosphates accumulation over basal levels (2356 ± 310 d.p.m. mg^{-1} protein over 288 ± 35 d.p.m. mg^{-1} protein, with 3.7 ± 0.2 mg protein per tube) with a $-\log EC_{50}$ of 4.93 ± 0.10 (means ± s.e.mean,

Table 1 Functional potencies of selective muscarinic antagonists on methacholine induced phosphoinositide (PI) metabolism and contraction in bovine tracheal smooth muscle

	PI metabolism		*Contraction*	
	pA_2 (−log M)	*Slope*	pA_2 (−log M)	*Slope*
AF-DX 116	6.32 ± 0.06 (22)	0.81 ± 0.10 (7)	6.30 ± 0.07 (25)	1.06 ± 0.02 (4)
4-DAMP methobromide	8.54 ± 0.08 (17)	0.90 ± 0.05 (4)	9.03 ± 0.05 (20)	1.12 ± 0.06 (4)
Pirenzepine	6.94 ± 0.07 (9)	0.97 ± 0.08 (3)	6.92 ± 0.08 (30)	1.10 ± 0.05 (4)

pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log([\text{antagonist}]/(\text{dose-ratio} - 1))$ since Schild analysis revealed no significant deviations of slopes from unity, indicating competitive antagonism. Results are expressed as means ± s.e.mean with number of determinations in parentheses. Data for contraction were included for comparison and were taken from Roffel *et al.* (1988).

$n = 15$). As shown in Figure 1 the three muscarinic antagonists tested were all able to shift the methacholine dose-response curve to the right in a parallel and concentration-dependent fashion. The slopes of the Schild plots derived from these data were not significantly different from unity, indicating competitive antagonism for all three antagonists. These slopes and the pA_2 values are given in Table 1. The pA_2 values indicate that 4-DAMP methobromide was 40 times more potent than pirenzepine and 166 times more potent than AF-DX 116 in antagonizing methacholine-induced stimulation of PI metabolism in bovine tracheal smooth muscle.

Discussion

Data on the pharmacological characterization of the muscarinic receptors that mediate PI breakdown in (airway) smooth muscle are relatively scarce and most of the information available has been obtained with only pirenzepine as the selective antagonist. Pirenzepine discriminates between M_1 and M_2 but not really between cardiac and smooth muscle/glandular receptor subtypes. The pA_2 value of 6.94 found for pirenzepine in bovine tracheal smooth muscle in the present study indicates an M_2 (i.e. non-M_1) character for the muscarinic receptors involved, in full agreement with previous studies on PI metabolism in this and other smooth muscle tissues (Grandordy *et al.*, 1986; Akhtar *et al.*, 1987; Noronha-Blob *et al.*, 1987; Gardner *et al.*, 1988).

In order to establish the subtype of muscarinic receptor that mediates PI metabolism in bovine tracheal smooth muscle (cardiac (M_2) or smooth muscle/glandular (M_3)), the selective muscarinic antagonists AF-DX 116 and 4-DAMP methobromide were used. These compounds possess opposite selectivity profiles, 4-DAMP methobromide being typically 5 to 10 fold more potent than AF-DX 116 at M_2 and 200 to 500 fold more potent than AF-DX 116 at M_3 receptors. In the present study 4-DAMP methobromide was found to be 166 times more potent than AF-DX 116, suggesting the involvement of M_3 rather than M_2 receptors. The pA_2 value of 6.32 found for AF-DX 116 is within the range of 6.0–6.6 obtained for antagonism of smooth muscle contraction (Batink *et al.*, 1987; Duckles *et al.*, 1987; Lazareno & Roberts, 1988a; Moore *et al.*, 1988; Roffel *et al.*, 1988), whereas pA_2 values obtained in the heart (6.9–7.5) (Batink *et al.*, 1987; Duckles *et al.*, 1987; Micheletti *et al.*, 1987) are 4 to 16 times higher than the value we measured. Similarly, the pA_2 value of 8.54 for 4-DAMP methobromide is close to the values found on various smooth muscle preparations (8.6–9.2) (Batink *et al.*, 1987; Gater *et al.*, 1987; Eglen *et al.*, 1987; Moore *et al.*, 1988; Roffel *et al.*, 1988) but outside the range observed with cardiac preparations (7.7–8.2) (Batink *et al.*, 1987; Gater *et al.*, 1987; Lazareno & Roberts, 1988a). The conclusion that PI metabolism in bovine tracheal smooth muscle is brought about by M_3 type muscarinic receptors concords with the M_3 character of the muscarinic receptors that mediate contraction (see Roffel *et al.*, 1988 and references cited therein). As shown in Figure 2a there is a significant correlation between the pA_2 values for pirenzepine, AF-DX 116 and 4-DAMP methobromide when contraction (shown for comparison in Table 1) and PI metabolism were measured ($r = 0.9984$, $P < 0.05$). This provides more evidence that the latter process is involved in the pharmacomechanical coupling of muscarinic receptor stimulation and (airway) smooth muscle contraction. Interestingly, a significant correlation ($r = 1.0000$, $P < 0.005$) was also noticed between the pA_2 values found here for PI metabolism in bovine tracheal smooth muscle and those obtained in human SK-N-SH neuroblastoma cells (Fisher & Heacock, 1988), in accordance with the putative M_3 character of the muscarinic receptors in that cell line (Figure 2b). In this context, it should be mentioned that the neuronal SH-SY5Y clone of SK-N-SH cells was very recently found to possess a homogeneous M_3 receptor population (Lambert *et al.*, 1989).

The finding that PI metabolism in bovine tracheal smooth muscle is mediated by M_3-type muscarinic receptors is not completely unexpected. Firstly, muscarinic receptors in exocrine glands (which are typically of the M_3 subtype, as assessed in binding (Lazareno & Roberts, 1988a) and secretion (Gater *et al.*, 1987) studies) are also coupled to this second messenger system (Ek & Nahorski, 1988), although the receptor involved in PI turnover has not, as yet, been pharmacologically identified. Secondly, expression studies with different muscarinic receptor genes have shown that the putative M_3 receptor subtype (HM4, mAChR III, m3) (Barnard, 1988) couples to PI turnover (but not to adenylate cyclase) in human kidney cells (Peralta *et al.*, 1988) and NG108-15 neuroblastoma cells (Fukuda *et al.*, 1988), whereas the M_2 subtype is efficiently coupled to adenylate cyclase in human kidney cells (Peralta *et al.*, 1988), chinese hamster ovary cells (Ashkenazi *et al.*, 1987) and A9L fibroblasts (Jones *et al.*, 1988), though it can, albeit poorly, also stimulate PI turnover in the first two of these cell types.

In conclusion, it has become clear that both PI metabolism and contraction in bovine tracheal smooth muscle are medi-

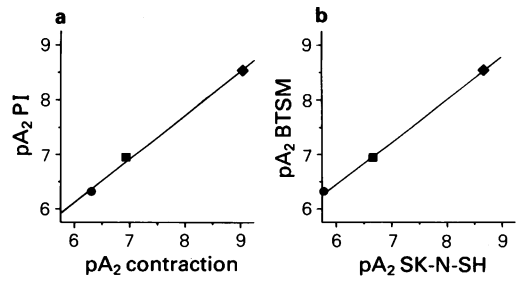


Figure 2 Correlation between the functional affinities (pA_2) of pirenzepine (■), AF-DX 116 (●), and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methobromide (◆) for phosphoinositide (PI) metabolism and contraction in bovine tracheal smooth muscle (a) and between the functional affinities for PI metabolism in bovine tracheal smooth muscle (BTSM) and those obtained in human SK-N-SH neuroblastoma cells (Fisher & Heacock, 1988) (b). Correlation coefficients were 0.9984 and 1.0000, respectively; probability values were <0.05 and <0.005, respectively.

ated by smooth muscle/glandular type (M_3) muscarinic receptors. As an important implication, the function of the major population of cardiac type (M_2) muscarinic receptors and the transduction mechanism involved remain to be elucidated.

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Characterisation of stereospecific binding sites for inositol 1,4,5-trisphosphate in airway smooth muscle

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1 A 'P_2' membrane fraction of bovine tracheal smooth muscle displays high affinity (K_D 3.8 ± 0.2 nM), saturable (B_{max} 1003 ± 170 fmol mg^{-1} protein) and reversible binding of D-*myo*[^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-Ins(1,4,5)P_3).

2 This binding site shows strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer with L-Ins(1,4,5)P_3, DL-Ins(1,3,4,5)P_4 and D-Ins(1,3,4)P_3 displacing [^{3}H]-Ins(1,4,5)P_3 with K_i values of 20 μM, 0.35 μM and 2.4 μM, respectively.

3 Specific binding of [^{3}H]-Ins(1,4,5)P_3 is enhanced at alkaline pH values (maximal at pH 7.75) and, in distinct contrast to [^{3}H]-Ins(1,4,5)P_3 binding in rat cerebellum membranes, is not inhibited by Ca^{2+} (5–500 μM).

4 Heparin displaces [^{3}H]-Ins(1,4,5)P_3 specific binding with an IC_{50} of 7.6 ± 1.0 μg ml^{-1}.

5 Comparative studies demonstrated specific [^{3}H]-Ins(1,4,5)P_3 binding in bovine cardiac atrial preparations (B_{max} 75 ± 5 fmol mg^{-1} protein) and very low specific [^{3}H]-Ins(1,4,5)P_3 binding in bovine cardiac ventricle and skeletal muscle membranes (≤25 fmol mg^{-1} protein).

Introduction

It is now well recognised that the activation of cell surface receptors that mobilize intracellular Ca^{2+} results in the release of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) into the cell through the action of phosphoinositidase C on phosphatidylinositol 4,5-bisphosphate (Downes & Michell, 1985; Berridge, 1987). In a wide variety of tissues, including airway smooth muscle (ASM), Ins(1,4,5)P_3 has been shown to release Ca^{2+} from non-mitochondrial, intracellular stores and in the latter tissue this results directly in the generation of tension (Hashimoto *et al.*, 1985). On the basis of limited structure-activity studies, it has been proposed that Ins(1,4,5)P_3 is recognised by a receptor protein which is linked either directly, or indirectly, to a Ca^{2+} channel located on a discrete portion of the endoplasmic reticulum (Berridge & Irvine, 1984; Nahorski & Potter, 1989). Indeed, a number of studies have demonstrated high affinity, saturable Ins(1,4,5)P_3 binding sites in a variety of peripheral and central tissues (Baukal *et al.*, 1985; Spät *et al.*, 1986a; Guillemette *et al.*, 1987). In cerebellum these sites have been shown to display strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer (Willcocks *et al.*, 1987) that corresponds to that associated with Ca^{2+} release (Strupish *et al.*, 1988; Nahorski, 1988). More recently it has been shown that in brain, submicromolar concentrations of Ca^{2+} potently inhibit Ins(1,4,5)P_3 binding (Worley *et al.*, 1987), an action possibly mediated via a neutral membrane protein termed 'calmedin' (Danoff *et al.*, 1988). This feedback inhibition of Ins(1,4,5)P_3 binding by calcium has been proposed as a mechanism allowing the generation of intracellular Ca^{2+} oscillations, which are observed in many cells following addition of agonists (Joseph *et al.*, 1989).

Although Ins(1,4,5)P_3 has now been shown to open single Ca^{2+} channels in aortic smooth muscle sarcoplasmic reticulum incorporated into planar lipid bilayers (Ehrlich & Watras, 1988), direct binding of Ins(1,4,5)P_3 has not been demonstrated previously in a smooth muscle preparation. Here we describe the binding of [^{3}H]-Ins(1,4,5)P_3 to a bovine tracheal smooth muscle preparation which displays high affinity and strict stereo- and positional specificity for the D-Ins(1,4,5)P_3 isomer. In addition, we demonstrate that, in marked contrast to cerebellum, Ca^{2+} in concentrations up to 1 mM does not inhibit [^{3}H]-Ins(1,4,5)P_3 binding in this tissue and discuss the importance of this observation in terms of the regulation of Ca^{2+} release in peripheral and central tissues.

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Methods

Preparation of tracheal smooth muscle membranes

The cervical trachealis muscle from freshly slaughtered cattle was dissected free of epithelium and surrounding connective tissue in ice-cold Krebs-Henseleit buffer. Pieces of tissue (15–20 g) were cross-chopped at 300 μm by a McIlwain tissue chopper. Tissue slices were washed in 20 mM $NaHCO_3$, 1 mM dithiothreitol (DTT), homogenised in 10 vol ice-cold buffer (Polytron, setting 6, 15 s) and centrifuged at 5000*g* for 15 min at 4°C. The supernatant was decanted and kept on ice and the pellet rehomogenised in 5 vol $NaHCO_3$/DTT buffer and recentrifuged as above. The pooled supernatants were then centrifuged at 38,000*g* for 20 min at 4°C and the pellet resuspended in homogenisation buffer at a protein concentration of ~5 mg ml^{-1}. This crude 'P_2' membrane fraction, which contains sarcoplasmic reticular and sarcolemmal membranes (Grover *et al.*, 1980) was used in binding studies. For the comparative studies, bovine adrenal cortex, cardiac atria and ventricle, skeletal muscle (sterno-maxillaris) and rat cerebella obtained from male Wistar rats were prepared in an identical manner (Challiss *et al.*, 1988). Protein concentrations were determined according to Lowry *et al.* (1951).

[^{3}*H*]-*Ins(1,4,5)*P_3 *binding*

Assays were all performed at 4°C in a final volume of 120 μl, containing 30 μl membranes (~200 μg protein), 30 μl D-*myo*[^{3}H]-Ins(1,4,5)P_3 (~2 nM), 30 μl 100 mM Tris-HCl, 4 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8 and 30 μl water or water containing 40 μM DL-Ins(1,4,5)P_3 to define non-specific binding (Challiss *et al.*, 1988). Assays were intermittently vortex-mixed and kept on ice for 30 min. Separation of bound and free [^{3}H]-Ins(1,4,5)P_3 was achieved by rapid

dilution (3 ml) and filtration through Whatman GF/B glass-fibre filters followed by 3 × 3 ml washes using ice-cold 25 mM Tris-HCl, 5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0. Radioactivity was subsequently determined after 12 h extraction in scintillation fluid.

Determination of [^{3}H]-*Ins(1,4,5)P_3 metabolism*

The metabolism of [^{3}H]-Ins(1,4,5)P_3 was determined by incubating ~2 nM [^{3}H]-Ins(1,4,5)P_3 with active or heat-inactivated (15 min, 100°C) ASM membranes (200 μg protein) at 4°C; reactions were terminated after 30 min by addition of an equal volume of 1 M trichloroacetic acid. Neutralized extracts were applied to Dowex AG1-X8 anion exchange columns and [^{3}H]-InsP_x fractions eluted (Berridge *et al.*, 1983; Batty *et al.*, 1985).

Materials

D-*myo*[^{3}H]-Ins(1,4,5)P_3 (17.0 Ci mmol^{-1}) was a kind gift from Du Pont-New England Nuclear. D-Ins(1,4,5)P_3 was purchased from Amersham. L-Ins(1,4,5)P_3 and DL-Ins(1,3,4,5)P_4 were produced synthetically in the Department of Chemistry, University of Leicester, and D-Ins(1,3,4)P_3 was generously provided by Dr R. Irvine (Department of Biochemistry, Babraham, Cambridge). Adenosine 5'-triphosphate (ATP) and heparin (Sodium salt: Grade I porcine intestinal mucosal) were obtained from Sigma. All other reagents were of analytical grade obtained from commercial sources.

Data analysis

Dissociation equilibrium constant (K_D) and maximal binding capacity (B_{max}) of [^{3}H]-Ins(1,4,5)P_3 were determined by Scatchard analysis of [^{3}H]-Ins(1,4,5)P_3 displacement with unlabelled D-Ins(1,4,5)P_3, following correction for isotopic dilution and non-specific binding. IC_{50} and slope factor values of displacement data were determined by computer-assisted curve fitting (ALLFIT) (De Lean *et al.*, 1978) following correction of data for non-specific binding. K_i values were calculated from IC_{50} values by the equation derived by Cheng & Prusoff (1973).

Results

Although binding experiments were performed in the absence of Mg^{2+}, which is essential for phosphorylation and dephosphorylation of Ins(1,4,5)P_3, preliminary experiments were performed to determine whether significant metabolism of [^{3}H]-Ins(1,4,5)P_3 occurred under the assay conditions described (see Methods section). Under normal incubation conditions, in the presence of 1 mM EDTA, >98% of the radioactivity remained in the InsP_3 fraction with <1.5% and <0.5% eluting as inositol bisphosphate (InsP_2) or inositol monophosphate (InsP_1) respectively.

In contrast to the rat cerebellum and bovine adrenal cortex preparations freezing the ASM 'P_2' fraction for subsequent use caused significant (>60%) loss of specific binding. This occurred irrespective of whether samples were frozen at −20°C or plunged into liquid nitrogen. Therefore all studies were performed on freshly prepared tissue. Comparison of centrifugation and rapid filtration techniques for separating bound and free radioligand revealed essentially similar total binding with the two methods. However, filtration gave lower and more consistent values for non-specific binding.

Saturation analysis of [^{3}H]-*Ins(1,4,5)P_3 binding in ASM membranes*

Scatchard analysis of [^{3}H]-D-Ins(1,4,5)P_3 displacement by unlabelled D-Ins(1,4,5)P_3 indicates a single binding site with a

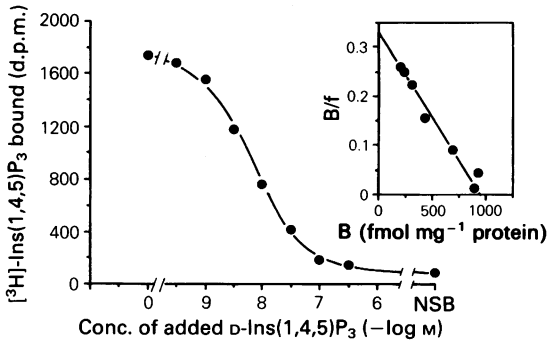


Figure 1 Saturation analysis of [^{3}H]-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. Assays were performed using 2 nM [^{3}H]-Ins(1,4,5)P_3 and 200 μg ASM membrane protein in a 25 mM Tris-HCl, 1 mM EDTA buffer (pH 7.8) together with increasing concentrations of unlabelled D-Ins(1,4,5)P_3 and incubated for 30 min at 4°C before rapid filtration. Non-specific binding (NSB) was determined with 10 μM DL-Ins(1,4,5)P_3. Values represent means of duplicate determinations from a single experiment with near identical results obtained in a further 2 separate experiments. The inset shows a Scatchard plot of bound/free (B/f) versus bound (B) ligand.

K_D of 3.8 ± 0.2 nM (mean ± s.e.mean) and B_{max} of 1003 ± 170 fmol mg^{-1} protein (n = 3) (Figure 1). Comparative data for [^{3}H]-D-Ins(1,4,5)P_3 binding to 'P_2' membrane fractions prepared in an identical manner from rat cerebellum, bovine adrenal cortex, cardiac atria and ventricle, and skeletal muscle are shown in Table 1. Binding of [^{3}H]-Ins(1,4,5)P_3 was also shown to be extremely rapid ($t_{1/2}$ on = 2 min) and reversible with a $t_{1/2}$ off of 5 min, determined by addition of D-Ins(1,4,5)P_3 (Figure 2).

Specificity of D-myo[^{3}H]-*Ins(1,4,5)P_3 binding*

The InsP_3 binding site in ASM appears highly selective for D-Ins(1,4,5)P_3 (Table 2). The pure L-enantiomer has a 4700 fold lower affinity for the site (Figure 3) which indicates that the binding site, as in cerebellum (Willcocks *et al.*, 1987), shows strict stereospecificity for the D-Ins(1,4,5)P_3 isomer. DL-Ins(1,3,4,5)P_4, which in its pure D-form has been shown to be

Table 1 Comparison of [^{3}H]-Ins(1,4,5)P_3 binding between tissues

Tissue	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n
Bovine			
Tracheal smooth muscle	3.8 ± 0.2	1003 ± 170	3
Adrenal cortex	3.7 ± 0.2	872 ± 70	4
Cardiac atria	4.3 ± 1.2	75 ± 5	3
Cardiac ventricle	—	≤25†	3
Skeletal muscle	—	≤25†	3
Rat			
Cerebellum	29.7 ± 4.1	14700 ± 1600	3

'P_2' membrane fractions from tissue homogenates were prepared as detailed in the Methods section with the cardiac, skeletal and smooth muscle cross-chopped (300 × 300 μm) on a McIlwain tissue chopper before homogenization. Incubation conditions were identical to those described in Figure 1 legend. B_{max} and K_D values were determined by Scatchard analysis of [^{3}H]-Ins(1,4,5)P_3 displacement by D-Ins(1,4,5)P_3 after correction for isotopic dilution and non-specific binding assessed in the presence of 10 μM DL-Ins(1,4,5)P_3. Values represent mean ± s.e.mean of n experiments.

† In the assay described using 2 nM [^{3}H]-Ins(1,4,5)P_3 and 250–350 μg membrane protein, specific binding was 45–70 d.p.m. This approximates to B_{max} values ≤25 fmol mg^{-1} protein.

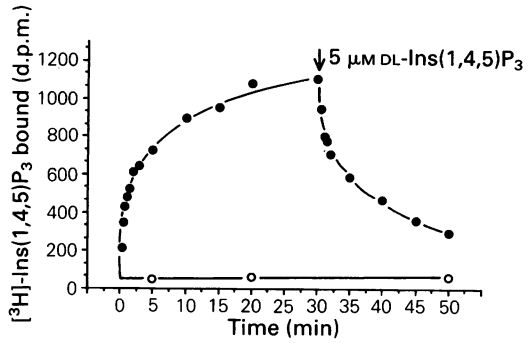


Figure 2 Rates of association and dissociation for [^{3}H]-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. Assay conditions were identical to those described in the legend to Figure 1. Reactions were started by the addition of 200 μg ASM membranes at 0 min and specific (●) and non-specific (○) [^{3}H]-Ins(1,4,5)P_3 binding determined at various times thereafter. The dissociation curve was obtained by adding D-Ins(1,4,5)P_3 (final concentration of 5 μM) to ASM membranes which had been incubated with [^{3}H]-Ins(1,4,5)P_3 for 30 min. Data from a single representative experiment are shown.

the most potent naturally occurring competitor for [^{3}H]-Ins(1,4,5)P_3 binding in other tissues, displayed an 80 fold lower affinity for this binding site. We have previously demonstrated that in bovine tracheal smooth muscle labelled with [^{3}H]-inositol and stimulated with the muscarinic receptor agonist carbachol, that [^{3}H]-Ins(1,3,4)-P_3 is the major [^{3}H]-$InsP_3$ accumulating in this tissue, even at relatively early timepoints (>85% at 1 min) (Chilvers *et al.*, 1988 and unpublished observations). It was therefore of considerable importance to assess the affinity of this $InsP_3$ isomer for the D-Ins(1,4,5)P_3 binding site in the same preparation. With a K_i value for Ins(1,3,4)P_3 of 2.4 μM it proved to have a 550 fold lower affinity for the binding site. Since incubations were performed at 4°C in the presence of 1 mM EDTA, and in the absence of added Mg^{2+} or ATP it is unlikely that any significant metabolism of the competing inositol polyphosphates occurred (Shears, 1989). Figure 3 also demonstrates that ATP had very weak displacing activity at this site.

Table 2 Inhibition of specific [^{3}H]-Ins(1,4,5)P_3 binding in airway smooth muscle (ASM) membranes

	IC_{50} (μM)	n
D-Ins(1,4,5)P_3	0.0062 ± 0.0004	4
L-Ins(1,4,5)P_3	29 ± 8	3
DL-Ins(1,3,4,5)P_4	0.49 ± 0.04	3
D-Ins(1,3,4)P_3	3.7, 3.0	2
ATP	1900 ± 300	3

Incubations were performed exactly as detailed in legend to Figure 1. Non-specific binding was determined using 10 μM DL-Ins(1,4,5)P_3. Values represent mean ± s.e.mean for n experiments, except for D-Ins(1,3,4)P_3 where, due to limited availability, $n = 2$ and hence individual values are given. The concentration of [^{3}H]-Ins(1,4,5)P_3 was 1.58 ± 0.17 nM for the 4 experiments performed to obtain all data-sets. Therefore IC_{50} values can be divided by (1 + 1.58/3.8) to obtain K_i values.

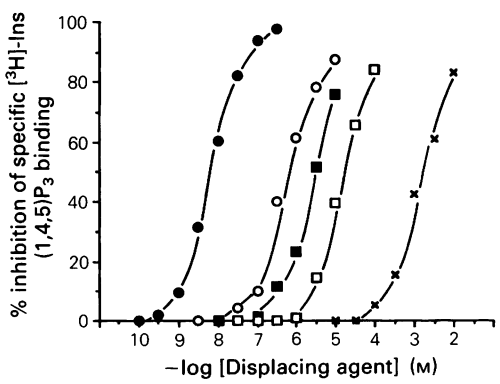


Figure 3 Specificity of [^{3}H]-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. Incubation conditions were identical to those in Figure 1 with specific binding determined by addition of 10 μM DL-Ins(1,4,5)P_3 to assess non-specific binding. Displacement of specific [^{3}H]-Ins(1,4,5)P_3 binding due to the presence of increasing concentrations of D-Ins(1,4,5) P_3 (●), DL-Ins(1,3,4,5) P_4 (○), D-Ins(1,3,4) P_3 (■), L-Ins(1,4,5)P_3 (□) and ATP (×) is shown, with data points representing means of 3 separate experiments each performed in duplicate, with the exception of Ins(1,3,4)P_3 where availability limited the number of experiments to 2; s.e.mean values (<5% of mean) were omitted for clarity.

Earlier studies have indicated that the glycosaminoglycan heparin is a potential Ins(1,4,5)P_3 receptor antagonist (Worley *et al.*, 1987; Cullen *et al.*, 1988; Ghosh *et al.*, 1988); furthermore, heparin-agarose affinity chromatography has been used to purify Ins(1,4,5)P_3 binding sites from rat cerebellum (Supattapone *et al.*, 1988). Therefore the ability of heparin to displace [^{3}H]-Ins(1,4,5)P_3 binding in ASM was examined. Figure 4 shows that heparin inhibited [^{3}H]-Ins(1,4,5)P_3 binding with a IC_{50} of 7.6 ± 1.0 μg ml^{-1} ($n = 4$), a value directly comparable to that seen in the rat cerebellum (6.6 ± 1.2 μg ml^{-1}; $n = 3$).

pH dependence and effect of Ca^{2+} on [^{3}H]-Ins(1,4,5)P_3 binding

The effect of pH on [^{3}H]-Ins(1,4,5)P_3 binding in ASM membranes was assessed by Tris-maleate (pH values 6.0–7.5) and Tris-HCl (pH values 7.5–9.0) buffers. Figure 5 demonstrates the marked increase in [^{3}H]-Ins(1,4,5)P_3 binding at pH values between 6 and 8. Assays were hence routinely conducted at pH 7.8.

Previous studies have indicated that the [^{3}H]-Ins(1,4,5)P_3 binding site in brain is potently inhibited by Ca^{2+} (EC_{50} ~ 300 nM) (Worley *et al.*, 1987; Joseph *et al.*, 1989). The inhibition by Ca^{2+} appears to be mediated by a protein termed 'calmedin' (Danoff *et al.*, 1988), since the purified cerebellum Ins(1,4,5)P_3 receptor is not Ca^{2+}-sensitive (Supattapone *et al.*, 1988). In the study by Danoff and colleagues it was also indicated that 'calmedin' activity is much lower in peripheral tissues, since 500 μM Ca^{2+} completely inhibited [^{3}H]-Ins(1,4,5) P_3 binding in various rat brain regions including spinal cord, yet caused only minor (15–20%) inhibition in testis, lung, liver and kidney preparations. Initial experiments, in which the

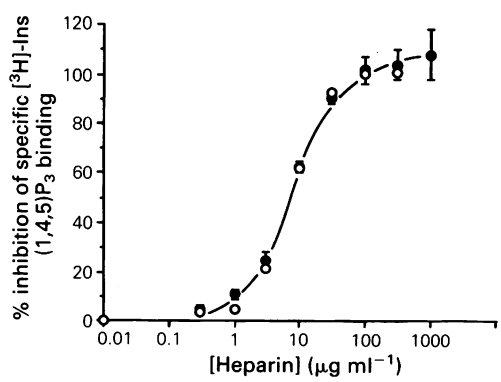


Figure 4 Displacement of [^{3}H]-Ins(1,4,5)P_3 binding by heparin. Cerebellum and airway smooth muscle (ASM) 'P_2' membrane fractions were prepared in an identical manner in 20 mM $NaHCO_3$, 1 mM dithiothreitol (pH 7.8) as described in the Methods section. With the exception that heparin (0.1–1000 μg ml^{-1}) was included instead of Ins(1,4,5)P_3, assays were performed as detailed in the legend for Figure 1. Values represent mean of 3 separate experiments each performed in duplicate; vertical lines show s.e.mean. (●) Airway smooth muscle; (○) cerebellum.

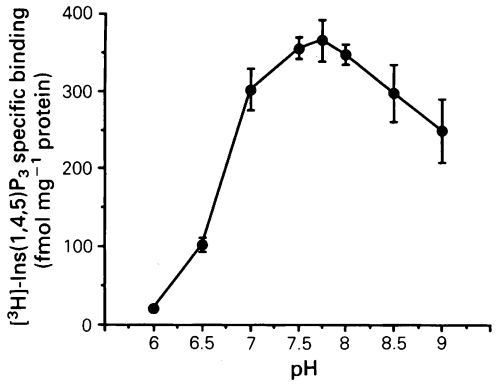


Figure 5 pH dependence of $[^3H]$-Ins(1,4,5)P_3 binding to airway smooth muscle (ASM) membranes. $[^3H]$-Ins(1,4,5)P_3 binding in ASM membranes was determined using 2 nM $[^3H]$-Ins(1,4,5)P_3 and 200 μg membrane protein in a range of 25 mM Tris (pH 7.5–9) and Tris-maleate (pH 6–7.5), 1 mM EDTA buffers incubated on ice for 30 min. Values represent mean of 3 separate experiments each performed in duplicate; vertical lines show s.e.mean.

effect of Ca^{2+} on $[^3H]$-Ins(1,4,5)P_3 binding to ASM membranes (prepared as described above) was examined, demonstrated that in the absence of EDTA substantial metabolism of the $[^3H]$-Ins(1,4,5)P_3 occurred during the 30 min incubation period (53 ± 3%; mean ± s.e.mean for 3 separate experiments). This was despite assays being performed at 4°C and the extent of metabolism clearly prevented interpretation of $[^3H]$-Ins(1,4,5)P_3 binding data. However, with further washing of the ASM membranes (2 × 50 ml 20 mM $NaHCO_3$, 1 mM DTT, pH 7.8), metabolism of the $[^3H]$-Ins(1,4,5)P_3, both in the absence of EDTA or in the presence of added $CaCl_2$ (1 mM) was considerably reduced (Table 3) with only minor inhibition of specific $[^3H]$-Ins(1,4,5)P_3 binding observed. In the presence of 1 mM EDTA the free $[Ca^{2+}]$, measured with a Ca^{2+}-sensitive electrode (Sigel & Affolter, 1987) was < 50 nM, whereas in the absence of EDTA the free $[Ca^{2+}]$ was 5–10 μM. In the presence of 1 mM $CaCl_2$ (no EDTA) the free $[Ca^{2+}]$ did not differ significantly from 1 mM.

Discussion

These data provide the first direct evidence for an Ins(1,4,5)P_3 binding site in smooth muscle. The binding site appears to represent the true physiological Ins(1,4,5)P_3 receptor rather than the inositol polyphosphate 5-phosphatase or 3-kinase enzymes since the affinity of Ins(1,4,5)P_3 binding in ASM membranes is at least 5000 fold greater than the K_m of the 5-phosphatase (Downes et al., 1982; Connolly et al., 1987), and at least 100 fold greater than the K_m of the 3-kinase enzyme which is also predominantly soluble (Irvine et al., 1986). In addition, the selectivity of the binding site for the various inositol polyphosphates tested, together with the affinity of heparin for this site, mirrors the relative potencies of these compounds in initiating/inhibiting Ca^{2+} release, as demonstrated in other tissues (Strupish et al., 1988; Nahorski, 1988; Ghosh et al., 1988).

The stereo- and positional specificity of $[^3H]$-Ins(1,4,5)P_3 binding in ASM membranes is very similar to that obtained in cerebellum (Willcocks et al., 1987), with the use of synthetic pure DL-Ins(1,3,4,5)P_3 and L-Ins(1,4,5)P_3 uncontaminated by D-Ins(1,4,5)P_3 greatly assisting in these determinations. The high affinity binding displayed by these sites in ASM (K_D 3.8 nM) is in close agreement with that demonstrated in particulate fractions of adrenal cortex (Baukal et al., 1985), anterior pituitary cells (Guillemette et al., 1987), liver (Spät et al., 1986b) and permeabilized hepatocytes and neutrophils (Spät et al., 1986a), but is of approximately 10 fold higher affinity than that obtained in cerebellum (K_D 20–40 nM) (Worley et al., 1987; Willcocks et al., 1987). The clear disparity in K_D values between neuronal and non-neuronal Ins(1,4,5)P_3 binding sites, together with the differences in Ca^{2+} regulation discussed above and preliminary findings that heparin may also have different affinities at these two sites (Willcocks & Nahorski, 1989), suggest possible heterogeneity of the Ins(1,4,5)P_3 receptor.

Joseph et al. (1989) have recently shown that the effects of Ca^{2+} on Ins(1,4,5)P_3 binding in cerebellum microsomes are exerted through a change in the apparent affinity of the receptor for Ins(1,4,5)P_3 rather than through effects on maximal binding. The sensitivity of $[^3H]$-Ins(1,4,5)P_3 binding in neuronal tissues to submicromolar Ca^{2+} concentrations is in marked contrast to the Ca^{2+} insensitivity of binding in ASM membranes, a property shared by a number of other peripheral tissues (Danoff et al., 1988). These different effects of Ca^{2+} on Ins(1,4,5)P_3 binding in different tissues may reflect the heterogeneous distribution of calmedin (Danoff et al., 1988) and may explain Ca^{2+}-induced inhibition of Ins(1,4,5)P_3-induced Ca^{2+} release, which is observed in neuronal (Joseph et al., 1989) but not peripheral tissue preparations (Burgess et al., 1984). These findings suggest that in ASM, Ca^{2+} release should parallel Ins(1,4,5)P_3 concentration in the cell, whereas in neuronal tissue Ca^{2+}, once released from intracellular stores, may inhibit further Ins(1,4,5)P_3-induced Ca^{2+} release. In line with this proposal, we have demonstrated recently that the muscarinic agonist carbachol induces a sustained increase in D-Ins(1,4,5)P_3 mass in cerebral cortex slices, compared to a transient increase in tracheal smooth muscle slices despite ongoing Ins(1,4,5)P_3 formation (Challiss et al., 1988; Chilvers et al., 1989). It appears therefore that the differential effect of Ca^{2+} on Ins(1,4,5)P_3 binding may be of fundamental importance in terms of differences in $[Ca^{2+}]_i$ regulation in neuronal and non-neuronal tissues.

In contrast to the different effects of Ca^{2+} on $[^3H]$-Ins(1,4,5)P_3 binding, the pH profile appears very similar between

Table 3 Effect of Ca^{2+} on $[^3H]$-Ins(1,4,5)P_3 binding and metabolism in airway smooth muscle (ASM) membranes

Incubation conditions	*Relative inhibition of specific $[^3H]$-Ins(1,4,5)P_3 binding* (% EDTA value)	n	*Metabolism of $[^3H]$-Ins(1,4,5)P_3* (% control)	n
+EDTA (1 mM)	0	3	0.6 ± 0.3	6
−EDTA	14.0 ± 3.3	3	24 ± 3	3
+$CaCl_2$ (1 mM)	8.0 ± 1.7	3	6 ± 1	3

Incubations were performed as described in the legend to Figure 1, except that ASM 'P_2' membrane fractions were washed in 2 × 50 ml 20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 7.8 before use and 1 mM EDTA or 1 mM $CaCl_2$ included as indicated. Free $[Ca^{2+}]$ in the absence of EDTA, measured using a Ca^{2+}-sensitive electrode, was 5–10 μM. $[^3H]$-Ins(1,4,5)P_3 metabolism was determined in parallel incubations by terminating reactions with 1 M trichloroacetic acid and sequential elution of $[^3H]$-Ins to $[^3H]$-InsP_3 in neutralised extracts by anion exchange chromatography. Values are means ± s.e.mean for *n* experiments. Control values for the metabolism experiments were determined by incubations containing heat-inactivated (100°C, 15 min) ASM membranes.

tissues at physiological pH values. Joseph *et al.* (1989) cite provisional data suggesting that pH-induced changes in ionization of the Ins(1,4,5)P_3 molecule closely parallel the effects of pH on Ins(1,4,5)P_3 binding. Since activation of Ca^{2+} mobilizing receptors on the surface of cells frequently leads to an increase in intracellular pH through protein kinase C stimulation of Na^+/H^+ exchange (Rozengurt, 1986; Frelin *et al.*, 1988), and given such a relationship between pH and Ins(1,4,5)P_3 binding, then even subtle changes in intracellular pH may have a physiological role in regulating Ins(1,4,5)P_3-induced Ca^{2+} release.

The IC_{50} values obtained for heparin at the Ins(1,4,5)P_3 binding site in ASM and cerebellum are directly comparable to values obtained previously in rat cerebellum (5 $\mu g\,ml^{-1}$, Worley *et al.*, 1987, and 16 $\mu g\,ml^{-1}$, Willcocks *et al.*, 1989) and rat liver microsomes (approx. 5 $\mu g\,ml^{-1}$, Tones *et al.*, 1989) and these correlate closely with the potency of heparin in inhibiting Ins(1,4,5)P_3-induced Ca^{2+} release (IC_{50} 5–16 $\mu g\,ml^{-1}$, Hill *et al.*, 1987; Cullen *et al.*, 1988). The activity of the heparin molecule appears to depend on an interaction between the negatively charged N-hexosamine sulphated residues and the Ins(1,4,5)P_3 receptor since the de-N-sulphated heparin is unable to compete with Ins(1,4,5)P_3 binding or affect Ins(1,4,5)P_3-induced Ca^{2+} release (Tones *et al.*, 1989). In permeabilized DDT_1MF-2 smooth muscle cells, heparin has been shown to be a competitive, reversible and potent antagonist of Ins(1,4,5)P_3-induced Ca^{2+} release (Ghosh *et al.*, 1988), with a heparin concentration of 10 $\mu g\,ml^{-1}$ completely inhibiting the response. In cerebellum membranes, Ins(1,4,5)P_3 binding has been shown to be approximately five times more sensitive to low molecular weight heparin (mol. wt. 4–6000) used in the above study compared with the non-cleaved heparin (mol. wt. >20,000) used in this study.

Ins(1,4,5)P_3 has been shown to result in Ca^{2+} release in permeabilized cardiac muscle (Suematsu *et al.*, 1984) and a range of contractile agonists, including noradrenaline, have been shown to increase [^{3}H]-InsP_3 formation in [^{3}H]-inositol prelabelled tissue (Poggioli *et al.*, 1986; Heathers *et al.*, 1988). However, the physiological importance of this second messenger in regulating contraction in this tissue remains controversial (Volpe *et al.*, 1988). Ehrlich & Watras (1988) failed to demonstrate Ins(1,4,5)P_3 activation of Ca^{2+} channels in cardiac sarcoplasmic reticulum vesicles incorporated into lipid bilayers and this, together with our demonstration of low specific [^{3}H]-Ins(1,4,5)P_3 binding in bovine cardiac atria and very low binding to cardiac ventricle membranes, supports the view that phosphoinositide hydrolysis may not play a central role in excitation-contraction coupling in this tissue. Indeed, it is possible that the very low specific [^{3}H]-Ins(1,4,5)P_3 binding detected in these cardiac muscle preparations reflects binding in contaminating vascular and neuronal elements.

In summary, we have identified a high affinity, stereo- and positionally specific binding site for D-*myo*[^{3}H]-Ins(1,4,5)P_3 in ASM membranes. This binding is maximal at pH 7.75, is displaced by heparin and is unaffected by physiological and supraphysiological Ca^{2+} concentrations. These findings provide an important link in the hypothesis that receptor-mediated increases in Ins(1,4,5)P_3 may, through interaction of this second messenger with specific intracellular receptors which mediate release of Ca^{2+} from intracellular stores, play a central role in pharmacomechanical coupling in smooth muscle (Somlyo *et al.*, 1988). In the airway, the development of an Ins(1,4,5)P_3 receptor antagonist, that can gain access to the interior of the ASM cell following topical administration via the inhaled route, may have therapeutic importance in diseases characterised by airways hyperreactivity.

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Endothelin-1 inhibits platelet aggregation *in vivo*: a study with ^{111}indium-labelled platelets

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1 A non-invasive technique for the scintigraphic determination of ^{111}indium-labelled platelet aggregation stimulated with submaximal doses of adenosine diphosphate (ADP, 56 μg kg^{-1} i.v.), collagen (100 μg kg^{-1} i.v.), platelet-activating factor (PAF, 0.1 μg kg^{-1} i.v.) or thrombin (18 iu kg^{-1} i.v.) was used to investigate the platelet-inhibitory effects of endothelin 1 (ET-1) in anaesthetized rabbits *in vivo*.

2 ET-1 (1 nmol kg^{-1} i.v.) inhibited ADP-stimulated platelet aggregation *in vivo*; a maximum inhibition of 78% of the control value was reached at 3 min, with 45% inhibition at 15 min, and a return to control values at 30 min after injection of the peptide.

3 ET-1 (1 nmol kg^{-1} i.v.) inhibited *in vivo* platelet aggregation in response to collagen or PAF by 86% and 52%, respectively, but had no effect on thrombin-induced platelet aggregation.

4 Indomethacin (5 mg kg^{-1} i.v.) abolished the ET-1-induced inhibition of ADP-stimulated platelet aggregation and significantly potentiated and prolonged the pressor response brought about by ET-1.

5 In conclusion, the data demonstrate that ET-1 potently inhibits platelet aggregation in the anaesthetized rabbit *in vivo* by releasing a hypotensive and anti-aggregatory cyclo-oxygenase product, presumably prostacyclin, into the circulation.

Introduction

The endothelins are a family of three structurally related, but pharmacologically distinct vasoactive peptides, containing 21 amino acid residues with 2 sets of intrachain disulphide bridges (Inoue *et al.*, 1989). Endothelin-1 (ET-1), the most widely studied member of this family, was isolated from conditioned medium of cultured vascular endothelial cells (Yanagisawa *et al.*, 1988a) and is now well characterized as a potent constrictor of arterial (Yanagisawa & Masaki, 1989) and venous vessels (D'Orleans-Juste *et al.*, 1989) both *in vitro* and *in vivo*. In addition, ET-1 releases vasodilator autacoids including prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF) from perfused organs and vessels of a variety of species (De Nucci *et al.*, 1988; Warner *et al.*, 1989) including the rabbit (Rae *et al.*, 1989).

We have previously demonstrated that ET-1 inhibits platelet aggregation in the anaesthetized rabbit when measured *ex vivo* in response to ADP (Thiemermann *et al.*, 1988). However, *in vitro* and *ex vivo* tests of platelet function correlate poorly with the *in vivo* activation of platelets and their participation in vascular thrombosis (Packham & Mustard, 1980; Smith, 1981; Dewanjee, 1984). More suitable experimental approaches for investigating platelet activation *in vivo* include: (1) determination of shortened platelet survival and/or increased turnover by means of radioactive-labelled platelets (Harker, 1978) or continuous platelet counting (Smith & Freuler, 1974); (2) measurement of circulating platelet aggregate ratios (Wu & Hoak, 1974); (3) the use of filter-loop techniques (Hornstra, 1980; Bee *et al.*, 1980) and (4) the determination of increased plasma levels of platelet factor 4, β-thromboglobulin (Kaplan & Osen, 1981), or thromboxane B_2 (Granstrom *et al.*, 1976).

In the present study a non-invasive technique for the scintigraphic determination of *in vivo* platelet aggregation with ^{111}indium-labelled platelets (Page *et al.*, 1982) was used to investigate the potential inhibitory effects of ET-1 on platelet aggregation induced by i.v. injection of adenosine 5'-diphosphate (ADP), collagen, platelet activating factor (PAF) or thrombin. A further aim was to determine whether the platelet-inhibitory or the haemodynamic effects of the peptide are mediated by an increased formation of endogenous prostaglandins *in vivo*.

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Methods

Animal selection and exclusion criteria

The study was carried out on male rabbits (New Zealand White SPF Rabbits, Regal, Great Bookham, U.K.) weighing 1.9 to 3.5 kg; they received a standard diet and water *ad libitum*. All data obtained from animals which died due to the injection of an agonist of platelet aggregation were excluded from the study.

^{111}*Indium-labelling of platelets*

Rabbit platelets were labelled *in vitro* with ^{111}In-oxine (Thakur *et al.*, 1976). Details of this procedure have been previously described (Page *et al.*, 1982; Oyekan & Botting, 1986) and validated for the rabbit (May *et al.*, 1989). Briefly, blood (15 ml) was obtained from the marginal ear vein, collected into trisodium citrate (3.8% w/v) and subsequently centrifuged for 12 min at 200 *g* to produce platelet-rich plasma (PRP). After the addition of prostaglandin E_1 (PGE_1, 300 ng ml^{-1}), the PRP was centrifuged for 15 min at 640 *g* and the platelet pellet resuspended in 1.5 ml of buffer solution (calcium-free Tyrode solution containing 300 ng ml^{-1} PGE_1). The platelets were incubated for 90 s at 37°C with ^{111}In-oxine (25–50 μCi). After a further centrifugation step (640 *g* for 15 min), the supernatent which contained free ^{111}In-oxine was removed and the platelet pellet resuspended in 1 ml of buffer.

Measurement of in vivo *platelet aggregation with an automated isotope monitoring system*

Animals were anaesthetized with a combination of diazepam (5 mg kg^{-1} i.p.) and Hypnorm (0.4 ml kg^{-1} i.m. containing 0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone). ^{111}In-labelled platelets (suspended in 1 ml buffer) were administered via a cannula in the marginal ear vein and allowed to equilibrate for 1 h.

Circulating ^{111}In-labelled platelets were continuously monitored in the thoracic and hind limb regions by one inch

crystal scintillation probes located over the thorax (to measure platelet accumulation in the pulmonary circulation) and over the hind limb (to measure peripheral thrombocytopenia). Counts were estimated with a dual channel spectrometer adjusted for the 174 keV and 247 keV peaks used (Nuclear Enterprises NE 461) and logged with the aid of a special application interface with a microcomputer.

In vivo platelet aggregation in response to i.v. administration of various platelet agonists was measured as an increase in ^{111}In platelet radioactivity in the pulmonary vasculature and as a concurrent fall in ^{111}In radioactivity in the femoral region (peripheral thrombocytopenia). Maximal changes in radioactive counts over thoracic and hind limb region were calculated, corrected for blood pool ^{111}In radioactivity (baseline value) and expressed as % increase and % decrease, respectively.

Stimulation of platelet aggregation in vivo

Pulmonary accumulation of ^{111}In-labelled platelets was monitored in response to an i.v. bolus injection (total volume 0.3 ml) of ADP, collagen, PAF or thrombin into the marginal ear vein of the anaesthetized animal. All agonists used in this study induce a dose-related increase in ^{111}In-platelet radioactivity in the pulmonary vasculature of the rabbit (May *et al.*, 1989). Thus, submaximal doses of ADP (56 μg kg^{-1} i.v.), collagen (100 μg kg^{-1} i.v.), PAF (0.1 μg kg^{-1} i.v.) or thrombin (18 iu kg^{-1} i.v.) were used to investigate the potential anti-aggregatory effects of ET-1. In animals that received several injections of ADP, a minimum time interval of 10 min was left between each injection. In animals that had more than one injection of collagen or PAF a time interval of at least 1 h was left between each injection. Only one dose of thrombin was given per animal, since the responses did not necessarily return to preinjection values within 60 min (May *et al.*, 1989).

Haemodynamic measurements

The haemodynamic effects of ET-1 were investigated in a separate group of rabbits to minimize the number of interventions (i.e. vascular trauma associated with surgery; vascular instrumentation by catheters) resulting in endothelial cell damage, enhanced thromboxane B_2 plasma levels, as well as thrombocytopenia which may therefore artificially influence platelet function *in vivo* (Schror & Thiemermann, 1986).

Ten minutes before surgery, the animals were premedicated with Hypnorm (0.1 ml kg^{-1} i.m.). General anaesthesia was induced with sodium pentobarbitone (30 mg kg^{-1} i.v.) and maintained with supplementary doses of the anaesthetic as required. The rabbits were intubated and ventilated with air from a Harvard ventilator at a rate of 36–40 strokes per min and a tidal volume of 18–20 ml. These ventilation parameters maintain physiological blood gas values (Thiemermann *et al.*, 1989a). Body temperature was maintained at 38 ± 1°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (CFP 8185). The left femoral vein was cannulated for drug administration. A catheter was placed in the left ventricle via the right common carotid artery for left ventricular pressure recordings made from a Bell & Howell 40-422-0001 pressure transducer.

Haemodynamic parameters, including heart rate, left ventricular systolic (LVP) and left ventricular contractile force dp/dt_{max} were continuously recorded on a 4-channel Grass 7D polygraph recorder (Quincy, Mass., U.S.A.). However, detailed analysis was only performed at time 0 (baseline measurement), 1, 5, 15, 30 and 60 min. Heart rate and contractile force (dp/dt_{max}) were automatically calculated from left ventricular systolic pulse curves by means of a Grass 7P4H tachograph and a Grass 7P20C differentiator, respectively.

Four experimental groups were studied: vehicle controls ($n = 3$); indomethacin controls ($n = 3$), ET-1 ($n = 6$) and ET-1 after indomethacin ($n = 6$).

Drugs

The following compounds were used: ADP, indomethacin, bovine serum albumin (BSA), Tyrode salt solution and thrombin (Sigma, Poole, Dorset); diazepam (Valium, Roche, Welwyn Welwyn Garden City); Hypnorm (Janssen Pharmaceutical Ltd., Oxford); collagen (Hormon-Chemie, Munich, F.R.G.); endothelin-1 (Peptide Research Institute, Osaka, Japan); PAF (Novabiochem, Nottingham); pentobarbitone sodium (Sagatal, May & Baker, Dagenham); ^{111}indium-oxine (Amersham International plc). ET-1 was dissolved in phosphate-buffered saline (pH 7.4). PAF was dissolved in 0.25% BSA and collagen was suspended in isotonic glucose solution (pH 2.7). Indomethacin was dissolved in 0.5% (w/v) sodium bicarbonate. All other compounds were prepared in physiological saline.

Statistical comparison

All values in the text, figures and tables are expressed as the mean ± s.e.mean of n observations. Where repeated measurements were taken over the course of the experimental period the results were analysed by a two-way analysis of variance, while end-point experiments were analysed by a one-way analysis of variance. Analysis of variance were followed by a least significance difference procedure (LSD) to determine the nature of the response (SPSS Inc., Chicago, Il., U.S.A.). A P value of <0.05 was considered statistically significant.

Results

Endothelin-1 inhibits platelet aggregation in vivo

The accumulation of ^{111}In-labelled platelets in the pulmonary vasculature in response to ADP (56 μg kg^{-1} i.v.; $n = 6$) was rapid and transient, reached a maximum within 30 s and returned to preinjection levels within 5 min. Subsequent injection of ADP (56 μg kg^{-1} i.v.; $n = 4$) at 15 min intervals (for 60 min) produced reproducible responses indicating that a desensitization of the platelets to repetitive injections of ADP did not occur (Table 1).

The time-course of the effects of ET-1 against ADP-induced platelet aggregation *in vivo* is deplicted in Figure 1. ET-1 (1 nmol kg^{-1} i.v.; $n = 6$) inhibited the maximal increase in ^{111}In-platelet radioactivity in the thoracic region in response to ADP by approximately 80% and 45% of the control value at 3 min and 15 min after injection of the peptide, respectively ($P < 0.01$; Figure 1a). However, at 30 min after injection of the peptide, the platelet-inhibitory effect of ET-1 was no longer evident ($P > 0.05$).

Table 1 Reproducibility of changes in ^{111}In-platelet radioactivity over thoracic and hind limb region in response to adenosine diphosphate (ADP) in anaesthetized rabbits

	^{111}*In-platelet radioactivity* (% change)	
Time (min)	*Thoracic region* (% increase in counts)	*Hind limb region* (% decrease in counts)
0	38 ± 4	26 ± 3
15	40 ± 5	27 ± 5
30	37 ± 4	25 ± 5
45	36 ± 6	24 ± 6
60	37 ± 5	26 ± 5

Values are expressed as mean ± s.e.mean of 4 observations. Successive injections of ADP (56 μg kg^{-1}; i.v. at 15 min intervals) produced consistent changes in ^{111}In-platelet radioactivity in both thoracic (accumulation in the lung) and hind limb regions (peripheral thrombocytopenia).

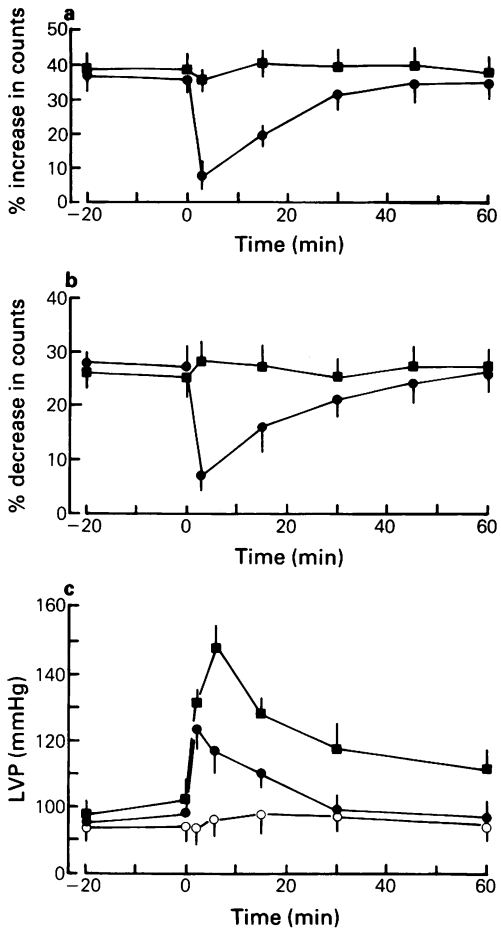


Figure 1 Time course of the effects of endothelin-1 (ET-1) on *in vivo* platelet aggregation induced by ADP (56 $\mu g\,kg^{-1}$, i.v.) in the anaesthetized rabbit. *In vivo* platelet aggregation was measured as % increase in ^{111}In-platelet radioactivity in the thoracic region (a) and % decrease in ^{111}In-platelet radioactivity in the femoral region (b) in response to successive injections of ADP (56 $\mu g\,kg^{-1}$, i.v. at 0, 3, 15, 30, 45 and 60 min). Different groups of animals received either ET-1 (●, 1 $nmol\,kg^{-1}$, $n = 6$) or indomethacin plus ET-1 (■, 5 $mg\,kg^{-1}$ i.v., $n = 4$). Indomethacin attenuated the anti-aggregatory effect of ET-1 at 3 and 10 min after injection of the peptide. (c) Depicts the changes in left ventricular systolic pressure (LVP) in animals that received either vehicle (○, control group; $n = 6$), ET-1 (●, 1 $nmol\,kg^{-1}$ i.v., $n = 6$) or indomethacin plus ET-1 (■, 5 $mg\,kg^{-1}$ i.v., $n = 6$). ET-1 was given at time 0. Data are expressed as mean of n observations; vertical lines show s.e.mean. $^*P < 0.05$ when compared to vehicle-control.

The ADP-induced accumulation of ^{111}In-labelled platelets in the pulmonary vasculature was associated with a significant fall in ^{111}In-platelet radioactivity in the hind limb region (Figure 1b). This peripheral thrombocytopenia in response to ADP was also inhibited by ET-1; the time-course of the ET-1-mediated reduction in both ADP-induced thrombocytopenia and accumulation of ^{111}In-platelet radioactivity in the lung showed a very similar pattern (Figure 1a and b).

To elucidate whether the inhibitory effect of ET-1 on platelet accumulation is mediated by the release of anti-aggregatory prostaglandins, a separate group of animals was treated with the cyclo-oxygenase inhibitor indomethacin (5 $mg\,kg^{-1}$, i.v.; $n = 6$) 20 min before injection of peptide. Indomethacin did not affect the responses to ADP alone (hind limb: 26 ± 4% and 25 ± 5%; lung: 39 ± 5% and 39 ± 6% before and after indomethacin, respectively), but abolished the inhibition of ADP-induced platelet aggregation brought about by ET-1 (Figure 1a and b).

Figure 2 shows the dose-dependence of the platelet-inhibitory effect of ET-1 *in vivo*. Lower doses of the peptide (0.01–0.1 $nmol\,kg^{-1}$) had no effect on the accumulation of ^{111}In-labelled platelets in the lung in response to ADP ($n = 4$; $P > 0.05$). However, 0.3 $nmol\,kg^{-1}$ ET-1 resulted in a 38% inhibition of ADP-induced platelet aggregation ($P < 0.05$), while 1 $nmol\,kg^{-1}$ of the peptide produced a near maximal effect. Higher concentrations of the peptide (3 $nmol\,kg^{-1}$) produce respiratory and/or cardiac failure in the rabbit (Thiemermann & Lidbury, unpublished results) and were therefore not investigated in the present study.

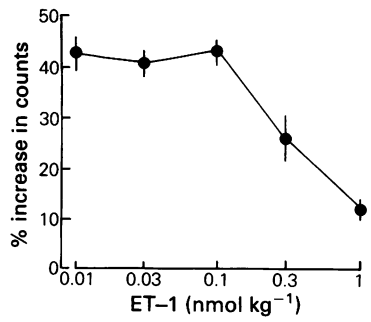


Figure 2 Dose-dependent inhibition of platelet accumulation in the lung by endothelin-1 (ET-1). *In vivo* platelet aggregation was measured as % increase in ^{111}In-platelet radioactivity in the thoracic region in response to ADP (56 $\mu g\,kg^{-1}$ i.v.). ET-1 was administered i.v. 3 min before ADP; the time interval between successive injections of ET-1 was at least 60 min. Concentrations of ET-1 below 0.1 $nmol\,kg^{-1}$ did not influence platelet aggregation, whereas concentrations of 0.3 $nmol\,kg^{-1}$ and 1 $nmol\,kg^{-1}$ of the peptide induced a significant inhibition. Data are expressed as mean of 4 observations; vertical lines show s.e.mean. $^*P < 0.05$ when compared to control.

The collagen (100 $\mu g\,kg^{-1}$; $n = 5$)-induced platelet accumulation *in vivo* was transient, reached a maximum (of 43 ± 6%) within 3 min and returned to preinjection levels within 10–25 min. ET-1 (3 min before injection of collagen) significantly attenuated the maximal increase in ^{111}In-labelled platelet radioactivity in the thoracic region and the fall in ^{111}In-platelet radioactivity in the hind limb region ($n = 6$; $P < 0.01$; Figure 3). Furthermore, ET-1 inhibited the PAF-stimulated (0.1 $\mu g\,kg^{-1}$; $n = 5$) accumulation of ^{111}In-platelet radioactivity in the pulmonary vasculature and the concurrent thrombocytopenia by about 50% and 40%, respectively ($n = 5$; $P < 0.01$; Figure 3).

The degree of ^{111}In-platelet accumulation in the lung after stimulation with thrombin (18 $iu\,kg^{-1}$; $n = 5$) reached a maximum of 35 ± 5% within 2 min. The corresponding fall in ^{111}In-platelet radioactivity in the hind limb region amounted to 28 ± 6%, but these responses returned to preinjection levels within 30–60 min. ET-1 reduced the thrombin-stimulated

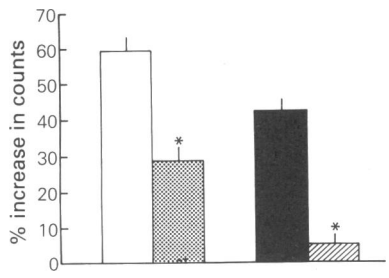


Figure 3 Endothelin-1 (ET-1)-induced inhibition of platelet accumulation in the lung induced by collagen and platelet-activating factor (PAF) in the anaesthetized rabbit. *In vivo* platelet aggregation was measured as % increase of ^{111}In-platelet radioactivity in the thoracic region. Different groups of animals received collagen (solid column; 100 $\mu g\,kg^{-1}$ i.v.), PAF (open column; 0.1 $\mu g\,kg^{-1}$ i.v.); ET-1 plus collagen (hatched column) or ET-1 plus PAF (stippled column). ET-1 (1 $nmol\,kg^{-1}$ i.v.) was given 3 min before PAF or collagen. Data are expressed as mean of 5–6 observations; vertical bars show s.e.mean. $^*P < 0.01$ when ET-1-treated animals were compared to the respective controls.

Table 2 Haemodynamic data in rabbits for heart rate (HR) and left ventricular contractile force (dp/dt_{max}) in control group (Con), endothelin-1 (ET-1) group and ET-1 plus indomethacin (Indo) group

Time (min)	HR (beats min^{-1}) Con	ET	Indo	dp/dt_{max} (10 mmHg s^{-1}) Con	ET-1	Indo
1	272 ± 5	263 ± 6	264 ± 5	408 ± 17	396 ± 35	383 ± 17
1	273 ± 4	251 ± 16	244 ± 13*	400 ± 16	488 ± 66*	504 ± 35*
5	272 ± 3	234 ± 18*	213 ± 7*	408 ± 17	450 ± 56	500 ± 47*
15	271 ± 3	237 ± 16*	220 ± 9*	409 ± 16	429 ± 51	458 ± 31
30	263 ± 2	238 ± 3*	235 ± 9*	400 ± 16	367 ± 37	413 ± 27
60	266 ± 4	255 ± 9	244 ± 7	396 ± 18	363 ± 35	375 ± 22

Values are given as mean ± s.e.mean of 6 observations for each group; *$P < 0.05$ when compared to control at the same time points.

platelet accumulation in the pulmonary vasculature and the respective thrombocytopenia to 22 ± 6% and 17 ± 3%, respectively, but this effect was not significant ($n = 7$; $P > 0.05$).

Haemodynamic effects of endothelin-1

Values for LVP, HR and left ventricular contractile force dp/dt_{max} measured during the course of the experiment are given in Figure 1c and Table 2. Baseline (time 0) haemodynamic data were similar in all animal groups studied ($P > 0.05$). In control animals, neither vehicle (phosphate-buffered saline; $n = 3$) nor indomethacin ($n = 3$) had any effect on any of the haemodynamic parameters measured. Thus, the results of both groups were combined and will be referred to as the control group. ET-1 caused a significant increase in LVP from 98 ± 6 mmHg to 124 ± 7 mmHg ($P < 0.01$; $n = 6$; Figure 1c) which returned to normal values within 30 min. Pretreatment of the animals with indomethacin both potentiated and prolonged the rise in LVP brought about by ET-1 ($P < 0.05$ at 5 to 60 min when compared to ET-1), so that the LVP was still significantly elevated in indomethacin-ET-1 treated animals at 30 min ($P < 0.01$ vs control) and 60 min ($P < 0.05$ vs control) after injection of the peptide (Figure 1c). In addition, ET-1 caused a reduction in heart rate and an increase in left ventricular contractile force, which were not significantly influenced by pretreatment of the animals with indomethacin (Table 2).

Discussion

In the present study, we investigated the platelet-inhibitory effects of ET-1 by measuring the accumulation of ^{111}indium-labelled platelets in the lung in response to various agonists of platelet aggregation. This technique is a measure of *in vivo* platelet aggregation (Page *et al.*, 1982; Oyekan & Botting, 1986; May *et al.*, 1989).

Our data clearly demonstrate that ET-1 inhibits *in vivo* platelet aggregation in response to ADP, collagen or PAF in the anaesthetized rabbit. The weak inhibition of thrombin-stimulated platelet aggregation by ET-1 was not unexpected, for thrombin-generated fibrin stabilizes the growing platelet mass resulting in deposition of irreversible platelet aggregates in the microcirculation (Niewiarowski *et al.*, 1972). This is reflected by our finding that the thrombin-induced accumulation of ^{111}In-labelled platelets in the pulmonary vasculature is not completely reversible and associated with a long-lasting thrombocytopenia.

The platelet-inhibitory effect of ET-1 *in vivo* is transient, lasting for about 15–30 min, and is most likely due to the release of anti-aggregatory autacoids, principally prostacyclin, into the circulation, for ET-1 does not inhibit platelet aggregation *in vitro* (De Nucci *et al.*, 1988). The view that the inhibition of platelet aggregation caused by ET-1 is mediated by prostacyclin is supported by our finding here that indomethacin abolished the anti-aggregatory effect of the peptide and by our previous finding that, like exogenous prostacyclin (Tateson *et al.*, 1977; Gorman *et al.*, 1977), inhibition of *ex vivo* platelet aggregation by ET-1 is associated with increased platelet cyclic AMP levels, and that this increase is ablated by indomethacin (Thiemermann *et al.*, 1989b). Bult *et al.* (1980) have shown that lower doses of indomethacin (2.5 mg kg^{-1} i.v.) are sufficient to inhibit prostacyclin formation (measured as 6-keto-$PGF_{1\alpha}$ by radioimmunoassay) in the rabbit. Even when the rabbits were subjected to endotoxin (a potent stimulus of endogenous prostacyclin formation), this low dose of indomethacin abolished the rise in plasma 6-keto-$PGF_{1\alpha}$ for at least 3 h.

In addition to inhibiting platelet aggregation, ET-1 caused a marked increase in left ventricular systolic pressure resulting in a reflex bradycardia. The pressor effect was potentiated and prolonged by indomethacin, substantiating that a concurrent release of vasodilator prostanoids, i.e. prostacyclin, into the circulation ameliorates the direct vasopressor effects of ET-1 (De Nucci *et al.*, 1988; Thiemermann *et al.*, 1988; Walder *et al.*, 1989).

Another possibility is that the platelet-inhibitory effects of ET-1 are mediated by endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), for ET-1 releases EDRF *in vitro* (Warner *et al.*, 1989) and EDRF inhibits platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987) and adhesion (Radomski *et al.*, 1987; Sneddon & Vane, 1988) *in vitro* and platelet aggregation *in vivo* (Bhardwaj *et al.*, 1988). However, it is unlikely that a stimulation of EDRF release by ET-1 *in vivo* accounts for the potent anti-platelet effects of the peptide for it is ablated by indomethacin, while the platelet-inhibitory effect of EDRF is not modified by indomethacin. Furthermore, inhibition of *ex vivo* platelet aggregation by ET-1 is not associated with an increase in platelet cyclic GMP levels (Thiemermann *et al.*, 1989b).

The deposition of ^{111}In-labelled platelets in the pulmonary vasculature is positively correlated with the number of circulating platelets and pulmonary blood flow (Hanson *et al.*, 1983). Thus, an ET-1 induced thrombocytopenia and/or a reduction in pulmonary blood flow may theoretically contribute to the observed platelet-inhibitory effect of the peptide.

Interestingly, the sequestration of platelets within the lung is not due to pulmonary vasoconstriction brought about by ET-1 for indomethacin potentiated the systemic pressor effects of the peptide, whilst preventing the effects of ET-1 on accumulation of ^{111}In-labelled platelets in the lung or the concurrent peripheral thrombocytopenia in response to ADP.

What mechanisms, then, can account for the ET-1-induced stimulation of endogenous prostacyclin formation? A variety of other pressor agents including angiotensin II (Cooper & Malik, 1986), vasopressin (Cooper & Malik, 1986), noradrenaline (Stewart *et al.*, 1984; Jeremy *et al.*, 1985b; Cooper & Malik, 1986) and the thromboxane A_2 analogue U46619 (Jeremy *et al.*, 1985a) stimulate vascular prostacyclin synthesis (Stewart *et al.*, 1984; Jeremy *et al.*, 1985a,b; Cooper & Malik, 1986), suggesting that prostacyclin release may occur as a defence mechanism in response to elevation of arterial blood pressure. However, in contrast to ET-1, endothelin-3 releases prostacyclin and inhibits *ex vivo* platelet aggregation in the anaesthetized rabbit without causing a significant pressor

effect (Lidbury *et al.*, 1989). Thus, it seems unlikely that the endothelin-induced release of prostacyclin *in vivo* occurs as a response to elevated blood pressure, but rather may be a direct receptor-mediated event. As there is very little difference between the platelet-inhibitory effects of ET-1 and ET-3 (Lidbury *et al.*, 1989), but a marked difference between them as pressor agents (Yanagisawa *et al.*, 1988b; Lidbury *et al.*, 1989; Walder *et al.*, 1989) it may well be that there is a functional difference between the putative endothelin receptors responsible for the release of prostacyclin and those mediating the constriction of vascular smooth muscle. A similar suggestion has been made about the receptors mediating vasoconstriction and EDRF release in response to ET-1 in perfused vascular beds (Warner *et al.*, 1989). Thus, our work reinforces the possibility that there are subclasses of endothelin receptors, some of which release anti-aggregatory and anti-hypertensive autocoids whereas others cause the pressor effects.

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Segregation of discrete $G_{s\alpha}$-mediated responses that accompany homologous or heterologous desensitization in two related somatic hybrids

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1 Prostacyclin and adenosine A_2 receptors activate adenylate cyclase in the neuroblastoma hybrid cell lines NG108-15 and NCB-20. Prolonged exposure of NG108-15 cells to iloprost (a stable analogue of prostacyclin) results in a subsequent reduction in the capacity for adenylate cyclase activation by iloprost, the adenosine analogue 5'-(N-ethyl)-carboxamidoadenosine (NECA) or NaF. In contrast prolonged exposure of NCB-20 cells to iloprost results only in the loss of iloprost responsiveness.

2 Iloprost pretreatment of NG108-15 cells also magnified the morphine-dependent inhibition of iloprost-stimulated adenylate cyclase activity from 36 to 48%. This change was not due to lower iloprost stimulation following desensitization, since the % inhibition of adenylate cyclase activity by morphine in control cells was constant irrespective of enzyme activity.

3 These heterologous effects observed in NG108-15 cells following iloprost pretreatment may involve changes in the $G_{s\alpha}$ protein, since there was a reduction of about 30% in the cholera toxin-induced [^{32}P]-ADP-ribosylation of a 45 kDa protein from cell membranes (corresponding to the extent of loss of NECA or NaF responsiveness). A similar reduction was not observed in NCB-20 cells.

4 These results indicate that iloprost pretreatment induces different forms of desensitization in NG108-15 and NCB-20 cell lines. The heterologous desensitization in the former may, like the human platelet, involve a functional loss of $G_{s\alpha}$ from the cell membrane. Changes in the activity of $G_{s\alpha}$ may also account for the heterologous effects on receptors that mediate inhibition of adenylate cyclase.

Introduction

Prostacyclin (PGI_2) is a naturally occurring derivative of the prostaglandin endoperoxides which mediates the inhibition of platelet aggregation (Moncada *et al.*, 1976). Other substances such as adenosine are also able to inhibit platelet aggregation (Born *et al.*, 1965). Both PGI_2 and adenosine interact with specific receptor sites on the platelet (PGI_2 and A_2 receptors respectively) and inhibit aggregation by activation of adenylate cyclase, thus raising intracellular levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Haslam & Rosson, 1975; Gorman *et al.*, 1977; Tateson *et al.*, 1977; Huttemann *et al.*, 1984).

Both adenosine and PGI_2 analogues have therapeutic potential as 'anti-platelet' agents. In clinical trials, however, it became evident that the use of PGI_2 agonists might be problematic since PGI_2 infusion was followed by a desensitization of platelet PGI_2 responsiveness (Sinzinger *et al.*, 1981). Desensitization *in vitro* of PGI_2 responsiveness has also been widely demonstrated in platelets (Miller & Gorman, 1979; MacDermot, 1986; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988), human fibroblasts (Gorman & Hopkins, 1980) and NCB-20 neuroblastoma hybrid cells (Blair *et al.*, 1982; Leigh & MacDermot, 1985). However, the characteristics of desensitization produced by prolonged activation of PGI_2 receptors are not the same in different cell types. In platelets, prolonged PGI_2 receptor activation leads to heterologous loss of responsiveness to adenylate cyclase-stimulating agents (Edwards *et al.*, 1987; Jaschonek *et al.*, 1988), whereas in NCB-20 cells the desensitization is homologous, affecting only subsequent responses to PGI_2 receptor stimulation (Leigh & MacDermot, 1985). These differences in desensitization probably relate to the different consequences of prolonged PGI_2 receptor activation upon the transmembrane signalling pathway (receptor, G-protein and adenylate cyclase) in each tissue (Gilman, 1984).

Since in practice platelets are a somewhat inconvenient preparation in which to perform long-term manipulations, we decided to use clonal cell lines as a more robust preparation to investigate in greater depth the mechanisms underlying PGI_2 receptor-mediated desensitization. In the present study we have identified two related neuroblastoma somatic hybrid cell lines which express receptors for both PGI_2 and adenosine, stimulation of which activates adenylate cyclase. The effects are described of prolonged incubation of these cells with iloprost, a stable structural analogue of PGI_2 (Skuballa & Vorbruggen, 1983), on the subsequent responsiveness of these cells to agents that activate or inhibit adenylate cyclase.

Methods

Cell culture

Cells of the NG108-15 and NCB-20 neuroblastoma somatic hybrid cell lines were both derived by fusions of the 6-thioguanine-resistant clone of N18TG2 mouse neuroblastoma. Fusions were made with (a) C6BU-1, a 5-bromodeoxyuridine-resistant clone of rat glioma to yield NG108-15, or (b) foetal Chinese hamster brain cells to yield NCB-20 (further details and references to these cells are given in MacDermot *et al.*, 1979). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Culture flasks (80 cm^2) were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. For iloprost pretreatment, this medium was replaced for 17 h with one containing only DMEM, to which iloprost was added (final concentration = 10 μM). Control cells were treated similarly except that iloprost was not included. Cells were harvested by agitation in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, and then washed three times in warm DMEM. The resultant pellets were frozen at −80°C until

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required. For each experiment, control and iloprost pretreated cells were always grown and harvested in parallel.

Adenylate cyclase

Adenylate cyclase activity was measured as described previously (Salomon *et al.*, 1974) with some modifications (Edwards *et al.*, 1987). Cell pellets were thawed and disrupted in a glass Dounce homogenizer using a homogenization buffer (25 mM Tris-HCl, 0.29 M sucrose, pH 7.4). Reaction mixtures of 100 μl contained 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride, 20 mM creatine phosphate disodium salt, 10 iu creatine kinase, 1 mM cyclic AMP sodium salt, 0.25 mM Ro20-1724 as a phosphodiesterase inhibitor, 1 mM [α-^{32}P]-ATP (2 μCi), 1 μM GTP and 200–400 μg of membrane protein. Reactions were incubated at 37°C for 15 min and then terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (about 20 000 c.p.m.) and the reaction mixtures centrifuged at 4°C for 20 min at 800 *g*. The [^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a two step chromatographic procedure (Salomon *et al.*, 1974) and the yield of [^{32}P]-cyclic AMP was corrected for losses on the columns by measurement of the recovery of [^{3}H]-cyclic AMP.

[^{3}H]-iloprost binding

[^{3}H]-iloprost binding was assayed in whole homogenates of control and desensitized cells. Cells were homogenized as before using 50 mM Tris-HCl, pH 7.4 containing 5 mM magnesium chloride. The homogenates (0.1–0.6 mg protein per sample) were incubated in a final volume of 250 μl of 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride containing 0.3–100 nM [^{3}H]-iloprost in the absence or presence of 10 μM unlabelled iloprost to determine non-specific binding. Samples were incubated for 90 min at 4°C and then filtered onto Whatman GF1B glass fibre filters and washed with 3 × 3.5 ml ice-cold buffer using a Brandel Cell Harvester. Scintillation fluid was added to the filters and they were left overnight before counting.

ADP-ribosylation of $G_{s\alpha}$

[^{32}P]-ADP ribosylation of cell membranes was measured as described previously (Edwards *et al.*, 1987) and based on earlier methods (Katada & Ui, 1982; Ribeiro-Neto *et al.*, 1985). Cell pellets were prepared as described above, and after thawing were homogenized in homogenization buffer, centrifuged at 500 *g* for 10 min and the supernatant retained. This was then microfuged for 6 min, and the resultant pellet resuspended in 100 μl of 50 mM Tris-HCl, pH 7.4. Reaction mixtures (volume = 100 μl) contained 30 μl of the membrane suspension (100–400 μg protein), 1 mM ATP, 15 mM glycine, 2.5 mM magnesium chloride, 10 mM thymidine, 100 μM GTP, 90 mM potassium phosphate buffer pH 7.0, 10 μM [^{32}P]-nicotinamide adenine dinucleotide (NAD 20 μCi) and 10 μg cholera toxin (A subunit, activated as described below). Incubations were performed at 37°C for 30 min, and the reactions terminated by the addition of 1 ml 20% (w/v) trichloroacetic acid. After 20 min at 4°C, the precipitate was pelleted by centrifugation (10 000 *g* for 10 min). The supernatants were discarded and the pellets washed once in 1 ml ether:ethanol (1:1 v/v), and once in 1 ml ether, then dried under a stream of N_2. The pellet was then dissolved in 200 μl of 0.0625 M Tris-HCl buffer pH 6.8, containing 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. The samples were heated in a boiling water bath for 5 min and then loaded onto a 10% SDS-polyacrylamide gel with a 3.75% polyacrylamide stacking gel. Molecular weights were estimated by comparison with protein standards in a Dalton Mark 7 kit (Sigma). After completion of electrophoresis, the gel was stained and fixed for 30 min in 0.2% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained in 25% (v/v) methanol and 7.5% (v/v) acetic acid until the background was clear. The gel was dried using a Bio-Rad Gel Drier and examined by autoradiography for 48 h at −80°C using Kodak X-Omatic RP film in a Kodak X-Omatic cassette. The autoradiographic plates were then scanned in a Schoeffel SD3000 Spectrodensitometer with SDC300 Density Computer with reflected light at 550 nm. From the resulting scan obtained by pen recorder, $G_{s\alpha}$ peaks were cut out and weighed. To account for differences in the protein content of samples added to each well, the peak produced by the protein front (see Figure 9) was also cut out and weighed. In each experiment, the size of the $G_{s\alpha}$ peak was normalized by expressing it as a percentage of the respective protein front peak.

The cholera toxin (A subunit) was activated after being dissolved in 250 μl H_2O, at a final concentration of 1 μg μl^{-1}. A 10 μl volume of the toxin solution was added to 10 μl of 50 mM dithiothreitol. The tube was capped and incubated at 37°C for 30 min.

The dose-response data for adenylate cyclase assays were analysed with a non-linear iterative curve fitting programme (Statgraphics, Statistical Graphics Corp., U.S.A.) according to a simple 2 parameter model that identifies a single receptor species, and gives V_{max} and EC_{50}. [^{3}H]-iloprost binding data were analysed by iterative non-linear regression analysis (Graph Pad).

Statistical analyses involved Student's *t* tests (unpaired, two-tailed) as appropriate.

Materials

[8-^{3}H]-adenosine 3':5'-cyclic monophosphate (23.6 Ci mmol^{-1}), [^{32}P]-ATP (40–50 Ci mmol^{-1}) and [^{3}H]-iloprost (14.7 Ci mmol^{-1}) were obtained from Amersham International; [^{32}P]-NAD (23.3 Ci mmol^{-1}) from New England Nuclear. Ro20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was a kind gift from Roche products and iloprost a generous gift from Schering AG Berlin. All other chemicals and drugs were obtained from Sigma Chemical Co. Ltd. or BDH Chemicals Ltd.

Results

In homogenates prepared from either NG108-15 or NCB-20 cells, iloprost, 5'-(N-ethyl)-carboxamidoadenosine (NECA) and NaF all produced concentration-dependent increases in adenylate cyclase activity (Figures 1–3). The EC_{50}s for adenylate cyclase activation by these agents in NG108-15 homogenates were 24 nM for iloprost, 420 nM for NECA and around 4 mM for NaF. Similar values were obtained in NCB-20 homogenates, the EC_{50}s being 28 nM for iloprost, 990 nM for NECA and again around 4 mM for NaF. Concentrations of NaF greater than 10 mM tended to reduce enzyme activity in both cell lines (Figure 3). Furthermore, iloprost produced around a 10 fold increase in adenylate cyclase activity in both cell lines, whereas NECA produced a smaller 2 to 4 fold increase (Figures 1 and 2).

Preincubation of cells with 10 μM iloprost for 17 h reduced basal adenylate cyclase activity in NG108-15 but not NCB-20 cells. In NG108-15 cells iloprost pretreatment reduced basal adenylate cyclase activity by 28% from 8.81 ± 0.78 to 6.34 ± 0.52 pmol cyclic AMP min^{-1} mg^{-1} protein, $n = 8$, $P < 0.05$. In NCB-20 cells iloprost pretreatment did not significantly affect basal adenylate cyclase activity, which was 4.58 ± 0.26 and 3.93 ± 0.40 pmol cyclic AMP min^{-1} mg^{-1} protein in control and iloprost pretreated cells respectively, $n = 8$. In both NG108-15 and NCB-20 cells, iloprost pretreatment greatly reduced the subsequent ability of iloprost to activate adenylate cyclase (Figure 1). This involved both a reduction in the maximum response to iloprost by about 70%, and an increase in EC_{50} by 6 fold and 4 fold in NG108-15 and

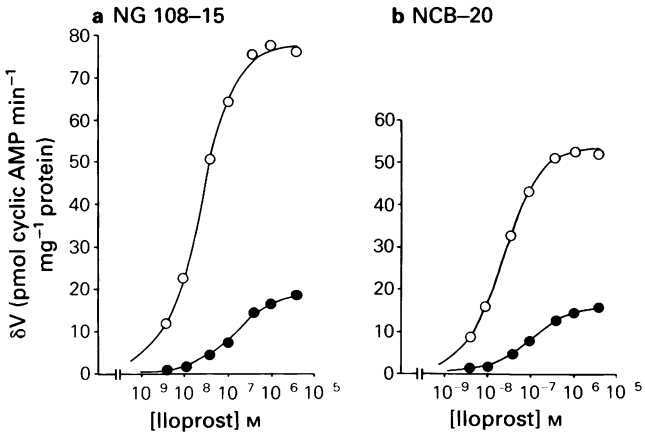


Figure 1 Activation of adenylate cyclase by iloprost in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). The increase in adenylate cyclase activity over basal is expressed as δV. The graphs give data from a single experiment, whereas the following data represent the mean values ± s.e.mean of three different experiments: NG108-15 cells, control EC_{50} = 24 ± 2 nM, iloprost desensitized EC_{50} = 144 ± 14 nM*, control δV_{max} = 103 ± 12 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 33 ± 8 pmol cyclic AMP $min^{-1} mg^{-1}$ protein*; NCB-20 cells, control EC_{50} = 28 ± 3 nM, iloprost desensitized EC_{50} = 119 ± 17 nM*, control δV_{max} = 69 ± 8 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 19 ± 3 pmol cyclic AMP $min^{-1} mg^{-1}$ protein*. δV_{max} indicates the maximum increase in adenylate cyclase over basal. In both cell lines, iloprost pretreatment reduced the δV_{max} and increased the EC_{50} of iloprost (*$P < 0.05$, Student's t test).

NCB-20 cells respectively (Figure 1). In NG108-15 cells iloprost pretreatment also reduced the NECA and NaF dependent increases in adenylate cyclase activity by around 30% (Figures 2 and 3). Similar decreases were not observed in NCB-20 cells. In the case of NECA-activated adenylate

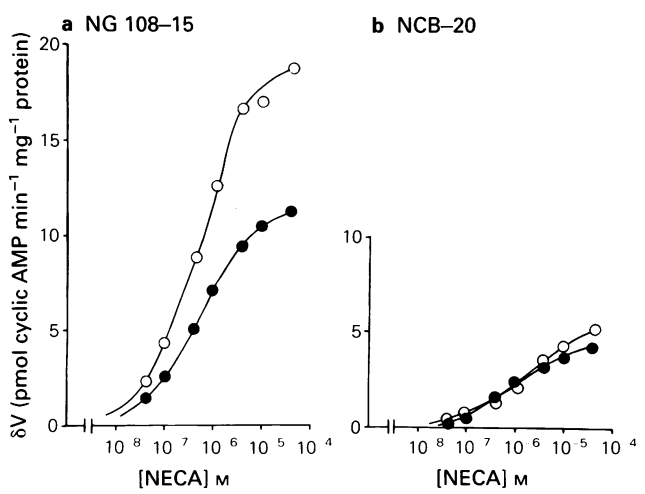


Figure 2 Activation of adenylate cyclase by 5'-(N-ethyl)-carboxamidoadenosine (NECA) in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). Details as in legend to Figure 1. The graphs give data from a single experiment whereas the following data represent the mean values ± s.e.mean of three different experiments: NG108-15 cells, control EC_{50} = 420 ± 10 nM, iloprost desensitized EC_{50} = 310 ± 80 nM, control δV_{max} = 26 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 17 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein *; NCB-20 cells, control EC_{50} = 990 ± 170 nM, iloprost desensitized EC_{50} = 570 ± 110 nM, control δV_{max} = 7 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein, iloprost desensitized δV_{max} = 7 ± 1 pmol cyclic AMP $min^{-1} mg^{-1}$ protein. In neither cell line did iloprost pretreatment alter the EC_{50} of NECA but it reduced δV_{max} for NECA in NG108-15 cells (*$P < 0.05$, Student's t test).

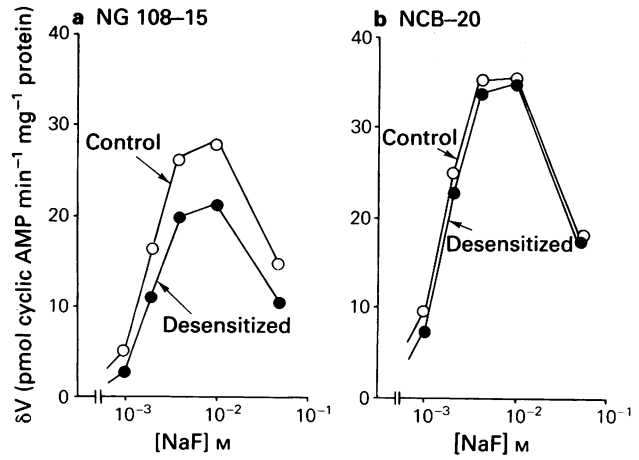


Figure 3 Activation of adenylate cyclase by NaF in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). Details as in the legend to Figure 1. In six separate experiments using a single (10 mM) concentration of NaF, iloprost pretreatment reduced δV by 28.7 ± 6.5% in NG108-15 cell homogenates, but only by 7.1 ± 3.3% in NCB-20 cell homogenates.

cyclase in NG108-15 cells, iloprost pretreatment reduced maximal enzyme activation without affecting the EC_{50} value of NECA (Figure 2).

Iloprost pretreatment of either cell line reduced the 'fold-stimulation' by iloprost of adenylate cyclase over basal activity (Figure 4). However, even in NG108-15 cells, iloprost pretreatment had no effect on the 'fold-stimulation' due to NECA or NaF.

Ligand binding experiments were performed to investigate the effects of iloprost pretreatment on the subsequent binding of [^{3}H]-iloprost to membrane preparations of the cell lines. We did not attempt to obtain full saturation curves for [^{3}H]-iloprost, as it is very difficult to obtain sufficient protein from the cultured cells. However, it has previously been shown (Leigh & MacDermot, 1985) that the binding of [^{3}H]-iloprost (D) to untreated NCB-20 cell membranes can be best described by a single population of high affinity sites (affinity,

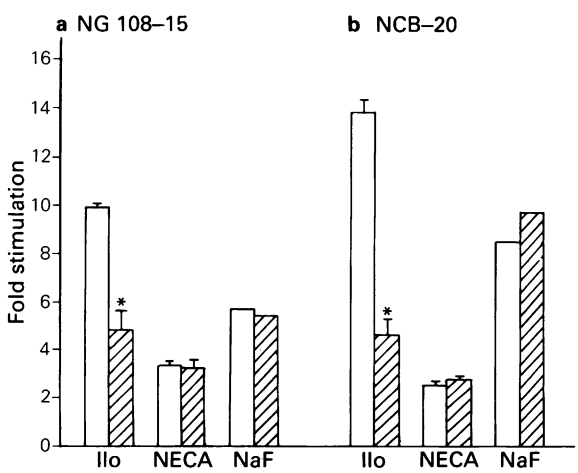


Figure 4 'Fold-stimulation' of adenylate cyclase over basal by different agents in (a) NG108-15 and (b) NCB-20 cell homogenates prepared from control cells (open columns) and cells that had been cultured for 17 h with 10 μM iloprost (hatched columns). 'Fold-stimulation' is defined as the maximum level of adenylate cyclase activation in the presence of drug divided by the level of adenylate cyclase activation in the absence of drug. Values are means ± s.e.mean from 3 separate experiments each, except for NaF which is displayed as the mean of 2 experiments. 'Fold-stimulation' was reduced only for iloprost (Ilo) following iloprost pretreatment (*$P < 0.05$, Student's t test). NECA, 5'-(N-ethyl)-carboxamidoadenosine.

K_D = 10 nM) with the addition of a low affinity, apparently non-saturable component of binding (C), which leads to significant deviation of the [^{3}H]-iloprost binding curve from a simple Langmuir isotherm at concentrations of [^{3}H]-iloprost above ~50 nM. In the present study iloprost displaceable [^{3}H]-iloprost binding to homogenates of untreated NG108-15 and NCB-20 cells could also be described by this model (goodness of fit assessed by actual distance: $r^2 > 0.95$).

$$\text{amount bound} = B_{max}\frac{[D]}{K_D + [D]} + C[D]$$

which yielded estimates of K_D in the range 5–15 nM, none of which differed significantly from 10 nM.

The iloprost displaceable binding of 4–6 concentrations of [^{3}H]-iloprost (0.3–100 nM) to homogenates of control and iloprost-pretreated NG108-15 and NCB-20 cells was measured as described in Methods (Figure 5). Iloprost pretreatment consistently resulted in a decrease in subsequent [^{3}H]-iloprost binding at all concentrations in the range 1–30 nM. This decrease was 77.6 ± 8.6% (mean ± s.d., n = 12) in NG108-15 cells and 67.6 ± 10.5% (mean ± s.d., n = 11) in NCB-20 cells. In all cases, the data could be described by the above two-component model with K_D constrained to be the same in control and treated preparations ($r^2 > 0.95$), suggesting that the iloprost pretreatment does not affect [^{3}H]-iloprost binding affinity. However, it was not possible to obtain independent estimates of [^{3}H]-iloprost affinity in homogenates of iloprost-treated cells, as the number of binding sites was so low. The B_{max} values obtained for [^{3}H]-iloprost binding to the high affinity sites in homogenates from untreated cells were 72.4 ± 11.3 fmol mg^{-1} protein in NG108-15 cells and 102.1 ± 26.8 fmol mg^{-1} protein in NCB-20 cells (mean ± s.e.mean, n = 6). The mean decrease in B_{max} of the high affinity site was 83.3 ± 2.0% in NG108-15 cells and 67.5 ± 12.7% in NCB-20 cells (n = 4). Iloprost pretreatment had no effect on the low affinity component of binding in either cell line; the value obtained for this component of binding was consistently 0.5 fmol mg^{-1} protein nM^{-1} in both cell lines.

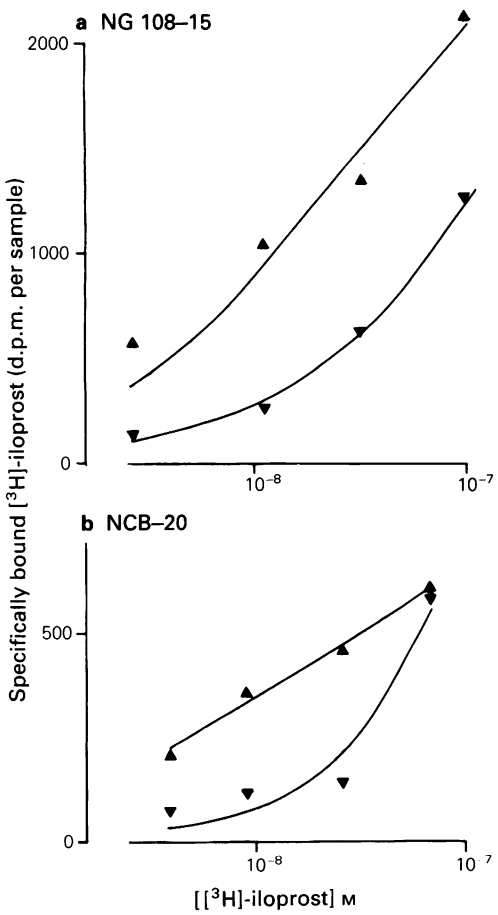


Figure 5 Specific binding of [^{3}H]-iloprost to (a) NG108-15 and (b) NCB-20 homogenates prepared from control cells (▲), and cells that had been cultured for 17 h with 10 μM iloprost (▼). The points on the graph are mean values from single representative experiments. Data are expressed as d.p.m. per sample and are not corrected for protein concentration. The solid lines represent the best fit of the two component model, amount bound = B_{max} ([D]/K_D + [D]) + C[D] to the data (see text), where K has been constrained to be the same in control and treated preparations. In NG108-15, K = 10 nM, B_{max}(control) = 1750 ± 304 d.p.m. per sample, B_{max}(treated) = 394 ± 67 d.p.m. per sample, C(control) = 5 ± 4 d.p.m. per sample nM^{-1} and C(treated) = 9 ± 1 d.p.m. per sample nM^{-1}. In NCB-20 homogenates, K = 5 nM, B_{max}(control) = 495 ± 11 d.p.m. per sample, B_{max}(treated) = 9 ± 88 d.p.m. per sample, C(control) = 2 ± 2 d.p.m. per sample nM^{-1}, C(treated) = 8 ± 2 d.p.m. per sample nM^{-1}.

Morphine produced a concentration-dependent inhibition of basal and iloprost-stimulated adenylate cyclase activity in NG108-15 cells (Figure 6). These effects were abolished in the presence of 100 μM of the opiate receptor antagonist naloxone (data not shown). In iloprost-pretreated NG108-15 cells, the maximum extent to which morphine could inhibit iloprost stimulated adenylate cyclase activity was increased by about one-third, with no change in the IC_{50} of morphine (Figure 6). The extent to which morphine could inhibit basal adenylate cyclase activity also appeared to be increased following iloprost pretreatment, but in this series of experiments the effect did not reach statistical significance (Figure 6). It is unlikely that the increase in the inhibitory capacity of morphine following iloprost pretreatment is due to the reduced iloprost stimulation, since in another experiment the percentage by which a given concentration of morphine inhibited iloprost-activated adenylate cyclase in NG108-15 cells was constant, and unrelated to initial enzyme activity (Figure 7).

Finally, experiments were performed to compare in control or iloprost pretreated cells the [^{32}P]-ADP-ribosylation of $G_{s\alpha}$ catalysed by cholera toxin. Autoradiography following ADP-ribosylation revealed reduced ^{32}P labelling of a 45 kDa protein in NG108-15 cell membranes after iloprost pretreatment when compared to control (Figure 8), and densitometer scanning of the autoradiographs revealed this to be a 31 ± 7% (mean ± s.e.mean from 4 experiments) decrease. However, it should be noted that the autoradiographic ADP-ribosylation analysis is only semi-quantitative, and relies upon the assumption that iloprost treatment does not change the labelling of the 'peak front' protein (see Methods). In two experiments, no similar change was observed in NCB-20 cells preincubated with iloprost (Figure 9), and in a single experiment pretreatment of NG108-15 cells with 100 μM NECA for 17 h also did not affect the ADP-ribosylation of $G_{s\alpha}$ (Figure 8). In two of the four experiments, two ^{32}P labelled bands were observed close to the 45 kDa marker (e.g. Figure 8), although we were unable to determine accurately their constituent molecular weights. The overall changes in cell function following iloprost pretreatment are collated in Table 1.

Table 1 Effects of iloprost desensitization on transmembrane signalling in NG108-15 and NCB-20 cells

	NG108-15	*NCB-20*
Iloprost activated adenylate cyclase	−68%	−73%
[^{3}H]-iloprost binding	−83%	−67%
Basal adenylate cyclase	−28%	−14%
NECA activated adenylate cyclase	−35%	0
NaF activated adenylate cyclase	−28%	−7%
$G_{s\alpha}$ (by ADP-ribosylation)	−31%	+4%
Morphine inhibited adenylate cyclase	+31%	

The values refer to % changes in function taken from the experiments described in the text. Decreased function is denoted by − and increased function by +, 0 refers to no change. The small decrease in basal adenylate cyclase in NCB-20 cells following iloprost pretreatment did not reach statistical significance.

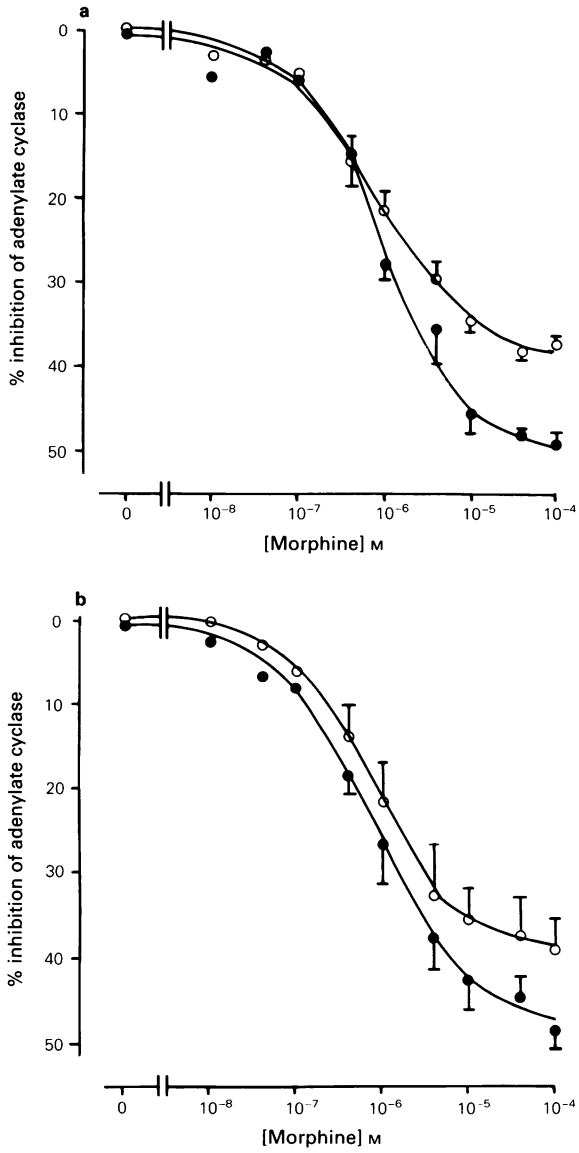


Figure 6 Inhibition of adenylate cyclase activity by morphine in NG108-15 cell homogenates prepared from control cells (○), and cells that had been cultured for 17 h with 10 μM iloprost (●). (a) Morphine inhibition of 1 μM iloprost-activated adenylate cyclase activity, whereas (b) shows morphine inhibition of basal adenylate cyclase activity. The points on the graph show mean values from three separate experiments, the data being normalized to give % inhibition of adenylate cyclase; vertical lines indicate s.e.mean. Mean parameters ± s.e.mean from these data were as follows: inhibition of iloprost-activated adenylate cyclase, control IC_{50} = 696 ± 233 nM, iloprost desensitized IC_{50} = 914 ± 218 nM, control maximum % inhibition = 36.7 ± 1.0%, iloprost desensitized maximum % inhibition = 48.0 ± 1.5%*; inhibition of basal adenylate cyclase, control IC_{50} = 1004 ± 502 nM, iloprost desensitized IC_{50} = 722 ± 199 nM, control maximum % inhibition = 38.6 ± 4.1%, iloprost desensitized maximum % inhibition = 46.1 ± 2.4%. Thus iloprost pretreatment did not affect the potency with which morphine inhibited adenylate cyclase. However, the extent to which morphine could inhibit iloprost-activated adenylate cyclase activity was increased following iloprost (*$P < 0.05$ by Student's t test).

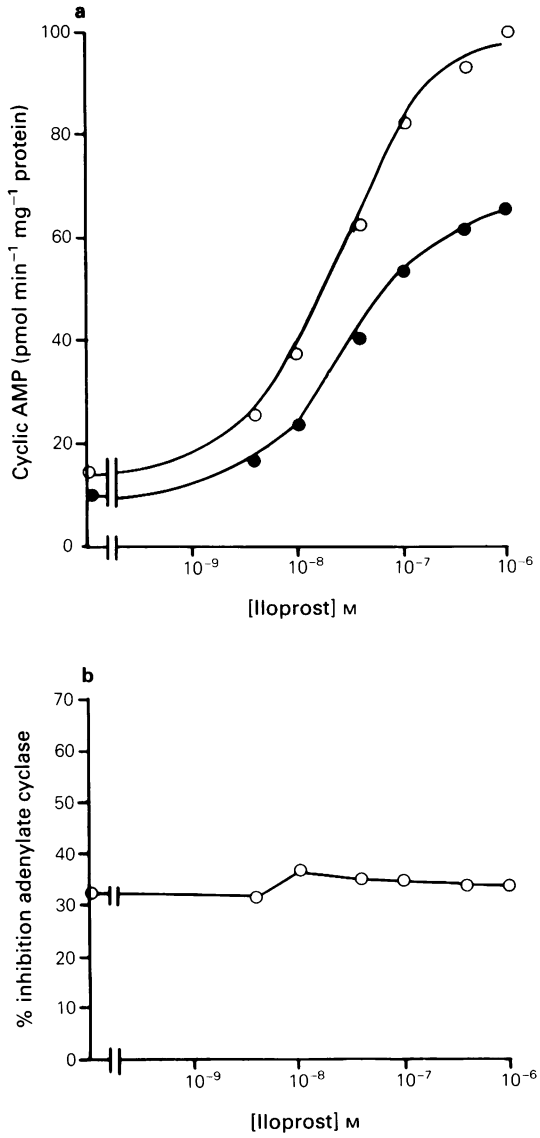


Figure 7 Inhibition of iloprost-activated adenylate cyclase by 10 μM morphine in a cell homogenate of control NG108-15 cells. (a) The activation of adenylate cyclase by increasing concentrations of iloprost in the absence (○) or presence (●) of 10 μM morphine. (b) Data transformed from (a) to show the % inhibition of adenylate cyclase by morphine at different iloprost concentrations. As can be seen, the extent of morphine inhibition was independent of adenylate cyclase activation by iloprost. The graphs contain data from a single experiment, each determination being done in triplicate. This experiment was repeated on one other occasion with the same result.

Discussion

Receptors such as PGI_2 and adenosine A_2 receptors, which mediate activation of adenylate cyclase, are coupled to the effector enzyme by the G_s protein. The G_s protein is a heterotrimer composed of α (45 kDa), β (35 kDa) and γ (10 kDa) subunits, although α and β subunits of other molecular weights have also been reported (for recent reviews see Gilman, 1987; Spiegel, 1987; Neer & Clapham, 1988). In the presence of agonist, the binding of guanosine 5'-triphosphate (GTP) to $G_{s\alpha}$ leads to its dissociation from $G_{s\beta\gamma}$. The $G_{s\alpha}$ GTP complex then binds to, and activates the adenylate cyclase enzyme. Finally, the GTP bound to $G_{s\alpha}$ is hydrolysed to GDP, and $G_{s\alpha}$ and $G_{s\beta\gamma}$ reassociate to terminate the activation of adenylate cyclase. Due to the apparent complexity of this system, desensitization of receptor responsiveness can occur in more than one way. Diminished responsiveness of a receptor that follows previous exposure to an agonist that occupies the same receptor is termed homologous desensitization. Homologous or 'agonist specific' desensitization is considered to involve mechanisms such as loss of receptor number, decrease in agonist affinity, or altered coupling between receptor and G protein (e.g. Clark & Butcher, 1979; Kassis & Fishman, 1982; Leigh & MacDermot, 1985; for review see Harden, 1983). More recently homologous desensitization of the β-adrenoceptor in some tissues has been shown to proceed by an agonist-induced phosphorylation of the β-adrenoceptor by

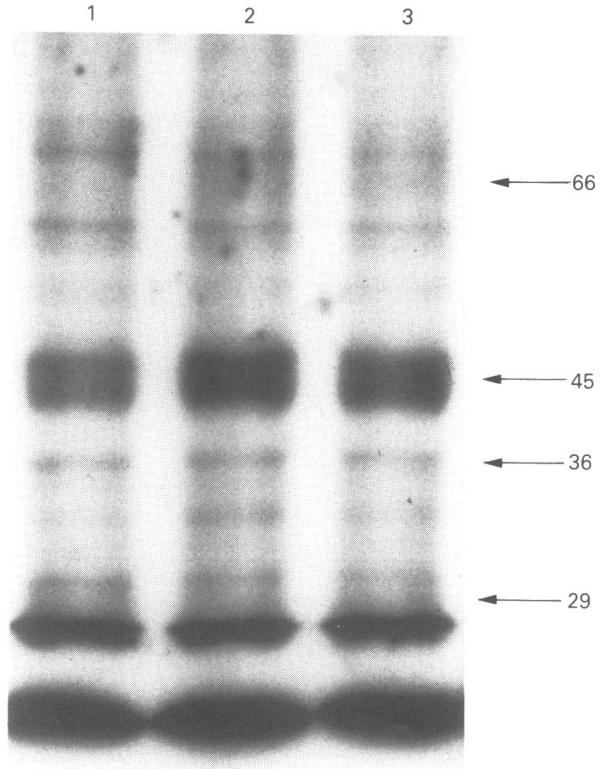


Figure 8 Autoradiograph of cholera toxin induced [^{32}P]-ADP-ribosylation of NG108-15 cell membrane proteins. Cells were incubated for 17 h with 10 μM iloprost (Lane 1), no drug (Lane 2), or 100 μM 5'-(N-ethyl)-carboxamidoadenosine (Lane 3) before membranes were prepared and ADP ribosylation carried out in the presence of [^{32}P]-nicotinamide adenine dinucleotide and cholera toxin. Iloprost pretreatment reduced the ^{32}P labelling of the protein bands around 45 kDa. As described in the results, two ADP-ribosylated protein bands are discernible close to 45 kDa in this experiment.

a specific kinase enzyme, which only phosphorylates the agonist-occupied receptor (Benovic *et al.*, 1986; for reviews see Sibley *et al.*, 1987 and Huganir & Greengard, 1987). On the other hand, heterologous desensitization describes the reduction in responsiveness of one receptor type that accompanies previous occupation by an agonist of a different receptor type. The mechanisms underlying heterologous or 'agonist non-specific' desensitization are less well characterized, but may well involve some modification of the G proteins, or their coupling to receptors or effector enzymes (Kassis & Fishman, 1982; Garrity *et al.*, 1983; Hsia *et al.*, 1985; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). The increased intracellular cyclic AMP probably mediates these changes for G_s coupled receptors, thus accounting for the non-specific nature of this type of desensitization (Harden, 1983). In the present study, we have investigated the characteristics of desensitization produced by the prostacyclin analogue, iloprost, in two closely related neuroblastoma hybrid cell lines. These cells were chosen because, like the human platelet, both express receptors for PGI_2 and adenosine which activate adenylate cyclase.

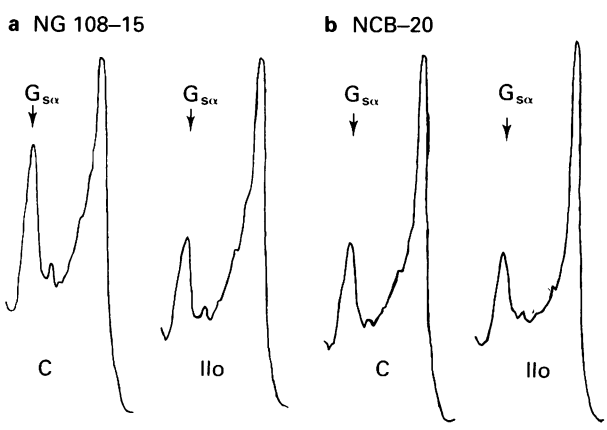


Figure 9 Densitometer scans of four lanes from an autogradiograph following cholera toxin induced [^{32}P]-ADP ribosylation of (a) NG108-15 and (b) NCB-20 cell membrane proteins in the absence (C) or presence (Ilo) of 17 h pretreatment with 10 μM iloprost. The peak to the right of each scan corresponded to the protein front at the base of each lane, whereas the peak to the left in each case corresponded to a 45 kDa protein and is identified as $G_{s\alpha}$. Iloprost pretreatment of NG108-15 cells produced a decrease in $G_{s\alpha}$ (almost 40% in this experiment), whilst having no effect on $G_{s\alpha}$ in NCB-20 cells.

Prolonged incubation of both NG108-15 and NCB-20 cells with iloprost reduced the subsequent ability of iloprost to activate adenylate cyclase. There was a large reduction in enzyme activation by iloprost, and an increase in the EC_{50} of iloprost in both cell lines. Similar results have been obtained following pretreatment of NCB-20 cells with prostacyclin agonists (Leigh & MacDermot, 1985), and we now describe a similar phenomenon in NG108-15 cells. Furthermore, in both cell lines [^{3}H]-iloprost binding was decreased by iloprost pretreatment, indicating reduced receptor numbers. It has previously been shown that the increase in EC_{50} for iloprost-activated adenylate cyclase following iloprost pretreatment of NCB-20 cells is due to the loss of spare receptors with no change in receptor affinity (Leigh & MacDermot, 1985; MacDermot, 1986). Our results here confirm this finding, since we also observed a decrease in prostacyclin receptor number and apparently no change in the affinity of the ligand-receptor interaction. The desensitization of iloprost-activated adenylate cyclase in NG108-15 and NCB-20 cells appears therefore to proceed by a similar mechanism, but the molecular events involved remain obscure at present. However, by analogy with the homologous loss of β-adrenoceptor responsiveness in other tissues, possibilities include agonist-induced receptor phosphorylation followed by internalization and/or down-regulation (Sibley *et al.*, 1987).

The two cell lines showed completely different patterns of responsiveness with regard to basal and NECA or NaF activated adenylate cyclase following iloprost pretreatment. In NG108-15 cells, iloprost pretreatment produced about a 30% reduction in basal adenylate cyclase activity, as well as in NECA and NaF activated enzyme activity. A similar heterologous loss of responsiveness has been demonstrated following PGE_1 pretreatment of NG108-15 cells (Kenimer & Nirenberg, 1981) and iloprost pretreatment of human platelets (Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). The heterologous loss of NECA or NaF responsiveness in NG108-15 cells was much less than the homologous loss of iloprost responsiveness, whereas the reduction in basal as well as NECA and NaF stimulated adenylate cyclase activity was very similar, suggesting a common mechanism for the heterologous effects. In marked contrast to the findings in NG108-15 cells, iloprost pretreatment of NCB-20 cells did not affect basal, or NECA and NaF activated adenylate cyclase activity. Thus, even though both cell lines share a common parent, the mouse N18TG2 neuroblastoma, and both express to a large extent the same receptors, iloprost-induced heterologous desensitization of adenylate cyclase-linked receptors is not seen in NCB-20 cells.

Interestingly, the differences between the two cell lines were not apparent when 'fold stimulation' over basal was taken into account. In both NG108-15 and NCB-20 cells, 'fold stimulation' produced by iloprost was reduced by iloprost pretreatment, whereas 'fold stimulation' produced by NECA and NaF was unchanged. This is not surprising for NCB-20 cells where no heterologous effects were apparent. However, in NG108-15 cells, although there were reductions in basal, NECA and NaF stimulated adenylate cyclase activities, they were all reduced proportionately, and thus no change in 'fold stimulation' was observed. This finding illustrates an inter-

esting difference between homologous and heterologous desensitization in these neuroblastoma cell lines, and may indicate the mechanism(s) involved. It seems that the loss of iloprost responsiveness following iloprost pretreatment (i.e. the reduction in maximal enzyme activation and 'fold stimulation') is due to a large reduction in prostacyclin receptor number in both cell lines. On the other hand, heterologous loss of sensitivity to NECA or NaF (with no change in 'fold stimulation') in NG108-15 cells pretreated with iloprost suggests a different mechanism perhaps involving the G_s protein.

This interpretation of the results is further elaborated by the following considerations. First, there was an iloprost-induced reduction in NaF-activated adenylate cyclase activity in NG108-15 cells. Since NaF activates adenylate cyclase by causing dissociation of the G_s heterotrimer to $G_{s\alpha}$ and $G_{s\beta\gamma}$ subunits, then the reduction in NaF activation of adenylate cyclase must be due to an effect at or beyond the level of the G_s protein. Secondly, the reduction in NECA-activated adenylate cyclase activity following iloprost pretreatment of NG108-15 cells was not accompanied by reduced 'fold stimulation'. This suggests that iloprost pretreatment of this cell line does not affect the adenosine A_2 receptor or its ability to couple to the G_s protein, but rather that the relative activity of G_s has changed. (We have attempted to characterize [^{3}H]-NECA binding to NG108-15 membranes to investigate this hypothesis, but have shown that the ligand predominantly labels a site that is not the A_2 receptor in these cells; Keen *et al.*, unpublished observations). Thirdly, in experiments to assess the abundance of the $G_{s\alpha}$ protein using cholera toxin, we observed the reduced [^{32}P]-ADP-ribosylation of a 45 kDa protein from NG108-15 cells pretreated with iloprost. A similar decrease was not evident in NCB-20 cells, and therefore a functional loss of $G_{s\alpha}$ from the NG108-15 cell membrane probably accounts for heterologous desensitization in this cell line. Reductions in $G_{s\alpha}$ have been observed in other cases of heterologous desensitization of G_s coupled receptors (Kassis & Fishman, 1982; Garrity *et al.*, 1983; Edwards *et al.*, 1987; Jaschonek *et al.*, 1988). Other mechanisms are, however, possible to account for some types of heterologous desensitization, such as an increase in G_i (Rich *et al.*, 1984). The means by which a functional loss of $G_{s\alpha}$ occurs upon desensitization is not known but possibilities include reduced transcription of $G_{s\alpha}$, covalent modification of the polypeptide (Jacquemin *et al.*, 1986), or the physical loss of $G_{s\alpha}$ from the cell membrane.

Some comment is also required about the protein bands labelled in the presence of cholera toxin and [^{32}P]-NAD. In some experiments using NG108-15 cells, two labelled bands could be observed close to the 45 kDa marker protein (Figure 8), although we were unable to deduce accurately the molecular weights of these bands. Cholera toxin-induced ADP-ribosylation of 45 and 42 kDa proteins has previously been observed in NG108-15 cell membranes (Milligan & McKenzie, 1988), so we may be observing the same phenomenon here. One intriguing possibility is that these two proteins represent different forms of $G_{s\alpha}$ from mouse and rat, sequencing of which has indicated small differences which might account for the variation in molecular weight (Sullivan *et al.*, 1986; Itoh *et al.*, 1986). However, some caution is required before accepting this interpretation, as $G_{s\alpha}$ does not align accurately on polyacrylamide gels according to its molecular weight(s) defined by cDNA sequence (for review see Gilman, 1987).

The potential involvement of cyclic AMP-dependent protein kinase in the functional loss of $G_{s\alpha}$ remains unresolved by these experiments. Since pretreatment of NG108-15 cells with NECA produces only homologous desensitization (Keen *et al.*, unpublished observations), it would appear that raised intracellular cyclic AMP is not responsible for heterologous desensitization in this case. However, not only does iloprost produce a much greater activation of adenylate cyclase in these cells than NECA, but we also do not know the relative time-courses of desensitization for these two agents. Thus, on the available evidence the involvement of cyclic AMP remains equivocal, and we are at present investigating these possibilities using non-hydrolysable cyclic AMP analogues, as well as different conditions for iloprost and NECA desensitization.

These experiments revealed an increased capacity of morphine to inhibit iloprost-activated adenylate cyclase in NG108-15 cells following iloprost pretreatment. Since the inhibitory capacity of morphine is unrelated to initial adenylate cyclase activity (see also Hsia *et al.*, 1984), then it appears that the enhanced inhibition caused by morphine is due to a real adaptive change in the cells rather than an artifact of the experimental protocol. However, these results were somewhat unexpected, since in human fibroblasts the ability of oxotremorine, a muscarinic agonist, to inhibit prostaglandin E_1 (PGE_1)-activated cyclic AMP accumulation was greatly reduced following PGE_1 pretreatment (Hsia *et al.*, 1985). These differences probably relate to qualitative differences between cell lines. The mechanism underlying the increased inhibitory capacity of morphine is unclear, but could involve an increase in the number of opiate receptors or G_i. In the present experiments the inhibitory capacity of morphine was increased to the same extent as the loss of NECA and NaF responsiveness following iloprost pretreatment of NG108-15 cells, suggesting that these effects may in some way be linked. If the level of adenylate cyclase activity in a cell is the vectorial sum of activated G_s and G_i proteins, then an agonist-induced loss of $G_{s\alpha}$ could lead to a related increase in G_i activity so accounting for the effects above. Pretreatment of NG108-15 cells with pertussis toxin, which inactivates amongst others the G_i protein, leads to an increase in basal adenylate cyclase activity (Hsia *et al.*, 1984). Thus, the level of adenylate cyclase activity in these cells may bear a direct relationship to the functional levels of G_s and G_i. It would also have been of interest to monitor the inhibition of adenylate cyclase by morphine following iloprost pretreatment of NCB-20 cells, but in a number of experiments the inhibition was small and we were thus unable to quantify modification of the response.

In conclusion, prolonged activation of prostacyclin receptors leads to different forms of desensitization of adenylate cyclase coupled receptors in NG108-15 and NCB-20 cells. The heterologous loss of responsiveness in NG108-15 cells probably relates to the functional loss of $G_{s\alpha}$ in these cells, and in this respect the characteristics of desensitization resemble those for human platelets following iloprost pretreatment. Furthermore, prolonged activation of prostacyclin receptors in this cell line alters the characteristics of adenylate cyclase inhibition by opiate receptors. These cell lines therefore provide convenient systems with which to study further the mechanisms of receptor desensitization, with particular reference to receptors considered important in the regulation of platelet function. It will be of particular interest to us to investigate why prolonged agonist occupation of a single receptor type leads to segregation of $G_{s\alpha}$-mediated responses that accompany homologous or heterologous desensitization in two closely related somatic hybrids.

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The action of atriopeptin III on renal function in two models of chronic renal failure in the rat

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1 The natriuretic and diuretic effects of atriopeptin III (125, 250 and 500 ng kg^{-1}, i.v.) were studied in groups of rats anaesthetized with pentobarbitone which were either sham controls, unilaterally nephrectomized controls, adenine-fed or subtotal nephrectomy chronic renal failure models.

2 Atriopeptin III given at these doses to the sham control animals had no effect on blood pressure, renal blood flow or glomerular filtration rate but reversibly increased urine flow, between 46% to 54%, absolute sodium excretion, between 52% to 61%, and fractional sodium excretion, between 48% to 54% (all P values <0.05) from the lowest to the highest dose. The adenine-fed chronic renal failure group of rats had a reduced renal blood flow of between 30 and 75%, and glomerular filtration rate of approximately 20%, compared to the sham controls. Administration of atriopeptin at 125, 250 and 500 ng kg^{-1} to the animals with renal failure increased water and sodium excretion to the same degree as observed in the sham group of rats.

3 In the group of unilaterally nephrectomized rats, atriopeptin III, at 125, 250 and 500 ng kg^{-1} increased urine flow by 36%, 47% and 72%, respectively, absolute sodium excretion by 37%, 57% and 106%, respectively, and fractional sodium excretion by 46%, 45% and 102%, respectively. A similar pattern of responses was observed in the subtotal nephrectomy, chronic renal failure group in which filtration rate was approximately 4 times less than the controls. Although the magnitude of the urine flow and absolute sodium excretion responses were the same, the increases in fractional sodium excretion were all significantly larger than in the unilaterally nephrectomized control group.

4 These results show that in two different models of chronic renal failure, atriopeptin III still caused a natriuresis and diuresis. This suggests that the nephrons retain sensitivity to the atrial natriuretic peptides in diseases such as chronic renal failure and that these compounds may be useful in mobilizing body fluids in this situation.

Introduction

The mechanisms underlying the renal actions of the atrial natriuretic peptides whereby they increase the excretion of fluid by the kidney (Raine *et al.*, 1989) is unclear. At relatively high doses they cause a selective dilatation of the renal vasculature (Hintze *et al.*, 1985), causing an increase in both renal blood flow and glomerular filtration rate which, by increasing filtered load, could increase the excretion of water and sodium. Nevertheless, at low doses, diuresis and natriuresis occur even though there are no measurable alterations in renal haemodynamics (Johns & Rutkowski, 1989). Investigations have been carried out to assess the possible tubular sites of action of these peptides; reports using both whole kidney and micropuncture methodologies have shown that the proximal tubular sodium reabsorption is minimally affected (Vinay *et al.*, 1987). However, there is increasing evidence that the fluid reabsorption along the loop of Henle and the inner medullary regions of the collecting tubule may be inhibited (Schnermann & Briggs, 1987) which could lead to increased water and sodium excretion.

The importance of these peptides in pathological situations in which there is excessive fluid retention and hypertension has not been defined. There are reports that in congestive cardiac failure (Saito *et al.*, 1987) and essential hypertension (Kohno *et al.*, 1987; Matsubara *et al.*, 1987) the circulating levels of the peptides are raised but they do not appear to contribute sufficiently to mobilize and remove fluid. Another situation in which they may play some role is that of chronic renal failure in which the number of functional nephrons is reduced and those which remain are hyperfiltering. In man, chronic renal failure is associated with increased plasma levels of atrial natriuretic peptide (Anderson *et al.*, 1986), hypertension and often volume expansion. The question arises as to whether the sensitivity of the remaining nephrons to the circulating atrial natriuretic peptide may be altered in some way. This problem was addressed in the present study by examining the effectiveness of a range of low doses of atrial natriuretic peptides in two different models of chronic renal failure. The first was induced by feeding high levels of adenine, which produces progressive blockage of nephrons, and the second was a subtotal nephrectomy in which the remaining nephrons underwent compensatory hypertrophy.

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Methods

Animal preparation

(a) *Sham control* These animals were intact rats which were untreated prior to use and fed a normal diet (Pilsbury 41B small animal diet), *ad libitum*.

(b) *Unilateral neplhrectomy control* Male albino Sprague-Dawley rats (260–280 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.), a 2–3 cm long incision was made on the right side of the dorsum, about 1 cm from the spine and caudally from the last rib, the right kidney was exposed and removed after ligation of its peduncle. The wound was closed in layers, and sutured with catgut for muscles and silk for skin. The animals were used 4–5 weeks later.

(c) *Chronic renal failure* Rats were taken at 270–280 g and fed a diet containing 0.75% adenine (weight for weight) for 4–5 weeks before use. Pellets of regular rat diet (Pilsbury 41B) were ground to a powder, the adenine (Sigma, U.K.) added and the mixture presented to the animals *ad libitum*. This diet has been shown to induce a pronounced reduction in renal function (Yokazawa *et al.*, 1986).

(d) *Chronic renal insufficiency* This was induced as previously described (Rutkowski *et al.*, 1981). Rats, at 270–280 g, were anaesthetized with sodium pentobarbitone, 60 mg kg^{-1}, i.p., and subjected to a right nephrectomy (see (b) above). The left kidney was exposed, 3/5 of the cortex was removed; bleeding was minimized by the application of a sterispon haemostatic sponge. The kidney was returned to the abdominal cavity and the musculature and skin sutured as described above. The animals were used 4–5 weeks later.

Experimental procedure

Male albino Sprague-Dawley rats (370–420 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and subjected to tracheostomy to provide clear airways. The right carotid artery was cannulated for measuring blood pressure (Statham P23Db pressure transducer linked to a Grass model 7 polygraph) and blood sampling. A cannula was inserted into the left jugular vein for saline infusion (NaCl 150 mmol) at 6 ml h^{-1} which was continued for the duration of the experiment. The left kidney was exposed using a mid-line abdominal approach, the ureter cannulated for urine collection and the renal artery separated from the renal vein and surrounding tissues to allow an electromagnetic flow probe (Skalar or Carolina) to be fitted for direct measurement of renal blood flow (SE medic flowmeter or a Carolina flowmeter linked to the Grass polygraph). Renal blood flow was not measured in the chronic renal insufficiency rats because of extensive connective tissue growth in that area resulting from the sub-total nephrectomy (see below). On completion of surgery, 2 ml of a solution containing 10 mg ml^{-1} inulin saline was given i.v. over 1 min and the infusion changed to this same solution for the remainder of the experiment (Johns, 1988). Measurements were begun 2 h later. Blood samples (0.6 ml) were removed, centrifuged, the plasma stored, while the cells were resuspended in an equal volume of saline and reinfused intra-arterially.

Experimental protocol

The experimental protocol consisted of 5 clearance periods each of 15 min duration, two before (basal) and two following (recovery) a period in which atriopeptin III (rat atrial natriuretic peptide 5-28 Bachem UK, Essex) was administered as a bolus dose of 0.3 ml, i.v., at the start of the urine collection. The two recovery clearances after the dose of atriopeptin III were taken 15 min following the end of the experimental period and therefore were used as the control clearances for the second dose of drug. Three doses of atriopeptin III were given, 125, 250 and 500 ng kg^{-1} in random order to each animal.

Renal blood flow was measured directly, glomerular filtration rate was estimated as the clearance of inulin (Johns *et al.*, 1976). Plasma and urinary sodium and concentrations were measured with a Corning flame photometer (410C).

Statistics

The changes in each variable were assessed by taking a mean of the two control clearances and the two recovery clearances and comparing it to the experimental period when the peptide was given. The absolute and percentage changes expressed in the tables and figures represent the means of the individual changes recorded in each animal. Mean values ± s.e. are used. Statistical analysis was undertaken using the paired Student's *t* test within groups and the unpaired Student's *t* test between groups. Differences were taken to be statistically significant when $P < 0.05$.

Results

Sham control rats

The results from this group of animals fed a normal diet are given in Table 1. Administration of atriopeptin III over this dose range had no effect on either blood pressure, renal blood flow or glomerular filtration rate. However, the doses of 125, 250 and 500 ng kg^{-1}, atriopeptin III produced significant reversible increases in urine flow of 47%, 46% and 54% (all P values <0.05), respectively, absolute sodium excretion, of 52%, 56% and 61% (all P values <0.01), respectively, and fractional sodium excretion, of 48%, 57% and 54% (all P values <0.05), respectively.

Chronic renal failure

Administration of the adenine diet caused a marked change in the appearance of the kidneys which was reflected in the left

Table 1 Effect of atriopeptin III (125, 250 and 500 ng kg^{-1}) on blood pressure and renal function in sham control rats ($n = 7$)

	125 ng kg^{-1}			250 ng kg^{-1}			500 ng kg^{-1}		
	Basal	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*
Blood pressure (mmHg)	126 ±8	131 ±3	123 ±7	129 ±6	130 ±7	120 ±11	128 ±6	124 ±8	122 ±9
Renal blood flow (ml min^{-1} kg^{-1})	13.2 ±1.3	13.1 ±1.5	13.4 ±1.2	12.9 ±1.4	12.5 ±1.1	12.5 ±1.1	11.9 ±0.9	11.6 ±1.0	11.8 ±1.0
Glomerular filtration rate (ml min^{-1} kg^{-1})	2.5 ±0.2	2.6 ±0.2	2.6 ±0.2	2.2 ±0.2	2.2 ±0.2	2.3 ±0.2	2.2 ±0.2	2.4* ±0.3	2.3 ±0.3
Urine flow (μl min^{-1} kg^{-1})	41.3 ±6.3	60.8* ±8.6	44.8 ±9.5	37.6 ±6.7	56.0* ±7.8	40.7 ±6.4	40.5 ±6.8	60.6* ±10.0	39.9 ±7.0
Absolute sodium excretion (μmol min^{-1} kg^{-1})	7.2 ±1.5	10.9* ±2.0	8.1 ±2.2	5.9 ±1.5	8.8* ±1.6	6.5 ±1.5	6.8 ±1.6	10.3* ±2.1	6.7 ±1.7
Fractional sodium excretion (%)	1.87 ±0.35	2.66* ±0.46	1.98 ±0.54	1.71 ±0.36	2.72* ±0.59	2.01 ±0.57	2.05 ±0.58	2.96* ±0.80	2.04 ±0.67

The P values represent a comparison between the mean of the two basal and recovery periods with the value obtained during atriopeptin III administration (Exptl) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

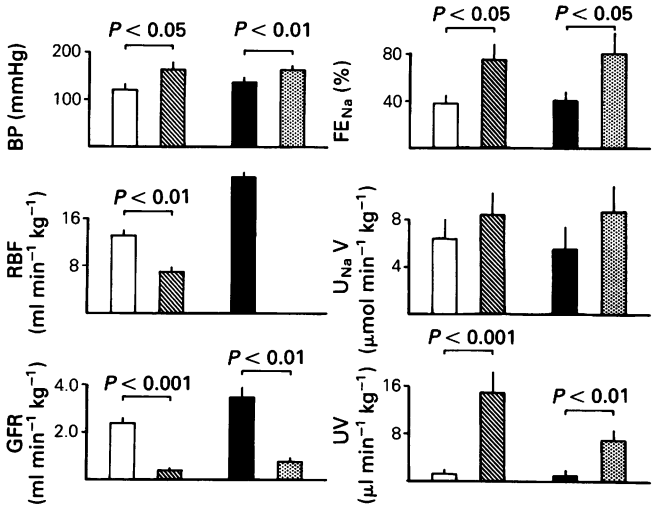


Figure 1 Comparison of the baseline levels of systemic blood pressure (BP), renal blood flow (RBF), glomerular filtration rate (GFR), urine flow (UV), absolute sodium excretion ($U_{Na}V$) and fractional sodium excretion (FE_{Na}) in sham control (open columns), adenine-fed chronic renal failure (hatched columns), unilateral nephrectomised control (solid columns) and subtotal nephrectomy chronic renal insufficiency (stippled columns) rats. The P values represent the significance levels using the unpaired Student's t test between groups.

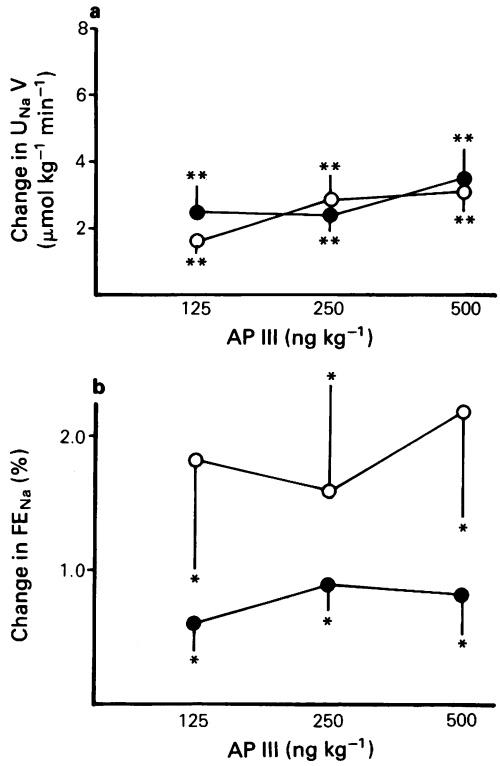


Figure 2 A comparison of the effects of atriopeptin III (APIII) at 125, 250 and 500 ng kg^{-1} on absolute sodium excretion ($U_{Na}V$) (a) and fractional sodium excretion (FE_{Na}) (b) in sham control rats (●) and adenine-fed chronic renal failure rats (○). The asterisks represent the significance levels using Student's t test comparisons within each group of rats. * $P < 0.05$; ** $P < 0.01$; $P < 0.001$.

kidney weight, of 3.58 ± 0.34 g compared with the sham of 1.43 ± 0.06 g, and in their functional characteristics as shown in Figure 1. Compared with the sham rats, the adenine-treated animals had a significantly elevated blood pressure (164 ± 14 versus 122 ± 6 mmHg, $P < 0.05$) and reduced renal blood flow (7.5 ± 1.1 versus 12.9 ± 1.1 ml min^{-1} kg^{-1}, $P < 0.01$) and glomerular filtration rate (0.4 ± 0.1 versus 2.4 ± 0.2 ml min^{-1} kg^{-1}, $P < 0.001$). Although urine flow was significantly greater in the adenine-treated group (75.2 ± 13 versus 38.3 ± 6.7 μl min^{-1} kg^{-1}, $P < 0.05$), absolute sodium excretion was not changed statistically, but fractional sodium excretion was markedly increased (15.0 ± 3.5 versus 1.7 ± 0.3%, $P < 0.001$).

The responses to atriopeptin III of these adenine-treated rats are given in Table 2 and show that the peptide had no effect on blood pressure or renal blood flow at these dose levels but increased glomerular filtration rate at 250 ng kg^{-1}, by 22% ($P < 0.05$). However, atriopeptin III significantly increased urine flow by 30% ($P < 0.01$) and 23% ($P < 0.05$) at 250 and 500 ng kg^{-1}, respectively. In these adenine-treated rats, atriopeptin III at 125, 250 or 500 ng kg^{-1} significantly increased absolute sodium excretion by 18%, 34% and 30% (all P values <0.01), respectively, and fractional sodium excretion, by 13%, 12% and 12% (all P values <0.05), respectively. A comparison of the changes in absolute and fractional sodium excretions caused by atriopeptin III in the sham and chronic renal failure rats are presented in Figure 2 and show that the responses in absolute sodium excretion were very similar. The increases in fractional sodium excretion in the failure rats were larger with greater variability than in the sham animals and the differences just failed to reach statistical significance.

Table 2 Effect of atriopeptin III (125, 250 and 500 ng kg^{-1}) on blood pressure and renal function in chronic renal failure (adenine) rats ($n = 5$)

	125 ng kg^{-1}			250 ng kg^{-1}			500 ng kg^{-1}		
	Basal	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*
Blood pressure (mmHg)	168 ±13	174 ±14	173 ±17	160 ±18	160 ±18	158 ±17	168 ±18	163 ±18	168 ±16
Renal blood flow (ml min^{-1} kg^{-1})	7.7 ±1.2	7.6 ±1.2	7.9 ±1.3	7.2 ±0.8	7.2 ±1.0	6.2 ±0.5	7.7 ±1.4	7.8 ±1.5	7.9 ±1.0
Glomerular filtration rate (ml min^{-1} kg^{-1})	0.38 ±0.09	0.42 ±0.09	0.44 ±0.12	0.39 ±0.06	0.47* ±0.10	0.37 ±0.08	0.34 ±0.07	0.40 ±0.09	0.35 ±0.08
Urine flow (μl min^{-1} kg^{-1})	78.1 ±9.7	96.5 ±15.2	91.7 ±13.2	80.3 ±12.6	99.8** ±17.2	73.5 ±12.9	91.6 ±11.5	109.0* ±13.0	84.7 ±10.1
Absolute sodium excretion (μmol min^{-1} kg^{-1})	8.4 ±1.3	11.3** ±2.1	10.7 ±2.1	8.9 ±2.1	11.9** ±2.6	9.0 ±2.1	10.6 ±2.0	13.3** ±2.4	10.0 ±1.8
Fractional sodium excretion (%)	15.9 ±2.8	19.1* ±4.2	17.9 ±4.3	16.9 ±4.8	19.0* ±5.1	17.8 ±5.1	21.0 ±2.9	23.3* ±3.8	21.0 ±5.0

The P values represent a comparison between the mean of the two basal and recovery periods with the value obtained during atriopeptin III administration (Exptl) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3 Effect of atriopeptin III (125, 250 and 500 ng kg^{-1}) on blood pressure and renal function in unilaterally nephrectomized rats ($n = 6$)

	125 ng kg^{-1}			250 ng kg^{-1}			500 ng kg^{-1}		
	Basal	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*
Blood pressure (mmHg)	142 ±7	144 ±7	138 ±4	143 ±6	148 ±4	144 ±4	143 ±7	138 ±5	133 ±5
Renal blood flow (ml min^{-1} kg^{-1})	22.7 ±1.6	22.7 ±1.9	23.1 ±1.7	23.8 ±1.4	21.8 ±1.6	22.8 ±1.6	23.8 ±1.4	21.8 ±1.1	21.9 ±1.1
Glomerular filtration rate (ml min^{-1} kg^{-1})	3.7 ±0.5	3.4 ±0.5	3.5 ±0.4	3.6 ±0.5	3.9 ±0.5	3.4 ±0.5	3.5 ±0.7	3.6 ±0.7	3.5 ±0.6
Urine flow (μl min^{-1} kg^{-1})	41.7 ±7.3	64.8* ±13.8	49.1 ±8.7	55.0 ±11.1	80.0* ±13.2	62.1 ±12.5	37.2 ±8.9	64.2* ±11.5	42.5 ±8.9
Absolute sodium excretion (μmol min^{-1} kg^{-1})	5.8 ±1.9	9.4* ±2.5	7.4 ±2.0	10.0 ±2.6	15.5* ±3.6	11.9 ±3.1	5.4 ±2.0	11.3* ±3.5	7.0 ±2.3
Fractional sodium excretion (%)	1.01 ±0.31	1.75* ±0.40	1.27 ±0.26	1.65 ±0.39	2.66* ±0.55	2.24 ±0.51	0.94 ±0.28	1.93* ±0.44	1.19 ±0.31

The P values represent a comparison between the mean of the two basal and recovery periods with the value obtained during atriopeptin III administration (Exptl) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Unilateral nephrectomized rats

In this group of animals in which one kidney had been removed and the remaining kidney had undergone hypertrophy (kidney weight 2.93 ± 0.1 g) blood pressure was similar to the sham animals. Both renal blood flow (12.9 ± 1.1 versus 23.0 ± 0.3 ml min^{-1} kg^{-1}) and glomerular filtration rate (3.5 ± 0.4 versus 2.4 ± 0.2 ml min^{-1} kg^{-1}) were significantly ($P < 0.01$ and $P < 0.05$, respectively) greater than the sham control rats but urine flow, absolute and fractional sodium excretions were very similar in the two groups.

Table 3 shows that administration of atriopeptin III had no effect on either systemic blood pressure, renal blood flow or glomerular filtration rate at any dose tested. However, atriopeptin III, at 125, 250 and 500 ng kg^{-1} given to the unilaterally nephrectomized animals, caused significant, reversible increases in urine flow, of 36%, 47% and 72% (all P values <0.05), respectively, absolute sodium excretion, of 37%, 57% and 106% (all P values <0.05), respectively and fractional sodium excretion of 46%, 45% ± 102% (all P values <0.05), respectively.

Chronic renal insufficiency

These animals had a much reduced renal mass (1.72 ± 0.04 g) compared to the unilateral nephrectomized rats and it was not feasible to measure renal blood flow due to extensive scarring from the preliminary surgery. Figure 1 shows that in comparison to the unilaterally nephrectomized control rats, blood pressure was higher (165 ± 5 versus 140 ± 5 mmHg, $P < 0.001$), glomerular filtration rate was lower (0.8 ± 0.1 versus 3.5 ± 0.4 ml min^{-1} kg^{-1}, $P < 0.01$) and while there were increased levels of urine flow (80 ± 18 versus 41 ± 7 μl min^{-1} kg^{-1}, $P < 0.05$) and fractional sodium excretion (7.0 ± 1.6 versus 1.0 ± 0.3%, $P < 0.001$) the absolute rate of sodium excretion was slightly but not significantly greater.

Table 4 presents the responses to atriopeptin III in the chronic renal insufficiency rats. Administration of the atriopeptin III had no measurable effect on either systemic blood pressure or renal haemodynamics at any dose tested. However, administration of atriopeptin III, at 125, 250 and 500 ng kg^{-1} caused significant reversible increases in urine flow, of 29%, 58% and 57% (all P values <0.005), respectively, absolute sodium excretion, of 31%, 73% and 78% (all P values <0.05), respectively, and fractional sodium excretion, of 16%, 51% and 71% (all P values <0.05), respectively. Figure 3 presents a comparison of the absolute changes induced by atriopeptin III in the unilaterally nephrectomized and the chronic renal failure rats. It shows that the changes in absolute sodium excretion were not significantly different at any dose level, although the response in the failure rats was slightly higher at the largest dose of atriopeptin III, but the increases in fractional sodium excretion were significantly (all P values <0.001) greater at every dose in the failure rats compared with the control unilaterally nephrectomised animals.

Discussion

The objective of this investigation was to define the action of the atrial natriuretic peptide, atriopeptin III, on renal haemodynamic and tubular function in chronic renal failure. This pathological situation is one in which the number of functioning nephrons may be severely reduced and in which the remaining nephrons are hyperfiltering with the consequence that their reabsorptive properties are severely disturbed

Table 4 Effect of atriopeptin III (125, 250 and 500 ng kg^{-1}) on blood pressure and renal function in chronic renal insufficiency rats ($n = 6$)

	125 ng kg^{-1}			250 ng kg^{-1}			500 ng kg^{-1}		
	Basal	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*	*Basal*	*Exptl*	*Recovery*
Blood pressure (mmHg)	171 ±7	171 ±6	163 ±5	166 ±8	169 ±6	166 ±5	156 ±6	160 ±4	161 ±7
Glomerular filtration rate (ml min^{-1} kg^{-1})	0.79 ±0.10	0.89 ±0.11	0.91 ±0.16	0.77 ±0.12	0.89 ±0.10	0.82 ±0.10	0.87 ±0.16	0.90 ±0.19	0.74 ±0.13
Urine flow (μl min^{-1} kg^{-1})	91.1 ±25.0	114.9* ±28.3	85.9 ±20.2	71.5 ±18.7	117.9* ±22.8	86.1 ±15.0	72.9 ±15.8	125.6* ±29.4	87.5 ±16.8
Absolute sodium excretion (μmol min^{-1} kg^{-1})	9.6 ±3.0	12.7* ±3.8	9.1 ±2.9	6.7 ±2.0	13.8* ±3.4	9.7 ±1.8	8.5 ±2.2	16.0* ±4.1	10.9 ±3.1
Fractional sodium excretion (%)	8.4 ±2.6	9.5* ±2.6	6.6 ±1.6	5.3 ±1.4	10.1* ±2.0	7.6 ±1.1	7.4 ±2.4	14.0* ±4.1	12.6 ±5.4

The P values represent a comparison between the mean of the two basal and recovery periods with the value obtained during atriopeptin III administration (Exptl) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

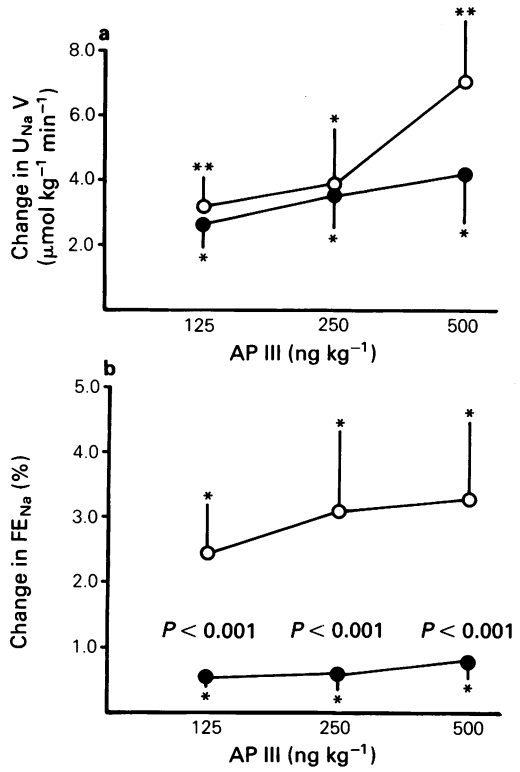


Figure 3 A comparison of the effects of atriopeptin III (APIII) at 125, 250 and 500 ng kg^{-1} on absolute sodium excretion ($U_{Na}V$) (a) and fractional sodium excretion (FE_{Na}) (b) in unilaterally nephrectomized control rats (●) and subtotal chronic renal insufficiency rats (○). The *P* values represent Student's unpaired *t* test comparisons between the two groups while the asterisks represent the significance levels using the Student's paired *t* test comparison within each group of animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(Hostetter *et al.*, 1981). Two different models of chronic renal failure were used in the present study, firstly, that arising from feeding adenine, and secondly, that consequent to subtotal nephrectomy.

Feeding adenine for 4–5 weeks caused an increase in kidney mass and was associated with a greatly reduced filtration rate, similar to that found by Yokazawa *et al.* (1986). These authors found that there was deposition of small crystals in the proximal tubular lumens, causing tubular obstruction, while in the plasma there were marked increases in the levels of urea nitrogen and uric acid. It was of interest that the adenine-fed rats had sodium excretion which were the same as in rats with normal renal function; thus, the greatly decreased filtration rate resulted in an extremely high fractional sodium excretion indicating that the reabsorption of water and sodium by the epithelial cells was much reduced.

Administration of atriopeptin III over the dose range studied in the sham control rats had no effect on renal haemodynamics but reversibly increased the excretion of water and sodium at every dose. The magnitude and pattern of these excretory responses were very similar to those observed by Luft *et al.* (1986b) using bolus administration of atriopeptin III over the same dose range in intact rats and most likely represented an action on tubular reabsorptive processes. There are now many studies using larger doses of these peptides which have reported much larger increases in water and sodium excretion, up to 10–15 times (Goetz, 1988), but under these circumstances there were often increases in glomerular filtration rate which, by increasing filtered load, could also have contributed to the natriuretic and diuretic response.

In the adenine-fed chronic renal failure rats, atriopeptin III induced increases in water and sodium excretion very similar in pattern or magnitude to those observed in the sham control rats and therefore these failing kidneys were still sensitive to the peptides. These observations support those of Hildebrant & Banks (1988) in rats with nephrotic syndrome, induced with aminonucleoside, and those of Sterzel *et al.* (1987), using rats with antiglomerular basement membrane nephritis, who found atrial natriuretic peptides were still able to increase the excretion of water and sodium. In this latter report there were concomitant increases in glomerular filtration rate. The increases in fractional sodium excretion which occurred in response to the atrial natriuretic peptide were not statistically different in the sham control rats as compared to the chronic renal failure rats. The baseline levels of fractional sodium excretion in the adenine-fed rats were larger compared to the sham animals, consequently the change in fractional sodium excretion to the atrial peptide in proportionate terms was smaller and therefore it is not possible to conclude whether a change in tubular sensitivity had occurred. Nonetheless, it is clear that in the adenine-induced chronic renal failure model, atrial natriuretic peptides at low, non-vasodilator levels, could still cause a natriuresis and diuresis.

In an attempt to study the chronic insufficiency model of renal failure the unilaterally nephrectomized rat was used as the appropriate control. The remaining kidney of this control was heavier and had greater blood flow and glomerular filtration rate compared with the kidney of the sham control rats but responded to all doses of atriopeptin III by increasing water, absolute and fractional sodium excretions to a similar degree to the sham control rats. Renal blood flow could not be measured in this insufficiency model, because of extensive scarring around the remnant kidney and although glomerular filtration rate was greatly reduced, water and sodium output were similar or slightly higher compared to the unilaterally nephrectomized controls. The consequence of these differences was that fractional sodium excretion was greatly elevated in the chronic renal insufficiency model. Infusion of atriopeptin III into the chronic renal insufficiency rats had no effect on glomerular filtration rate but significantly increased urine flow and sodium output, which in size and pattern were almost identical to those found in the unilaterally nephrectomized controls. The increases in fractional sodium excretion were significantly larger in absolute terms in the insufficiency rats than the control rats at every dose tested, and one interpretation would be that there was an increased sensitivity to the peptide. Together these results show that the insufficiency animals remain responsive to the peptides and these observations are consistent with and support those of Luft *et al.* (1986a), who used a similar model of chronic renal insufficiency, and with the adenine-fed model described above using comparable doses of atriopeptin III.

It would seem that in both models of chronic renal failure, adenine feeding and subtotal nephrectomy, the nephrons retained or possibly increased their sensitivity to the atrial natriuretic peptide as its action on many fewer nephrons elicited the same magnitude of response. This apparent increase in sensitivity to atriopeptin III in these states of sodium and water retention is similar to that previously reported in DOCA-salt induced hypertension (Johns & Rutkowski, 1989) in which fluid expansion also exists. However, it is important to recognise that, although the same dose of peptide was given to both control and failure rats, in the latter groups the metabolism of the peptide may have been slower and its action prolonged as has been suggested by Predel and coworkers (1987) in progressive renal failure in man. Indeed, Luft *et al.* (1986b) have shown that the kidney accounts for almost 60% of the clearance by atriopeptin III from the plasma. The consequence of this would be an apparent equivalence of action in both control and failure rats which might not accurately reflect the sensitivity of the nephrons to the peptide.

This investigation has examined the changes in renal function which occur when atriopeptin III was given to two different models of chronic renal failure, one induced by feeding adenine, one resulting from subtotal nephrectomy. The dose levels of atriopeptin III used had no effect on blood pressure

or renal haemodynamics in either the control or renal failure groups of rats but it did increase water and sodium output at every dose tested. The magnitudes of the natriuresis and diuresis following atriopeptin III were similar in both the control and chronic renal failure groups which indicated that the nephrons present within the failing kidneys retained or even increased their sensitivity to the peptides. These findings suggest that administration of atrial natiuretic peptides may be useful in mobilizing body fluids in those patients who have chronic renal failure.

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Resiniferatoxin, a potent capsaicin-like stimulator of peripheral nociceptors in the neonatal rat tail *in vitro*

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1 A spinal ventral root response was measured following the activation of peripheral fibres by noxious (heat at 48°C, capsaicin, bradykinin) and innocuous (brush) stimuli in a preparation of the neonatal rat spinal cord-tail maintained *in vitro*.

2 Following superfusion of the tail with 0.1–1.0 nM of the potent irritant, resiniferatoxin (RTX), brief, irregular depolarization and a selective loss of capsaicin sensitivity was produced. RTX 10–100 nM evoked a tonic response, initiated transient irregular depolarizations and densitization to further applications of RTX and capsaicin but not to other stimuli. Following RTX 1 μM a prolonged loss of sensitivity to all noxious stimuli was produced.

3 When a selective densitization to capsaicin was produced by a long application of capsaicin, RTX was also ineffective.

4 Superfusion of the tail with 4β-phorbol, 12, 13-dibutyrate (PDBu), a protein kinase C activator, stimulated capsaicin-sensitive peripheral fibres. Prolonged administration of PDBu attenuated or abolished further responses to PDBu and bradykinin but responses to RTX and capsaicin were unchanged. The protein kinase C inhibitor staurosporine (50–200 nM), attenuated the effects of PDBu and bradykinin but not those of RTX or capsaicin.

5 The present data suggest that neither RTX nor capsaicin act on peripheral nociceptors via a phorbol ester-like stimulation of protein kinase C. Rather, RTX acts on nociceptors by a similar mechanism to capsaicin. These effects may be the basis for the irritant properties of RTX and may further relate to the antinociceptive actions observed *in vivo*. RTX is therefore a potent new tool with which to investigate the properties of nociceptive neurones and provides a prototype for further development of antinociceptive agents.

Introduction

Resiniferatoxin (RTX), a diterpene derivative obtained from the plant family *Euphorbiaceae*, is a potent irritant whose mechanism of action is not understood (Hergenhahn *et al.*, 1974; Williamson *et al.*, 1980). RTX has structural analogy with other irritants, including tumour promoting phorbols (Evans & Schmidt, 1979) which produce their effects via the activation of cellular protein kinase C (Castagna *et al.*, 1982). RTX appears not to be a tumour promoter, is a poor activator of brain protein kinase C (Ellis *et al.*, 1987) and does not compete with phorbol esters for protein kinase C binding sites (Driedger & Blumberg, 1980). It is known that the 4-OH, 3-MeO phenylacetic acid substituent at C20 of RTX is an important determinant of its activity (Williamson *et al.*, 1980). This moiety also features in the structure of capsaicin, a compound known for its irritant and algesic properties. The actions of capsaicin have been studied more extensively and this vanillylamide appears to produce its effects via the selective activation of a subset of sensory neurones including polymodal nociceptors and warm thermoceptors (Fitzgerald, 1983; Buck & Burks, 1986; Bevan *et al.*, 1987; Szolcsanyi, 1989).

In the present study we have tested whether RTX activates peripheral nociceptors and whether its effects are produced via a capsaicin-like or phorbol ester-like mechanism of action. For these studies we have used an *in vitro* preparation of the neonatal spinal cord-tail (Yanagisawa & Otsuka, 1984; Dray *et al.*, 1988; 1989) where peripheral fibres may be activated by a number of noxious stimuli (Yanagisawa *et al.*, 1984; Fitzgerald, 1985; 1986) to evoke a spinal nociceptive response.

Methods

In these experiments activation of peripheral fibres in an *in vitro* preparation of the spinal cord with attached tail was assessed. The spinal cord and tail were isolated from 1–2 day old rats following decapitation. The most superficial layer of the skin was carefully removed from the distal four fifths of the tail with fine forceps. This exposed cutaneous fibres and their endings to allow activation by bradykinin and to facilitate activation by capsaicin. Damage to deeper skin layers and tissue severely compromised chemical sensitivity. Both the spinal cord and tail were separately superfused with physiological salt solution of the following composition at 24°C (mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10) and equilibrated with 95% O_2, 5% CO_2. Activation of fibres in the tail by peripheral stimuli was assessed by recording the depolarizing response produced in a spinal ventral root (L_3–L_5). Recordings were made with a low impedence extracellular glass pipette filled with electrolyte and placed in an electrolyte-filled well containing the selected ventral root. The ventral root potential was recorded d.c. with respect to the spinal cord which was earthed. Signals were amplified by conventional means and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder. Drugs were administered in the tail superfusate, usually for a 10 s period to test for agonistic properties or for a 5 min period to study densitization. The noxious heat stimulus consisted of a 10 s superfusion with physiological salt solution heated to 48°C and the innocuous stimulus was lightly brushing the surface of the tail with a fine sable-hair paint brush. For noxious chemical stimulation submaximal doses of capsaicin (0.3–0.7 μM) or bradykinin (0.1–0.4 μM) were used. Test stimuli were repeated following an interval of 15 min or 60 min between doses of bradykinin to prevent the occurrence of tachyphylaxis.

In the first series of experiments the effects of RTX and its interactions with capsaicin and other stimulants were assessed. The next series tested whether RTX has similar properties to the selective protein kinase C activator 4β-phorbol 12,13-dibutryrate (PDBu) and whether the effects of RTX were sensitive to the potent protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986). In these experiments the tail superfusate contained no added calcium to enhance the responses produced by PDBu (Dray *et al.*, 1988).

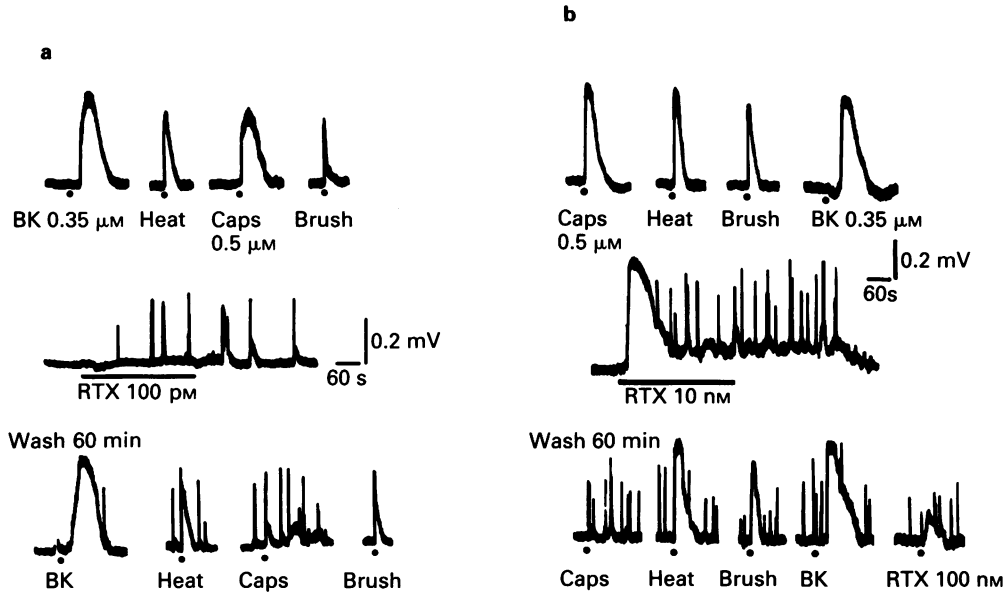


Figure 1 (a) Resiniferatoxin (RTX) did not produce a tonic depolarization but could induce cross-desensitization to capsaicin. The top traces show reproducible nociceptive responses to bradykinin (BK, 0.35 μM), noxious heat (48°C), capsaicin (Caps, 0.5 μM) and an innocuous response to light brush. The prolonged administration of RTX 100 pM (middle trace) did not evoke a tonic response but initiated a series of transient irregular depolarizations. These lasted longer than 4–6 h after the end of the RTX administration. The bottom traces show that following a 60 min wash period, responses to capsaicin were abolished whereas those to other stimuli were unaffected. (b) Depolarization of peripheral fibres by RTX. The top traces show control ventral root responses following application of noxious heat (48°C), a submaximal dose of capsaicin (0.5 μM) or bradykinin (0.5 μM) and innocuous brushing of the tail. The middle trace shows the initial tonic response produced by a prolonged administration of RTX (10 nM). The response was not sustained in the continuous presence of RTX and a series of irregular and transient depolarizations were initiated. These lasted for many hours. Following a 60 min wash period (bottom traces) there was a complete loss of sensitivity to capsaicin and almost complete abolition of RTX sensitivity (tested at 100 nM) whereas responsiveness to other stimuli was unchanged. The administration of brief stimuli are indicated by the dot below the traces while more prolonged administrations are shown as a bar below the trace. The calibration bars are indicated in this and other figures.

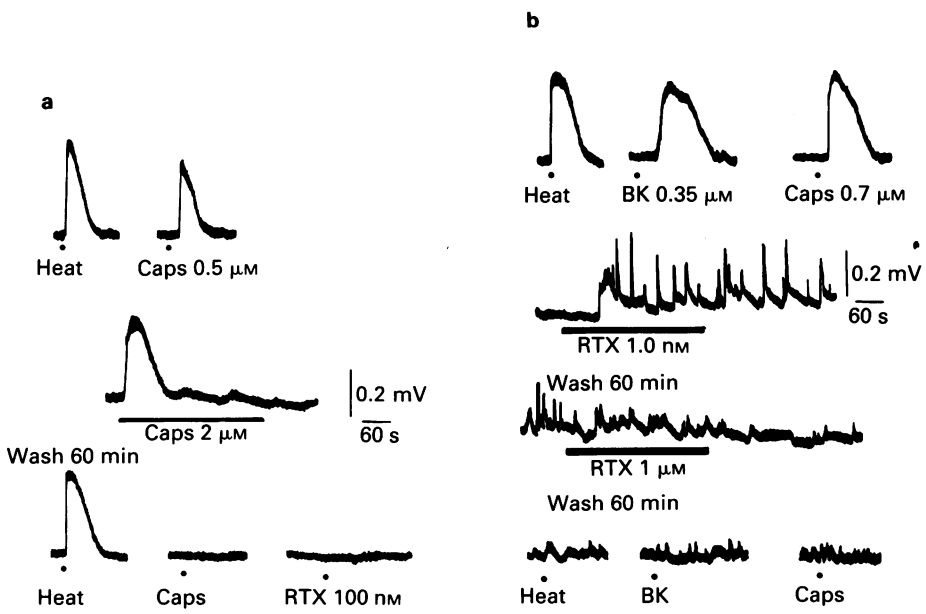


Figure 2 (a) Loss of resiniferatoxin (RTX) sensitivity following selective desensitization with capsaicin. The top traces show depolarizing responses to noxious heat and a brief administration of capsaicin (Caps, 0.5 μM). In the middle trace, the response evoked by capsaicin was not sustained during the continuous presence of capsaicin (2 μM). When retested 60 min later (bottom trace), the response to noxious heat was unchanged but capsaicin sensitivity was completely lost. Moreover neither a tonic depolarization nor transient irregular depolarizations were initiated following the administration of RTX (100 nM). (b) Non-selective impairment of nociceptive responses following RTX. The top traces show control responses to noxious heat (48°C), bradykinin (BK, 0.35 μM) and capsaicin (0.7 μM). Superfusion with RTX, (1 nM) produced a response of small amplitude and initiated a series of irregular, transient depolarizations. A higher dose of RTX (1 μM) did not evoke a subsequent depolarization but following this application the responsiveness to all noxious stimuli was lost (bottom traces) for more than 4–6 h.

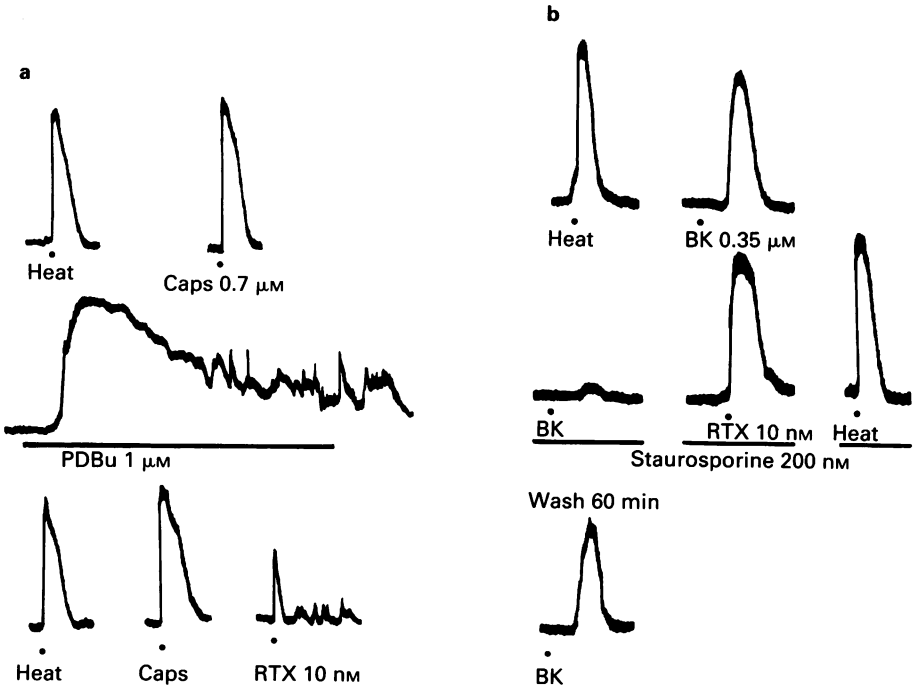


Figure 3 (a) Resiniferatoxin (RTX) evokes a response following desensitization by a phorbol ester. The top traces show control responses to noxious heat and capsaicin. During the prolonged superfusion of the tail with 4β-phorbol 12,13-dibutyrate (PDBu, 1 μM) there was a large depolarization which was not sustained throughout the presence of PDBu. The subsequent administration of PDBu was ineffective (not shown). Following a 60 min wash period, further tests showed that the responses to noxious heat and capsaicin were unchanged and RTX was also able to evoke a response. (b) Staurosporine did not affect the response to RTX. As before, the top traces show control responses to noxious heat and bradykinin (BK). Staurosporine (200 nM) was superfused for 15 min to equilibrate with the tissue before each stimulus was retested. The middle traces show almost complete abolition of the response to bradykinin but responses to RTX and noxious heat were still produced. The response to bradykinin partially recovered (bottom trace) after a 60 min wash period.

Drugs were obtained from the following sources: capsaicin (Sigma, 10 mM stock solution made up in DMSO and diluted with physiological solution immediately before use); resiniferatoxin (Fluka); bradykinin (synthesized at Sandoz); 4β-phorbol 12,13-dibutyrate (Avanti Polar Lipids); staurosporine (Fluka).

Results

In confirmation of previous observations (Dray *et al.*, 1989), brief (10 s) applications of noxious heat or administrations of capsaicin at a submaximal dose (EC_{50} approx 500 nM) evoked brief (50–103 s) responses which were reproducible for several hours. A prolonged (5 min) administration of capsaicin 1–2 μM also produced a response but this response was not sustained in the continued presence of capsaicin. Following this, capsaicin was ineffective for many hours but the responsiveness to other noxious stimuli (heat, bradykinin), as well as responses to innocuous light brushing of the tail were unchanged.

At the lowest doses of RTX that were tested (1 and 10 pM, 10 s to 5 min of 5 experiments), no detectable responses were observed and neither were the responses to other simuli subsequently affected. However, following a prolonged administration at 0.1–1.0 nM (5 min, $n = 8$) transient and irregular depolarizations were initiated and responses to further test doses of capsaicin or RTX were abolished without notable changes in the responses produced by other stimuli (Figure 1a). No recovery of RTX or capsaicin sensitivity was observed up to 6 h afterwards.

At doses of 10 to 100 nM applied for 10 s ($n = 6$ preparations) (Figure 1b), a ventral root depolarization was observed following the first administration of RTX. A dose-response profile for RTX was not obtained as the amplitude of the response was attenuated upon repeated administrations, probably due to the rapid occurrence of tachyphylaxis (Figure 1b). A potency estimate for RTX and capsaicin was obtained by comparing the response to a submaximal dose of capsaicin with that produced by a subsequent single administration of RTX. These data indicated that RTX was approximately 50–100 times more potent than capsaicin.

Following the tonic phase of the RTX-evoked response, brief and irregular depolarizations occurred repeatedly for more than 4–6 h, despite continuous washing of the tissue (Figure 1b). During this period the response to capsaicin, administered to the tail, was abolished but the response to bradykinin, noxious heat or innocuous brush was unchanged (Figure 1a, b). At the highest doses of RTX used in this study (1–10 μM) responsiveness to all noxious peripheral stimuli was subsequently abolished and no recovery was observed within 4–6 h (Figure 2a).

When capsaicin was administered for a prolonged period (2 μM, 5 min) to produce a selective desensitization (Dray *et al.*, 1989), the subsequent administration of RTX (100 nM, $n = 5$) did not evoke a tonic depolarization or any transient, irregular depolarizations (Figure 2b).

Superfusion of the tail with PDBu (1 μM, $n = 5$) produced an activation of capsaicin-sensitive fibres and a ventral root response as described previously (Dray *et al.*, 1988). During a prolonged superfusion with PDBu (1 μM, 5 min) the response was not sustained throughout the application (Figure 3a) and an acute desensitization to subsequent administrations of PDBu was produced, possibly due to inactivation of intracellular protein kinase C. However, subsequent administration of RTX or capsaicin was still able to evoke a response (4 of 4 experiments, Figure 3a). Alternatively prolonged superfusion with the potent protein kinase C inhibitor, staurosporine (50–200 nM, 15–20 min, $n = 4$) (Tamaoki *et al.*, 1986) abolished the effect of PDBu, reversibly attenuated the responses to bradykinin (Figure 3b) but did not affect the responses to noxious heat, RTX or capsaicin (Figure 3b).

Discussion

Several features of the effects induced by RTX suggest that it produces potent interactions with capsaicin-sensitive peripheral fibres by mechanisms closely resembling those of capsaicin rather than a phorbol ester. Thus RTX produced a tonic depolarization of peripheral fibres followed by a period of transient, irregular depolarizations. On repeated administration apparent densitization to RTX and cross-desensitization to capsaicin but not to other stimuli occurred. Indeed desensitization to RTX and cross-desensitization to capsaicin could be seen after doses of RTX too low to produce a substantial tonic response but sufficient to induce transient, irregular depolarizations. Conversely, desensitization following a prolonged administration of capsaicin (2 μM) was accompanied by a selective inability of RTX to induce either tonic or transient depolarizations. The rapid occurrence of desensitization to RTX precluded an accute determination of its potency as an activator of peripheral fibres. Nevertheless, the comparison of a submaximal response to capsaicin with a subsequent response to RTX suggested that its potency is some 50–100 times greater.

Higher doses of capsaicin (20–50 μM) have also been shown to produce a tonic response followed by irregular depolarizations, but these subside within 10–20 min after the termination of the administration of capsaicin (Dray *et al.*, 1989). Such concentrations of capsaicin also induced a prolonged non-selective impairment of nociceptive responses (Dray *et al.*, 1989). Indeed exposure to higher doses of RTX (1 μM) also produces a prolonged and non-selective impairment of responsiveness to noxious stimuli.

The present data indicate a number of similarities between the effects of RTX and those of capsaicin. The basis for these effects and why RTX is more potent and longer lasting than capsaicin is not presently understood. However, the similarities of action are consistent with other studies showing that RTX and capsaicin act on the same population of sensory neurones maintained in culture and increase membrane permeability to cations, elevate intracellular cyclic AMP concentrations and produce selective sensory neurotoxicity (Winter *et al.*, 1989). *In vivo* studies further show that RTX, like capsaicin, produced pain, neurogenic inflammation and hypothermia (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989), while acute systemic administration of these substances produces antinociceptive and anti-inflammatory activity (Campbell *et al.*, 1989; Szallazi & Blumberg, 1989).

It was clear from the present experiments that the pattern of activity observed with RTX was unlike that seen following activation of peripheral fibres with a phorbol ester (Dray *et al.*, 1988). In these experiments tonic depolarization and tachyphylaxis induced by PDBu attenuated the response to bradykinin rather than that to RTX or capsaicin. Moreover, the protein kinase C inhibitor staurosporine consistently attenuated the effects of bradykinin and PDBu but not those of capsaicin or RTX. The present experiments therefore support the likelihood that the irritant properties of RTX are due to the activation of capsaicin-sensitive nociceptive fibres through an interaction with a capsaicin-recognition site and/or through activation of a similar transduction mechanism. Capsaicin-like actions of RTX may also relate to the antinociceptive properties of RTX observed *in vivo*. Finally, the remarkable potency and selectivity of RTX observed in this and other studies indicate that it will be a valuable tool for studying sensory neurone function.

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Developmental changes in β-adrenergic and cholinergic interactions on calcium-dependent slow action potentials in rat ventricular muscles

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1 Developmental changes in the effect of isoprenaline (Iso) and acetylcholine (ACh) interactions on Ca^{2+}-dependent slow action potentials (APs) were studied in the ventricular muscles of foetal (12–20 days post-gestation), neonatal (0–20 days old), and adult (2–3 months old) rats. The slow APs were recorded at 0.2 Hz in partially depolarized preparations (an extracellular K^+ concentration of 25 mM).

2 Iso (1 nM to 10 μM) began to increase the $\dot{V}_{max}$ of the slow APs (an approximate indicator of Ca^{2+} current) on foetal day 18; its potentiating effect became greater with age and reached the adult level about 2 weeks after birth.

3 ACh (10 μM) abolished the Iso (1 μM)-induced increased in the $\dot{V}_{max}$ observed in the late foetal and neonatal periods.

4 The inhibitory effect of ACh on the $\dot{V}_{max}$ was antagonized by atropine but not by pirenzepine, suggesting that ACh reduces Ca^{2+} current (in the presence of β-adrenoceptor agonists) by stimulating muscarinic (M_2) cholinoceptors.

5 These results suggest that developmental changes in the modulatory effects of β-adrenoceptor and cholinoceptor agonists on Ca^{2+} channels occur from a few days before birth to 2 weeks after birth and that the functional coupling between muscarinic cholinoceptors and Ca^{2+} channels has already been established when the coupling between β-adrenoceptors and Ca^{2+} channels starts to operate.

Introduction

Ontogenic development in the neural control of cardiac (ventricular) Ca^{2+} channels has attracted much attention because the Ca^{2+} current (I_{Ca}) through these channels is partly responsible for the plateau phase of the cardiac action potential (AP) and is closely linked to the contractile activation of the myocardium (for review, Pappano, 1977; Sperelakis & Pappano, 1983). The sympathetic and parasympathetic nerve regulate the intensity of the cardiac I_{Ca}, and therefore the force of cardiac contraction, by releasing adrenergic and cholinergic transmitters, respectively, from their nerve terminals. The cascade mechanism following stimulation of β-adrenoceptors by their agonists is summarized as follows: binding of a β-adrenoceptor agonist to its receptor → increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) level via adenylate cyclase activation mediated by the stimulatory guanosine triphosphate (GTP)-binding (G_s) protein → increase in phosphorylation of Ca^{2+} channel-regulatory protein by cyclic AMP-dependent protein kinase → increase in Ca^{2+} influx through Ca^{2+} channels (for review, Reuter, 1983; Sperelakis, 1984; Tsien *et al.*, 1986; Sperelakis & Wahler, 1988).

Stimulation of muscarinic (M) cholinoceptors by their agonists has the opposite effect. In brief, activation of muscarinic cholinoceptors reverses the stimulation of adenylate cyclase, produced by the activation of β-adrenoceptors, by acting via the inhibitory GTP-binding (G_i) protein. The net result is a lowering of the cyclic AMP level back to the basal level. The activation of muscarinic cholinoceptors may also stimulate guanylate cyclase, the resulting cyclic GMP acting either directly on cyclic GMP-activated phosphodiesterase or via stimulation of cyclic GMP-dependent protein kinase (Waldman & Murad, 1987; Sperelakis & Wahler, 1988; Bode & Brunton, 1988).

Many reports have described the developmental changes in the pharmacological and biochemical properties of β-adrenoceptors and muscarinic cholinoceptors and of Ca^{2+} channels in the rat heart. These include chronotropic or inotropic responses to β-adrenoceptor or cholinoceptor agonists (Nukari-Siltovuori, 1977; Marvin *et al.*, 1980), β-agonist-stimulated adenylate cyclase activity (Martin *et al.*, 1973), binding sites for β-adrenoceptor and muscarinic cholinoceptor blockers (Lau & Slotkin, 1982; Nedoma *et al.* 1986), and binding sites for Ca^{2+} channel blockers (Kazazoglou *et al.*, 1983). In contrast, much less information is available on the developmental changes in the electrophysiological properties of cardiac Ca^{2+} channels in rats, especially in relation to the modulation of the channel properties by β-adrenoceptor and muscarinic cholinoceptor agents.

The main purpose of the present study was to examine the developmental changes in the modulatory effects of isoprenaline (Iso) and acetylcholine (ACh) on Ca^{2+}-dependent slow APs in rat ventricular muscle in various stages of differentiation. Preliminary results of this study have been published in abstract form (Kojima *et al.*, 1986; 1987).

Methods

Animals

Foetal (12–20 days post-gestation), neonatal (0–20 days old), and adult (2–3 months old) rats of the Wistar strain were used in these experiments. For breeding, adult female rats in oestrus were mated with adult male rats overnight. The finding of sperm in vaginal smears the next morning was considered to be evidence of pregnancy, and the embryonic age was based on this as day 0 of gestation. When required, foetuses were removed from the uterus of their mother at determined gestational ages (under pentobarbitone anaesthesia, 30 mg kg^{-1}, i.p.).

Prepartion and bath solution

The animals were decapitated or stunned by a blow on the head, and hearts were quickly removed. Ventricular strips

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were dissected from 12 to 20 day-old foetuses and 0 day-old neonates, and papillary muscles were dissected from 0 to 20 day-old neonates and adults. There were no significant differences in the AP parameters between the ventricular strips and the papillary muscles in the 0 day-old neonates. Therefore, the data obtained from these two prepartions (for neonatal day 0) were pooled.

The preparation was placed in an organ bath (2 ml volume), and superfused with a modified Tyrode solution which was gassed with a mixture of 95% O_2 : 5% CO_2 (pH 7.3–7.4). The bath temperature was maintained at 36.5 ± 0.5°C. Tyrode solution had the following composition (in mmol l^{-1}): NaCl 136.0, KCl 5.4, $CaCl_2$ 1.8. $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.7 and glucose 10.0.

Action potential recordings

After an equilibration period of at least 1 h, transmembrane potentials were recorded by conventional glass microelectrodes filled with a 3 M KCl (resistance, 10–20 MΩ). The potentials were amplified with a high input impedance amplifier with capacitance neutralization (M-707, WP Instruments, New Haven, CT, U.S.A.). The preparations were electrically stimulated by a stimulation-isolation unit (SEN-7103, Nihon Kohden, Tokyo, Japan) with a unipolar point electrode (tip diameter 0.2 mm); the second electrode was at a distance from the preparation. Cathodal stimuli (1 ms duration, 0.5 Hz) were used to elicit fast APs and anodal stimuli (2–5 ms duration, 0.2 Hz) to elicit slow APs. The stimulus intensity was increased as necessary to evoke the fast and slow APs whose $\dot{V}_{max}$ became maximal. After the recording of the fast APs, the external K^+ concentration ($[K]_o$) was elevated from 5.4 to 25 mM to depolarize the resting potential to about −43 mV and to inactivate the fast Na^+ channels. A resultant change in osmolarity with increasing $[K]_o$ were not corrected because no appreciable osmotic effect on AP parameters was observed. Both AP and dV/dt (first time derivative of AP, obtained by an RC differentiator) were displayed on a dual-beam oscilloscope (VC-9, Nihon Kohden, Tokyo) and photographed with a kymograph camera. Calibration with a sawtooth generator showed that this differentiator was linear over the desired range of 0–500 V s^{-1}.

The AP parameters measured were $\dot{V}_{max}$, AP amplitude, and AP durations at 50% and 90% repolarization (APD_{50} and APD_{90}, respectively); the resting potential was also measured.

Data analysis

The data are presented as means ± s.e.mean, unless otherwise indicated. Student's paired t test was used in the comparison between the means of two groups. A one-way analysis of variance and Dunnett's test were used in comparisons among the multigroup means of the different age groups (Wallenstein *et al.*, 1980). A P value of <0.05 was considered statistically significant. Calculations and statistical analyses were done with the aid of a personal computer (PC-9801RX, NEC, Tokyo, Japan).

Drugs

The drugs used in the present study were: (−)-isoprenaline hydrochloride (Nikken Chemicals, Tokyo, Japan), acetylcholine chloride (Daiichi Seiyaku, Tokyo, Japan), atropine sulphate (Nakarai Chemicals, Kyoto, Japan), and pirenzepine hydrochloride (Nippon Boehringer Ingelheim, Kawanishi, Japan). All drugs were dissolved in Tyrode solution except Iso, which was dissolved in Tyrode solution containing 0.1 mM ascorbic acid to prevent its oxidation.

Results

Developmental changes in fast action potentials

Developmental changes in the fast APs of rat ventricular muscles were studied to compare with those in the slow APs (refer to the next section). Figure 1 represents typical APs recorded at 0.5 Hz from the foetal, neonatal, and adult hearts. Figure 2 summarizes the developmental changes in the $\dot{V}_{max}$ (a), resting potential (b), overshoot (c), AP amplitude (d), and AP duration (e) of fast APs.

Spontaneous activity was recorded from some hearts of 12 day-old (4 out of 7 preparations, Figure 1a) and 14 day-old foetuses (4 out of 8 preparations), but not from the hearts of 16 day-old or older foetuses. Figure 1b–j shows APs evoked by electrical stimulation of the non-active preparations. $\dot{V}_{max}$, AP amplitude, and resting potential of the fast APs increased (hyperpolarized) with age and reached their adult levels around the day of birth. In contrast, APD_{90} first increased until foetal day 14, then decreased markedly, and reached the adult value by about 2 weeks after birth. APD_{50} changed in parallel with APD_{90}. Overshoot remained almost constant during the developmental period covered by the examination. Afterhyperpolarization was observed only in 12 to 16 day-old foetal hearts: 6–16 mV hyperpolarization on foetal day 12 (Figure 1b), 3–12 mV hyperpolarization on foetal day 14 (Figure 1c), and 0–5 mV hyperpolarization on foetal day 16.

Developmental changes in isoprenaline-induced slow action potential

Developmental changes in Iso-induced slow APs were studied in foetal, neonatal, and adult hearts. The slow APs were recorded at 0.2 Hz in the presence and absence of increasing concentrations (1 nM to 10 μM) of Iso. Figure 3 shows typical examples of these experiments. The slow APs could be evoked by electrical stimulation even in the absence of Iso. In the slow APs in the absence of Iso (controls, left column in Figure 3), $\dot{V}_{max}$ remained almost constant during development (see also the open circles in Figure 5a). APD_{90}, AP amplitude, and overshoot, however, showed a biphasic change: they first increased until post-gestation days 16–20, and then decreased with age (see also the open circles in Figures 5b–d).

When increasing concentrations (1 nM to 10 μM) of Iso were applied to the preparations at different developmental ages (three right columns in Figure 3), clear dose-dependent increases in $\dot{V}_{max}$, AP amplitude, and overshoot were observed after birth. Figure 4 summarizes the dose-response curve for $\dot{V}_{max}$ at representative ages of foetuses and neonates; the maximum response was observed with 1 μM Iso. On the basis

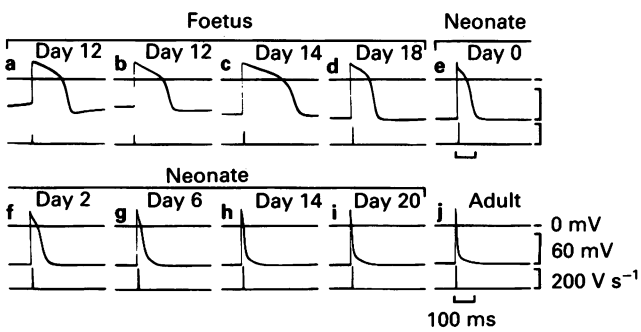


Figure 1 Developmental changes in fast action potentials (APs) in rat ventricular muscles at various ages. (a–d) Fast APs recorded on post-gestation days 12 (a, b), 14 (c), and 18 (d). (e–i) Fast APs recorded on neonatal days 0 (e), 2 (f), 6 (g), 14 (h), and 20 (i). (j) Fast AP recorded in the adult (a: spontaneous AP and b–j: APs evoked by electrical stimulation at 0.5 Hz). In each panel, the upper, middle, and lower traces represent zero potential, AP, and dV/dt (the peak excursion of dV/dt gives $\dot{V}_{max}$), respectively.

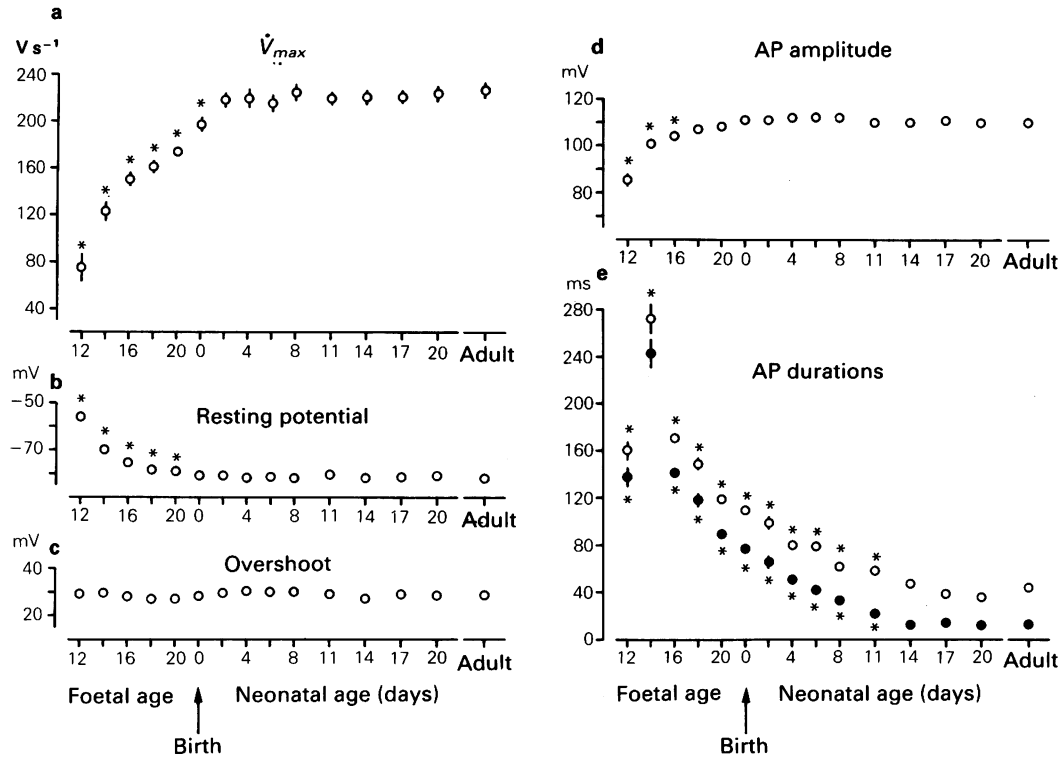


Figure 2 Summary of developmental changes in parameters of the fast action potentials (APs) in rat ventricular muscles. (a) Changes in $\dot{V}_{max}$. (b) Resting potential changes. (c) Overshoot values. (d) AP amplitude changes. (e) Changes in APD_{50} (●) and APD_{90} (○) (AP durations at 50 and 90% repolarization levels, respectively). Each point represents the mean ± s.e.mean (the s.e. bars are not shown when the s.e. mean is smaller than the size of symbol.) The number of experiments for each point was 12–30 (usually about 20). The asterisk denotes statistical difference (*$P < 0.05$) from the adult value (Dunnett's test).

of this finding, a concentration of 1 μM Iso was used in the following experiments.

Figure 5 summarizes the developmental changes in $\dot{V}_{max}$ (a), AP amplitude (b), overshoot (c), and APD_{90} (d) of the slow APs in the presence (filled circles) and the absence (open circles) of 1 μM Iso. Iso significantly increased $\dot{V}_{max}$, AP amplitude, and overshoot from post-gestation day 18 onwards ($P < 0.05$, Student's paired t test, $n = 3$–9): a small increase during foetal life and a greater increase soon after birth. A significant increase in APD_{90}, however, was observed in both

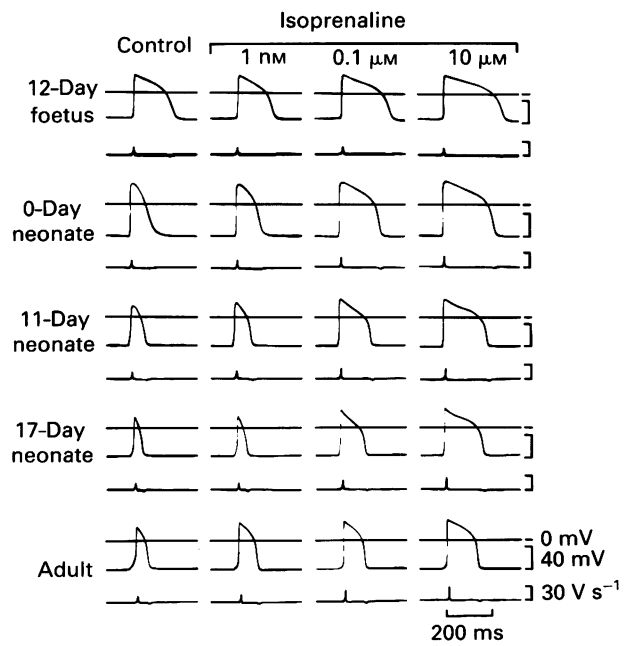


Figure 3 Developmental changes in isoprenaline (Iso)-induced slow action potentials (APs) in rat ventricular muscles. Bathing solution contained 25 mM $[K]_o$. The AP traces and dV/dt traces in the left column are in the absence of Iso (Control). The AP records in the three right-hand columns are in the presence of Iso (1 nM, 0.1 μM, and 10 μM). Taken from the same single cells for each age. Note that Iso did not increase $\dot{V}_{max}$ of slow APs in the 12 day foetal age, but increased $\dot{V}_{max}$ in the neonatal and adult ages in a dose-dependent manner.

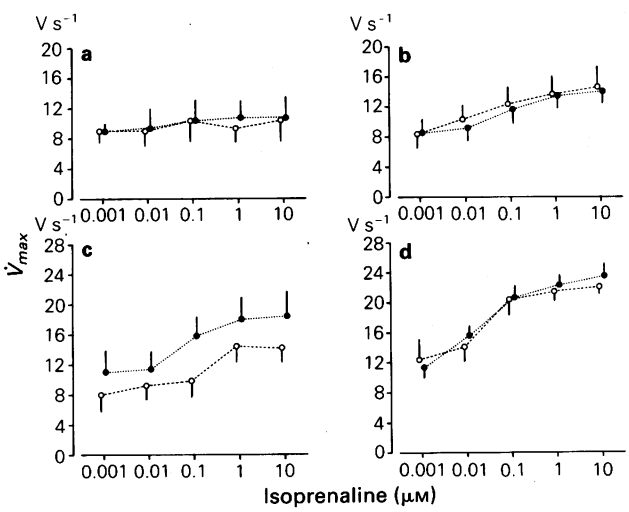


Figure 4 Dose-response curves for $\dot{V}_{max}$ of isoprenaline (Iso)-induced slow action potentials (APs) in rat ventricular muscles ($[K]_o$ = 25 mM). The slow APs were recorded in the presence of Iso (1 nM to 10 μM), whose concentration was increased every 10 min. (a) Curves obtained on post-gestation days 12 (○, $n = 3$) and 20 (●, $n = 3$). (b) Curves observed on neonatal days 0 (○, $n = 7$) and 4 (●, $n = 5$). (c) Curves obtained on neonatal days 8 (○, $n = 5$) and 11 (●, $n = 5$). (d) Curves observed on neonatal day 17 (○, $n = 6$) and in the adult (●, $n = 7$). Each point represents the mean with s.e.mean shown by vertical lines. Note that the slope of the curves becomes more steep with age at the late foetal and neonatal ages. A (near) maximum value is observed at 1 μM Iso.

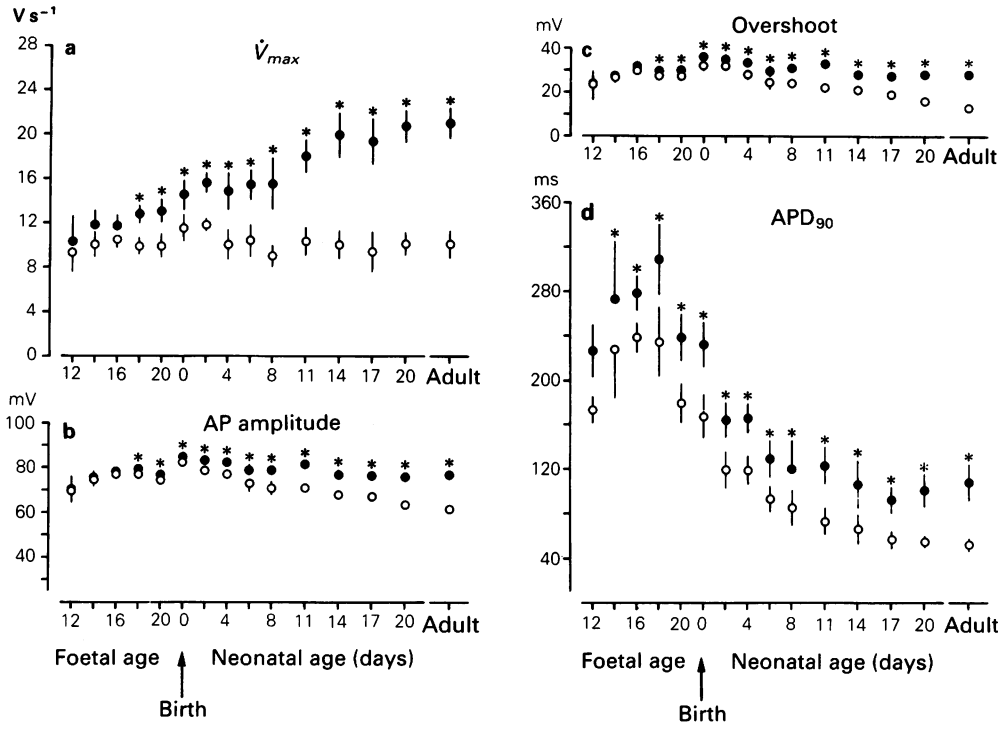


Figure 5 Graphic summary of the developmental changes in parameters of the isoprenaline (Iso, 1 μM)-induced slow action potentials (APs) in rat ventricular muscles ($[K]_o$ = 25 mM). (a) Changes in $\dot{V}_{max}$. (b) Changes in AP amplitude. (c) Overshoot values. (d) Changes in APD_{90}. Open circles represent the data in the absence of Iso (Control) and filled circles the data in the presence of 1 μM Iso. Each point represents the mean with s.e. mean shown by vertical lines. (The s.e. lines are not shown when the s.e.mean was smaller than the size of the symbol.) The number of experiments for each point was 3–9 (usually 5–8). The asterisk denotes statistical difference (*$P < 0.05$) from the control value (Student's paired t test).

the foetal and neonatal rats: APD_{50} changes in parallel with APD_{90} (data not shown). $\dot{V}_{max}$ attained its adult level around neonatal day 14, APD_{90} around neonatal day 14, and AP amplitude and overshoot around the day of birth (see the filled circles in Figure 5).

Developmental changes and inhibition by acetylcholine of isoprenaline-induced slow action potentials

The inhibitory action of ACh on Iso-induced slow APs was studied on foetal, neonatal, and adult hearts. In this study, slow APs were first recorded at 0.2 Hz in the presence of 1 μM Iso alone, and then 10 min after the addition of 10 μM ACh (in the continuing presence of 1 μM Iso). Figure 6 summarizes the developmental changes in the reduction by ACh of $\dot{V}_{max}$ (a) and APD_{90} (b) of the slow APs. The $\dot{V}_{max}$ values in the presence of Iso increased with age, as can be seen in Figure 6a (open circles). When ACh was added in the continuing presence of Iso, there was a significant reduction in $\dot{V}_{max}$ from post-gestation day 18 onwards ($P < 0.05$, Student's paired t test, n = 4–6; filled circles in Figure 6a). It appears that ACh reduced $\dot{V}_{max}$ to almost the same level (10–12 Vs^{-1}) at all developmental ages; i.e., the apparently greater effect of ACh with age is related to the greater effect of Iso with age.

The APD_{90} values in the presence of Iso alone first increased and then decreased with age, as can be seen in Figure 6b (open circles). ACh added in the continuing presence of Iso was effective in shortening APD_{90} during both the foetal and neonatal ages examined (filled circles). The percentage inhibition of APD_{90} by ACh varied between 30 and 40% at all ages studied, except on post-gestation day 16 (16%).

Muscarinic cholinoceptor subtypes involved in inhibition by acetylcholine of isoprenaline-induced slow action potentials

To determine what subtype of muscarinic cholinoceptor participates in the effect of Iso–ACh interaction on slow APs, the actions of pirenzepine (a blocker of M_1-cholinoceptors) and atropine (a blocker of M_1- and M_2-cholinoceptors) on the ACh-induced reductions in $\dot{V}_{max}$ and APD_{90} of the Iso-induced slow APs were examined on neonatal days 0, 6, and 12: neonatal day 0 is when Iso-induced change in $\dot{V}_{max}$ became significant; neonatal day 12 is when Iso-induced changes in $\dot{V}_{max}$ and APD_{90} reached almost adult levels (see the filled circles in Figure 6a, b).

In these studies, the slow APs were recorded at 0.2 Hz first in the presence of 1 μM Iso alone (Iso), second in the presence of 1 μM Iso plus 10 μM ACh (Iso + ACh), third in the presence of 1 μM Iso plus 10 μM ACh plus 10 μM pirenzepine (Iso + ACh + Pz), and fourth in the presence of 1 μM Iso plus 10 μM ACh plus 1 μM atropine (Iso + ACh + Atr). That is, all four trials were carried out on a single impalement. Changing from one trial to the next was done every 10 min.

Figure 7 summarizes the results of these experiments on neonatal days 0 (a and c, n = 6) and 12 (b and d, n = 5). ACh significantly reduced the $\dot{V}_{max}$ and APD_{90} of the Iso-induced slow APs at both ages ($P < 0.05$, Dunnett's test). Atropine (1 μM) was effective ($P < 0.05$), but pirenzepine (10 μM) was always ineffective ($P > 0.05$) in antagonizing the ACh-induced effects on $\dot{V}_{max}$ and APD_{90} (Dunnett's test). Similar results were also obtained on neonatal day 6 (data not shown).

Discussion

Developmental changes in cardiac fast action potentials

In the present study on rats aged 12 days post-gestation to neonatal day 20, the $\dot{V}_{max}$ of the fast APs and the resting potential increased with age and reached their adult levels around the day of birth. APD_{90}, however, first increased, then decreased progressively from post-gestation day 16 onwards, and attained its adult level around neonatal day 14. These results are qualitatively consistent with the findings reported by Couch *et al.* (1969), (11.5 day-old foetuses to newborn),

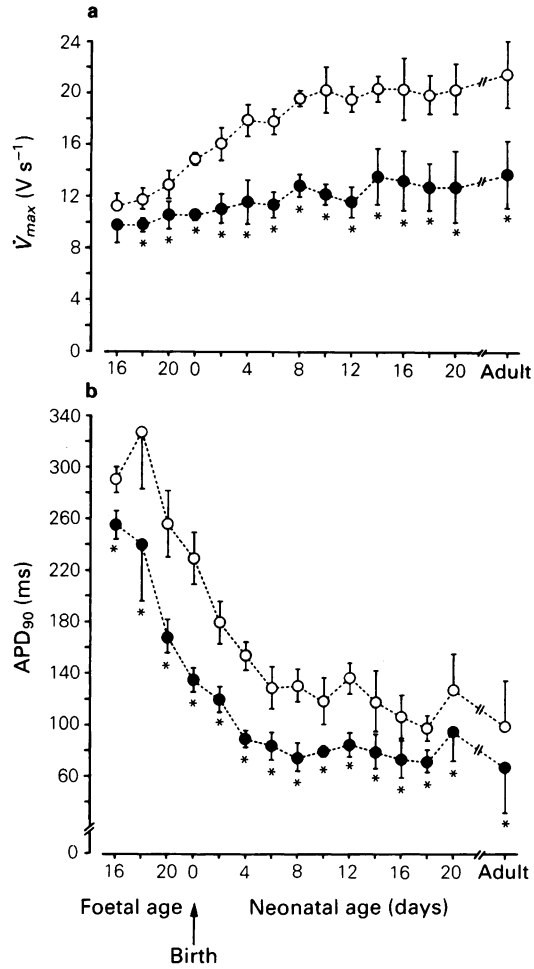


Figure 6 Summary of developmental changes in the inhibitory action of acetylcholine (ACh) on $\dot{V}_{max}$ (a) and APD_{90} (b) of isoprenaline (Iso)-induced slow action potentials (APs) in rat ventricular muscles ($[K]_o$ = 25 mM). Open circles represent the data in the presence of 1 μM Iso, and filled circles the data in the presence of 1 μM Iso plus 10 μM ACh. Each point represents the mean and vertical bars show s.e.mean. The number of experiments for each point was 4–6. The asterisk denotes statistical difference (*$P < 0.05$) from the Iso-treated value (Student's paired t test).

Bernard (1975), (10 to 21 day-old foetuses), and Legrand *et al.* (1981), (13.5 day-old foetuses to 2 day-old neonates). These previous studies, however, provided no information on when each AP parameter reaches its adult level, because they were performed on 10 days post-gestation to 2 day-old neonatal rats. To our knowledge, this is the first report in which five fast AP parameters of rat ventricular muscles were studied over a wide range of foetal and neonatal ages.

The large increase in resting potential during the foetal period may be due mainly to an increase in K^+ permeability (for details, see Sperelakis & Shigenobu, 1972). This increase, in turn, may be partly or mostly responsible for an increase in $\dot{V}_{max}$ (a measure of I_{Na}) and a decrease in APD_{90}. In connection with the developmental change in K^+ channels, we have recently observed that in single channel recordings (cell-attached patch clamp), the inward rectifying K^+ channel was missing in young 3 day-old embryonic chick hearts, but present in older 11 and 19 day-old hearts (unpublished observations). A similar developmental change in the inward rectifying K^+ channel may also have occurred in the rat hearts in the present experiments.

To explain the underlying ionic mechanism for the developmental changes we noted in fast AP parameters, voltage clamp or patch clamp studies are required. However, these studies on single cardiac cells of foetal rats have not yet been successful because these cells are too fragile to allow the gigaohm seal of patch electrodes.

Developmental changes in isoprenaline-induced slow action potentials

In the present study, the $\dot{V}_{max}$ values of slow APs in the absence of Iso (basal $\dot{V}_{max}$) showed no development changes between post-gestation day 12 and postnatal months 2–3 (adult age). An increase in $\dot{V}_{max}$ caused by Iso appeared on post-gestation day 18 and reached its adult level around neonatal day 14. These two findings suggest that some I_{Ca} (corresponds to basal $\dot{V}_{max}$ and is essential for basal cardiac contraction) is already present before post-gestation day 12, whereas more I_{Ca} (corresponds to Iso-induced increase in $\dot{V}_{max}$ and is necessary for more forceful cardiac contraction) appears on post-generation day 18. Developmental changes in the cardiac sympathetic nerve innervation, in the β-adrenoceptor-adenylate cyclase system, and in the number of Ca^{2+} channels may be related to the above findings.

As described in the Introduction, the cascade mechanism involved in the stimulation of β-adrenoceptors by agonists, leading to an increase in I_{Ca}, includes several steps. It is necessary, therefore, to know the developmental changes in these cascade steps in the rat heart. For this purpose, developmental changes in the number of β-adrenoceptors assessed as [^{3}H]-dihydroalprenolol binding sites and of Ca^{2+} channels assessed as [^{3}H]-nitrendipine binding sites have been studied in the accompanying paper (Kojima *et al.*, 1990). In addition, the levels of three substrates (45, 47, and 52 kDa proteins), identified for cholera toxin-catalyzed APD-ribosylation, were previously examined at various ages of foetuses and neonates (Kojima *et al.*, 1988). The present finding that the increase in $\dot{V}_{max}$ of slow APs (or I_{Ca}) caused by Iso appeared on post-gestation day 18 and reached its adult level at around two weeks after birth, corresponds more closely to our findings on the developmental time courses of the G_s levels and the number of Ca^{2+} channels than to the finding that the number of β-adrenoceptors reaches its adult level on neonatal day 6 (for detail, see Kojima *et al.*, 1988; 1990).

Previous studies showed that fast AP duration or slow AP amplitude recorded from foetal and neonatal rat hearts was dependent on $[Na]_o$ (Bernard, 1975; Langer *et al*, 1975; Yamamoto *et al.*, 1984), suggesting some contribution of a slow inward Na^+ component to the $\dot{V}_{max}$ of the present slow APs. In the present studies, the overshoot of Iso-induced slow APs on post-gestation day 14 to neonatal day 12 varied by 26–31 mV per decade when $[Ca]_o$ was changed between 0.25 and 4 mV [27 ± 1 mV per decade ($n = 4$) for adult preparation]; there was no age-dependent change in these values (unpublished observations). The ideal change as a calcium electrode at 37°C expected from the Nernst equation would be 30.7 mV per decade. This finding, therefore, suggests that the $\dot{V}_{max}$ of the present slow APs are dependent mainly on slow inward Ca^{2+} current and maybe not or a little on slow inward Na^+ current (for slow inward Na^+ current, see Sperelakis & Pappano, 1983).

Developmental changes in isoprenaline-acetylcholine interaction on Ca^{2+}-dependent slow action potential

The present results indicate that ACh started, on post-gestation day 18, to reduce the $\dot{V}_{max}$ of Iso-induced slow APs to the level before the administration of Iso (10–12 V s^{-1}), and the effect of ACh became greater as the $\dot{V}_{max}$ of Iso-induced slow APs increased with age. In rat ventricular muscles, developmental changes in the number of muscarinic cholinoceptors assessed as [^{3}H]-quinuclidinyl benzilate binding sites and in the level of single substrate (41 kDa protein), identified for pertussis toxin-catalyzed ADP-ribosylation, have been studied in the accompanying paper (Kojima *et al.*, 1990) and in an earlier one (Kojima *et al.*, 1988), respectively. The present findings

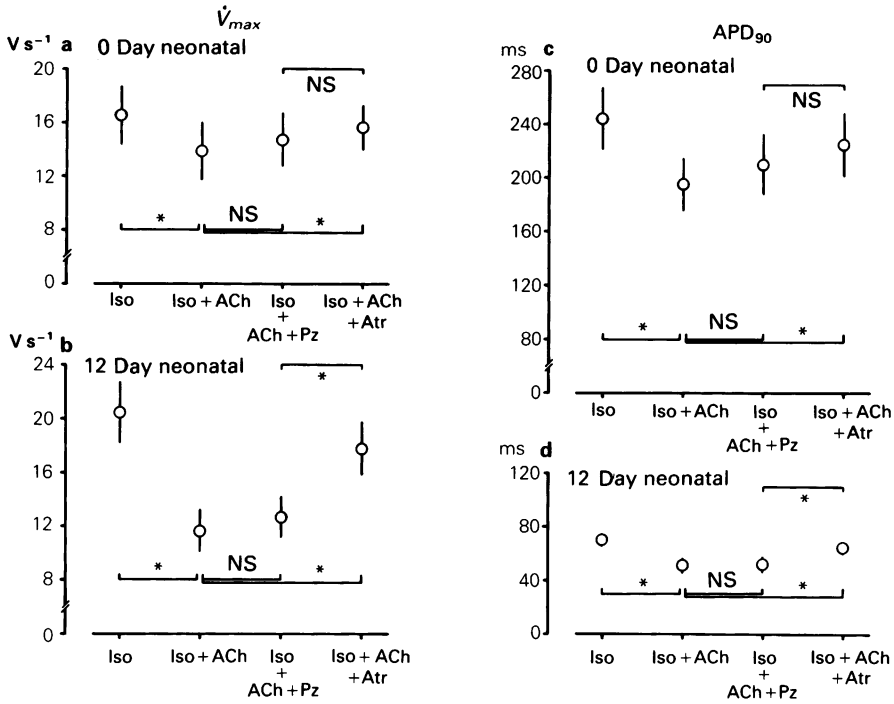


Figure 7 Antagonism by pirenzepine or atropine of $\dot{V}_{max}$ and APD_{90} of isoprenaline (Iso) plus acetylcholine (ACh)-induced slow action potentials (APs) in rat ventricular muscles on neonatal days 0 and 12 ($[K]_o$ = 25 mM). (a and c): $\dot{V}_{max}$ and APD_{90}, respectively, on neonatal day 0. (b and d) $\dot{V}_{max}$ and APD_{90}, respectively, on neonatal day 12. Drugs used and their concentrations were 1 μM isoprenaline (Iso), 10 μM acetylcholine (ACh), 10 μM pirenzepine (Pz), and 1 μM atropine (Atr). The numbers of experiments for neonatal days 0 and 12 were 6 and 5, respectivly. The asterisk denotes statistical difference (*$P < 0.05$) from the value in the presence of 1 μM Iso and 10 μM ACh (Dunnett's test).

described above are supported by the fact that the number of muscarinic cholinoceptors and the level of G_i protein reached their maximum levels about one week earlier than did the number of β-adrenoceptors and the level of G_s proteins (for detail, see Kojima *et al.*, 1988; 1990).

In connection with the developmental changes described here in the effect of Iso-ACh interaction on slow APs, the following findings have been described in embryonic chick hearts. An inhibitory effect of ACh on Iso-induced slow APs did not occur on embryonic days 3–4, but did occur on embryonic days 7–18 (Biegon & Pappano, 1980). Liang *et al.* (1986) have proposed the following limiting factors related to the development of muscarinic cholinergic inhibition of adenylate cyclase in embryonic chick hearts. During early embryonic days 2.5–10, the availability of the 39 kDa protein (a substrate for pertussis toxin-catalyzed ADP-ribosylation) may limit the coupling of the muscarinic cholinoceptors to inhibition of adenylate cyclase activity. After embryonic day 10, however, the number of muscarinic cholinoceptors may limit the physiological response to muscarinic stimulation.

In the present study, Iso and ACh were similarly effective in increasing and reducing, respectively, the APD_{90} of slow APs during both the foetal and neonatal periods (Figure 5d and Figure 6b). These developmental changes in APD_{90} caused by Iso and ACh are different from those in $\dot{V}_{max}$ caused by Iso and ACh; Iso increases and ACh decreases $\dot{V}_{max}$ slightly in the foetal period, but more actively with age in the neonatal period (compare Figure 5a with Figure 5d, and Figure 6a with Figure 6b). This finding suggests that Iso and ACh have different mechanisms for changing the $\dot{V}_{max}$ and APD_{90} of slow APs. Modifications of I_{Ca} by Iso and ACh do not seem to be enough to explain these developmental changes in the $\dot{V}_{max}$ and APD_{90} of slow APs.

This is the first description of the developmental changes in the effect of β-adrenergic and cholinergic interactions on Ca^{2+}-dependent slow APs in rat ventricular muscle. The present information is essential for an understanding of how neurotransmitters transfer signals from membrane receptors to cardiac ionic channels or intracellular effectors during development. The present findings are also useful for electropharmacologists who wish to use foetal and neonatal rat hearts in their experiments.

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Developmental changes in β-adrenoceptors, muscarinic cholinoceptors and Ca^{2+} channels in rat ventricular muscles

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1 In an attempt to explain the previous electrophysiological data on the ontogeny of β-adrenergic and muscarinic cholinergic interactions on cardiac Ca^{2+} current, biochemical studies were performed on the ontogeny of β-adrenoceptors, muscarinic cholinoceptors and Ca^{2+} channels in cardiac muscle of developing rats: 16–20 days old foetuses, 0–20 days old neonates, and 2–3 months old adults.

2 Developmental changes in cardiac β-adrenoceptors, muscarinic cholinoceptors, and Ca^{2+} channels were determined with the use of specific radioligands, [^{3}H]-dihydroalprenolol (DHA), [^{3}H]-quinuclidinyl benzilate (QNB), and [^{3}H]-nitrendipine (NTD), respectively.

3 The B_{max} value (fmol mg^{-1} tissue) for [^{3}H]-DHA binding started to increase on post-gestation day 20, reached almost its maximum level on neonatal day 6, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level.

4 The B_{max} value (fmol mg^{-1} tissue) for [^{3}H]-QNB binding started to increase on post-gestation day 16, reached almost its maximum level on neonatal day 0, remained almost constant until neonatal day 15, and then decreased to its adult level.

5 The B_{max} value (fmol mg^{-1} tissue) for [^{3}H]-NTD binding increased with age between post-gestation day 18 and neonatal day 15, stayed almost constant until neonatal day 20, and then decreased to its adult level.

6 The K_d values for [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD bindings remained almost constant during the developmental period examined.

7 Isoprenaline (Iso) increased the $\dot{V}_{max}$ of slow action potentials (APs) from post-gestation day 18, and the adult level was reached at about 2 weeks after birth; this developmental time course is similar to that of Ca^{2+} channels. The number of β-adrenoceptors also started to increase a few days before birth, but attained its peak about one week earlier than did the $\dot{V}_{max}$ of slow APs or the number of Ca^{2+} channels.

8 Acetylcholine (ACh) almost completely abolished the Iso-induced increase in $\dot{V}_{max}$ observed from post-gestation day 18 to neonatal day 20; this developmental time course for the ACh effect is consistent with the finding that the number of muscarinic cholinoceptors started to increase on post-gestation day 16 and reached a peak on the day of birth.

9 Previous electrophysiological and the present biochemical findings strongly suggest that the functional coupling between muscarinic cholinoceptors and Ca^{2+} current is already established when the coupling between β-adrenoceptors and Ca^{2+} current starts to operate in developing rat hearts.

Introduction

The development of the neuronal control of the cardiac Ca^{2+} current attracts much attention, because a knowledge of its ontogenesis provides information as to how the complex physiological function of Ca^{2+} currents is gradually put together by the integration of several specific components during development. The main findings from our previous study (Kojima *et al.*, 1990) were: (i) isoprenaline (Iso) increased $\dot{V}_{max}$ [the maximum rate of rise of action potential (AP)] of slow APs from post-gestation day 18, and its increasing effect reached the adult level about 2 weeks after birth; (ii) acetylcholine (ACh) became effective on post-gestation day 18 in reducing the $\dot{V}_{max}$ of Iso-induced slow APs to the level before the administration of Iso, and its inhibitory effect became greater as the $\dot{V}_{max}$ of Iso-induced slow APs increased with age. As described previously (Kojima *et al.*, 1990), the transmembrane signalling mechanism following stimulation of β-adrenoceptors and muscarinic cholinoceptors by their agonists, which increase and decrease Ca^{2+} current, respectively, depends on the components of the receptors, guanosine triphosphate (GTP)-binding proteins, adenylate cyclase, guanylate cyclase, adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase, and Ca^{2+} channels. Our electrophysiological findings require confirmation by biochemical data on the developmental changes in the components related to β-adrenergic and muscarinic cholinergic interactions on Ca^{2+} current.

Several biochemical studies using rat hearts have addressed the developmental changes in the components mentioned above (see Discussion). Most investigators, however, have examined the developmental changes of only one component. As a result, there are differences in the developmental stages or time periods used and some discrepancies exist among their data even when the same component was examined. The B_{max} values are also often expressed in different units. The 'fmol mg^{-1} protein' unit is most commonly used, but sometimes it is the 'fmol mg^{-1} tissue' unit. The biochemical findings previously reported, therefore, cannot adequately explain our previous electrophysiological data on the developmental

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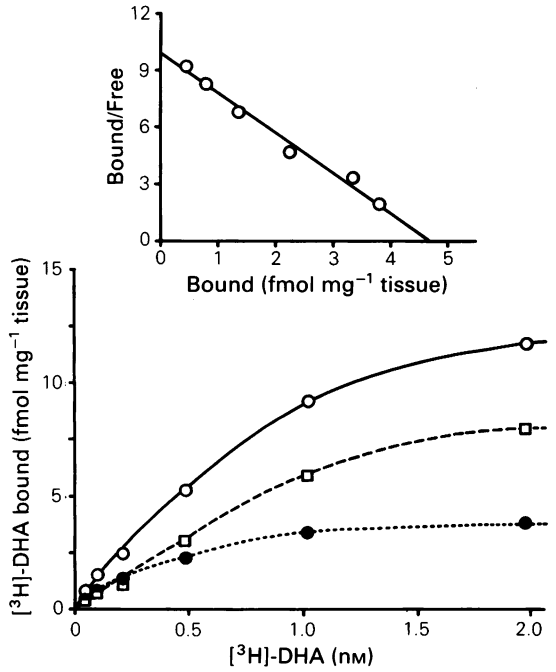


Figure 1 Analysis of [^{3}H]-dihydroalprenolol (DHA) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [^{3}H]-DHA ranging from 0.05 to 2.0 nM for 30 min at 25°C: (○), total binding; (□), non-specific binding; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled propranolol and subtracted from total binding. Duplicate estimations were used for each point. Scatchard analysis of the specific binding is represented in the inset: B_{max} = 4.754 fmol mg^{-1} tissue and K_d = 0.481 nM. The results shown are representative of 6 experiments.

changes in β-adrenergic and muscarinic cholinergic interactions on Ca^{2+} current.

To clarify such discrepancies, we have investigated the developmental changes in β-adrenoceptors and muscarinic cholinoceptors and in Ca^{2+} channels in rat ventricular muscles by using the specific radioligands [^{3}H]-dihydroalprenolol (DHA), [^{3}H]-quinuclidinyl benzilate (QNB), and [^{3}H]-nitrendipine (NTD), respectively. We have obtained some biochemical data to explain our previous electrophysiological ones, i.e., the developmental changes in Iso-ACh interaction on cardiac slow APs (Kojima *et al.*, 1990).

Methods

Animals and tissue preparations

Foetal (16–20 days post-gestation), neonatal (0–20 days old), and adult (2–3 months old) Wistar rats were used in the present experiments. The method of obtaining foetuses and neonates of specified ages has been described (Kojima *et al.*, 1990). The hearts were rapidly excised and transferred into an ice-cold oxygenated modified Tyrode solution (for composition, see Kojima *et al.*, 1990). Ventricles dissected from the hearts were quickly frozen and kept at −80°C until required for binding assay.

The frozen ventricles were weighed, minced with scissors, and then homogenized in 40 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a polytron apparatus (2 × 20 s, setting 5: NS-310E, Niti-on, Tokyo, Japan). The homogenates were centrifuged at 40,000*g* for 20 min at 4°C (18PR-5, Hitachi, Tokyo, Japan). The resultant pellets were washed twice in the ice-cold Tris-HCl buffer. They were finally resuspended in 40 volumes of the buffer and used as tissue homogenates for the following binding assays.

Radioligand binding assays

Binding of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD to cardiac β-adrenoceptors, muscarinic cholinoceptors and Ca^{2+} channels, respectively, were assayed by the methods of Yamada *et al.* (1984), Yamamura & Snyder (1974), and Erman *et al.* (1983). Briefly, the tissue homogenates (2.5 mg tissue for [^{3}H]-DHA and [^{3}H]-NTD binding assays and 5 mg tissue for [^{3}H]-QNB binding assay) were incubated with different concentrations of: (i) [^{3}H]-DHA (0.05–2 nM) in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4) for 30 min at 25°C; (ii) [^{3}H]-QNB (0.01–0.4 nM) in a final volume of 1 ml of 50 mM Na/K-phosphate buffer (pH 7.4) for 60 min at 25°C; and (iii) [^{3}H]-NTD (0.05–1 nM) in a final volume of 1 ml of 50 mM Tris-HCl buffer (pH 7.4) for 90 min at 25°C. After a steady state had been reached, the binding reaction was terminated by rapid filtration under reduced pressure through Whatman GF/B glass-fibre filters using a Brandel cell harvester (M-24R, Brandel, MD, U.S.A.). The filters were rinsed immediately three times with 3 ml of the ice-cold assay buffers for [^{3}H]-DHA, [^{3}H]-QNB and [^{2}H]-NTD binding assays.

Tissue-bound radioactivity was extracted from the filters for 12–24 h in 10 ml of scintillation fluid (Emulsifier-Scintillator 299™, Packard, IL, U.S.A.). The radioactivity was determined in a liquid scintillation counter (TRI-CARB 4640, Packard, IL, U.S.A.) at a counting efficiency of 30–40%. All binding measurements were made in duplicate in 3–8 independent experiments (except 2 experiments for [^{3}H]-DHA and [^{3}H]-QNB bindings on post-gestation day 16). [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD bindings displaced by 1 μM propranolol, 1 μM atropine, and 1 μM nifedipine, respectively, were considered to be specific. All data in the text refer to specific binding. For the [^{3}H]-NTD binding assay, all experiments were carried out under dim light because of the light sensitivity of the dihydropyridine derivative. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine albumin as the standard.

Data analysis

Binding data were analysed as described previously (Yamada *et al.*, 1984). Receptor or channel binding data from the saturation studies were analysed by a modified Scatchard equation corresponding to the linear equation:

$$B = K_d(B/F) + B_{max}$$

where B is the specifically bound ^{3}H-ligand, F is the free concentration of ^{3}H-ligand, K_d is the apparent dissociation equilibrium constant, and B_{max} is the total receptor or channel density. When the binding data are plotted in this manner, the negative of the slope of the regression line gives K_d, and the Y-intercept is B_{max}. The curve fitting programme was obtained from General Statistics (Hewlett-Packard, CO, U.S.A.) for use with a microcomputer (HP9825A, Hewlett-Packard, CO, U.S.A.).

Data are expressed as means ± s.e.mean. A one-way analysis of variance (ANOVA) and Dunnett's test were used for comparison among the means of the different age groups (Wallenstein *et al.*, 1980). A P value of < 0.05 was considered statistically significant. Calculations and analysis were done with the aid of a personal computer (PC-9801RX, NEC, Tokyo, Japan).

Drugs and chemicals

[^{3}H]-DHA (specific activity, 95 Ci mmol^{-1}), [^{3}H]-QNB (43.6 Ci mmol^{-1}), and [^{3}H]-NTD (73.7 Ci mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A.). Propranolol hydrochloride and atropine sulphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and nifedipine from Yodogawa Seiyaku Co. (Osaka, Japan). Other drugs and chemicals were obtained from commercial sources (Sigma Chemical Co. or Nakarai Chemicals, Kyoto, Japan).

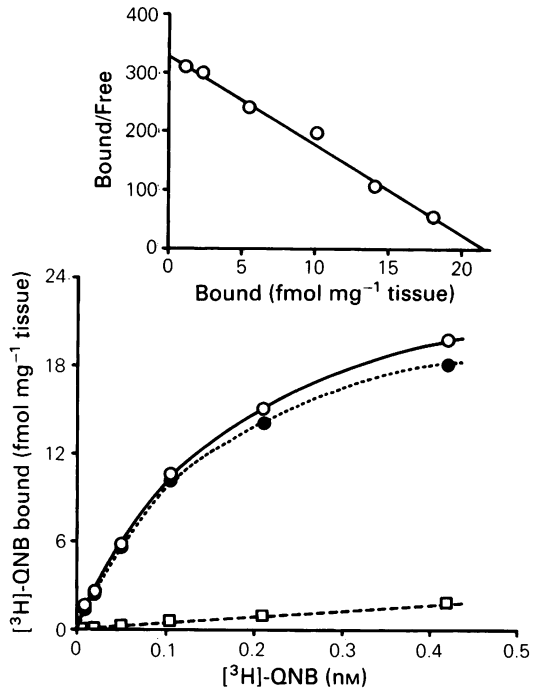


Figure 2 Analysis of [^{3}H]-quinuclidinyl benzilate (QNB) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [^{3}H]-QNB ranging from 0.01 to 0.4 nM for 60 min at 25°C: (○), total binding; (□), non-specific bindings; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled atropine and subtracted from total binding. Duplicate estimations were used for each point. Scatchard analysis of the specific binding is represented in the inset: B_{max} = 21.80 fmol mg^{-1} tissue and K_d = 0.0657 nM. The results shown are representative of 6 experiments.

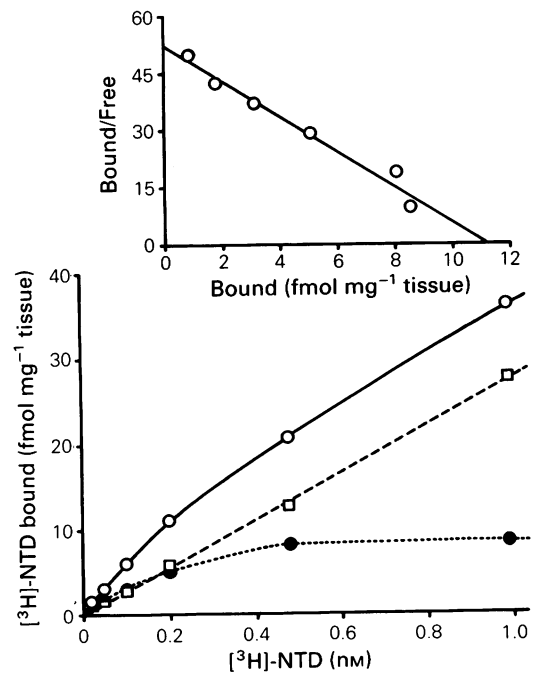


Figure 3 Analysis of [^{3}H]-nitrendipine (NTD) binding to adult (2–3 months old) rat ventricular muscle. Cardiac membranes were incubated with increasing concentrations of [^{3}H]-NTD ranging from 0.05 to 1.0 nM for 90 min at 25°C: (○), total binding; (□), non-specific binding; (●), specific binding. Non-specific binding was determined in the presence of 1 μM unlabelled nifedipine and subtracted from total binding. Duplicate estimates were used for each point. Scatchard analysis of the specific binding is represented in the inset: B_{max} = 11.31 fmol mg^{-1} tissue and K_d = 0.216 nM. The results shown are representative of 7 experiments.

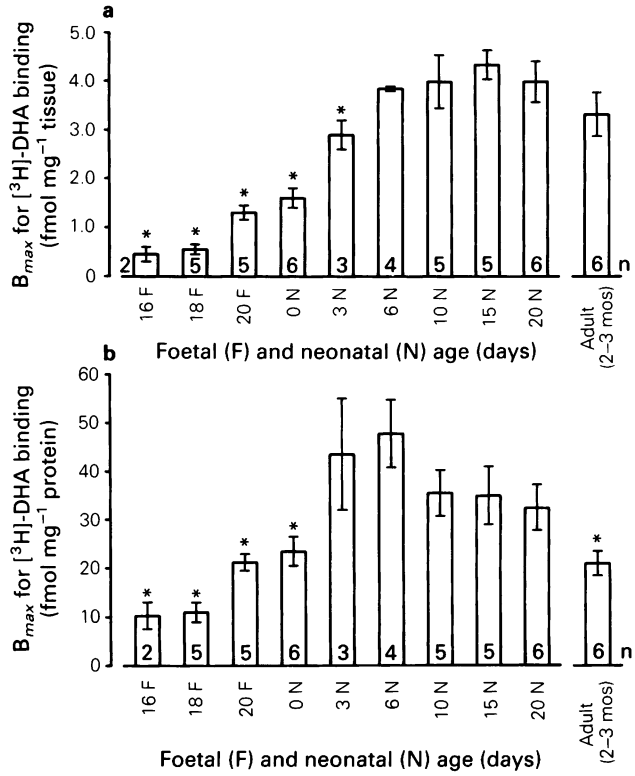


Figure 4 Developmental changes in B_{max} for specific binding of [^{3}H]-dihydroalprenolol (DHA) (β-adrenoceptors) to rat cardiac cell membrane. The B_{max} values are expressed in units of fmol mg^{-1} tissue (a) and of fmol mg^{-1} protein (b). Column heights represent mean values and vertical bars show s.e.mean of 2–6 separate determinations from rats at various ages. The number of experiments is given at the bottom of columns. On the abscissae, F and N denote foetal and neonatal age, respectively. These abbreviations are used in subsequent legends. * Significantly different from the highest value on neonatal day 15 (a) or that on neonatal day 6 (b), $P < 0.05$, Dunnett's test.

Results

Equilibrium bindings of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD to heart homogenates

Radioligands of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD were used as biochemical markers for the differentiation of β-adrenoceptors, muscarinic cholinoceptors and Ca^{2+} channels, respectively. In preliminary experiments, we confirmed that ligand binding to heart homogenates reached equilibrium at 25°C by 30 min for [^{3}H]-DHA, 60 min for [^{3}H]-QNB, and 90 min for [^{3}H]-NTD under the present experimental conditions. The main panels of Figures 1, 2, and 3 depict the total, specific, and non-specific bindings of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD, respectively, to the homogenates of adult rat ventricles. The specific binding, i.e., the difference between the total and non-specific bindings, is a saturable process. Specific bindings of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD at a saturable concentration were 28 ± 6% (at 0.5 nM, n = 6), 95 ± 1% (at 0.1 nM, n = 6), and 47 ± 2% (at 0.2 nM, n = 7), respectively.

The insets in Figures 1, 2, and 3 show Scatchard plots for the specific bindings of [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD, respectively, to their receptors or channels. The linear curve of Scatchard plots demonstrates the presence of a single class of binding site for each ligand under the present conditions. This is also true of the homogenates from foetal and neonatal ventricles (data not shown).

Developmental changes in [^{3}H]-DHA binding sites (β-adrenoceptors)

The density and affinity of β-adrenoceptors for [^{3}H]-DHA were determined by computing B_{max} (the maximum number of

Table 1 Developmental changes in K_d values for [^{3}H]-dihydroalprenolol (DHA), [^{3}H]-quinuclidinyl benzilate (QNB), and [^{3}H]-nitrendipine (NTD) binding to rat cardiac cell membrane at various ages

Age (days)	K_d *values* (nM) [^{3}H]-*DHA binding*	[^{3}H]-*QNB binding*	[^{3}H]-*NTD binding*
16 F	0.227 ± 0.094 (2)	0.075 ± 0.005 (2)	0.175 ± 0.029 (4)
18 F	0.391 ± 0.099 (5)	0.111 ± 0.013 (4)	0.145 ± 0.033 (5)
20 F	0.378 ± 0.081 (5)	0.107 ± 0.003 (5)	0.147 ± 0.011 (7)
0 N	0.354 ± 0.059 (6)	0.138 ± 0.011 (6)	0.153 ± 0.021 (7)
3 N	0.352 ± 0.056 (3)	0.128 ± 0.012 (4)	0.186 ± 0.014 (6)
6 N	0.444 ± 0.047 (4)	0.113 ± 0.009 (5)	0.214 ± 0.019 (6)
10 N	0.378 ± 0.047 (5)	0.118 ± 0.011 (5)	0.198 ± 0.009 (7)
15 N	0.385 ± 0.040 (5)	0.122 ± 0.012 (5)	0.233 ± 0.015 (7)
20 N	0.430 ± 0.064 (6)	0.102 ± 0.005 (6)	0.239 ± 0.014 (6)
Adults	0.413 ± 0.064 (6)	0.094 ± 0.011 (6)	0.212 ± 0.012 (7)

K_d values were obtained from the Scatchard analysis of saturation experiments. Values are means ± s.e.mean of 2–7 preparations in each group (figures in parentheses give the number of experiments). ANOVA indicates that there are no significant differences in K_d values of each ligand group ($P > 0.05$). 'F' and 'N' in the age column mean foetal and neonatal age, respectively.

binding sites) and apparent K_d (dissociation constant) values, respectively, from Scatchard plots of binding data obtained. Developmental changes in B_{max} and K_d for [^{3}H]-DHA binding were studied in foetal (16–20 days old), neonatal (0–20 days old), and adult (2–3 months old) rat ventricles.

Figure 4 gives a summary of the present results obtained for [^{3}H]-DHA binding (n = 2–6). The time courses of developmental changes in B_{max} are different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the 'fmol mg^{-1} tissue' unit (Figure 4a), the B_{max} value started to increase steeply on post-gestation day 20, reached almost a maximum level on neonatal day 6, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level. In the case of the 'fmol mg^{-1} protein' unit (Figure 4b), the B_{max} value increased steeply with age between post-gestation day 18 and neonatal day 6, and then decreased moderately to its adult level.

In Table 1, developmental changes in K_d values for [^{3}H]-DHA binding are summarized. Analysis of variance of the data given in Table 1 shows that there are no significant differences in the apparent K_d values for specific [^{3}H]-DHA binding among the different age groups examined ($P > 0.05$, ANOVA, $n = 2$–6).

Developmental changes in [^{3}H]-QNB binding sites (muscarinic cholinoceptors)

[^{3}H]-QNB binding was examined as a measure of muscarinic cholinoceptors. Developmental changes in B_{max} and K_d for [^{3}H]-QNB binding were also examined in rat hearts of the same pre- and post-natal ages as those in the case of [^{3}H]-DHA binding assay.

Figure 5 gives a summary of the developmental changes in B_{max} for [^{3}H]-QNB binding ($n = 2$–6). Their developmental time courses are also different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the 'fmol mg^{-1} tissue' unit (Figure 5a), the B_{max} value showed a steady-state increase from post-gestation day 16, reached almost a maximum level on neonatal day 0, remained almost constant until neonatal day 15, and then decreased slightly to its adult level. In the case of the 'fmol mg^{-1} protein' unit (Figure 5b), the B_{max} value increased with age between days 16 and 20 post-gestation, kept almost a constant level until neonatal day 3, and then decreased greatly to its adult level.

In Table 1, developmental changes in K_d values for [^{3}H]-QNB binding are summarized. The values of K_d remained

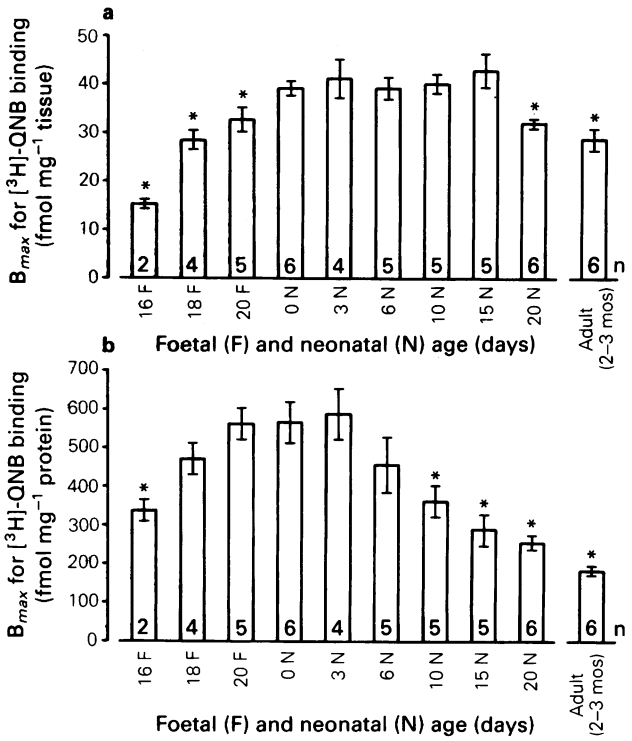


Figure 5 Developmental changes in B_{max} for specific binding of [^{3}H]-quinuclidinyl benzilate (QNB) (muscarinic cholinoceptors) to rat cardiac cell membrane. The B_{max} values are expressed in units of fmol mg^{-1} tissue (a) and of fmol mg^{-1} protein (b). Column heights represent mean values and vertical bars show s.e.mean of 2–6 separate measurements from rats at various ages. The number of experiments is given at the bottom of columns. * Significantly different from the highest value on neonatal day 15 (a) or that on neonatal day 3 (b), $P < 0.05$, Dunnett's test.

almost constant at all developmental stages studied ($P > 0.05$, ANOVA, $n = 2$–6).

Developmental changes in [^{3}H]-NTD binding sites (Ca^{2+} channels)

[^{3}H]-NTD binding was studied as a measure of NTD-sensitive Ca^{2+} channels. Developmental changes in B_{max} and K_d for [^{3}N]-NTD binding were also examined in rat hearts of the same pre- and post-natal ages as those in the case of [^{3}H]-DHA binding assay.

Figure 6 summarizes the developmental changes in B_{max} for [^{3}H]-NTD binding ($n = 4$–7). Their developmental time courses are also different when the B_{max} values are expressed in units of fmol mg^{-1} tissue and of fmol mg^{-1} protein. In the case of the 'fmol mg^{-1} tissue' expression (Figure 6a), the B_{max} value increased with age between post-gestation day 18 and neonatal day 15, kept almost the same level until neonatal day 20, and then decreased slightly to its adult level. In the case of the 'fmol mg^{-1} protein' expression (Figure 6b), the B_{max} value started increasing on post-gestation day 16, reached almost a maximum level on neonatal day 6, remained almost constant until neonatal day 20, and then decreased slightly to its adult level.

In Table 1, developmental changes in K_d values for [^{3}H]-NTD binding are summarized. The values of K_d remained almost constant at all developmental stages studied ($P > 0.05$, ANOVA, $n = 4$–7).

Discussion

Developmental changes in β-adrenoceptors, muscarinic cholinoceptors, and Ca^{2+} channels

In the present study (Figure 4), the number of [^{3}H]-DHA binding sites (β-adrenoceptors; fmol mg^{-1} tissue) started to

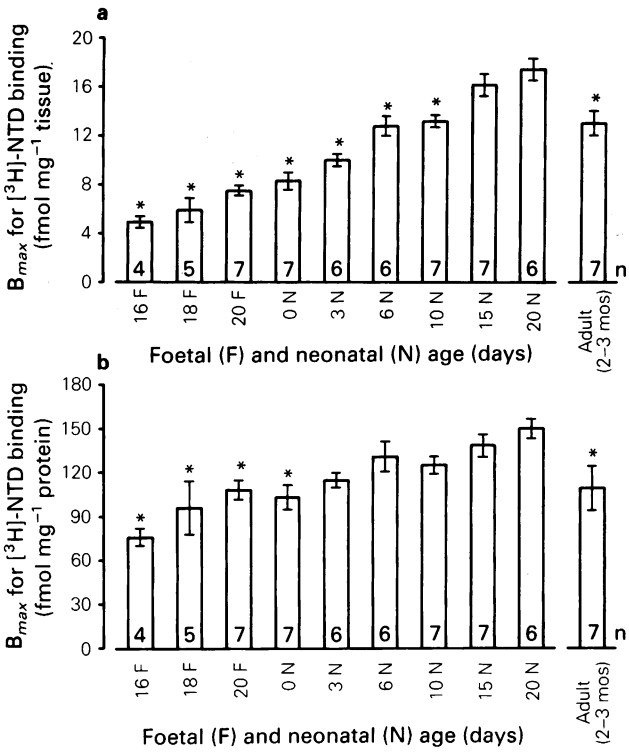


Figure 6 Developmental changes in B_{max} for specific binding of [^{3}H]-nitrendipine (NTD) (Ca^{2+} channels) to rat cardiac cell membrane. The B_{max} values are expressed in units of fmol mg^{-1} tissue (a) and of fmol mg^{-1} protein (b). Column heights represent mean values and vertical bars show s.e.mean of 4–7 separate determinations from rats at various ages. The number of experiments is given at the bottom of columns. * Significantly different from the highest value on neonatal day 20 (a and b), $P < 0.05$, Dunnett's test.

increase on day 20 post-gestation, reached nearly a maximum level on neonatal day 6, and remained almost constant thereafter. The number of [^{3}H]-DHA binding sites (fmol mg^{-1} protein), however, increased rapidly between post-gestation day 18 and neonatal day 6, and decreased gradually from neonatal day 10 to the adult level. Systematic studies on postnatal development of β-adrenoceptors have been reported, but none on their pre-natal development. Lau & Slotkin (1982) found that specific binding of [^{3}H]-DHA (pmol g^{-1} tissue) approximately doubled from 3 to 28 days after birth, increasing steeply between 3 and 6 days of age and then gradually between 6 and 28 days of age. Noguchi *et al.* (1981) and Noguchi and Whitsett (1983) showed that [^{3}H]-DHA binding sites (fmol mg^{-1} protein) were most numerous at 1 day of age and gradually decreased with age to the adult level. The developmental pattern of [^{125}I]-pindolol binding sites (fmol mg^{-1} protein), however, showed a large increase between neonatal days 5 and 15 followed by a decline between 15 and 40 days of age (Slotkin *et al.*, 1988). Cros *et al.* (1988) reported that the B_{max} of [^{125}I]-iodocyanopindolol (fmol mg^{-1} protein) was half that of adults during neonatal days 0–10 and increased progressively during days 15 to 60. It is clear that there are vast discrepancies among the data in the literature. It is also noted that these ontogenic data on radioligand binding for β-adrenoceptors are incomplete because these data are lacking for the pre-natal developmental changes of age.

In the present study (Figure 5), the number of receptor binding sites for [^{3}H]-QNB (muscarinic cholinoceptors; fmol mg^{-1} protein) started increasing on day 16 post-gestation, reached nearly a peak level 4 days later, remained almost constant until neonatal day 3, and then gradually decreased to the adult level. Nedoma *et al.* (1986) reported that the density of [^{3}H]-QNB binding sites (pmol g^{-1} protein) increased steeply between the 15th and the 19th days of pre-natal development, and then gradually decreased to the adult level. Ho *et al.* (1986) observed that the number of [^{3}H]-QNB binding sites (fmol mg^{-1} protein) showed an increase between post-gestation day 16 and the day of birth, remained at this level during the first week of post-natal life, and reached a peak on day 9. This was followed by a gradual decline over the next 6 months. These two groups, however, gave no information about developmental changes in β-adrenoceptors and Ca^{2+} channels, although Ho *et al.* (1986) demonstrated developmental changes in the content of calmodulin.

In the present study (Figure 6), the number of [^{3}H]-NTD binding sites (Ca^{2+} channels; fmol mg^{-1} protein) gradually increased starting on day 16 post-gestation, reached almost a maximum level on neonatal day 6, remained almost constant until neonatal day 20, and then decreased slightly to the adult level. In regard to the developmental changes in Ca^{2+} channels, Kazazoglou *et al.* (1983) reported that the number of [^{3}H]-NTD receptors (fmol mg^{-1} protein) was low in the foetal stage and increased markedly between 17 days pre-natal and 15 days post-natal. Then B_{max} values remained constant between 15 days post-natal and the adult stage (2 months). Our data are essentially similar to those of Kazazoglou *et al.* (1983).

B_{max} values expressed in units of fmol mg^{-1} tissue and fmol mg^{-1} protein

As is evident from Figures 4–6, developmental time courses of B_{max} for [^{3}H]-DHA, [^{3}H]-QNB, and [^{3}H]-NTD bindings are different when the B_{max} values are expressed in fmol mg^{-1} tissue and fmol mg^{-1} protein units. In general, the B_{max} values expressed in 'fmol mg^{-1} protein' units reached a maximum level earlier in their developmental time courses than did those expressed in 'fmol mg^{-1} tissue' units. Furthermore, the former B_{max} values (fmol mg^{-1} protein) showed a greater decrease in magnitude from the maximum level to the adult level than the latter B_{max} values (fmol mg^{-1} tissue).

These discrepancies between the B_{max} data expressed in different units may be due largely to the following: (i) the ratio of 'mg protein/mg tissue' increases with age, i.e., 0.055 ± 0.005 ($n = 6$) on day 16 post-gestation, 0.076 ± 0.003 ($n = 13$) on the day of birth, 0.111 ± 0.004 ($n = 12$) on neonatal day 10, 0.123 ± 0.006 ($n = 11$) on neonatal day 20, and 0.144 ± 0.006 ($n = 12$) at adult ages of 2–3 months (the present data); (ii) the ratio of 'sarcolemmal protein/cell protein' or 'surface area/fibre mass' decreases with age because of the development of intracellular organs (Baker & Potter, 1980; Whitsett & Darovec-Beckerman, 1981; Nedoma *et al.*, 1986).

The developmental time course of B_{max} for [^{3}H]-DHA and [^{3}H]-NTD bindings (present data) and of GTP-binding protein levels (Kojima *et al.*, 1988) are more similar to those of Iso-induced increases in $\dot{V}_{max}$ (Kojima *et al.*, 1990) when the former biological data are expressed in units of fmol mg^{-1} tissue than when fmol mg^{-1} protein is used. In the following section, therefore, we used the B_{max} data expressed in 'fmol mg^{-1} tissue' units for comparison with our electrophysiological data.

A comparison between the present biochemical findings and the preceding electrophysiological findings on the effect of β-adrenergic and muscarinic cholinergic interactions on Ca^{2+} current

Iso increases the $\dot{V}_{max}$ of slow APs gradually from post-gestation day 18 and the adult level is reached about 2 weeks after birth (Kojima *et al.*, 1990). This developmental time course correlates well with that of Ca^{2+} channels (present data) and of stimulatory GTP-binding (G_s) proteins (Kojima *et al.*, 1988). The number of β-adrenoceptors started to increase on post-gestational day 20 when the G_s protein, the number of Ca^{2+} channels, and the $\dot{V}_{max}$ of Iso-induced slow APs also started to increase. The number of β-adrenoceptors, however, reached its peak about one week earlier than did the other three parameters (Figure 7).

Acetylcholine (ACh) almost completely abolished the Iso-induced increase in $\dot{V}_{max}$ observed on post-gestation days 18–20 and neonatal days 0–20 as well as in adult rats (Kojima *et al.*, 1990). This finding can be explained by the following facts: (i) the density of muscarinic cholinoceptors is higher than that of β-adrenoceptors and of Ca^{2+} channels during the developmental period examined, and (ii) the number of muscarinic cholinoceptors reaches a peak 1–2 weeks earlier than does that of β-adrenoceptors, of Ca^{2+} channels (present data) and of G_s protein levels (Kojima *et al.*, 1988).

In summary, we have studied the ontogeny of the following parameters to elucidate developmental changes in the transmembrane signalling system responsible for the control of cardiac Ca^{2+} current: β-adrenoceptors and muscarinic cholinoceptors, G_s and G_i proteins, Ca^{2+} channels, and influence of Iso-ACh interactions on $\dot{V}_{max}$ of slow APs (Kojima *et al.*, 1988; 1990, and the present paper). In general, the transmembrane signalling mechanism related to muscarinic cholinergic responses reached a peak level about one week earlier than did the transmembrane signalling mechanism related to β-adrenergic responses. From the viewpoint of the relationship of developmental time courses and β-adrenergic responses (Figure 7), there is a good parallel relationship among the parameters of G_s proteins, Ca^{2+} channels, and $\dot{V}_{max}$ of slow APs but not of β-adrenoceptors: these receptors attained a peak level about one week earlier than the others. These findings strongly suggest that the functional coupling between muscarinic cholinoceptors and Ca^{2+} current is already established when the coupling between β-adrenoceptors and Ca^{2+} current starts to operate. The nature of the neuronal factors which regulate the levels of β-adrenoceptors, muscarinic cholinoceptors, and NTD-sensitive Ca^{2+} channels in cardiac cells remains to be determined.

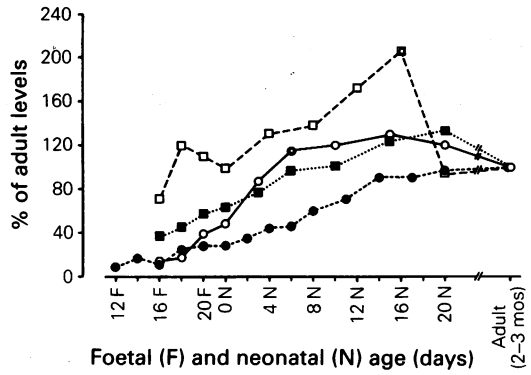


Figure 7 Developmental changes in β-adrenoceptor-related transmembrane signalling system for Ca^{2+} current in rat hearts. Curves are plotted for [^{3}H]-dihydroalprenolol (DHA) binding sites (β-adrenoceptors: ○), substrates for cholera toxin (CTX)-catalyzed ADP-ribosylation (G_s protein: □), [^{3}H]-nitrendipine (NTD) binding sites (Ca^{2+} channels: ■), and isoprenaline (Iso)-induced increase of $\dot{V}_{max}$ (Ca^{2+} current: ●), relative to adult levels at 100%. The enclosed symbols (◎, ▣, ■, ◉) on each curve indicate the time when each parameter reaches a (near) maximum level. The data for CTX substrates and for potentiating effect of Iso on Ca^{2+} current are taken from Kojima *et al.* 1988 and 1990, respectively, with permission.

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Effect of neuropeptide Y on cardiac output, its distribution, regional blood flow and organ vascular resistances in the pithed rat

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1 The effects of neuropeptide Y on cardiac output, its distribution and organ vascular resistances were determined with tracer microspheres in pithed rats.

2 Neuropeptide Y increased blood pressure by increasing both cardiac output and total peripheral resistance. The increase in cardiac output was due to an increase in stroke volume as heart rate was not changed. Increased vascular resistance in the splenic, renal, testicular, epididimidal, skeletal muscle, large intestinal and mesenteric vascular beds contributed to the increase in total peripheral resistance. Vasoconstriction was most pronounced in the mesenteric bed.

3 This study indicates that neuropeptide Y increases blood pressure by increasing cardiac output and total peripheral resistance. The increased cardiac output is possibly due to an increase in venous return, whilst the increased total peripheral resistance was due to regional vasoconstriction, particularly in the mesenteric bed.

Introduction

Neuropeptide Y is a 36 amino acid peptide, originally isolated from porcine brain (Tatemoto, 1982). There is increasing evidence that this peptide is involved in the sympathetic vasoconstrictor response; it is co-stored and co-released with noradrenaline from perivascular sympathetic nerve endings and from the adrenal medulla in most species including man (Lundberg *et al.*, 1983; 1984). Low doses of neuropeptide Y, which alone do not induce vasoconstriction, have been shown to facilitate vasoconstrictor responses to noradrenaline, α-adrenoceptor agonists and sympathetic nerve stimulation in pithed and anaesthetized rats (Dahlöf *et al.*, 1985; Revington *et al.*, 1987). Higher doses of neuropeptide Y induce a direct pressor effect in pithed rats (Dahlöf *et al.*, 1985). Previous studies have been unable to ascertain whether neuropeptide Y-induced pressor responses are due to effects on cardiac output, total peripheral resistance or both. In the present study by use of tracer microspheres, we have examined the effects of neuropeptide Y on cardiac output, cardiac output distribution, total peripheral resistance and organ vascular resistance in the pithed rat.

Methods

Determination of cardiac output, its distribution and regional vascular resistance

Male Wistar rats, weighing 250–300 g (Bantin & Kingman Ltd, Hull) were pithed under halothane anaesthesia using a 16 gauge steel needle. Immediately after pithing, the rats were respired with air through a tracheal cannula by means of a respiratory pump (BioScience, Sheerness, U.K.) operating at 54 cycles min^{-1} with a volume of 20 ml kg^{-1}. The right femoral artery was cannulated and connected to a Bell & Howell type 4-422-0001 pressure transducer to measure systemic arterial blood pressure which was recorded on a Grass 7D polygraph; heart rate was determined from the systemic blood pressure trace by a Grass 7P44 cardiotachograph. The left femoral artery was also cannulated and connected to a Braun Perfusor IV pump (Melsungen, F.R.G.) for the withdrawal of blood. With the aid of pressure monitoring, a cannula was passed down the right common carotid artery into the left ventricle. Rectal temperature was maintained at 37°C by means of a homeothermic blanket system (BioScience, Sheerness, Kent). Neuropeptide Y (500 pmol bolus injection (0.5 ml) followed by a 200 pmol min^{-1} infusion (0.1 ml min^{-1})) [porcine neuropeptide Y, Neosystems S.A., Strasbourg, France]) or saline (0.5 ml bolus followed by an 0.1 ml min^{-1} infusion) were administered through a cannula previously placed in the left external jugular vein. Pressor responses to neuropeptide Y were always maximal and sustained 1 min after injection, and at this time (in both the neuropeptide Y-treated and saline-treated animals), 60,000–80,000 ^{113}Sn labelled microspheres (15 ± 3 μm; NEN, Boston, MA, U.S.A.), suspended by ultrasonication in saline containing 0.01% Tween 80, were injected into the ventricle over 20 s. Blood was withdrawn from the left femoral artery at a rate of 0.5 ml min^{-1} during and, for 70 s after, the microsphere injection. The circulation was stopped with an air embolism and the organs dissected out, weighed and placed in scintillation vials for counting in a Packard Autogamma 500 γ-scintillation counter.

The number of counts in the blood sample was also determined in order that cardiac output and organ vascular resistances could be determined as described by McDevitt & Nies (1976) and by us previously (MacLean & Hiley, 1988a,b). All results are given as the mean ± s.e.mean. The statistical significance between groups was assessed by one-way random block, analysis of variance followed by the least significant difference procedure (Snedecor & Cochran, 1980).

Results

Table 1 shows that there were no significant differences in basal blood pressure or basal heart rate between the control and neuropeptide Y-treated groups. In the neuropeptide Y-treated group, the peptide increased both mean arterial and diastolic blood pressure by 78%. This was due to the combined effects of a 40% greater cardiac output and 38% greater total peripheral resistance than that of the saline-treated group. The enhanced cardiac output was due to a 34% increase in stroke volume, since heart rate was not affected by this dose of neuropeptide Y. Increased vascular resistance in the splenic, renal, testicular, epididimidal, skeletal muscle,

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Table 1 Effects of neuropeptide Y (500 pmol bolus + 200 pmol min^{-1} infusion) on blood pressure, cardiac index, heart rate and organ vascular resistance in the pithed rat as determined by use of tracer microspheres

	Saline control	*Neuropeptide Y*
Basal mean arterial pressure (mmHg)	36.9 ± 3.5	37.5 ± 2.5
Change in mean arterial pressure	−1 ± 1	29.4 ± 4.0***
Basal diastolic pressure (mmHg)	27.5 ± 3.5	30.6 ± 2.0
Change in diastolic pressure	−1 ± 1	24 ± 3***
Basal heart rate (beats min^{-1})	318 ± 9	329 ± 14
Change in heart rate	−3 ± 4	−3 ± 5
Cardiac index (ml min^{-1} × 100 g^{-1} body wt.)	11.3 ± 0.7	15.8 ± 1.0**
Stroke volume (μl)	104 ± 8	139 ± 10*
TPR (mmHg ml^{-1} min × 100 g body wt.)	3.13 ± 0.21	4.33 ± 0.46*
Organ vascular resistances (mmHg ml^{-1} min g)		
Heart	23.3 ± 2.7	29.8 ± 2.6
Lungs	86.6 ± 16.0	35.2 ± 6.4**
Liver	313 ± 36	316 ± 47
Spleen	60.0 ± 5.6	89.2 ± 10.6*
Kidneys	15.7 ± 1.8	42.5 ± 6.9**
Testes	224 ± 12	327 ± 46*
Fat	3232 ± 674	6622 ± 1830
Epididimides	506 ± 45	746 ± 92*
Skeletal muscle	527 ± 71	821 ± 117*
Skin	743 ± 127	832 ± 151
Stomach	94 ± 13	103 ± 11
Small intestine	33.7 ± 2.8	45.1 ± 4.9
Large intestine	38.7 ± 6.0	69.6 ± 10.9*
Pancreas & mesentery	90.1 ± 11.1	366 ± 57***

For all groups, $n = 8$.
Significant differences between the saline (0.5 ml bolus + 0.1 ml min^{-1} infusion) control group and the neuropeptide Y-treated group were determined by one-way analysis of variance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
Basal values of heart rate, diastolic and mean arterial blood pressures were obtained before the administration of either saline or neuropeptide Y. Changes are those occurring between these values and the midpoint of the microsphere injection. Cardiac index is the absolute value determined by the tracer microsphere method. TPR is the total peripheral resistance calculated from cardiac index and mean arterial pressure during the microsphere injection assuming central venous pressure to be zero.

large intestinal and mesenteric vascular beds (49%, 171%, 46%, 47%, 56%, 79% and 306% increases respectively, compared to the saline-treated control group) contributed to the increased total peripheral resistance (Table 1).

Neuropeptide Y induced a decrease in the vascular resistance in the lungs (59% compared with the saline control group) and an increase in the fractional distribution of cardiac output to these organs (by 235% compared to the saline control group). It also induced an increase in the proportion of the cardiac output passing to the small intestine (by 17%) while causing a decrease in that passing to the kidneys and the pancreas/mesentery by 42% and 62% respectively (Figure 1).

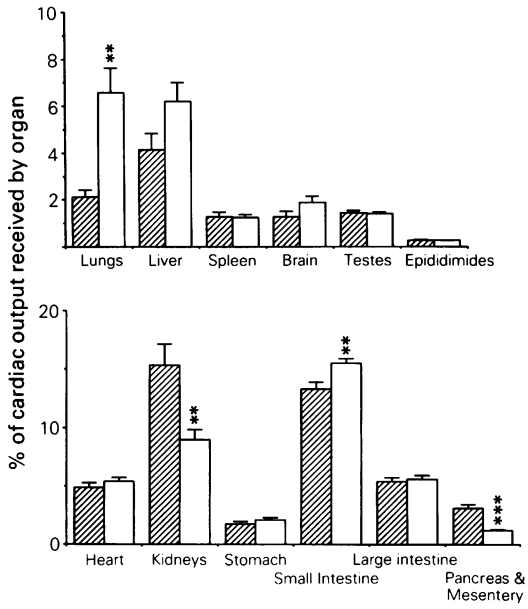


Figure 1 Effect of neuropeptide Y on the fractional distribution of cardiac output in pithed rats. The hatched columns represent the results from rats given saline (0.5 ml bolus + 0.1 ml min^{-1} infusion) and the open columns are data from rats given neuropeptide Y (500 pmol bolus + 200 pmol min^{-1} infusion). $n = 8$ in both groups. Significant differences between the saline control group and the neuropeptide Y-treated group were determined by one-way analysis of variance. $**P < 0.01$; $***P < 0.001$.

Discussion

This study shows that neuropeptide Y exerts its pressor effect in the pithed rat by increasing both cardiac output and total peripheral resistance. The increased cardiac output was due to an increase in stroke volume since heart rate was not changed by neuropeptide Y administration. Pressor responses to neuropeptide Y which were not accompanied by bradycardia have previously been observed in the pithed rat (Dahlöf *et al.*, 1985). In the pithed rat, increased stroke volume could be due to greater venous return, an increase in cardiac contractility or both. Coronary arteries are richly innervated by neuropeptide Y-containing nerve fibres (Allen *et al.*, 1983) and neuropeptide Y has been shown to cause both coronary vasoconstriction and a reduction in cardiac contractility in isolated heart preparations and in the conscious rabbit (Minson *et al.*, 1987). Thus it is likely, that in the pithed rat, the increased stroke volume observed after neuropeptide Y administration is due to an increase in venous return. Increased vascular resistance in the splenic, renal, testicular, epididimidal, skeletal muscle, large intestinal and mesenteric vascular beds contributed to the increase in total peripheral resistance, the increase being most profound in the mesenteric bed. This is compatible with reports that neuropeptide Y induces pressor responses in the perfused mesenteric arterial bed of the rat (Westfall *et al.*, 1987). Neuropeptide Y also induced a decrease in resistance in the lungs and a concomitant increase in the percentage of cardiac output passing to these organs. It should be noted that the microspheres detected in the lungs represent not only those passing through the bronchial arteries but also those passing through arteriovenous anastomoses and which are subsequently trapped in the lungs after passage along the great veins. Thus any effects of neuropeptide Y on lung vascular resistance could be due to actions on either the anastomoses or the bronchial arteries.

Increased renal and mesenteric vascular resistance decreased the proportion of cardiac output sent to these vascular beds and increased that sent to other organs such as the small intestine and lungs. It is of interest that neuropeptide Y caused renal vasoconstriction in this study. Plasma neuropeptide Y levels are increased after haemorrhage in conscious rats (Morris *et al.*, 1987) and in the pig (Lundberg *et al.*, 1987) and renal vasoconstriction is severe in both clinical and experimental hypovolaemic shock (Hock *et al.*, 1982; Hardaway, 1985; MacLean *et al.*, 1989b). Increased levels of circulating neuropeptide Y may, therefore, exacerbate the renal vasoconstriction observed in haemorrhagic shock.

It is becoming apparent that the pharmacological profile of neuropeptide Y has some similarity to that of the vasoactive peptide endothelin-1, recently isolated from endothelial cells in culture (Yanagisawa *et al.*, 1988). We have previously shown that endothelin-1 (300 pmol) increases total peripheral resistance mainly by vasoconstricting the mesenteric vascular bed (MacLean *et al.*, 1989a) and others have reported that more potent doses of endothelin-1 also increase vascular resistance in the kidneys and in skeletal muscle (Thomas *et al.*, 1989; Walder *et al.*, 1989). Furthermore, we have shown that

in conditions of moderate hypoxia, hypercapnia and acidosis, the potency of both endothelin-1 (MacLean *et al.*, 1989a) and neuropeptide Y (MacLean & Hiley, unpublished observations) in the blood-perfused mesenteric arterial bed of the pithed rat is increased and we have observed that both neuropeptide Y (MacLean & Hiley, unpublished observations) and endothelin-1 (MacLean *et al.*, 1989a) induce a unique fluctuation of blood pressure and heart rate in such hypoxic conditions. Neuropeptide Y-induced vasoconstriction in the rabbit ear artery is endothelium-dependent and it has been suggested that neuropeptide Y could promote the release of an endothelial derived vasoconstricting factor (Daly & Hieble, 1987). Whilst there are differences in the pharmacological profiles of neuropeptide Y and endothelin in the conscious rat (Zukowska-Grojec *et al.*, 1987; Gardiner *et al.*, 1988; 1989; Han *et al.*, 1989) it is interesting to speculate that, in some vascular beds, the activities of these two peptides may be related in some way.

It has been observed by others that in many ways, neuropeptide Y mimics the effects of noradrenaline in that it inhibits release of noradrenaline by acting on prejunctional sites, induces vasoconstriction and bradycardia *in vivo* and contracts isolated veins and arteries *in vitro* (Dahlöf *et al.*, 1985; Pernow *et al.*, 1986). Indeed, the effects of neuropeptide Y on cardiac output, total peripheral resistance and organ vascular resistance described here are similar to those induced by α-adrenoceptor agonists as described in previous microsphere studies (Hiley & Thomas, 1987; MacLean & Hiley, 1988b).

In conclusion, this study shows that neuropeptide Y exerts its pressor effect in the pithed rat by increasing cardiac output and total peripheral resistance. The increased cardiac output is possibly through an increase in venous return, and the increased total peripheral resistance mainly due to mesenteric vasoconstriction. Neuropeptide Y, endothelin and the noradrenergic nervous system may interact in the control of blood pressure.

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Characterization of MDL 73005EF as a 5-HT_{1A} selective ligand and its effects in animal models of anxiety: comparison with buspirone, 8-OH-DPAT and diazepam

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1 With radioligand binding techniques, MDL 73005 EF (8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl-methylamino)ethyl]-8-azaspiro[4,5]decane-7,9-dione methyl sulphonate) shows high affinity (pIC_{50} 8.6) and selectivity (>100 fold compared to other monoamine and benzodiazepine receptor sites) for the 5-hydroxytryptamine $(5\text{-HT})_{1A}$ recognition site; it was both more potent and more selective than buspirone in this respect.

2 In rats pretreated with reserpine, 8-hydroxy-2-(di-n-propyl-amino) tetralin (8-OH-DPAT) induced forepaw treading and flat body posture; in the same model, MDL 73005EF and buspirone showed minimal agonist activity and at high doses MDL 73005EF inhibited responses to 8-OH-DPAT.

3 In rats trained to discriminate 8-OH-DPAT from saline in a drug discrimination paradigm, both MDL 73005EF and buspirone generalized dose-dependently and completely to the 8-OH-DPAT cue.

4 To define the anxiolytic potential of MDL 73005EF, it was examined in the elevated plus-maze test and in the water-lick conflict test in comparison with diazepam and buspirone. In both tests MDL 73005EF induced effects similar to those seen following diazepam. Buspirone had similar effects to both MDL 73005EF and diazepam in the water-lick conflict test but opposite effects in the elevated plus-maze. 8-OH-DPAT also had opposite effects in the elevated plus-maze test to MDL 73005EF and diazepam.

5 The anti-conflict effects of MDL 73005EF were reversed by low doses of the 5-HT_{1A} receptor agonist, 8-OH-DPAT; those of buspirone were neither antagonised nor mimicked by 8-OH-DPAT.

6 These results suggest that an interaction with 5-HT_{1A} receptors is the basis of the anxiolytic-like activity of MDL 73005EF. However, its mechanism of action is clearly different from that of buspirone, possibly reflecting a greater selectivity for the 5-HT_{1A} receptors located presynaptically on central 5-hydroxytryptaminergic neurones.

Introduction

The involvement of 5-hydroxytryptamine (5-HT) in anxiety has been the subject of many studies (Gardner, 1985) and the recent appearance of non-benzodiazepine anxiolytic compounds, such as buspirone and ipsapirone, has rekindled interest in this involvement (Chopin & Briley, 1987; Traber & Glaser, 1987). Both these compounds show high affinity for the 5-HT_{1A} subtype of 5-HT receptors (Peroutka, 1985) and are considered to act as partial agonists at this receptor (De Vivo & Maayani, 1986; Martin & Mason, 1987). In many tests the agonist effects predominate and ipsapirone and buspirone mimic the effects of the 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) (Sprouse & Aghajanian, 1986; Tricklebank *et al.*, 1987; Gilbert & Dourish, 1987). However, antagonism of 8-OH-DPAT effects can be clearly seen in other tests (Smith & Peroutka, 1986; Goodwin *et al.*, 1986). It is therefore not clear whether buspirone and ipsapirone are acting primarily as 5-HT_{1A} receptor agonists or as antagonists to exert their anxiolytic effects. Further problems of interpretation arise from their rapid metabolism to 1-(2-pyrimidyl) piperazine (Gammans *et al.*, 1983; Traber & Glaser, 1987), an antagonist at α_2-adrenoceptors (Gower, 1986), which has been suggested to play a role in the anticonflict properties of buspirone (Gower & Tricklebank, 1988).

MDL 73005EF (8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl-methylamino)ethyl]-8-azaspiro[4,5]decane-7,9-dione methyl sulphonate; Figure 1) evolved from a programme aimed at the rational design of 5-HT_{1A} receptor ligands by graphics computer-assisted technology (Hibert *et al.*, 1988a). This paper describes the characterization of MDL 73005EF as a potent and highly selective ligand for 5-HT_{1A} receptors. As such, it was of interest to compare its activity with 8-OH-DPAT and buspirone in two animal tests considered predictive of anxiolytic activity and to examine the role of 5-HT_{1A} receptors in the activities of these compounds in these tests. Some of these

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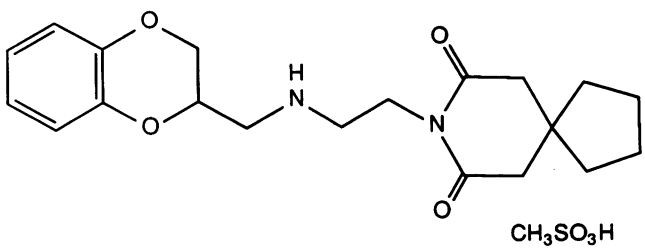


Figure 1 Structure of MDL 73005EF.

data have been presented to the British Pharmacological Society in preliminary form (Hibert *et al.*, 1988b; Moser *et al.*, 1988).

Methods

Animals

Male Sprague-Dawley rats (Charles River, France) weighing 200–300 g were used throughout. Before experimental use they were housed in wire cages, in groups of 6, in temperature (22 ± 1°C) and humidity (55% relative humidity) controlled animal quarters on a 12 h light-dark cycle with lights on between 6 h 00 min and 18 h 00 min. Animals had free access to food and tap water except where indicated in the methods.

Radioligand binding studies

Radioligand binding studies for central neurotransmitter and benzodiazepine recognition sites were carried out with brain membranes from Sprague-Dawley rats as described in detail previously (Mir *et al.*, 1988).

Behavioural effects in reserpine-treated rats

Following the method described by Tricklebank *et al.* (1984), rats were treated with reserpine (1 mg kg^{-1}) 18 h before the test. For observation of behaviours the animals were placed singly in clear plastic boxes, 430 × 90 × 160 mm high, 5 min before injection of the compound under test. Observation sessions of 45 s duration began 5 min later and were repeated every 3 min over a 12 min period. Flat body posture and forepaw treading were scored by a ranked intensity scale where 0 = absent, 1 = equivocal, 2 = present and 3 = intense. These values were summed for each rat over the 12 min observation period to give a maximum possible score of 15. The antagonist effects of MDL 73005EF or buspirone against the behaviours induced by a submaximal dose of 8-OH-DPAT (0.125 mg kg^{-1} s.c.) were quantified by pretreating the animals with the compounds 30 min before administration of 8-OH-DPAT. The observer was aware of the pretreatment each rat had received.

Drug discrimination

Tests were carried out in five standard operant cages (LI 830, Letica Scientific Instruments, Barcelona) with a food dispenser centred between two levers. The cages were housed separately in sound and light attenuating chambers and illuminated by a house light. The apparatus was programmed by solid state logic modules driven by an Apple IIe computer.

The training and testing procedures have been described in detail previously (Tricklebank *et al.*, 1987). Rats were deprived of food to 75–80% of their free-feeding weight and trained to press either right or left levers for food reinforcement (Dustless Precision Pellets, Bio Serv. Inc., New Jersey). Once the lever pressing response was established by continuous reinforcement, the schedule was changed to fixed ratio 10 (one food pellet per 10 responses). Thirty minutes before daily (Monday–Friday) 14 min test sessions, animals were injected s.c. with either saline or 8-OH-DPAT (0.05 mg kg^{-1}). 8-OH-DPAT (D) or saline (S) was given according to a repeating sequence: S, D, D, S, S, D, S, S, D, D. Each day the total number of responses made on both levers before the first food pellet was obtained and the total number of responses on the left and right levers during the whole 14 min test session were recorded. The criterion for correct discrimination was that the rats received the first food pellet before making more than 14 responses.

Experiments were carried out on Tuesdays and Fridays and commenced once the animals were achieving the discrimination criterion in 80% or more of the training sessions of the preceding 3 week period. On experimental days, sessions were of 3 min duration and without reinforcement. The number of responses obtained on the left and right levers during the 3 min test session and the total number of responses when the rat first made 10 responses on one or other lever were recorded. The rat was considered to have selected the lever on which it first made 10 responses. Data are presented as the percentage of rats selecting the 8-OH-DPAT appropriate lever from those responding i.e. making 10 responses on one or other lever. Experimental groups consisted of not less than six animals and the same animals were used to study different doses of the test drug over a number of experimental sessions.

Shock suppressed drinking test

In a modified version of the Vogel drinking test (Vogel *et al.*, 1971) rats were deprived of drinking water for 48 h before the test and placed in groups of 6 in the test room 24 h after deprivation.

The test apparatus consisted of a plastic cage (20 × 15 × 15 cm) with a metal grid floor and a water spout set into one of the side walls which was insulated except for the first 5 mm. The number of licks made by a rat was monitored (Columbus Instruments Anxiometer, Model 102) and after each 20 licks the rat received a shock via the drinking spout of 0.4 mA which lasted 1 s (Lafayette Instrument Co., Model 82400). On the test day all rats first underwent a pre-test session to select those to be used for the experimental session. If, on being placed individually in the test cage, the rats did not find the drinking spout within 1 min or made less than 40 or more than 180 licks during the 2 min following the first lick, they were excluded from further testing. Rats thus selected were arranged in treatment groups pseudo-randomly so that the mean number of licks made in the pre-test was approximately equal between treatment groups. Test compounds were given 30 min before the experimental session which lasted 3 min. The number of licks made during this period was recorded.

The effect of compounds on unpunished drinking was studied by essentially the same method, except that no shock was used and the rats were not deprived of water; previous experiments had shown that non-shocked deprived rats drank almost continuously during the 3 min test session making it impossible to study dipsogenic effects.

Elevated plus-maze test

All behavioural testing was carried out between 13 h 30 min and 18 h 00 min in an elevated plus-maze constructed in grey plastic according to the design of Pellow *et al.* (1985) with arms 50 cm long and 10 cm wide. The two enclosed arms had side walls 50 cm high and the maze was raised 50 cm from the floor. For testing, the rats were placed individually in the centre of the maze in a darkened room and observed for 5 min by an observer in another room via a closed circuit T.V. camera. The number of open- and closed-arm entries (defined as entry of all four limbs into an arm of the maze) during the observation period was recorded and the maze was cleaned after each rat. Rats that fell from the maze were excluded from the experiment. The results were expressed as the mean of the ratio of open-arm to total arm entries for individual rats and as the mean number of open-arm entries and mean total entries made by the rats during the 5 min observation period. In all experiments diazepam (2 mg kg^{-1} s.c.) was used as a positive control.

Tail-flick test

Groups of 5 rats were treated with MDL 73005EF (1 or 3 mg kg^{-1} s.c.) 30 min before testing. Measurement of tail-flick latency was carried out with a focussed light source directed at a point half-way along the length of the tail. The timing of the latency to tail flick was determined automatically by the

apparatus (Apelex Tail Flick Model-SD 20), from the moment the light source was turned on, to the rat's removal of its tail from the light beam, by means of a photocell located under the tail. A cut off time of 10 s was used.

Drugs

Buspirone HCl (Bristol-Myers), 8-OH-DPAT HBr (Research Biochemicals Incorporated) and MDL 73005EF (synthesized at Merrell Dow Research Institute) were dissolved in 0.9% (w/v) saline. Diazepam (Roche) was suspended in 0.9% saline containing 0.01% (v/v) Brij 35 (Sigma). All drugs were prepared immediately before use and given either orally (p.o.) or subcutaneously (s.c.) in a volume of $2\,ml\,kg^{-1}$ or $1\,ml\,kg^{-1}$, respectively. All doses refer to the salt except for 8-OH-DPAT where the doses were calculated as base.

Statistics

All results are presented as means and standard errors of the means (s.e.mean). In the punished drinking test the presence of shock strongly biases the results towards multiples of 20 licks and for this reason the data from this test were analysed by the Mann-Whitney U-test. The results of the elevated plus-maze test and from the 5-HT behavioural syndrome experiments were also analysed by the Mann-Whitney U-test following a significant result with the Kruskall-Wallis one-way analysis of variance for non-parametric data. Differences between groups were considered as significant if $P < 0.05$.

Results

Affinity and selectivity for 5-HT_{1A} binding sites

In a variety of radioligand binding assays, MDL 73005EF proved a highly selective displacer of binding to the 5-HT_{1A} recognition site (Table 1). In this regard it was both more potent and more selective (vis-à-vis dopamine D_2 receptor binding) than buspirone (Table 1). It should also be noted that MDL 73005EF has negligible affinity for α_2-adrenoceptors, unlike 8-OH-DPAT.

Behavioural effects in reserpine-treated rats

Subcutaneous injection of 8-OH-DPAT ($0.06–2\,mg\,kg^{-1}$) into reserpine-treated rats rapidly induced forepaw-treading and a flat body posture. In contrast to 8-OH-DPAT, both MDL 73005EF ($0.25–8\,mg\,kg^{-1}$ s.c.) and buspirone ($0.5–16\,mg\,kg^{-1}$ s.c.) induced only mild to moderate behavioural effects in this model, buspirone being slightly more potent than MDL 73005EF in this regard (Figure 2).

When tested as an antagonist of the behaviours induced by 8-OH-DPAT, MDL 73005EF ($0.25–16\,mg\,kg^{-1}$ s.c.) was weakly effective against the forepaw-treading component, but had no dose-related effects on flat body posture. Buspirone ($0.5–2\,mg\,kg^{-1}$) significantly reduced forepaw treading but not in a dose-dependent manner and at $2\,mg\,kg^{-1}$ actually increased the intensity of flat body posture (Table 2).

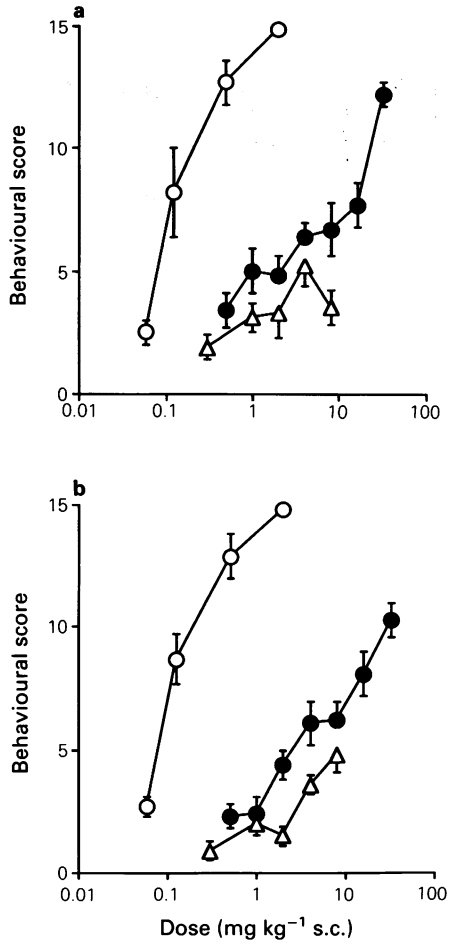


Figure 2 Comparison of the behavioural response to MDL 73005EF (△) with that of 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) (○) and buspirone (●) in reserpine-treated rats. The intensity of (a) forepaw treading and (b) flat body posture was evaluated between 5 and 20 min after drug administration. All values represent the mean for groups of 6–12 rats; vertical lines show s.e.mean.

Drug discrimination

Substitution of MDL 73005EF ($0.1–3\,mg\,kg^{-1}$ s.c.) for 8-OH-DPAT produced a dose-dependent increase in the number of

Table 1 Affinity of MDL 73005EF, 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) and buspirone for central neurotransmitter recognition sites

Site	*^3H ligand*	*MDL 73005EF*	*8-OH-DPAT*	*Buspirone*
5-HT_{1A}	8-OH-DPAT	8.6 ± 0.1	8.5 ± 0.1	7.5 ± 0.2
5-HT_{1B}	5-HT	4.5 ± 0.3	5.4 ± 0.1	4.9 ± 0.3
5-HT_2	Ketanserin	5.5 ± 0.1	<5	5.6 ± 0.2
α_1	Prazosin	5.6 ± 0.1	5.9 ± 0.1	ND
α_2	Clonidine	5.4 ± 0.1	6.9 ± 0.1	<5
β	Dihydroalprenolol	4.6 ± 0.2	<5	ND
D_1	Flupenthixol	4.3 ± 0.2	ND	<4
D_2	Domperidone	6.4 ± 0.1	5.3 ± 0.3	6.5 ± 0.1
Benzodiazepine	Flunitrazepam	<4	ND	<4

Radioligand binding assays for central neurotransmitter recognition sites were carried out in brain membranes from Sprague-Dawley rats as described in the Methods section. Results in the table are expressed as pIC_{50} values mean ± s.e.mean ($pIC_{50} = \log_{10}$ concentration giving 50% inhibition of specific binding) for $n = 3–6$ independent determinations. 5-HT, 5-hydroxytryptamine: α_1 and α_2, α-adrenoceptors; D_1 and D_2, dopamine receptors; β, β-adrenoceptors. ND = not determined. 5-HT_{1B} binding was performed with [^{3}H]-5-HT in the presence of 8-OH-DPAT and mesulergine and D_1 binding with [^{3}H]-flupenthixol in the presence of domperidone.

Table 2 The effects of MDL 73005EF and buspirone on behaviours induced by 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) in reserpine-treated rats

Compound	*Dose* (mg kg^{-1} s.c.)	n	*Forepaw treading*	*Flat body posture*
Saline		9	7.0 ± 0.2	7.4 ± 0.3
MDL 73005EF	0.25	6	7.2 ± 0.8	7.1 ± 0.9
	0.5	6	6.5 ± 0.7	6.0 ± 0.7
	1	6	6.0 ± 1.1	6.8 ± 0.7
	2	6	5.0 ± 0.6*	4.2 ± 0.9*
	4	6	4.2 ± 0.9**	6.0 ± 1.1
	8	6	4.2 ± 0.6**	5.7 ± 0.5*
	16	6	2.5 ± 0.7**	6.7 ± 1.3
Saline		9	9.4 ± 0.6	12.2 ± 0.7
Buspirone	0.5	9	7.1 ± 1.5	13.6 ± 0.6
	1	9	5.4 ± 0.7**	12.8 ± 0.9
	2	9	7.7 ± 1.3	14.9 ± 0.1*

MDL 73005EF or buspirone was given 30 min before 8-OH-DPAT (0.125 mg kg^{-1} s.c.). All values are the mean behavioural score ± s.e.mean and *n* is the number of rats per group.
* $P < 0.05$; ** $P < 0.01$ compared to saline treated controls by the Mann-Whitney U-test.

rats selecting the 8-OH-DPAT appropriate lever (Table 3A). Complete generalisation to the 8-OH-DPAT cue was obtained with 1 mg kg^{-1} s.c. MDL 73005EF. Over the same dose range MDL 73005EF, when given 30 min before administration of 8-OH-DPAT, did not alter the ability of rats to select the 8-OH-DPAT appropriate lever correctly (Table 3B). In both the agonism and antagonism studies there was some suppression of responding at the highest dose used, although the rats did not appear overtly sedated nor to have a marked 5-HT syndrome.

Table 3 The effects of MDL 73005EF in rats trained to discriminate 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT, 50 μg kg^{-1} s.c.) from saline

Compound	*Dose* (mg kg^{-1} s.c.)	n	*% selecting the 8-OH-DPAT appropriate lever*
A Agonist effects of MDL 73005EF			
8-OH-DPAT	0.05	17/20	88
MDL 73005EF	0.1	6/6	0
	0.3	14/17	50
	1	13/17	85
	3	7/17	100
B Antagonist effects of MDL 73005EF			
8-OH-DPAT	0.05	15/17	100
MDL 73005EF	0.1	8/8	75
	0.3	16/17	94
	1	13/16	77
	3	9/17	100

n: number of rats responding/number of animals tested.

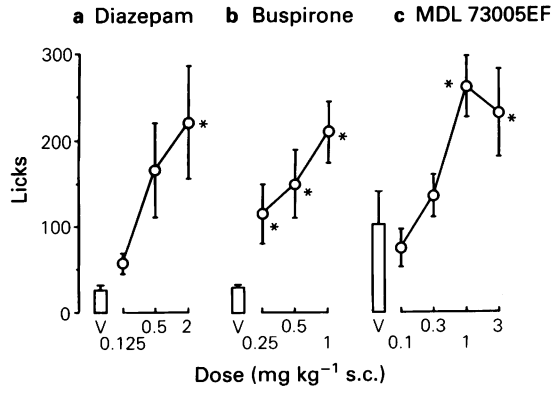


Figure 3 The effect of (a) diazepam, (b) buspirone and (c) MDL 73005EF on punished drinking in water-deprived rats following s.c. administration. All compounds were given 30 min before testing. Points represent mean values and vertical lines show s.e.mean. * $P < 0.05$ compared to vehicle control group (V) by the Mann-Whitney U-test.

Shock-suppressed drinking test

Following subcutaneous administration, diazepam, MDL 73005EF and buspirone dose-dependently increased punished licking (Figure 3). MDL 73005EF was also active following oral administration, with the maximal effect at 5 mg kg^{-1} (saline 33 ± 11 licks, MDL 73005EF 197 ± 41, $n = 11$, $P < 0.01$ Mann-Whitney U-test). Neither buspirone, 8-OH-DPAT nor MDL 73005EF increased unpunished drinking in non-deprived rats at the doses that were maximally effective in the punished drinking test (data not shown).

In order to explore the role of 5-HT$_{1A}$ receptors in the responses to diazepam, buspirone and MDL 73005EF in this test, we examined the effects of 8-OH-DPAT (30–150 mg kg^{-1} s.c.), both alone and in combination with the other three drugs. 8-OH-DPAT had no significant effects on punished

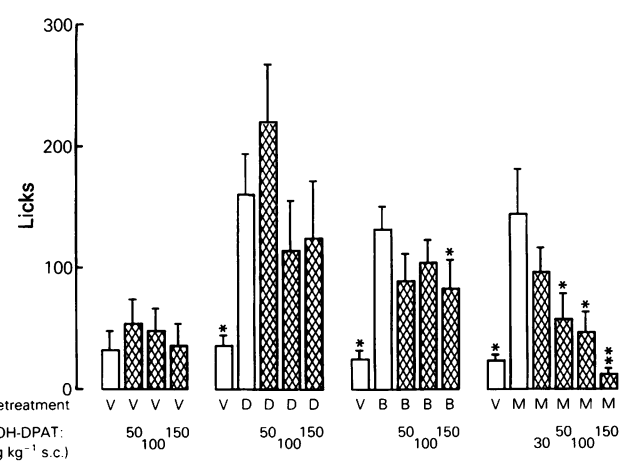


Figure 4 Antagonism of the anticonflict effects of buspirone (B; 1 mg kg^{-1} s.c.), MDL 73005EF (M; 1 mg kg^{-1} s.c.) and diazepam (D; 2 mg kg^{-1} s.c.) by 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT). V = vehicle-treated animals; the hatched columns represent those groups that received 8-OH-DPAT. All values are the mean of 11–23 observations. Vertical bars show s.e.mean. Drug treatments were given 30 min before testing except 8-OH-DPAT which was given 15 min before testing. * $P < 0.05$; ** $P < 0.01$, compared to drug treatment alone by the Mann-Whitney U-test.

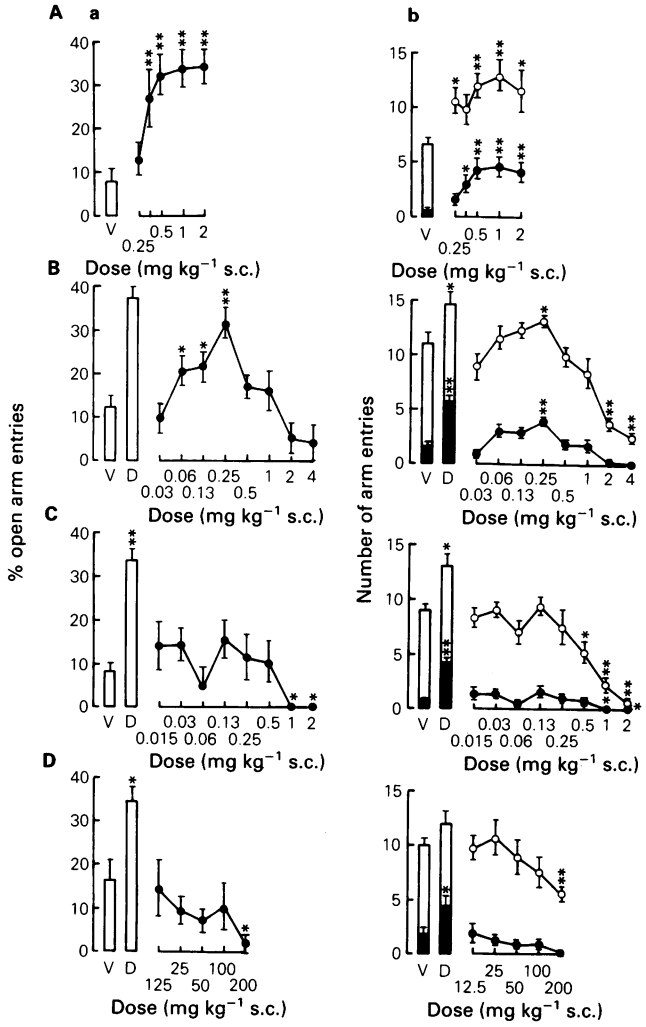


Figure 5 The effect of (A) diazepam, (B) MDL 73005EF, (C) buspirone and (D) 8-hydroxy-2-(di-n-propyl-amino)tetralin (8-OH-DPAT) in the elevated plus-maze test. (a) Shows the effect on the percentage of open-arm entries. The effect of vehicle (V) pretreatment is shown by the columns. (b) Shows the effect on the number of open-arm entries and total arm entries. The solid areas of the columns and (●) represent open-arm entries while the open columns and (○) represent total arm entries. All values are the mean of 10 observations. Vertical bars show s.e.mean. D represents the effect of diazepam (2 mg kg^{-1} s.c.) pretreatment included as a positive control. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to vehicle treated control by the Mann-Whitney U-test.

drinking when given alone, but when given in combination with either diazepam (2 mg kg^{-1} s.c.), buspirone (1 mg kg^{-1} s.c.) or MDL 73005EF (1 mg kg^{-1} s.c.), clear differences in its effects became apparent (Figure 4). Thus, the antipunishment effects of MDL 73005EF were dose-dependently inhibited by 8-OH-DPAT, with complete reversal occurring at 150 μg kg^{-1}. In contrast, when either buspirone or diazepam pretreated rats were administered 8-OH-DPAT, there was no significant inhibition of punished drinking except in the case of buspirone where a small, but significant reduction was observed following 150 μg kg^{-1} 8-OH-DPAT (Figure 4).

Elevated plus-maze test

Following saline pretreatment approximately 10% of the total entries made by the rats were into the open arms. Diazepam dose-dependently and significantly increased this percentage to a maximum of between 30 and 40% (Figure 5Aa). This increase in the percentage of open arms entered was accompanied by a significant increase in the total number of arms entered (Figure 5Ab), indicating an increase in maze activity despite the sedation that was clearly evident in rats in their home cages following the higher doses used.

MDL 73005EF (0.03–0.25 mg kg^{-1} s.c.) showed a similar profile of activity to diazepam in the elevated plus-maze (Figure 5B). Unlike diazepam, there was no evidence of overt sedation following MDL 73005EF. Nor was sedation observed at any of the higher doses tested (0.5–4 mg kg^{-1} s.c.), although these induced smaller increases in the percentage of open arm entries than was obtained with 0.25 mg kg^{-1}. There was no obvious behavioural change that could have accounted for this reversal as the rats appeared essentially normal at all doses tested.

Both buspirone and 8-OH-DPAT induced only decreases in the percentages of open arm entries and in total entries (Figure 5C and D). In the case of buspirone these effects reached statistical significance at doses (0.5–2 mg kg^{-1} s.c.) similar to those that had significant anti-conflict effects in the punished licking test. The effect of buspirone in the plus-maze was not likely to have been due to the appearance of the 5-HT behavioural syndrome as this was only weak at the doses used (see Figure 2). Moreover, 8-OH-DPAT induced a more marked 5-HT syndrome than buspirone, but this did not impair the ability of rats to move between arms.

Tail-flick test

MDL 73005EF (1–3 mg kg^{-1} s.c.) had no significant effect on tail-flick latency in response to radiant heat (data not shown).

Discussion

The present results establish MDL 73005EF as a potent ligand at 5-HT$_{1A}$ recognition sites, with at least 1000 fold greater selectivity for these sites over other 5-HT receptor subtypes or benzodiazepine receptors and at least 100 fold selectivity over α_1-, α_2- or β-adrenoceptors, dopamine D_1 or D_2 receptors. MDL 73005EF is both more potent and more selective for 5-HT$_{1A}$ receptors than buspirone, which is only 10 times more active at 5-HT$_{1A}$ than at dopamine D_2 receptors.

Two *in vivo* models were used to explore the functional effects of MDL 73005EF at 5-HT$_{1A}$ receptors. Induction of forepaw treading and flat body posture in the reserpine-treated rat is considered to reflect activation of 5-HT$_{1A}$ receptors present on postsynaptic (i.e. non-5-HT-containing) neurones (Tricklebank *et al.*, 1984; Mir *et al.*, 1988). Both MDL 73005EF and buspirone (in confirmation of earlier results of Smith & Peroutka, 1986) were poor inducers of forepaw treading and flat body posture compared to 8-OH-DPAT. Since buspirone appears to be a partial agonist at postsynaptic 5-HT$_{1A}$ receptors (De Vivo & Mayaani, 1986) both compounds were also tested for their ability to inhibit the behavioural effects of 8-OH-DPAT. Although MDL 73005EF dose-dependently inhibited 8-OH-DPAT-induced forepaw treading, this occurred only at moderate to high doses and no dose-related antagonism of flat-body posture was observed. Buspirone was even less active as an antagonist than was MDL 73005EF, with no dose-related effects against forepaw treading and even a significant potentiation of flat-body posture.

In contrast to the data obtained in the reserpine-treated rat model, MDL 73005EF acted in a manner consistent with its being a full agonist when tested in rats trained to discriminate 8-OH-DPAT from saline. Thus it generalised completely, and at low doses, to the stimulus effects of 8-OH-DPAT. Similar results have been obtained previously for buspirone (Tricklebank *et al.*, 1987). Over the same dose range which resulted in generalisation, MDL 73005EF was unable to inhibit the stimulus effects of 8-OH-DPAT. Activation of 5-HT$_{1A}$ receptors is strongly implicated as the basis of the 8-OH-DPAT-induced stimulus effects (Tricklebank *et al.*, 1987) and the clear agonist effects of MDL 73005EF in this test compared to its weak antagonist effects against 8-OH-

DPAT in the reserpine-treated rat model suggest that, like buspirone, it is best described as a partial agonist at 5-HT_{1A} receptors.

Recent work with electrophysiological techniques has shown that postsynaptic 5-HT_{1A} receptor partial agonists behave as full agonists on presynaptic 5-HT_{1A} receptors, i.e. those located on raphé cell bodies (Sprouse & Aghajanian, 1988). Thus, it might be expected that agents such as MDL 73005EF and buspirone would strongly reduce central 5-HT neuronal activity by a combination of presynaptic suppression of neurone firing and postsynaptic partial agonist activity (Andrade & Nicoll, 1987). There is much evidence to suggest that such a reduction in 5-HT neuronal activity should result in an anxiolytic action (Iversen, 1984; Gardner, 1985; Johnston & File, 1986; Thiebot, 1986; Chopin & Briley, 1987) and, indeed, clear evidence of anxiolytic-like activity has been obtained with both MDL 73005EF and buspirone in the present study. However, what is surprising is that despite similar activities in the tests for 5-HT_{1A} receptor activity, there were major differences between the two compounds in terms of their effects in different animal models of anxiety and their interaction with 8-OH-DPAT in these tests.

In the shock-suppressed drinking test MDL 73005EF, buspirone and diazepam showed anxiolytic-like activity. Although the effect of buspirone in conflict tests is inconsistent, positive effects have been obtained by many authors (see Gardner, 1986) and this result confirms previous findings from this laboratory (Gower & Tricklebank, 1988). The interpretation of results from anticonflict tests, such as the Vogel test, that use appetite motivated behaviour and punishment, can be confounded by compounds having either dipsogenic or analgesic properties. The increase in punished licking was unlikely to have been due to an effect on thirst as none of these compounds increased unpunished drinking in non-deprived rats. Similarly, an effect on pain sensitivity was excluded as MDL 73005EF did not have an analgesic effect in the tail-flick test. It is interesting to note the clear reversal of the anticonflict activity of MDL 73005EF by 8-OH-DPAT, at doses that did not affect similar responses to buspirone or diazepam pretreatment. This reversal by 8-OH-DPAT is unlikely to have been due to disruption of drinking behaviour by the induction of the 5-HT syndrome as 8-OH-DPAT had no clear effect against either buspirone or diazepam. In some experiments the highest dose of 8-OH-DPAT did significantly reduce the effects of diazepam, but this effect was inconsistent, as was the effect against buspirone. We do not, at present, have any clear indication why 8-OH-DPAT should have this selective interaction with MDL 73005EF. One possibility is that the anticonflict effects of MDL 73005EF are heavily dependent on its agonist effects in the raphé nuclei, whereas those of buspirone and diazepam involve other mechanisms (see below). Clearly further experiments are needed to verify such a hypothesis. Previous data have suggested that 8-OH-DPAT has anti-conflict activity (Engel *et al.*, 1984), but we found no evidence for this, in agreement with the results of Deacon & Gardner (1986).

Although the anxiolytic actions of buspirone are believed to be mediated by an effect at 5-HT_{1A} receptors (Peroutka, 1985), the inability of 8-OH-DPAT to either mimic, or to inhibit consistently, the antipunishment effects of buspirone in the shock-suppressed drinking test suggests that 5-HT_{1A} receptors are not involved in this particular response in rats. This conclusion would be consistent with the results of Gower & Tricklebank (1988) which suggest that the α_2-adrenoceptor antagonist effects of the metabolite of buspirone, 1-(2-pyrimidyl)-piperazine, mediate its effects in this test. Despite having structural similarities with buspirone, MDL 73005EF lacks the aryl piperazine moiety, and therefore cannot be metabolized to 1-(2-pyrimidyl)-piperazine.

In the elevated plus-maze test 8-OH-DPAT and buspirone were found to have opposite effects to those induced by MDL 73005EF and diazepam in that they decreased both the total number and the percentage of open arm entries made. A similar effect of 8-OH-DPAT has been obtained by Critchley & Handley (1987) and as other 5-HT_{1A} receptor agonists have a similar profile of activity in this test (Moser, 1989), their anxiogenic-like effect would seem to be due to an action at this receptor subtype. It seems unlikely that the reduction in total arm entries following 8-OH-DPAT or buspirone could be a result of non-specific effects such as sedation or ataxia. Thus, diazepam is more sedative than buspirone (Goa & Ward, 1986), but in the elevated plus-maze test, activity was increased by diazepam whereas 8-OH-DPAT, which normally increases locomotor activity (Tricklebank *et al.*, 1984), significantly decreased total arm entries. The 5-HT behavioural syndrome cannot be ruled out as contributing to the decrease in open arm entries. However, such an effect should have been more apparent following 8-OH-DPAT than buspirone, which at the doses used in this study induced only mild symptoms of the 5-HT behavioural syndrome compared to 8-OH-DPAT (Figure 2).

The dose-range over which MDL 73005EF exhibited anxiolytic-like activity in the elevated plus maze test (0.03–0.25 $mg\,kg^{-1}$ s.c.) was lower than that used in the Vogel test (0.3–1 $mg\,kg^{-1}$ s.c.). We have no explanation for this difference, but it may reflect a different degree of involvement of 5-HT receptor mechanisms in the two tests. The qualitatively different effects of 8-OH-DPAT in the two tests supports this suggestion. An anxiogenic-like effect following 5-HT_{1A} receptor stimulation may be the basis of the bell-shaped dose-response curve obtained with MDL 73005EF, as it shows weak postsynaptic 5-HT_{1A} agonist effects (Figure 2). However, some caution should be attached to such a conclusion for the basis of the difference between the tests, as it is a common finding that 5-HT_{1A} ligands have different effects in different anxiolytic tests (Chopin & Briley, 1987; Gardner, 1988).

The results presented here do not provide any direct indication for the site of action of MDL 73005EF in these animal models of anxiety. However, the substantial selectivity of MDL 73005EF for 5-HT_{1A} recognition sites *in vitro* (Table 1), the fact that 5-HT_{1A} receptor activation can be demonstrated at anxiolytic doses (Table 3) and the selective interaction with 8-OH-DPAT (Figure 4) all point to an active involvement of the 5-HT_{1A} receptor. 5-HT_{1A} receptors are present both presynaptically on raphé neurones, where activation results in decreased raphé cell firing (Sprouse & Aghajanian, 1986; VanderMaelen *et al.*, 1986), and postsynaptically in many other areas, such as cortex and hippocampus (Verge *et al.*, 1985; Pazos & Palacios, 1985). As MDL 73005EF shows negligible agonist activity at postsynaptic 5-HT_{1A} receptors at 'anxiolytic' doses (Figure 2), it seems unlikely that such a mechanism can be involved in its anxiolytic-like effects. This conclusion is supported by the lack of anxiolytic-like effects of 8-OH-DPAT at doses which clearly activate postsynaptic 5-HT_{1A} receptors (Figure 2) in both the Vogel and elevated plus-maze tests.

In fact, the potent suppression of the anticonflict activity of MDL 73005EF by 8-OH-DPAT suggests that MDL 73005EF is reducing activity at 5-HT_{1A} receptors located postsynaptically. Theoretically, such an effect of MDL 73005EF could be caused either by an agonist effect on raphé cell body 5-HT_{1A} receptors or by an antagonist effect at postsynaptic receptors, or even a combination of the two. In view of the weak effects of MDL 73005EF at postsynaptic sites, which require doses well in excess of those that are active in animal models of anxiety, we would suggest that an agonist effect at cell body 5-HT_{1A} receptors which leads to a reduction in raphé cell firing is responsible for its 'anxiolytic' effects. The clear difference between MDL 73005EF and both 8-OH-DPAT and buspirone, which also powerfully suppress raphé neuronal firing (Sprouse & Aghajanian 1986; 1988), can be attributed to either a different balance of pre- and postsynaptic activities (8-OH-DPAT) or a general lack of selectivity (due both to properties of the parent molecule and its principle metabolite) at the doses used (buspirone).

In summary, these results clearly demonstrate that MDL

73005EF is capable of reversing shock-suppressed drinking in thirsty rats and also of increasing their exploration of open arms in an elevated plus-maze. Conflict tests have been widely used for many years to detect novel anxiolytic agents (Geller & Seifter, 1960; Vogel *et al.*, 1971) and the elevated plus-maze test (Handley & Mithani, 1984) has been extensively validated as a model of anxiety (Pellow *et al.*, 1985). The activity that we observe for MDL 73005EF is very similar to that seen with conventional benzodiazepine anxiolytics and suggests, therefore, that MDL 73005EF has the potential for clinical anxiolytic activity. Furthermore, the data presented in this paper suggest that this is the result of a reduction in central 5-HT neuronal function, most probably as a result of a selective agonist effect on 5-HT_{1A} receptors located on 5-HT neuronal cell bodies in the raphé nuclei.

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Anti-inflammatory activity of bee venom peptide 401 (mast cell degranulating peptide) and compound 48/80 results from mast cell degranulation *in vivo*

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1 The relationship between the anti-inflammatory activity of the bee venom peptide 401 in the carrageenin-induced oedema of the rat hind paw and its mast cell degranulating activity has been re-investigated.

2 Mast cell degranulation caused by compound 48/80 ($10\,mg\,kg^{-1}$) or by allergen challenge in rats sensitized to *Nippostrongylus brasiliensis* also suppressed rat hind paw oedema in the same test.

3 The anti-inflammatory activities of peptide 401 and compound 48/80 were partially suppressed by pretreatment of rats with mepyramine and methysergide, at doses ($2.5\,mg\,kg^{-1}$) that completely suppressed skin reactions to these mast cell-derived amines. Pretreatment of rats with compound 48/80 also suppressed the apparent anti-inflammatory actions of peptide 401 and of compound 48/80.

4 Injection of peptide 401 together with carrageenin increased the inflammatory response in the rat hind paw.

5 The anti-inflammatory activity of peptide 401 and of compound 48/80 in the carrageenin-induced swelling of the rat hind paw arises from mast cell degranulation *in vivo*.

Introduction

Peptide 401 (MCD peptide) contains 22 residues and occurs in the venom of the common European honey bee to the extent of about 2% by weight (Gauldie *et al.*, 1976; Breithaupt & Habermann, 1968). It has powerful anti-inflammatory activity (at doses as low as $0.1\,mg\,kg^{-1}$) in a variety of animal models, i.e., hind paw oedema in the rat induced by carrageenin or turpentine, adjuvant arthritis in the rat, and increased skin permeability in the rat resulting from subcutaneous injection of bradykinin, prostaglandin E_1 kallikrein, histamine and 5-hydroxytryptamine (5-HT) (Billingham *et al.*, 1973; Hanson *et al.*, 1974). It has a powerful degranulating effect on mast cells *in vitro* and this, of course, involves the release of histamine and other pharmacologically active agents. It was originally reported (Billingham *et al.*, 1973; Hanson *et al.*, 1974) that the anti-inflammatory activity of the peptide was distinct from and did not depend on its action on mast cells. On this basis, it was suggested (Billingham *et al.*, 1973) that the peptide might be a useful therapeutic agent in some arthritic conditions. This suggestion is perhaps an echo of the long-held belief that bee venom has some curative effects in rheumatoid arthritis (Beck, 1935).

We made a number of chemically defined derivatives of the peptide in the hope of decreasing its ability to degranulate mast cells while retaining the anti-inflammatory activity. We found, however, that the two activities changed roughly in parallel (Banks *et al.*, 1980; Dempsey, 1981). We also failed to demonstrate any direct effect of peptide 401 on rat polymorphonuclear leukocytes which might mediate anti-inflammatory activity (Dempsey *et al.*, 1985).

These results led us to re-examine the pharmacology of the anti-inflammatory activity of peptide 401 in the rat paw oedema test. We find that the conclusions drawn by previous workers were incorrect. The peptide is not a true anti-inflammatory agent but exerts its activity through degranulation of mast cells, i.e., it is a counter-irritant. Preliminary accounts of this work have been published (Banks *et al.*, 1980; 1983).

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Methods

Carrageenin hind paw oedema

Male Sprague-Dawley rats weighing 125 to 150 g were randomly caged in groups of five and given food and water *ad libitum*. The volumes of both hind paws of each rat were measured with a calibrated volume-differential meter (Ugo Basile, Milan). Each rat was injected, subplantar, with 0.1 ml of a 1% solution of carrageenin in physiological saline, unless otherwise stated. Rats receiving drug treatments were injected subcutaneously with 0.1 ml of the test solution immediately after the carrageenin injection. The control groups were injected subcutaneously with the same volume of 0.9% saline. After injections, the volumes of the rat hind paws were measured over a period of 5 h as indicated in the figures. The effects of antagonists to histamine (H_1, mepyramine; H_2, cimetidine), or 5-HT (methysergide) were determined by injecting the antagonist subcutaneously 45 min before the carrageenin injection.

The increase in paw volume was calculated in the following way. The meter reading was first converted to a volume following calibration and, at each time, t, the increase in volume $V_t - V_0$ (where V_t is the volume at time t and V_0 is the initial volume before treatment) was determined. Mean V_t is the sum of all V_t in a group divided by n, the number of animals in the group. Volumes of both the hind paw injected with carrageenin (the right paw, R) and the uninjected hind paw (L) were measured. Significance values were calculated by Student's t test.

In some experiments, peptide 401 was injected with carrageenin in a total volume of saline of 0.1 ml.

Pre-depletion of mast cells

Rats were depleted of mast cell-derived mediators according to the regimen of Di Rosa *et al.* (1971). Rats received $0.6\,mg\,kg^{-1}$ of compound 48/80 intraperitoneally (0.1% w/v in physiological saline) twice daily for 3 days and $1.2\,mg\,kg^{-1}$ twice on the fourth day. The efficacy of the pretreatment was evident from the course of the carrageenin oedema in depleted rats compared with normal animals, swelling in the first hour

after carrageenin injection being virtually absent in the depleted animals.

Sensitization of rats with the nematode Nippostrongylus brasiliensis

Rats were injected sub-cutaneously with third stage larvae of *Nippostrangylus brasiliensis* (L3 1800) in 0.4 ml sterile physiological saline. Three weeks later, the rats were used for testing the response to carrageenin oedema by challenge with allergen containing one worm equivalent per μl. The larvae and allergen were gifts from Dr F.L. Pearce (Chemistry Department, University College, London).

The effectiveness of sensitization was determined from the ability of allergen to induce degranulation of peritoneal mast cells from sensitized rats (White & Pearce, 1982).

Materials

Peptide 401 was purified from freeze-dried bee venom (Bulgarcoop, Sofia, Bulgaria) as described previously (Banks *et al.*, 1981). Compound 48/80 was from Sigma Chemical Co. (Poole, Dorset). Mepyramine maleate (Antihisan) was from May and Baker (Manchester). Methysergide maleate was from Sandoz (London) and cimetidine (Tagamet) was from Smith, Kline and French (Welwyn Garden City, Herts.). Carrageenin (sodium salt) was from BDH Chemicals Ltd. (Poole, Dorset).

Results

Inhibition of carrageenin oedema by peptide 401 and compound 48/80

Both peptide 401 and compound 48/80 suppress the normal response to subplantar carrageenin over a 4 h period, as shown in Figure 1a and c. Peptide 401 is more active, on a weight basis, showing significant activity at 0.1 mg kg^{-1} while compound 48/80 is active at 1 mg kg^{-1}. The dose-response curve for compound 48/80 is complicated because at higher doses (up to 15 mg kg^{-1}) the inhibition of swelling is less than it is at 2 mg kg^{-1}. There is also a considerable mortality among animals treated with compound 48/80 at doses higher than 2 mg kg^{-1} and we have, therefore, omitted doses of 10 and 15 mg kg^{-1} from Figures 1c and d.

The effect of a single sub-cutaneous dose of peptide 401 (1 mg kg^{-1}) on the response to carrageenin in animals depleted of histamine and 5-HT by pretreatment with compound 48/80 is shown in Figure 2. The reduction in swelling of the injected foot is much less in depleted animals than in normal animals but the swelling of the contralateral foot due to peptide 401 is greater, over the first 2 h, in the depleted animals than in normal animals. The same is true in depleted animals treated with single doses of compound 48/80 (15 mg kg^{-1}) as compared with non-depleted animals (data not shown).

Pretreatment with compound 48/80

Pretreatment of rats with compound 48/80 virtually eliminated the initial swelling in response to sub-plantar carrageenin but was without significant effect on the later stages (1.5 to 5 h). This is consistent with the observations made by Vinegar *et al.* (1976).

Effects of mepyramine and methysergide on the anti-inflammatory activities of peptide 401 and compound 48/80

Mepyramine (a histamine H_1 antagonist) and methysergide (a 5-HT antagonist) in doses that completely suppressed the skin responses to intradermal injection of histamine and 5-HT

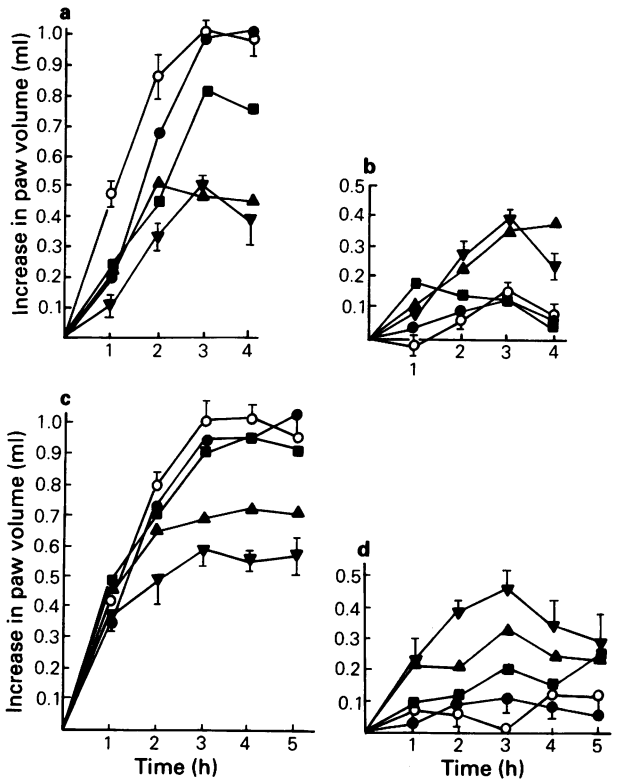


Figure 1 The effects of peptide 401 (a, b) or compound 48/80 (c, d) at different doses on the swelling induced by sub-plantar carrageenin (0.1 ml, 1% w/v in 0.9% saline) in the right foot (a, c) and on the volume of the contralateral foot (b, d). Drugs were given s.c. in 0.1 ml 0.9% saline at the same time as the irritant ($t = 0$). Experimental points are the means of measurements in 5 animals; error bars denote ±s.e.mean and are included only for control and highest dose values for the sake of clarity. Standard errors did not exceed 20% of mean. (○) Control animals injected s.c. with 0.1 ml 0.9% saline; (●) 0.05 mg kg^{-1}; (■) 0.1 mg^{-1}; (▲) 1 mg kg^{-1}; (▼) 2 mg kg^{-1}.

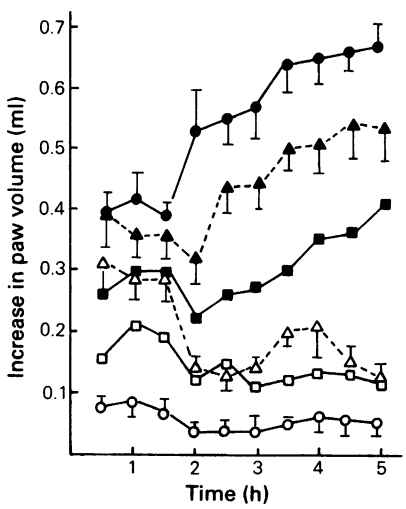


Figure 2 The effect of peptide 401 on the response to subplantar carrageenin in normal rats and in rats depleted of mast cell contents by pretreatment with compound 48/80 0.6 mg kg^{-1} twice daily for 3 days and 1.2 mg kg^{-1} twice on day 4. A single dose of peptide 401 (1 mg kg^{-1}) was given (s.c.) at the same time as carrageenin. Open symbols for volumes of uninjected (left) hind feet; filled symbols for injected feet. (○, ●) Control animals receiving 0.1 ml 0.9% saline, s.c.; (□, ■) normal animals receiving peptide 401; (△, ▲) depleted animals receiving peptide 401. Dashed lines, depleted animals, complete lines for normal animals. Experimental points are means of measurements from five animals and error bars are ±s.emean. Where not shown, standard errors did not exceed 20% of the mean.

Table 1 Inhibition of carageenin-induced oeema in the rat hind paw by peptide 401 and by compound 48/80 and the effects of antagonists

Degranulator ($mg\,kg^{-1}$)	*Antagonist* ($mg\,kg^{-1}$)	n	% *Inhibition of oedema* *t* (h) 1	2	4
—	H_1 and 5-HT[a] (2.5)	5	13 ± 4.0	21 ± 4.0	9.5 ± 1.3
Peptide 401 (1)	—	15	−3 ± 0.27	50*** ± 5.0	50*** ± 9.2
401 (1)	H_1 and 5-HT (2.5)	15	−27† ± 2.1	11†† ± 1.2	29†† ± 3.8
Compound 48/80 (10)	—	11	−30 ± 3.0	35*** ± 3.8	54*** ± 6.8
48/80 (10)	H_1 and 5-HT (2.5)	10	−17 ± 2.5	11† ± 0.9	25†† ± 1.3
—	H_2[b] (50)	10	−14 ± 2.5	−4 ± 0.6	−5 ± 0.4
Peptide 401 (1)	—	10	37 ± 3.4	53 ± 4.4	53 ± 5.3
401 (1)	H_2 (50)	10	63† ± 9.7	71† ± 14	67† ± 13
Compound 48/80 (10)	—	6	−28 ± 1.8	13 ± 0.8	36 ± 1.8
48/80 (10)	H_2 (50)	5	3 ± 0.2	22 ± 1.7	40 ± 5.2

[a] Mepyramine and methysergide (H_1 and 5-HT antagonists respectively).
[b] Cimetidine (H_2 antagonist).
Significance levels for drug compared with drug-free control (*) or antagonist compared with antagonist-free control (†). *** and †††, $P < 0.001$; ** and ††, $P < 0.005$; * and †, $P < 0.01$

respectively ($2.5\,mg\,kg^{-1}$ for each antagonist) reduced the anti-inflammatory activities of both peptide 401 and compound 48/80 by about 50% in animals when given 45 min prior to carrageenin injection. For the sake of clarity, only data obtained at 1, 2 and 4 h have been included in Table 1. The swelling of the contralateral paw was also suppressed (Table 2). Treatment with mepyramine and methysergide alone partly suppressed the initial phase of carrageenin oedema but did not significantly alter the later phase (Table 1). This observation is also consistent with the suggestions made earlier by Vinegar *et al.* (1976).

Effects of pretreatment with cimetidine on the anti-inflammatory activities of peptide 401 and compound 48/80

Pretreatment of rats with cimetidine (a histamine H_2-receptor antagonist) 45 min before carrageenin injection, slightly, though significantly ($P < 0.05$), enhanced the anti-inflammatory effects of peptide 401 and compound 48/80. Cimetidine alone ($50\,mg\,kg^{-1}$) had no significant effect on the course of carrageenin oedema (Table 1).

Effects on carrageenin oedema of sensitization followed by allergen challenge

Rats that had been sensitized to the nematode, *Nippostrongylus brasiliensis*, were challenged subcutaneously with allergen, extracted from the worms, immediately following subplantar injection of carrageenin. The sensitized animals showed approximately 40% reduction in swelling compared with control animals (Figure 3). In contrast to peptide 401 and compound 48/80, allergen challenge did not cause swelling of the contralateral paw. The anti-inflammatory activity resulting from allergen challenge was suppressed in sensitized

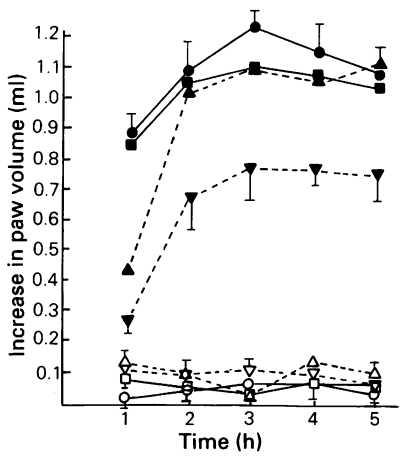


Figure 3 Effect of allergen challenge in rats sensitized to *N. brasiliensis* on the response to sub-plantar carrageenin. Complete lines are for normal animals; dashed lines for sensitized animals. Open symbols are for uninjected (left) hind feet; closed symbols for injected hind feet. (○, ●) Normal animals injected with saline (0.1 ml, 0.9%, s.c.) at the same time as carrageenin; (□, ■) normal animals injected with allergen (1000 worm equivalents, s.c.) at $t = 0$; (△, ▲) sensitized animals injected with saline; (▽, ▼) sensitized animals injected with allergen. Measurements are means on groups of five animals; error bars ± s.e.mean; where not shown, standard errors did not exceed 20% of the mean.

Table 2 Swelling in the uninjected paw induced by peptide 401 and compound 48/80 during the course of carrageenin-induced oedema and the effects of antagonists

Degranulator ($mg\,kg^{-1}$)	*Antagonist* ($mg\,kg^{-1}$)	n	*Increase in volume of uninjected foot* (ml) *t* (h) 1	2	3	4	5
Saline (control)	—	14	0.08 ± 0.02	0.09 ± 0.01	0.07 ± 0.02	0.09 ± 0.03	0.03 ± 0.03
—	H_1 and 5-HT[a] (2.5)	5	0.02 ± 0.04	0.01 ± 0.03	0.01 ± 0.04	0.01 ± 0.03	0.01 ± 0.02
Peptide 401 (1)	—	15	0.26*** ± 0.02	0.27*** ± 0.03	0.19*** ± 0.03	0.21*** ± 0.04	0.17** ± 0.05
Peptide 401 (1)	H_1 and 5-HT (2.5)	15	0.15† ± 0.02	0.15†† ± 0.02	0.13†† ± 0.02	0.13†† ± 0.03	0.11†† ± 0.03
Saline (control)	—	10	0.03 ± 0.01	0.03 ± 0.01	0 ± 0.01	−0.02 ± 0.01	−0.03 ± 0.01
Compound 48/80 (10)	—	11	0.23*** ± 0.02	0.21*** ± 0.02	0.16*** ± 0.02	0.15*** ± 0.02	0.16*** ± 0.02
Compound 48/80	H_1 and 5-HT (2.5)	10	0.11††† ± 0.02	0.08††† ± 0.03	0.03††† ± 0.02	0.02††† ± 0.02	0.02††† ± 0.02

[a] Mepyramine and methysergide (H_1 and 5-HT antagonists respectively).
Levels of significance are for the effect of drug compared with drug-free control (*) and for the effect of inhibitor compared with inhibitor-free control (†), at significance levels of *** and †††, $P < 0.001$; ** and ††, $P < 0.005$; * and †, $P < 0.01$.

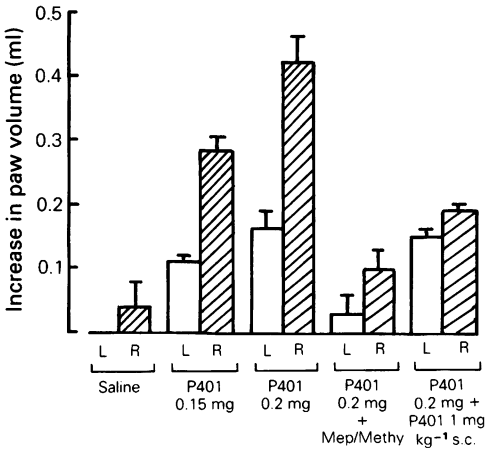


Figure 4 Increase in paw volume at 90 min following subplantar injections of peptide 401 (P401) (in 0.1 ml 0.9% saline) into the right hind feet of normal animals or animals pretreated 45 min previously with mepyramine (Mep) and methysergide (Methy) (2.5 mg kg^{-1}), or treated with peptide 401 (1 mg kg^{-1}) at the same time as sub-plantar injection of peptide 401. Measurements are means from groups of five animals; error bars ± s.e.mean.

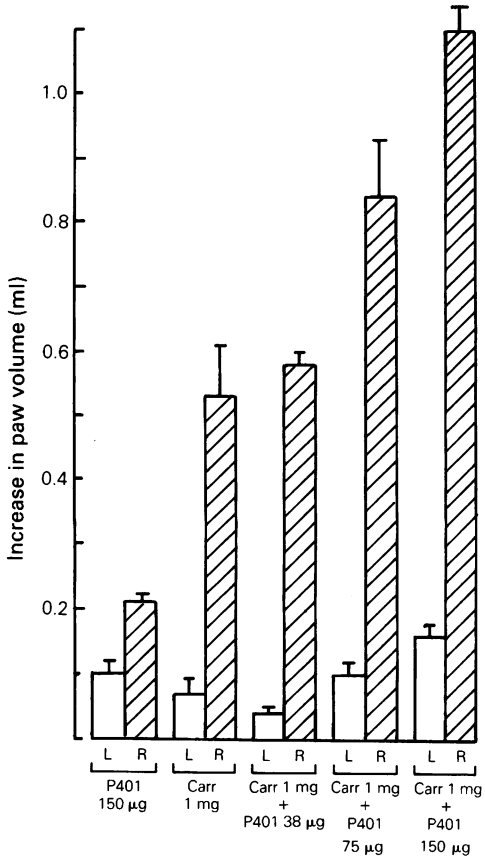


Figure 5 Increases in paw volumes of uninjected (L) and injected (R) feet 3 h after injection into the hind paw of carrageenin (Carr) with and without addition of varying amounts of peptide 401 (P401). Volume injected, 0.1 ml, 1% carrageenin ± peptide 401 in 0.9% saline. Also shown is the increase in volumes of the hind feet 90 min after injection of the highest dose of peptide 401 alone. Measurements are means from groups of five animals; error bars ± s.e.mean.

animals pretreated with mepyramine and methysergide (each at 2.5 mg kg^{-1}) 45 min before carrageenin injection and allergen challenge (data not given). Animals tested before sensitization was complete (as judged by lack of degranulation of isolated mast cells induced by allergen *in vitro*) did not show a reduction in carrageenin-induced oedema when injected with allergen, as compared with saline-treated controls.

The irritant activity of peptide 401

Direct injection of peptide 401 into the rat hind paw produced swelling both in the injected (R) and uninjected (L) feet (Figure 4). Pretreatment of animals with mepyramine and methysergide as before almost abolished the swelling in response to subplantar 401 both in the injected and the uninjected paws. Sub-cutaneous injection of peptide 401 45 min before subplantar injection of the same peptide significantly suppressed the irritant action in the foot (Figure 4).

Co-administration of peptide 401 with carrageenin into the right hind paw produced a dose-dependent enhancement of the inflammatory response over the entire time course. The enhancement of swelling over the first 2 h is presumably due to the mast cell degranulating properties of the peptide, releasing vasoactive constituents locally. The effect of the peptide on the later phase of the oedema (>2 h) is of more interest (see Discussion). The effect of co-injection of peptide 401 and carrageenin at 3 h is shown in Figure 5. The oedema produced by peptide 401 and carrageenin together was greater than the sum of the swellings produced by the irritants given separately (Figure 5).

Discussion

The results indicate that there is a strong connection between mast cell degranulation and suppression of carrageenin-induced oedema in the rat hind paw. This finding is in sharp contrast to earlier reports (Billingham *et al.*, 1973; Hanson *et al.*, 1974) in which the apparent anti-inflammatory effects of peptide 401 in a number of animal models were held not to depend on mast cell degranulation. The synthetic histamine releaser, compound 48/80, has now been shown to be nearly as effective as peptide 401, in suppressing carrageenin-induced oedema but the dose-response curve is bell shaped, (see Results section). This could have led Hanson *et al.* (1974) to underestimate the anti-inflammatory activity of compound 48/80 in the earlier study. We have now shown that the activities of peptide 401 (and derivatives thereof) and of compound 48/80 (Figures 1a and c), parallel the abilities of these compounds to degranulate rat peritoneal mast cells *in vitro* (Banks *et al.*, 1980; Dempsey, 1981; present work). The activities of both peptide 401 and compound 48/80 were equally affected by such compounds as mepyramine, chlorpheniramine (results not shown), methysergide and cimetidine (Table 1).

Mast cell depletion results in a marked attenuation of the anti-inflammatory activity of both peptide 401 (Figure 2) and of compound 48/80 (results not shown). Pretreatment of rats with antagonists of histamine and of 5-HT also significantly reduced the anti-inflammatory effects of both degranulators (Table 1). Together, these findings suggest that the anti-inflammatory effects are at least in part mediated by mast cell-derived species. That depletion is rather more effective than is pretreatment with H_1-receptor and 5-HT antagonists suggests that species other than histamine and 5-HT may be involved in the anti-inflammatory activity of mast cell degranulators. The slight enhancement of the anti-inflammatory effects of the two degranulators caused by pretreatment of animals with the H_2-receptor antagonist, cimetidine (Table 1) is best understood as due to antagonism of feedback inhibition of mast cell degranulation by histamine acting on H_2-receptors (Lichtenstein & Gillespie, 1975).

Vinegar *et al.* (1976) proposed that the early stage of carrageenin oedema in the rat hind paw was mediated by mast-cell

derived species. Consistently, we found in depleted animals that oedema formation in the first hour after injection of carrageenin was almost totally suppressed though the later phase (1.5–5 h) was unaffected (results not shown). On the other hand, neither H_1 nor 5-HT antagonists suppressed the early phase of oedema (Table 1) and this too is consistent with the work of Vinegar *et al.* (1982).

The effects of peptide 401 and of compound 48/80 on the uninjected feet require comment. At the same time as the swelling in response to sub-plantar carrageenin is reduced by these basic degranulators, injected sub-cutaneously, the contralateral feet swell. The effect is most marked in the early phase of the oedematous response in depleted animals (Figure 2). Were anti-inflammatory activity to be assessed from the difference in swelling of injected and uninjected feet (Billingham *et al.*, 1973; Hanson *et al.*, 1974) this might well give rise to an overestimate of activity. The origin of the swelling is obscure but might be associated with complexing of the basic degranulators with heparin that would also be released on degranulation in non-depleted animals and this could prevent access to the general circulation.

Atkinson & Hicks (1975) pointed out that true anti-inflammatory agents should always reduce the irritation produced by an inflammatory stimulus no matter how administered. 'Counter irritants', on the other hand, may reduce the inflammation at one site by producing an irritant effect elsewhere. To distinguish the two effects, it was noted that if a counter irritant were injected with, that is at the same site as a known irritant, inflammation would either be unaffected or enhanced whereas a true anti-inflammatory agent injected with the irritant should reduce the inflammatory response. It can be seen from Figure 5 that a subplantar injection of peptide 401 together with carrageenin produces a considerable and dose-dependent enhancement of the oedematous response. The peptide is not, therefore, a true anti-inflammatory agent.

Final confirmation that mast cell degranulation can attenuate inflammation comes from experiments in rats sensitized to the nematode, *Nippostrongylus brasiliensis*. Subcutaneous allergen challenge at the same time as carrageenin is injected into the hind paw of sensitized animals reduces the swelling to the same extent as do the basic degranulators (Figure 3 compared with Figures 1 and 2). The only difference is that in the sensitized animals there is no swelling of the contralateral feet. This could be because the allergen remains bound, close to the site of injection and is not free to act systematically.

Mast cell degranulation may release chemotactic factors that mobilize or densitize circulating neutrophils (Atkins *et al.*, 1978; Soter, 1980; Tannenbaum *et al.*, 1980) while access of the neutrophils to the site of inflammation (Atherton & Born, 1972) may be enhanced as a result of increased vascular permeability caused by histamine and 5-HT. Such a role for the biogenic amines would be consistent with the partial antagonism observed with mepyramine and methysergide.

It therefore appears that peptide 401 is a counter irritant. It reduces both the chemically mediated and cell-mediated phases of inflammation, being active in both the early and the later phases of carrageenin oedema and also against adjuvant arthritis (Billingham *et al.*, 1973) but it is most unlikely to be of any therapeutic value.

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The central and regional cardiovascular responses to intravenous and intracoronary administration of the phenyldihydropyridine elgodipine in anaesthetized pigs

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1 The central and regional cardiovascular responses to intravenous (0.3, 1.0, 3.0 and 10.0 $\mu g\,kg^{-1}\,min^{-1}$) and intracoronary (0.3, 0.9, 3.0 and 4.5 $\mu g\,kg^{-1}\,min^{-1}$) infusions of elgodipine, a phenyldihydropyridine, and its solvent were studied in anaesthetized pigs.

2 Elgodipine (i.v.) caused dose-dependent decreases in arterial blood pressure (up to 44%) and systemic vascular resistance (up to 48%), whereas heart rate, LV dP/dt_{max}, left ventricular filling pressure, cardiac output and segment length shortening did not change. The absence of a negative inotropic effect with the employed doses was confirmed by the intracoronary infusions; with the lowest dose (0.3 $\mu g\,kg^{-1}\,min^{-1}$) both LV dP/dt_{max} and segment length shortening decreased by less than 10%. With 0.9 $\mu g\,kg^{-1}\,min^{-1}$ (intracoronary) the negative inotropic properties of the drug became apparent as LV dP/dt_{max} and segment length shortening decreased by 20% and 33%, respectively, whereas heart rate and left ventricular filling pressure were not affected.

3 Transmural myocardial blood flow did not change during intravenous infusion of elgodipine, as vasodilatation, more pronounced in the subepicardial than in the subendocardial layers, compensated for the decrease in arterial perfusion pressure. The intracoronary infusions revealed that the decrease in normalized subendocardial/subepicardial blood flow ratio was not secondary to the fall in arterial blood pressure.

4 Myocardial oxygen consumption decreased during both the i.v. and the intracoronary administration of elgodipine. With the i.v. administration the decrease was secondary to the hypotensive action of the drug, whereas with the intracoronary administration the negative inotropic properties played the dominant role.

5 Elgodipine (i.v.), although not affecting total cardiac output, caused a redistribution in favour of the nutritional blood flow at the expense of the arteriovenous anastomotic (AVA) blood flow. Up to an infusion rate of 3.0 $\mu g\,kg^{-1}\,min^{-1}$ the decrease in AVA-flow was due to a fall in arterial blood pressure, but at the highest infusion rate both the decrease in arterial perfusion pressure and an increase in their resistance contributed to a further decrease in AVA blood flow.

6 The skeletal muscles benefited most from the elgodipine(i.v.)-induced increase in nutritional blood flow, but vasodilatation was not uniform for all muscle groups. Up to an infusion rate of 3 $\mu g\,kg^{-1}\,min^{-1}$ the vasodilatation in the renal vascular bed was more pronounced in the inner than in the outer cortex, but, at 10 $\mu g\,kg^{-1}\,min^{-1}$, vascular resistances of both cortical layers returned to baseline values. In all regions of the brain, blood flow was maintained until the highest infusion rate was given. With 10 $\mu g\,kg^{-1}\,min^{-1}$ only flow to the vital parts of the brain (diencephalon and brain stem) was maintained. Blood flows to the skin and various abdominal organs were well maintained up to 3 $\mu g\,kg^{-1}\,min^{-1}$ but, at the highest dose, a decrease was observed in blood flow to the adrenals and spleen. Vascular resistances of all these organs and tissues decreased dose-dependently.

7 The potent systemic and coronary vasodilator actions of elgodipine during i.v. administration, which were not accompanied by negative inotropic and positive chronotropic properties or decreases in the perfusion of vital organs, warrant further study as this compound could be useful in the treatment of essential hypertension, myocardial ischaemia and, possibly, moderate chronic heart failure.

Introduction

The effectiveness of the first generation of calcium antagonists (nifedipine, verapamil and diltiazem) in the treatment of a large number of cardiovascular disorders has led to the development of a new generation of calcium entry blockers, with more selective properties. Elgodipine (IQB-875) is a newly synthesized drug with calcium entry blocking properties (Tejerina *et al.*, 1989), belonging to the phenyldihydropyridine group (Figure 1). *In vitro* the drug proved to be 100 fold more potent in causing vasodilatation than in causing negative inotropic and chronotropic effects (Tejerina *et al.*, 1989). The *in vivo* effects of elgodipine on the cardiovascular system have not yet been evaluated and, thus, the aim of the present study was two fold.

Firstly, we studied the effects of elgodipine on systemic haemodynamics and on regional blood flows in anaesthetized pigs. Apart from the effects of elgodipine on the various layers of the myocardium, we also studied the effects of this drug on the several parts of the brain and the different layers of the kidney. As a calcium antagonist may have differential effects on skeletal muscles of different regions (Duncker *et al.*, 1986a), we also estimated elgodipine-induced changes in blood flow in six different muscle groups. Since it has been well established that, in anaesthetized animals, a large fraction of the cardiac output is shunted through arteriovenous anastomoses (AVA: Kaihara *et al.*, 1968; Verdouw *et al.*, 1980; 1982; Saxena & Verdouw, 1985a), which are abundantly present in the skin (Hales & Cliff, 1977; Saxena & Verdouw, 1985b), and that antihypertensive drugs may exert differential effects on the flow through these anastomoses (Hof & Hof, 1989), we paid

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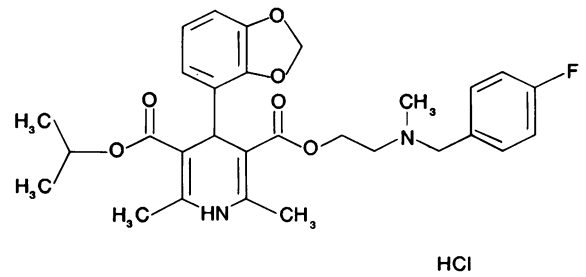


Figure 1 Chemical structure of elgodipine (IQB 875) a phenyldihydropyridine derivative with a molecular weight of 561.06. Isopropyl (2-(N-methyl-N-(4-fluorobenzyl)-amine)-ethyl,2,6-dimethyl-4-(2',3'-methylenedioxyphenyl)-1.5-dihydropyridine-3,5-dicarboxylate, monohydrochloride).

special attention to the effects of elgodipine on the division of cardiac output into its nutritional and non-nutritional fractions using the radioactive microsphere technique. As the microspheres (of 15 μm diameter), which pass through the AVA, will be trapped by the lungs, this non-nutritional fraction can be determined by counting the radioactivity in the lungs (Saxena & Verdouw, 1982; Hof & Hof, 1989).

Secondly an attempt was made to separate the direct from the indirect (that is, those secondary to changes in systemic haemodynamics) effects of elgodipine on myocardial contractility and on distribution of coronary blood flow. This was done by infusing the drug directly into a coronary artery.

Methods

After an overnight fast, cross-bred Landrace × Yorkshire pigs of either sex (22–28 kg, $n = 27$) were sedated with 120 mg azaperone (Stresnil) i.m., anaesthetized with 150 mg metomidate (Hypnodil) i.v., intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrous oxide (1:2). Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range: $7.35 < pH < 7.45$; $35\,mmHg < PCO_2 < 45\,mmHg$ and $110\,mmHg < PO_2 < 180\,mmHg$. Catheters (7F) were placed in the superior vena cava for administration of 160 mg kg^{-1} α-chloralose followed by an infusion of a low dose of sodium pentobarbitone (5 mg kg^{-1} h^{-1}); for administration of the muscle relaxant pancuronium bromide (4 mg) before thoracotomy; and for administration of haemaccel (Behringwerke A.G., Marburg, F.R.G.) to replace blood loss. Catheters were also positioned in the descending aorta for withdrawal of blood samples and measurement of central aortic blood pressure. A microtipped catheter (7F Millar), inserted via the left carotid artery, was used to measure left ventricular pressure and its first derivative (LV dP/dt). After thoractomy, an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta, while the great cardiac vein was cannulated for collection of blood in which the haemoglobin concentration and oxygen saturation were determined (by an OSM2 system, Radiometer, Copenhagen, Denmark). In 13 of the animals the proximal left anterior descending coronary artery was dissected free for placement of an electromagnetic flow probe around the vessel, while a cannula was inserted for intracoronary infusions of elgodipine.

In order to determine regional blood flows, the left atrial appendage was cannulated for injection of a batch of 1–2 × 10^6 carbonized plastic microspheres (15 ± 1 μm (s.d.) in diameter) labelled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce. Fifteen seconds before the injection of microspheres, blood was withdrawn from a femoral artery at a rate of 10 ml min^{-1} until 60–65 s after completion of the injection of the microspheres. At the end of each experiment in the animals which received intracoronary infusions of drug or solvent, the area perfused by the left anterior descending coronary artery was identified by intracoronary injection of patent blue violet (Sigma, St. Louis, MO, U.S.A.). After the i.v. treated animals had been killed with an overdose of sodium pentobarbitone, various organs (adrenals, bladder, liver, stomach, small intestine, brains and kidneys) and tissues (abdominal skin, various muscle groups) were excised, weighed and put into vials. In the animals given intracoronary infusions only the heart was excised. The hearts were fixed with formaldehyde (10% v/v) and 48 h later divided into atria, right and left ventricle. The myocardium of the left ventricle was divided into 3 layers of equal thickness: subepicardium, mesocardium and subendocardium. The kidneys were divided into the medulla and 3 cortical layers of equal thickness. Of the brains, the cerebral hemispheres, the cerebellum, the diencephalon and the brain stem were removed in order to obtain blood flow data for the different parts of the brain.

The radioactivity was counted and the amount of blood flow to the various tissues (Q_{tis}) calculated as:

$$Q_{tis}(ml\,min^{-1}) = (I_{tis}/I_{art}) \times Q_{art},$$

where I_{tis} and I_{art} are, respectively, the radioactivity (c.p.m.) in a particular tissue and that of the arterial blood sample, while Q_{art} is the rate of withdrawal of the blood sample. The resistance of a particular tissue was calculated as the ratio between the mean arterial blood pressure and the Q_{tis}. Full details of the procedures and the calculation of flow data with this technique have been described previously (Duncker *et al.*, 1986a).

Myocardial oxygen consumption ($M\dot{V}O_2$) was calculated as the product of coronary blood flow and the difference in the oxygen contents of the arterial and coronary venous blood.

Regional myocardial segment length shortening was measured by sonomicrometry (Triton Technology Inc., San Diego, CA, U.S.A.) by a pair of ultrasonic crystals implanted approximately 10–15 mm apart in the subepicardial layer. From the tracings, segment length shortening (SLS) was calculated as:

$$SLS(\%) = 100 \times (EDL - ESL)/EDL,$$

in which EDL and ESL are the segment length at end-diastole and end-systole, respectively.

Experimental protocols

Four consecutive 10 min i.v. infusions of elgodipine (0.3, 1.0, 3.0 and 10 μg kg^{-1} min^{-1}; $n = 7$) and equal volumes of the solvent ($n = 7$) or 4 consecutive 10 min intracoronary infusions of elgodipine (0.3, 0.9, 3.0 and 4.5 μg kg^{-1} min^{-1}; $n = 7$) or equal volumes of the solvent ($n = 6$) were administered. Systemic haemodynamics, regional myocardial function and the distribution of coronary blood flow were determined in all 4 series of experiments, but the distribution of cardiac output was only determined during the i.v. infusions of elgodipine or its solvent.

Statistical analysis

All data have been presented as the arithmetic mean ± s.e.mean. The significance of the effects of the solvent or elgodipine on the different variables was evaluated by Duncan's new-multiple range-test once an analysis of variance (randomized block design) had revealed that the samples represented different populations. The baseline values and the effects of the solvent were compared similarly but, in this case, one-way analysis of variance was used. Statistical significance was defined as $P < 0.05$ (two-tailed).

Drugs

Except for the anaesthetics given during surgery the only drugs used in this study were elgodipine (by courtesy of Dr A. Galiano, Instituto de Investigacion y Desarrollo, Madrid, Spain). For the i.v. infusions in the anaesthetized pigs, elgodipine was dissolved in 3% (v/v) ethanol for the highest

Table 1 Systemic haemodynamics after cumulative 10 min intravenous infusions of elgodipine or its solvent in anaesthetized pigs

			Elgodipine ($\mu g\,kg^{-1}\,min^{-1}$) *or equal volumes of its solvent* ($2\,ml\,min^{-1}$)				
		Baseline values	*0.3*	*1.0*	*3.0*	*10.0*	*Recovery*
HR	Solvent	92 ± 7	91 ± 7	92 ± 7	92 ± 7	89 ± 7	
	Elgodipine	102 ± 7	101 ± 7	100 ± 9	103 ± 9	106 ± 11	112 ± 9†
MAP	Solvent	88 ± 5	86 ± 4	85 ± 5	85 ± 5	84 ± 5†	
	Elgodipine	91 ± 3	88 ± 4	80 ± 5*	63 ± 5*	51 ± 3*	72 ± 4†
CO	Solvent	2.4 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	
	Elgodipine	2.5 ± 0.3	2.4 ± 0.3	2.4 ± 0.3	2.5 ± 0.3	2.7 ± 0.4	2.6 ± 0.3
SVR	Solvent	38 ± 3	37 ± 3	38 ± 3	38 ± 3	38 ± 2	
	Elgodipine	40 ± 5	41 ± 5	38 ± 4*	28 ± 2*	21 ± 2*	32 ± 3†
LV dP/dt_{max}	Solvent	2230 ± 180	2240 ± 190	2230 ± 190	2180 ± 180	2180 ± 170	
	Elgodipine	2780 ± 350	2880 ± 330	2820 ± 340	2640 ± 360	2540 ± 470	3220 ± 410
LVEDP	Solvent	7 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1	
	Elgodipine	8 ± 1	7 ± 1	8 ± 1	7 ± 1	7 ± 1	6 ± 1

Data are presented as means ± s.e.mean, $n = 7$ for both groups. HR = heart rate (beats min^{-1}); MAP = mean arterial blood pressure (mmHg); CO = cardiac output ($l\,min^{-1}$); SVR = systemic vascular resistance ($mmHg\,min\,l^{-1}$); LV dP/dt_{max} = maximal rate of rise of left ventricular blood pressure ($mmHg\,s^{-1}$); LVEDP = left ventricular end-diastolic blood pressure (mmHg); Recovery = data obtained 45 min after the highest infusion rate had been stopped.

* Drug-induced changes from baseline significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

† $P < 0.05$ vs baseline values (only presented for solvent and Recovery).

($10\,\mu g\,kg^{-1}\,min^{-1}$) infusion rate and subsequently further diluted with distilled water to give the lower doses. The infusion rate was $2\,ml\,min^{-1}$ for all doses. For the intracoronary infusions the elgodipine was dissolved in 1% (v/v) ethanol and the required doses were reached by adjusting the infusion rate (from $0.13\,ml\,min^{-1}$ to $2\,ml\,min^{-1}$). Preparation of the solutions, and infusion of the drug, took place while the drug was protected from light.

Results

Intravenous infusions of elgodipine

Systemic haemodynamics No significant changes occurred during infusion of the solvent in any of the systemic haemodynamic variables, except for a slight decrease (<4%) in arterial blood pressure (Table 1). The most striking effect of elgodipine was a dose-dependent lowering of the arterial blood pressure, which was already significant during infusion of $1\,\mu g\,kg^{-1}\,min^{-1}$. The fall in arterial blood pressure (up to 44%) was caused by systemic vascular dilatation as cardiac output was not affected. Heart rate, left ventricular dP/dt_{max} and left ventricular end-diastolic blood pressure were also unaffected. Forty-five min after the infusions had been stopped, arterial blood pressure and systemic vascular resistance showed signs of recovery, but were still lower (21% and 20%, respectively) than baseline.

Myocardial blood flow and performance The solvent had no effect either on the transmural blood flow and its distribution over the subendocardial and the subepicardial layers or on coronary vascular resistance during the course of the experiments (Figure 2). Elgodipine had a minor effect on left ventricular myocardial blood flow; only the increases (up to 30%) in the subepicardial layers during the two highest infusion rates reached levels of statistical significance (Figure 2). In view of the decrease in arterial perfusion pressure, it can be calculated that vasodilatation occurred in all layers of the myocardium since there were decreases in the coronary vascular resistance (that is, the ratio of mean arterial blood pressure and local blood flow); the decrease in the subepicardium (up to 50%) was more pronounced than the decrease (up to 25%) in the subendocardium (Figure 2). Forty-five min after termination of the highest infusion rate, overall coronary vascular resistance was still 35% lower than during baseline.

In the solvent-treated animals there was a minor decrease (5%, $P < 0.05$) in myocardial oxygen extraction, reflected by the small increase (from 22 ± 1% to 27 ± 2%, $P < 0.05$) in the oxygen saturation in the great cardiac vein. With elgodipine, the decrease (35% after the highest infusion rate) in oxygen extraction was considerably larger as the coronary venous oxygen saturation more than doubled (from 22 ± 3% to 48 ± 4%, $P < 0.05$). The decrease in myocardial oxygen extraction was secondary to the decrease in oxygen demand (Figure 3).

Myocardial segment length shortening did not change either during infusion of the solvent (19 ± 1%, 18 ± 1%, 19 ± 1%, 19 ± 1% and 20 ± 2% at baseline and after 10, 20, 30 and 40 min, respectively) or during infusion of the drug (19 ± 1%, 19 ± 2%, 20 ± 1%, 19 ± 2%, 18 ± 1% and 19 ± 2% at baseline, during 0.3, 1.0, 3.0, 10.0 $\mu g\,kg^{-1}\,min^{-1}$ of elgodipine and after 45 min of recovery).

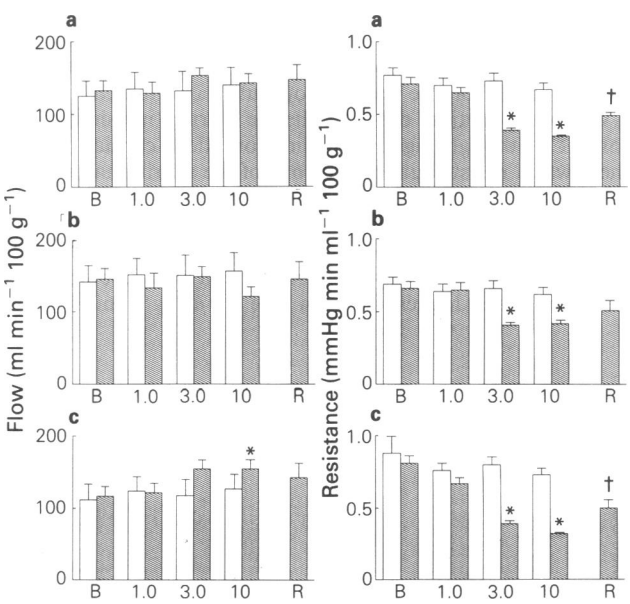


Figure 2 Effect of cumulative 10 min infusions of elgodipine (hatched columns, $n = 7$; 1, 3 and $10\,\mu g\,kg^{-1}\,min^{-1}$) and equal volumes ($2\,ml\,min^{-1}$) of its solvent (open columns, $n = 7$) on (a) transmural left ventricular, (b) subepicardial and (c) subendocardial blood flows and resistances in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean.* The drug-induced changes from baseline are significantly different from the solvent-induced changes from baseline. †$P < 0.05$ vs baseline (determined only for the solvent-treated animals and after 45 min of recovery in the elgodipine-treated animals). B = baseline. R = data obtained 45 min after the infusions had been stopped.

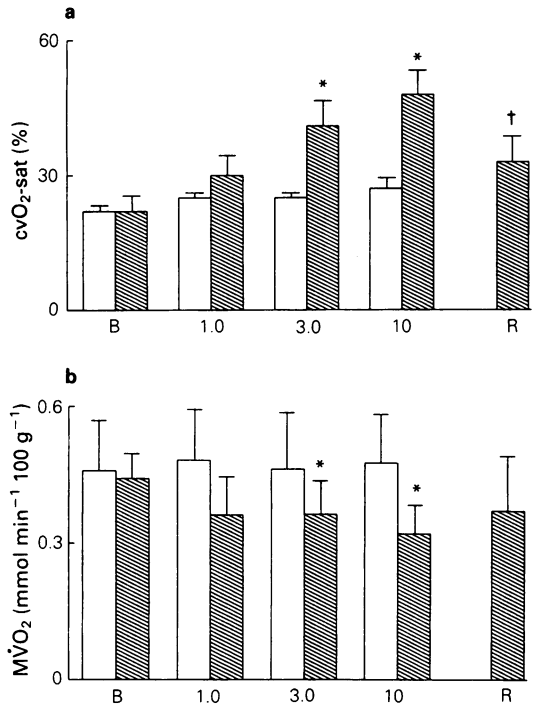


Figure 3 Effect of cumulative 10 min infusions of elgodipine (hatched columns, $n = 7$) or equal volumes (2 ml min^{-1}) of its solvent (open columns, $n = 7$) on (a) coronary venous oxygen saturation (cvO_2-sat) and (b) left ventricular oxygen consumption ($M\dot{V}O_2$). Data are presented as mean and the bars show s.e.mean.* The drug-induced changes from baseline are significantly different from the solvent-induced changes from baseline. †$P < 0.05$ vs baseline (determined only for the solvent-treated animals and after 45 min of recovery in the elgodipine-treated animals). B = baseline. R = data obtained 45 min after the infusions had been stopped.

Distribution of cardiac output

Fractionation of cardiac output into nutrient and non-nutrient flows Figure 4 illustrates that, under baseline conditions, a large fraction of the cardiac output was shunted through AVA. This fraction did not change during infusion of the solvent. Although elgodipine did not affect cardiac output, there was a redistribution in favour of the nutritional fraction. Forty-five min after the infusions were stopped, AVA-flow and resistance had returned to values close to baseline. At 1 and 3 μg kg^{-1} min^{-1} elgodipine, the decrease in the non-nutrient, AVA, flow paralleled the decrease in mean arterial blood pressure, but at the highest infusion rate (10 μg kg^{-1} min^{-1}) constriction of the AVA also contributed to the lower AVA-flow (Figure 5).

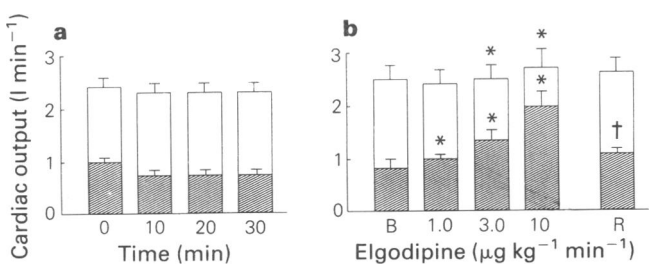


Figure 4 Fractionation of cardiac output in arteriovenous anastomotic (open column) and capillary (hatched column) blood flow during cumulative 10 min infusions of (b) elgodipine ($n = 7$) or (a) equal volumes (2 ml min^{-1}) of its solvent ($n = 7$) in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. *The drug-induced changes from baseline are significantly different from the solvent-induced changes from baseline. †$P < 0.05$ vs baseline (determined only for the solvent-treated animals and after 45 min of recovery in the elgodipine-treated animals). B = baseline. R = data obtained 45 min after the infusions had been stopped.

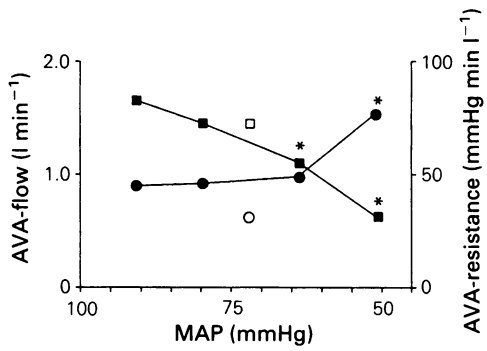


Figure 5 Arteriovenous anastomotic (AVA) blood flow (■, □) and resistance (●, ○) of the elgodipine-treated animals ($n = 7$) are plotted against the mean arterial blood pressure (MAP), measured at the end of each infusion rate. The open symbols are the data obtained 45 min after the infusions had been stopped. *$P < 0.05$ vs baseline.

Distribution of nutritional blood flow Table 2 shows that, in the kidneys, the normalized flow both to the inner cortex and to the outer cortex, in which it was twice that of the inner cortex, did not change during solvent infusion. No effect of elgodipine on cortical flows was observed until the highest dose was reached. With the highest infusion rate (i.e. at lowest renal perfusion pressure) both cortical flows decreased, the largest effect being observed in the outer cortex. Medullary blood flow was not affected by any rate of infusion of elgodipine. Lowering of the renal vascular resistances with the two lower infusion rates prevented a fall in blood flow due to the lower perfusion pressure. During infusion of 10 μg kg^{-1} min^{-1} elgodipine, renal vascular resistances started to increase and this, together with the lower perfusion pressure, caused a decrease in renal blood flow at this dose. All renal vascular resistances had returned to pre-drug values when the measurements were repeated after the 45 min recovery period. The outer cortical flow was, however, still significantly lower than the pre-drug values due to the lower perfusion pressure.

Elgodipine had no effect on regional cerebral blood flows until the highest dose was reached (Table 3). With the highest infusion rate there was a 10–15% decrease ($P < 0.05$) in blood flow to all regions of the brain. All regional cerebral blood flows except that in the cerebellum returned to baseline during the recovery period, although mean arterial blood pressure remained 20 mmHg below baseline.

Flows to a number of other organs, except for the adrenals and spleen, were also well maintained until the highest infusion rate of elgodipine (Table 4). The blood flow to the abdominal skin tended to increase, but the increase did not reach levels of significance.

The skeletal muscles benefited more from the increase in nutritional blood flow than any other tissue or organ. Figure 6 illustrates that elgodipine did not exert the same effect on all muscle groups. With the highest dose the increases in blood flow to the masseter (10 fold), to the iliopsoas (7 fold) and to the diaphragm (5 fold) were all significantly higher than to the abdominal muscle (2 fold). Forty-five min after the infusion had stopped, all muscle flows had decreased by approximately 50%. As the increases in muscle flows occurred in spite of the lower mean arterial blood pressures, the decreases in the resistances were even more pronounced than the increases in flow (Figure 7).

Intracoronary infusions of elgodipine

Systemic haemodynamics Infusion of the solvent had no effect on systemic haemodynamics (Table 5). The lower infusion rates of elgodipine (up to 0.9 μg kg^{-1} min^{-1}) only affected left ventricular dP/dt_{max} (−20%, $P < 0.05$). At the higher infusion

Table 2 Effect of cumulative 10 min infusions of elgodipine or its solvent on renal blood flow and resistance in anaesthetized pigs

		Elgodipine ($\mu g\,kg^{-1}\,min^{-1}$) *or equal volumes of its solvent* ($2\,ml\,min^{-1}$)				
		Baseline values	*1.0*	*3.0*	*10.0*	*Recovery*
Blood flow ($ml\,min^{-1}\,100\,g^{-1}$)						
Kidneys	Solvent	235 ± 25	232 ± 16	223 ± 19	226 ± 16	
	Elgodipine	231 ± 19	246 ± 28	256 ± 25	151 ± 24*	192 ± 23
Outer Cortex	Solvent	362 ± 33	360 ± 21	354 ± 25	357 ± 23	
	Elgodipine	349 ± 28	350 ± 38	347 ± 31	201 ± 32*	278 ± 35†
Inner Cortex	Solvent	207 ± 24	206 ± 18	194 ± 23	194 ± 20	
	Elgodipine	206 ± 19	226 ± 26	240 ± 25	142 ± 22*	174 ± 22
Medulla	Solvent	63 ± 6	61 ± 4	56 ± 5	55 ± 4	
	Elgodipine	66 ± 5	69 ± 7	79 ± 7	51 ± 6	58 ± 7
Resistance ($mmHg\,min\,ml^{-1}\,100\,g$)						
Kidneys	Solvent	0.40 ± 0.05	0.38 ± 0.04	0.40 ± 0.04	0.39 ± 0.04	
	Elgodipine	0.41 ± 0.04	0.36 ± 0.05	0.26 ± 0.03*	0.38 ± 0.06*	0.43 ± 0.09
Outer Cortex	Solvent	0.25 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.24 ± 0.02	
	Elgodipine	0.28 ± 0.02	0.25 ± 0.03	0.19 ± 0.02*	0.29 ± 0.04*	0.31 ± 0.08
Inner Cortex	Solvent	0.47 ± 0.07	0.44 ± 0.05	0.47 ± 0.06	0.47 ± 0.06	
	Elgodipine	0.47 ± 0.04	0.39 ± 0.05	0.28 ± 0.03*	0.40 ± 0.05	0.47 ± 0.09
Medulla	Solvent	1.55 ± 0.28	1.47 ± 0.18	1.63 ± 0.26	1.61 ± 0.21	
	Elgodipine	1.45 ± 0.11	1.26 ± 0.14	0.87 ± 0.13*	1.07 ± 0.11*	1.46 ± 0.32

Data are presented as means ± s.e.mean, $n = 7$ for both groups.
Recovery = data obtained 45 min after the highest rate of infusion was stopped.
* Drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.
† $P < 0.05$ vs baseline values (only presented for solvent and Recovery).

Table 3 Effect of cumulative 10 min intravenous infusions of elgodipine or solvent on cerebral blood flow in anaesthetized pigs

		Elgodipine ($\mu g\,kg^{-1}\,min^{-1}$) *or equal volumes of its solvent* ($2\,ml\,min^{-1}$)				
		Baseline values	*1.0*	*3.0*	*10.0*	*Recovery*
Total brain	Solvent	29 ± 2	30 ± 2	30 ± 2	30 ± 2	
	Elgodipine	32 ± 1	31 ± 2	31 ± 2	27 ± 2*	28 ± 2†
Cerebral hemispheres	Solvent	29 ± 2	30 ± 2	29 ± 2	30 ± 1	
	Elgodipine	32 ± 1	31 ± 2	31 ± 2	27 ± 2*	28 ± 2
Diencephalon	Solvent	28 ± 2	30 ± 3	28 ± 2	28 ± 2	
	Elgodipine	27 ± 1	27 ± 2	28 ± 2	25 ± 1	26 ± 1
Cerebellum	Solvent	33 ± 2	35 ± 2	35 ± 3	34 ± 2	
	Elgodipine	38 ± 2	38 ± 3	37 ± 3	33 ± 3*	33 ± 2†
Brain stem	Solvent	26 ± 2	26 ± 2	26 ± 2	27 ± 2	
	Elgodipine	28 ± 2	25 ± 3	28 ± 2	26 ± 2	25 ± 2

Data are presented as mean ± s.e.mean, $n = 7$ for both groups. Flow data are presented in $ml\,min^{-1}\,100\,g^{-1}$. Recovery = data obtained 45 min after the highest rate of infusion was stopped.
* Drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.
† $P < 0.05$ vs baseline values (only presented for solvent and Recovery).

Table 4 Effect of cumulative 10 min intravenous infusions of elgodipine or solvent on regional blood flows in anaesthetized pigs

		Elgodipine ($\mu g\,kg^{-1}\,min^{-1}$) *or equal amounts of its solvent* ($2\,ml\,min^{-1}$)				
		Baseline values	*1.0*	*3.0*	*10.0*	*Recovery*
Adrenals	Solvent	135 ± 15	149 ± 11	147 ± 16	154 ± 21	
	Elgodipine	184 ± 14	159 ± 8	178 ± 18	134 ± 18*	209 ± 29
Skin	Solvent	1.1 ± 0.4	1.1 ± 0.5	0.7 ± 0.1	0.9 ± 0.4	
	Elgodipine	1.1 ± 0.3	1.1 ± 0.3	1.4 ± 0.3	1.7 ± 0.5	0.9 ± 0.2
Bladder	Solvent	4.1 ± 0.4	4.6 ± 0.3	4.6 ± 0.4	4.9 ± 0.5	
	Elgodipine	4.7 ± 0.5	4.9 ± 0.4	5.1 ± 0.7	3.7 ± 0.6	3.7 ± 0.8
Liver	Solvent	43 ± 4	34 ± 7	28 ± 7	27 ± 6	
	Elgodipine	39 ± 9	24 ± 7	17 ± 6	10 ± 2	14 ± 6†
Stomach	Solvent	20 ± 3	22 ± 3	20 ± 3	20 ± 3	
	Elgodipine	19 ± 2	23 ± 5	26 ± 6	21 ± 3	26 ± 2
Small intestine	Solvent	38 ± 5	35 ± 3	34 ± 4	35 ± 3	
	Elgodipine	39 ± 3	38 ± 3	35 ± 3	32 ± 3	38 ± 2
Spleen	Solvent	247 ± 45	241 ± 48	222 ± 48	246 ± 49	
	Elgodipine	273 ± 25	271 ± 27	257 ± 45	144 ± 31*	251 ± 51

Data are presented as mean ± s.e.mean, $n = 7$ for both groups. Flow data are presented in $ml\,min^{-1}\,100\,g^{-1}$. Recovery = data obtained 45 min after the highest dose was stopped.
* Drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.
† $P < 0.05$ vs baseline values (only presented for solvent and Recovery).

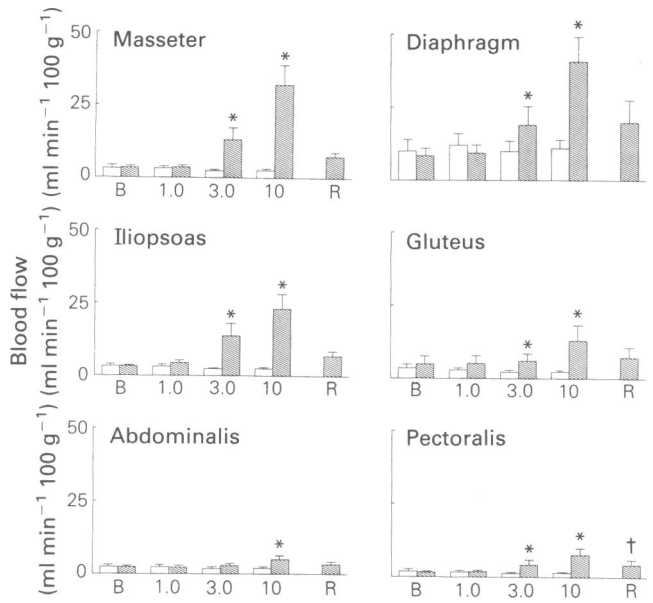


Figure 6 Effect of cumulative 10 min infusions of elgodipine (hatched columns, $n = 7$) or equal volumes (2 ml min^{-1}) of its solvent (open columns, $n = 7$) on blood flow to different muscle groups in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. *The drug-induced changes from baseline are significantly different from the solvent-induced changes from baseline. †$P < 0.05$ vs baseline (determined only for the solvent-treated animals and after 45 min of recovery in the elgodipine-treated animals). B = baseline. R = data obtained 45 min after the infusions had been stopped.

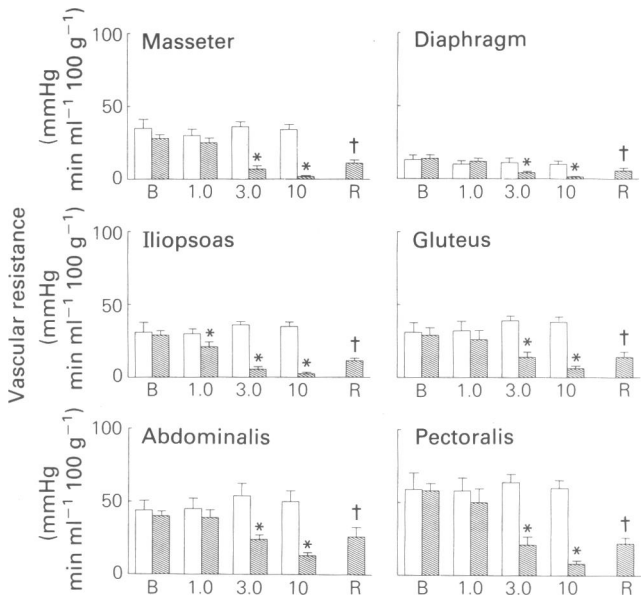


Figure 7 Effect of cumulative 10 min infusions of elgodipine (hatched columns, $n = 7$) or equal volumes (2 ml min^{-1}) of its solvent (open columns, $n = 7$) on the resistances of different muscle groups in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. *The drug-induced changes from baseline are significantly different from the solvent-induced changes from baseline. †$P < 0.05$ vs baseline (determined only for the solvent-treated animals and after 45 min of recovery in the elgodipine-treated animals). B = baseline. R = data obtained 45 min after the infusions had been stopped.

rates there were not only further decreases in LV dP/dt_{max} (−30%, $P < 0.05$), but also cardiac output (−15%), mean arterial blood pressure (−35%) and systemic vascular resistance (−25%) were significantly affected (Table 5). Sixty min after the highest rate of elgodipine infusion had stopped, all parameters had returned to baseline except for mean arterial blood pressure, which was still depressed (−10%).

Myocardial blood flow Intracoronary infusions of the solvent had neither an effect on myocardial perfusion (Figure 8), nor on myocardial resistance. Elgodipine increased transmural blood flow of the myocardium perfused by the left anterior descending coronary artery by up to 200% during infusion of 0.9 μg kg^{-1} min^{-1}. When the infusion rate was increased to 3 μg kg^{-1} min^{-1}, blood flow started to decrease and, at 4.5 μg kg^{-1} min^{-1}, transmural blood flow was not different from baseline (Figure 8). Although all layers benefited from the initial increase in transmural flow, the increments in the subepicardium (140 ml min^{-1} 100 g^{-1}) were considerably larger than in the subendocardium (100 ml min^{-1} 100 g^{-1}). The decreases in subendo- and subepicardial blood flows at the higher infusion rates were very similar. In the segment adjacent to the myocardium perfused by the left anterior descending coronary artery, the only significant effect was a 25% decrease in subendocardial blood flow at the highest infusion rate of elgodipine.

Table 5 Systemic haemodynamics after cumulative 10 min intracoronary infusions of elgodipine or its solvent in anaesthetized pigs

			Elgodipine (μg kg^{-1} min^{-1}) or equal volumes of its solvent (0.13, 0.39, 1.3, 2.0 ml min^{-1})				
		Baseline values	*0.3*	*0.9*	*3.0*	*4.5*	*Recovery*
HR	Solvent	103 ± 5	102 ± 4	102 ± 4	102 ± 4	106 ± 3	104 ± 3
	Elgodipine	102 ± 7	103 ± 8	104 ± 9	108 ± 9	108 ± 10	98 ± 9
MAP	Solvent	85 ± 6	83 ± 6	81 ± 5	81 ± 7	86 ± 7	83 ± 7
	Elgodipine	86 ± 5	83 ± 4	76 ± 6	63 ± 4*	57 ± 4*	77 ± 3*
CO	Solvent	2.8 ± 0.4	2.8 ± 0.3	2.7 ± 0.3	2.7 ± 0.3	2.8 ± 0.3	2.5 ± 0.4
	Elgodipine	2.8 ± 0.1	2.7 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.4 ± 0.2*	2.6 ± 0.2
SVR	Solvent	32 ± 3	30 ± 3	31 ± 3	31 ± 3	32 ± 4	38 ± 5
	Elgodipine	31 ± 2	31 ± 3	32 ± 3	26 ± 2*	24 ± 2*	32 ± 3
SV	Solvent	27 ± 3	28 ± 3	27 ± 3	26 ± 3	27 ± 3	24 ± 4
	Elgodipine	28 ± 2	27 ± 3	25 ± 2	24 ± 2	22 ± 2*	27 ± 3
LV dP/dt_{max}	Solvent	2660 ± 280	2570 ± 260	2700 ± 360	2830 ± 440	3000 ± 460	2790 ± 330
	Elgodipine	2490 ± 329	2330 ± 280	2000 ± 290*	1970 ± 230*	1750 ± 240*	2460 ± 400
LVEDP	Solvent	6 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1
	Elgodipine	8 ± 1	8 ± 1	9 ± 1	10 ± 1	10 ± 1	8 ± 1

Data are presented as mean ± s.e.mean, $n = 7$ for the elgodipine group and 6 for the solvent group.
HR = heart rate (beats min^{-1}); MAP = mean arterial blood pressure (mmHg); CO = cardiac output (l min^{-1}); SVR = systemic vascular resistance (mmHg min l^{-1}); SV = stroke volume (ml); LV dP/dt_{max} = maximal rate of rise of left ventricular blood pressure (mmHg s^{-1}); LVEDP = left ventricular end-diastolic blood pressure (mmHg); Recovery = data were obtained 60 min after infusion at the highest rate had been stopped.
* Drug-induced change from baseline significantly different ($P < 0.05$) from the solvent-induced change from baseline.

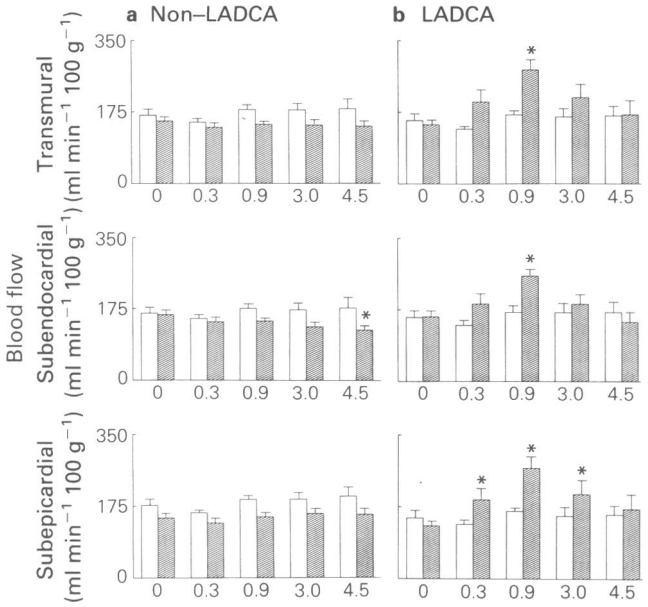


Figure 8 Myocardial blood flow after cumulative (0.3, 0.9, 3.0, 4.5 $\mu g\,kg^{-1}\,min^{-1}$) intracoronary infusions of elgodipine (hatched columns, $n = 7$) or its solvent (open columns, $n = 6$) directly into the left anterior descending coronary artery (LADCA) (b). (a) Non-LADCA = area adjacent to the LADCA-perfused myocardium. Data have been presented as mean and the bars show s.e.mean. *Elgodipine-induced changes from baseline are significantly different from solvent-induced changes from baseline.

Oxygen consumption and function of the myocardium perfused by the left anterior descending coronary artery The solvent had no effect on either the myocardial oxygen consumption (515 ± 26, 432 ± 27, 510 ± 36, 463 ± 25, 507 ± 67 $\mu mol\,min^{-1}\,100\,g^{-1}$ at baseline and after 10, 20, 30 and 40 min of solvent infusion, respectively) or regional segment length shortening (22 ± 1%, 22 ± 1%, 21 ± 1%, 21 ± 1% and 21 ± 1% at baseline and after 10, 20, 30 and 40 min of solvent infusion). The oxygen saturation in the great cardiac vein was 23 ± 2%, 49 ± 4%, 66 ± 3%, 71 ± 2% and 78 ± 3% (all $P < 0.05$ versus baseline) at baseline and after 0.3, 0.9, 3.0 and 4.5 $\mu g\,kg^{-1}\,min^{-1}$ of elgodipine, respectively, and returned to 29 ± 3% 60 min after the infusions had been stopped. The decrease in myocardial oxygen extraction was caused by a decrease in myocardial oxygen consumption (470 ± 48, 410 ± 42, 366 ± 46, 274 ± 47 and 200 ± 44 $\mu mol\,kg^{-1}\,min^{-1}$ at baseline and after 0.3, 0.9, 3.0 and 4.5 $\mu g\,kg^{-1}\,min^{-1}$ of elgodipine, respectively). A representative example of the effects of elgodipine on segment length shortening is shown in Figure 9. At the lowest dose there was a slight but significant decrease in segment length shortening (from 18 ± 2% to 16 ± 2%, $P < 0.05$). With increasing infusion rates there were further dose-dependent decreases (to 12 ± 2% and 7 ± 3% after 0.9 and 3.0 $\mu g\,kg^{-1}\,min^{-1}$ of elgodipine, respectively) and, after the highest dose, segment length shortening was almost completely abolished (3 ± 2%). Figure 10 illustrates that the reduction in myocardial oxygen consumption was closely related to the reduction in segment length shortening ($r = 0.99$, $P < 0.05$). During the wash-out period segment length shortening recovered gradually (10 ± 2% and 15 ± 3% after 15 and 30 min, respectively) and had returned to pre-drug values (18 ± 2%) after 60 min.

Discussion

Systemic haemodynamics

The present study shows that elgodipine is a potent arterial vasodilator. With the same model, we have previously evaluated the effects not only of a number of other dihydropyridines such as nifedipine (Wolffenbüttel & Verdouw, 1983), nimodipine (Duncker *et al.*, 1986a), nisoldipine (Duncker *et al.*, 1986b) and bepridil (Verdouw & Scheffer, 1984) but also of vasodilators such as nicorandil (Verdouw *et al.*, 1987a), pimobendan (Verdouw *et al.*, 1986) and its *O*-demethyl metabolite UD-CG 212 Cl (Verdouw *et al.*, 1987b). Comparison of these studies reveals that the potency of elgodipine in reducing sys-

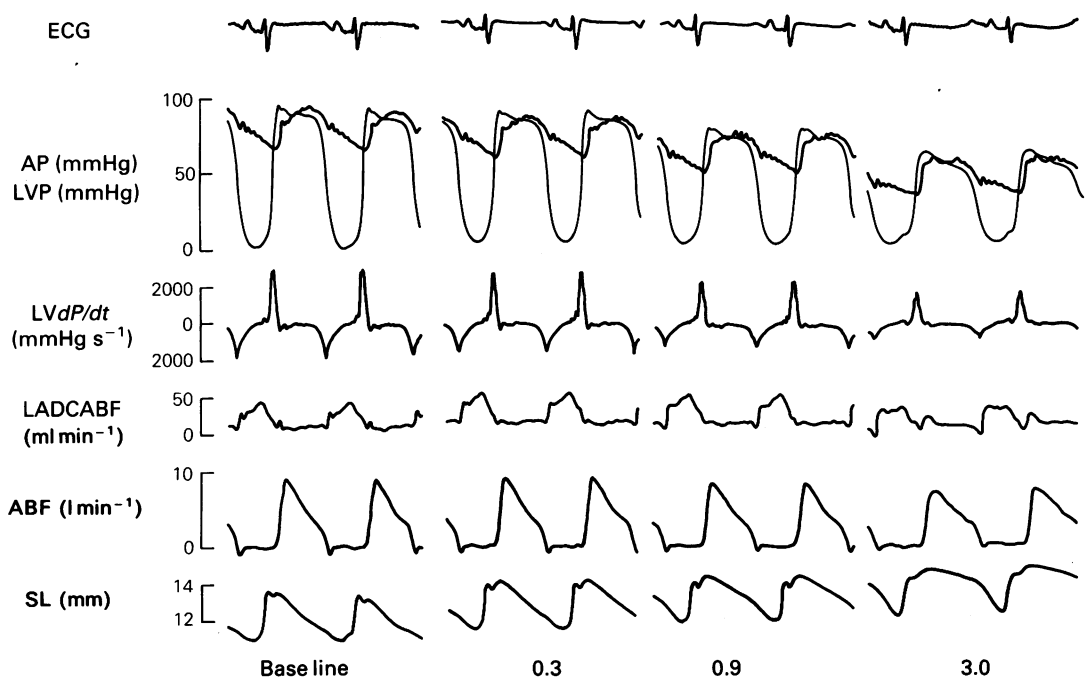


Figure 9 Representative tracing of the effects of intracoronary (left anterior descending coronary artery) infusions of elgodipine on systemic haemodynamics and regional myocardial blood flow and function in an anaesthetized pig. AP and LVP are central aortic and left ventricular blood pressure, respectively; LV *dP/dt* first derivative of LVP; LADCABF = left anterior descending coronary artery blood flow; ABF = ascending aortic blood flow; SL = segment length. The infusion rates of elgodipine ($\mu g\,kg^{-1}\,min^{-1}$) are given at the bottom of the figure.

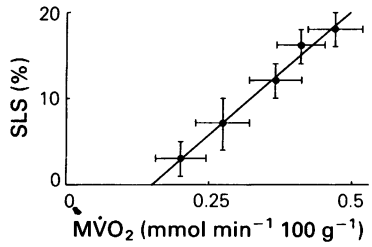


Figure 10 During the intracoronary infusion of elgodipine the reduction in local myocardial oxygen consumption ($M\dot{V}O_2$) is closely related ($r = 0.99$) to the decrease in segment length shortening (SLS). Data are presented as mean and the bars show s.e.mean.

temic vascular resistance is slightly less than that of nifedipine, nisoldipine and nimodipine, but considerably larger (10–50 fold) than that of bepridil, nicorandil, pimobendan and UD-CG 212 Cl. As mean arterial blood pressure was considerably lowered by elgodipine, a reflex mediated tachycardia would be expected to compensate for the hypotensive action of the drug. The absence of this tachycardia can partially be explained by the anaesthetic regimen used. It must, however, be kept in mind that, in the same preparation, nisoldipine, while causing similar decreases in arterial blood pressure, increased heart rate by up to 25% (Duncker *et al.*, 1986b). It is therefore likely that, as observed with nimodipine (Duncker *et al.*, 1986a), elgodipine possesses some negative chronotropic actions (Tejerina *et al.*, 1989) which counterbalance the reflex tachycardia. Interestingly, LV dP/dt_{max} was not affected by elgodipine despite the fall in arterial pressure. As heart rate and left ventricular filling pressure were also not affected it can be concluded that, in doses up to $10\,\mu g\,kg^{-1}\,min^{-1}$ *in vivo*, the compound does not exert negative inotropic actions. The absence of a negative inotropic effect of elgodipine became even more evident during the intracoronary infusions, as $0.3\,\mu g\,kg^{-1}\,min^{-1}$ substantially dilated the vascular bed of the left anterior descending coronary artery but had only a minor effect on regional contractile function, as reflected by the changes in local segment length shortening and LV dP/dt_{max}. With intracoronary administration of $0.9\,\mu g\,kg^{-1}\,min^{-1}$, the negative inotropic properties of elgodipine became apparent. In view of the small fraction (3–4%) of the cardiac output used for myocardial perfusion, a 30 times higher dose (approximately $30\,\mu g\,kg^{-1}\,min^{-1}$) may be needed to observe a negative inotropic effect with i.v. infusions. These doses will most likely not be clinically applicable because of severe hypotension.

Myocardial blood flow and function

Despite the decrease in arterial blood pressure, left ventricular blood flow was well maintained during the elgodipine infusions as a result of a direct vasodilator response to the drug, the effect being slightly more pronounced in the subepicardial layer (45% decrease in vascular resistance) than in the subendocardial layer (30% decrease in the vascular resistance). The preferential vasodilatation of the subepicardial layers by antihypertensive drugs, in particular when the fall in blood pressure is accompanied by a tachycardia, has been well established (Domenech & Goich, 1976; Feigl, 1983). The observation that intracoronary infusion of $0.3\,\mu g\,kg^{-1}\,min^{-1}$ elgodipine, which did not affect arterial blood pressure, exclusively increased blood flow to the subepicardial layers of the area perfused by the left anterior descending coronary artery indicates a preferential effect of the drug to give subepicardial vasodilatation.

With the i.v. infusions, the decrease in myocardial oxygen consumption was secondary to the hypotensive action of elgodipine. The much larger decrease in myocardial oxygen consumption with the intracoronary infusions was caused by the additional decrease in regional contractile function (Figure 10). Intracoronary administration of drugs, which locally decrease myocardial oxygen consumption while maintaining a global left ventricular function, may be useful during coronary angioplasty as they improve ischaemic tolerance during this procedure (Amende *et al.*, 1987). The data from the present study indicate that elgodipine can be a useful addition to the armament for such interventions.

Arteriovenous anastomotic blood flow

More than 20 years ago, Kaihara *et al.* (1968) showed that anaesthesia may open AVA, but relatively little attention has been paid to the effects of pharmacological agents on the fractionation of cardiac output into arteriovenous anastomotic and capillary blood flow (Verdouw *et al.*, 1980; Hof & Hof, 1989). The latter group studied the effect of several antihypertensive drugs on nutritional and non-nutritional blood flow and concluded that isradipine, a related compound to elgodipine, which decreased mean arterial blood pressure from 75 to 50 mmHg, also decreased AVA blood flow, but slightly increased the resistance of the AVA, probably due to sympathetic reflex activation (Hof & Hof, 1989). In the present study elgodipine had no effect on cardiac output but the fraction shunted through the anastomoses decreased dose-dependently. Up to $3\,\mu g\,kg^{-1}\,min^{-1}$ this was entirely due to a decrease in arterial blood pressure (Figure 4) as the resistance of the anastomoses was not affected. However, at the highest infusion rate, a doubling of the resistance of the AVA also contributed to a further fall in AVA-flow. As suggested by Hof & Hof (1989), this increase in resistance may be reflex-mediated, but as this increase occurs at the lowest mean arterial blood pressure (Figure 5), we cannot entirely exclude the possibility that the lumen diameter of the AVA decreased due to hypotension.

Regional blood flows

The largest increases in capillary blood flow were found in the skeletal muscle although, as with nimodipine (Duncker *et al.*, 1986a), we observed some regional differences.

With similar decreases (25–30%) in perfusion pressure elgodipine, like nisoldipine (Duncker *et al.*, 1986b) did not affect cerebral blood flow, whereas nimodipine caused a 20% decrease and nicorandil doubled cerebral blood flow. When, after elgodipine infusions, mean arterial blood pressure fell below 60 mmHg, there was a decrease in cerebral blood flow which was limited to the evolutionary higher parts of the brain (the cerebellum and the cerebral hemispheres), while sparing the more vital parts of the brain.

Nimodipine (Duncker *et al.*, 1986a), nisoldipine (Duncker *et al.*, 1986b) and nicorandil (Verdouw *et al.*, 1987b), while inducing similar hypotensive effects, decreased total renal blood flow by 20–50%, whereas no change was observed with elgodipine, demonstrating a renal vasodilator action by the latter drug. To our knowledge the effects of calcium antagonists on regional renal blood flows have not yet been investigated. Elgodipine (up to $3\,\mu g\,kg^{-1}\,min^{-1}$) caused vasodilatation in both the inner and outer cortex, the effect being more pronounced in the inner cortex. This is in accordance with earlier studies on other vasodilator drugs (Stein *et al.*, 1971; De Bermudez & Haysleet, 1972; Hardaker & Wechsler, 1973; Kirschenbaum *et al.*, 1974). Although it has been suggested that the diminution in perfusion pressure in itself, by causing intrarenal production and liberation of angiotensin (Lachance *et al.*, 1974), might be responsible for the intrarenal blood flow changes (Carrière & Daigenault, 1970), the importance of redistribution of renal blood flow with elgodipine cannot yet be assessed.

The potent vasodilator actions of i.v. administered elgodipine, which were not accompanied by negative inotropic and positive chronotropic properties or decreases in the perfusion of vital organs, warrant further study as this compound could be useful in the treatment of essential hypertension, myocardial ischaemia and, possibly, moderate chronic heart failure.

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Cyclic GMP release and vasodilatation induced by EDRF and atrial natriuretic factor in the isolated perfused kidney of the rat

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1 Guanosine 3':5'-cyclic monophosphate (cyclic GMP) release and vascular tone was measured in the isolated kidney of the rat perfused at constant flow with Krebs-Henseleit solution. The effects of 3 vasodilators, acetylcholine (ACh), atrial natriuretic factor (ANF) and sodium nitroprusside (SNP) on the renal release of cyclic GMP and vascular tone were examined. The ability of the endothelial-derived relaxing factor (EDRF) inhibitors, haemoglobin and gossypol, to modify vasodilatation and vasodilator-induced changes in cyclic GMP releases from the kidney was also investigated.

2 Renal cyclic GMP release was elevated 8 fold by ANF (0.01 μM), 5 fold by SNP (1 μM) and 3 fold by ACh (0.3 μM).

3 For ACh, both the increase in renal cyclic GMP release and the vasodilatation were reduced by the EDRF inhibitors, haemoglobin (1 μM) and gossypol (15 μM). For SNP, neither the increase in renal cyclic GMP release nor vasodilatation were inhibited by gossypol (15 μM).

4 For ANF, neither the increase in cyclic GMP release from the kidney nor its vasodilator activity were affected by haemoglobin (1 μM).

5 EDRF inhibitors reduced the basal release of cyclic GMP from 0.32 ± 0.06 pmol min^{-1} to 0.18 ± 0.03 pmol min^{-1}, gossypol being more effective than haemoglobin.

6 The results are consistent with the ability of ACh to induce EDRF-mediated vasodilatation in the isolated perfused kidney of the rat. Basal EDRF release appears to contribute approximately 50% to the basal release of cyclic GMP from this preparation. The renal vasodilator action of ANF however, is independent of EDRF, although the renal vascular endothelium cannot be discounted as a site at which ANF stimulates cyclic GMP production.

Introduction

The arterial vasodilator activity of endothelial-derived relaxing factor (EDRF) is mediated by activation of the soluble (cytosolic) form of guanylate cyclase elevating guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Forstermann *et al.*, 1986). Atrial natriuretic factor (ANF) which also elevates cyclic GMP is thought to activate the particulate (membrane bound) form of guanylate cyclase through an endothelium-independent mechanism (Winquist *et al.*, 1984). The vascular endothelium may, however, contain functional receptors for ANF since endothelial removal reduced the ability of ANF to stimulate cyclic GMP from rabbit aorta (Linz *et al.*, 1986) and in cultured bovine endothelial cells ANF-induced elevation of cyclic GMP content was 10 fold greater than in vascular smooth muscle (Schenk *et al.*, 1985).

In marked contrast to ANF, little is known about the renal properties of EDRF and its role in the control of renal vascular resistance. In other vascular beds including, the rabbit isolated ear (Griffith *et al.*, 1988) and the rat mesentery (Byfield *et al.*, 1986, Randall & Hiley, 1988), angiographic techniques or the use of detergents have indicated a role for EDRF in the control of arteriolar resistance. In the rat isolated perfused kidney, we have previously been unable to produce selective inhibition of acetylcholine (ACh)-induced vasodilatation with deoxycholate (Burton *et al.*, 1987), although some degree of selectivity was achieved with the detergent CHAPS. Bhardwaj & Moore (1988) employing a similar rat kidney preparation, however, demonstrated that the potentiation of renal vasoconstriction produced by CHAPS could not be reproduced by EDRF inhibitors, questioning the selectivity of detergents for the endothelium in the rat isolated kidney. While EDRF release in the kidney, unlike the heart (Amecuza *et al.*, 1988), has yet to be demonstrated, initial experiments with EDRF inhibitors (Burton *et al.*, 1988; Bhardwaj & Moore, 1988) are consistent with the presence of EDRF-mediated renal vasodilatation.

The present study was performed to establish (a) whether measurements of renal cyclic GMP synthesis were consistent with the presence of EDRF-mediated vasodilatation in the kidney and (b) whether EDRF may mediate either the increase in renal cyclic GMP synthesis or the renal vasodilatation induced by ANF. Experiments were performed in the isolated perfused kidney of the rat to examine the effect of the EDRF inhibitors haemoglobin (Edwards *et al.*, 1986) and gossypol (Alheid *et al.*, 1987) on vasodilator-induced changes in renal cyclic GMP release. The vasodilators studied were ACh, ANF and sodium nitroprusside (SNP).

Methods

Isolated perfused kidney of the rat

Male Wistar rats (200–300 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.), the jugular vein cannulated and heparin (200 units kg^{-1}) administered by i.v. bolus injection. Following a flank incision the left renal artery was cannulated via an incision made in the aorta, the left adrenal artery being tied when necessary. The kidney was removed to a thermostatically controlled perfusion cabinet (37°C) and perfused with a Krebs-Henseleit solution of composition (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2 and glucose 10, containing theophylline (1 mM) and gassed with 95% O_2 and 5% CO_2. Renal

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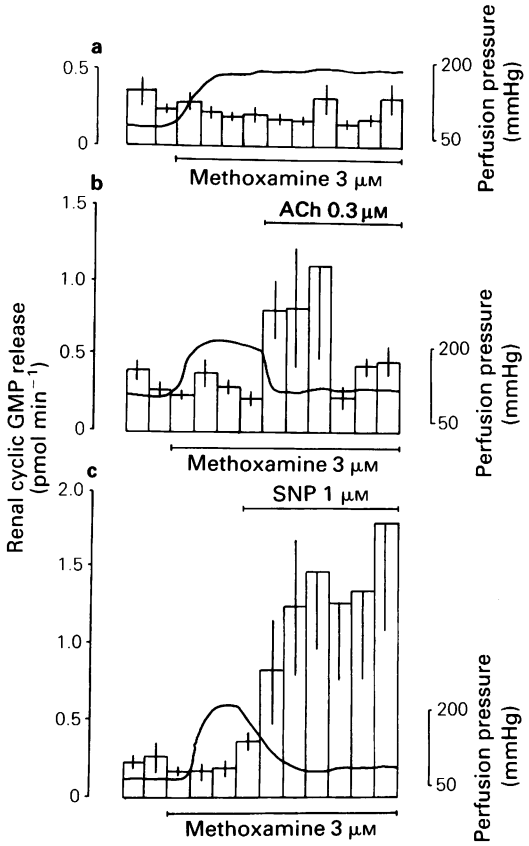


Figure 1 Effects of (a) methoxamine (3 μM) alone ($n = 6$) and together with either (b) acetylcholine (ACh, 0.3 μM, $n = 6$), or (c) sodium nitroprusside (SNP 1 μM, $n = 6$) on the time course of renal cyclic GMP release from the isolated perfused kidney of the rat following vasoconstriction with methoxamine (3 μM). Representative traces of renal perfusion pressure obtained from single kidneys are shown by the continuous line.

perfusion pressure was recorded electronically and the combined renal venous and tubular effluent collect for cyclic GMP assay.

Cyclic GMP assay

Following collection of renal effluent, the samples were immediately boiled (2 min) to inhibit phosphodiesterase activity and stored at −20°C. Samples were extracted and concentrated before assay as follows: 4 ml of each sample was acidified with 1 M HCl and subjected to ion exchange chromatography using a Dowex cation exchange resin. The column sizes were 9 × 0.6 cm and the resin was regenerated after each sample with 1 M HCl. Columns were eluted with distilled water and control experiments using renal effluent 'seeded' with known concentrations of cyclic GMP showed that fractions eluted between 7–14 ml diluted parallel to a standard curve of cyclic GMP in distilled water. Control experiments using [^{3}H]-cyclic GMP demonstrated that 47.7 ± 4.1% ($n = 4$) of the ^{3}H added could be recovered in the fraction assayed for cyclic GMP.

All samples were freeze-dried and re-constituted in 250 μl distilled water and a 200 μl aliquot acetylated by the addition of 2 μl acetic anhydride and 4 μl triethylamine. The derivatised nucleotide was immunoassayed using guanosine 3'5'-cyclic phosphoric acid 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester, 5nCi per tube. Calibration binding curves were constructed by use of standard concentrations of cyclic GMP (7.8 fmol–2 pmol per tube) dissolved in Krebs-Henseleit solution which had previously been subjected to ion exchange chromatography.

Experimental protocol

Each kidney received only a single dose of each vasodilator which was infused for a 6 min period during renal vasoconstriction with the α_1-adrenoceptor agonist methoxamine (3 μM). In initial time course experiments, cyclic GMP was assayed in renal effluent collected at 1 min intervals during vasodilator infusion. The time of maximum cyclic GMP release was 0–3 min during infusion with ACh (0.3 μM) but, 4–6 min during infusion with SNP (1 μM) or ANF (0.01 μM). Values for cyclic GMP quoted in the text for ACh were therefore obtained in 0–3 min, while those for SNP or ANF were obtained in 4–6 min. Doses of vasodilators were chosen to produce approximately 80% of the maximum vasodilator response obtained to each agent in the absence of theophylline. The use of the EDRF inhibitors, gossypol and haemoglobin, required different protocols. Kidneys were pretreated with a 10 min infusion of gossypol (15 μM) 30 min before dilator stimulation. In contrast, haemoglobin (1 μM) was infused 1 min before and throughout the period of vasodilator administration.

Materials

Dowex 50 (H^+ form 4% cross-linked 200–400 dry mesh) (Sigma); guanosine 3'5'-cyclic phosphoric acid 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester (New England Nuclear); atrial natriuretic factor (rat 8–33 amino acids) (Pennisula); acetylcholine chloride, sodium nitroprusside, gossypol (all Sigma). Cyclic GMP antisera were obtained from Professor B. Brown, Department of Human Metabolism and Clinical Biochemistry, Sheffield. Methoxamine hydrochloride was a gift from the Wellcome Foundation.

Haemoglobin was prepared by a modification of the method of Edwards *et al.* (1986). Rat blood was spun at 3000 *g* for 20 min the supernatant discarded and the red cell concentrate then washed 3 times with 10 ml Krebs-Henseleit solution: 2 ml of the remaining red cell concentrate was then lysed with 2 ml water and spun to remove the cellular debris. The supernatant was chromatogrammed on a Sephadex G75 column (2.5 × 25 cm) and was eluted with Krebs-Henseleit solution at 30 ml h^{-1}. The haemoglobin fraction was assayed by the cyanmethaemoglobin method (Drabkin & Austin, 1935).

Statistics

Results were evaluated by either paired or unpaired Student's *t* test where appropriate.

A preliminary account of some of the results presented in this paper was communicated to the British Pharmacological Society Meeting in London, December 1988.

Results

Renal vasoconstriction

Methoxamine (3 μM) increased the renal perfusion pressure from 61.7 ± 4.8 to 153.3 ± 9.9 mmHg ($P < 0.001$, $n = 6$). The renal release of cyclic GMP was not significantly different ($n = 6$) in the absence (0.26 ± 0.07 pmol min^{-1}) or in the presence (0.20 ± 0.08 pmol min^{-1}) of methoxamine-induced vasoconstriction.

Renal vasodilators

The vasoconstrictor response to methoxamine was inhibited 77.8 ± 9.5% ($n = 6$) by ACh (0.3 μM), 57.2 ± 7.1% ($n = 5$) by ANF (0.01 μM) and 46.8 ± 12.7% ($n = 5$) by SNP (1 μM) and these vasodilator responses were sustained over at least a 6 min period during which time renal effluent was collected for cyclic GMP assay. All three vasodilators increased the renal release of cyclic GMP but there were differences in both the

extent and the duration of the increases obtained. Release was increased from 0.33 ± 0.04 to 0.91 ± 0.23 pmol min^{-1} ($P < 0.05$, $n = 6$) by ACh (0.3 μM, 0–3 min), however this increase declined during the 4–6 min period of the infusion despite the dilator response being sustained (Figure 1). Both vasodilator response and the increase in cyclic GMP release produced by SNP were slower in onset than those obtained with ACh. The increase in renal cyclic GMP release induced by SNP (1 μM, 4–6 min) from 0.23 ± 0.07 to 1.34 ± 0.47 pmol min^{-1} ($P < 0.05$, $n = 5$) did not reach a maximum until the 4–6 min period of its infusion (Figure 1). ANF (0.01 μM) produced an immediate and sustained effect on cyclic GMP release which had increased from 0.63 ± 0.36 to 5.80 ± 1.96 pmol min^{-1} ($P < 0.05$, $n = 5$) during the 4–6 min period of the ANF infusion.

EDRF inhibitors

The influence of haemoglobin on both vascular tone and renal cyclic GMP release measured in the presence of ACh and ANF is demonstrated in Figure 2 using non-paired statistics. In the presence of haemoglobin (1 μM), the vasodilator response to ANF remained unchanged and was accompanied by a significant increase in cyclic GMP release from 0.16 ± 0.06 to 8.44 ± 1.60 pmol min^{-1} ($P < 0.001$, $n = 5$). However, haemoglobin (1 μM) markedly reduced dilator responses to ACh which were unaccompanied by any increase in renal cyclic GMP release from 0.34 ± 0.16 to 0.23 ± 0.06 pmol min^{-1} ($n = 5$).

The effect of gossypol (15 μM) on vascular tone and cyclic GMP release produced in the presence of ACh and SNP is demonstrated in Figure 3 using non-paired statistics. Following treatment with gossypol (15 μM), the vasodilator response to SNP was not inhibited and was associated with a significant increase ($P < 0.001$) in renal cyclic GMP release from 0.16 ± 0.03 to 1.09 ± 0.24 pmol min^{-1}. However, following gossypol, there was a marked reduction in the vasodilator response to ACh which was not associated with any increase in cyclic GMP release, from 0.09 ± 0.01 to 0.11 ± 0.02 pmol min^{-1} ($n = 6$).

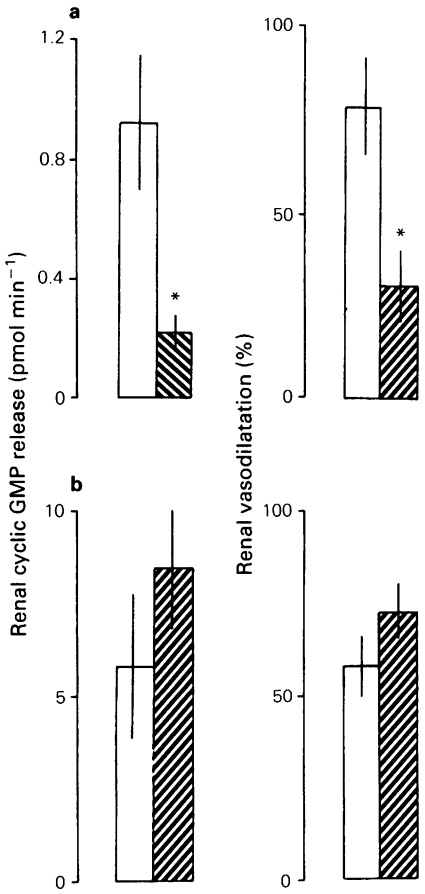


Figure 2 Effect of haemoglobin (1 μM) on renal vasodilatation and renal cyclic GMP release in the presence of either (a) acetylcholine (0.3 μM) or (b) atrial natriuretic factor (0.01 μM). Paired histograms denote vasodilatation or cyclic GMP release measured in the absence (open) or presence (hatched) of haemoglobin. Vertical bars represent the s.e.mean. * $P < 0.001$ (non-paired).

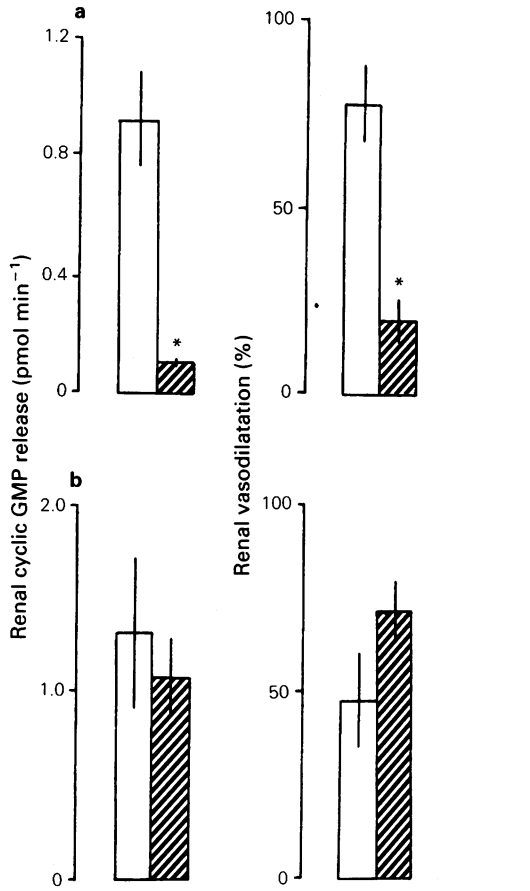


Figure 3 Effect of gossypol (15 μM) on renal vasodilatation and renal cyclic GMP release in the presence of either (a) acetylcholine (0.3 μM) or (b) sodium nitroprusside (1 μM). Paired histograms denote vasodilatation or cyclic GMP release measured either without (open) or with (hatched) gossypol pretreatment. Vertical bars represent s.e.mean. * $P < 0.001$ (non-paired).

Basal cyclic GMP release

The basal release of cyclic GMP from the kidney was 0.33 ± 0.06 pmol min^{-1} ($n = 33$). The basal release was significantly lower ($P < 0.05$) in kidneys receiving an EDRF inhibitor at 0.18 ± 0.03 pmol min^{-1} ($n = 26$). In kidneys which later received either ACh or SNP, the basal release of cyclic GMP (0.27 ± 0.05 pmol min^{-1}, $n = 11$) was significantly lower ($P < 0.01$) when pretreated with gossypol (15 μM, 0.12 ± 0.02 pmol min^{-1}, $n = 11$). In kidneys which later received either ACh or ANF, although the mean basal release of cyclic GMP (0.46 ± 0.16 pmol min^{-1}, $n = 11$) was lower in the presence haemoglobin (1 μM) (0.30 ± 0.07 pmol min^{-1}, $n = 10$) the difference was not significant at the 5% level.

Discussion

This paper contains the first report of measurements of cyclic GMP release from the isolated kidney and required the presence of theophylline in the perfusate to bring the basal release rate to within the limit of detection of the assay system. Cyclic GMP synthesis is normally assessed from the

cyclic GMP content of tissue homogenates rather than whole organ release. Evidence that renal cyclic GMP release may also reflect the level of cyclic GMP synthesis in the rat isolated kidney was provided by the increase in renal cyclic GMP release produced by ANF and SNP, standard stimulators of the particulate (Winquist *et al.*, 1984) and soluble (Forstermann *et al.*, 1986) forms of guanylate cyclase respectively. The use of the isolated perfused kidney allowed the vascular reactivity of the kidney to be assessed in parallel with renal cyclic GMP synthesis.

In arterial smooth muscle ACh activates soluble guanylate cyclase indirectly by stimulating the synthesis of EDRF (Forstermann *et al.*, 1986). Renal vasodilatation induced by ACh in the isolated kidney was accompanied by a 3 fold increase in cyclic GMP release although, the vascular reactivity of the isolated kidney was partially depressed by the presence of the phosphodiesterase inhibitor, theophylline. The increase in cyclic GMP release was maximal after 2 min but declined towards basal levels after 6 min despite a sustained vasodilator response. A similar time course has been demonstrated for the effects of ACh on the tissue content of cyclic GMP and generated tension in arterial rings (Gruetter & Lemke, 1986). The ability of ACh to increase renal cyclic GMP release was abolished by the structurally dissimilar EDRF inhibitors, haemoglobin and gossypol, which also inhibited ACh-induced renal vasodilatation. Such data are consistent with the hypothesis that EDRF-mediated vasodilatation can be stimulated in the isolated perfused kidney by acetylcholine. It is also consistent with the chemical identity of EDRF being nitric oxide (Palmer *et al.*, 1987) since nitric oxide is known to activate soluble guanylate cyclase in the kidney (Arnold *et al.*, 1977).

Both the increase in cyclic GMP release produced by ANF in the isolated perfused kidney and its renal vasodilator activity remained unaffected by treatment with haemoglobin, which acts as an EDRF inhibitor by binding nitric oxide (Gibson & Roughton, 1957). EDRF synthesis would not therefore appear to be involved in either the renal vasodilator activity of ANF or its ability to stimulate cyclic GMP synthesis. While the renal vasodilator response to ANF at the dose employed was smaller than that produced by ACh, the increase in cyclic GMP release was considerably larger. In isolated arteries, the ability of ANF to stimulate cyclic GMP is markedly enhanced by the presence of the endothelium (Linz *et al.*, 1986) and in cultured endothelial cells cyclic GMP elevation by ANF was some 10 fold greater than that in vascular smooth muscle (Schenk *et al.*, 1985). The renal vascular endothelium therefore could also represent a site for ANF to stimulate cyclic GMP synthesis in the isolated perfused kidney. However, since ANF stimulates a different form of guanylate cyclase from EDRF or the nitrovasodilators, such a difference could equally be explained by a greater synthetic capacity or a wider distribution of the particulate form of the enzyme. It has been proposed that ANF stimulates cyclic GMP in a variety of renal cell types (Takeda *et al.*, 1986). At the present time the importance of cyclic GMP to endothelial cell function and, the importance of the endothelium to the renal properties of ANF remain uncertain.

In isolated arterial rings, endothelial removal reduces the tissue content of cyclic GMP (Rapoport & Murad, 1983), indicating the presence of basal EDRF synthesis. Evidence for the presence of basal EDRF release from the isolated perfused kidney was provided by the results obtained with the EDRF inhibitors. Combination of the data obtained for both gossypol and haemoglobin showed a significant reduction in basal cyclic GMP release. Gossypol, in particular, reduced the basal outflow of cyclic GMP from the kidney by 60% to 0.1 pmol min^{-1} indicating that the kidney may continuously synthesize and/or release EDRF. The precise mechanism of action of gossypol as an EDRF inhibitor is unknown. While gossypol may, like other antioxidants, act by generating superoxide ions to break down nitric oxide (Moncada *et al.*, 1986), it potentiates rather than inhibits the dilator response to nitroprusside, thought to be a nitric oxide generating agent. Due to the irreversible nature of its effect and prolonged exposure time, gossypol was considered to be a better agent than haemoglobin to detect a change in basal cyclic GMP release.

However, the contribution which the basal EDRF release may make to the basal perfusion pressure in the rat isolated kidney is less certain, since although EDRF inhibitors produce a small increase in the basal perfusion pressure, ACh-induced vasodilatation cannot be detected in the absence of generated tone. Methoxamine was employed to elevate the renal vascular tone on the assumption that the endothelial α-adrenoceptor was of the α_2-subtype. Methoxamine therefore, being an α_1-selective agonist, would be unlikely to stimulate cyclic GMP release through the endothelium. Although as expected, methoxamine did not increase cyclic GMP release from the isolated perfused kidney, an interaction between the vascular effects of methoxamine and EDRF is still possible in the kidney since it has been proposed that the basal EDRF release inhibits the contractile activity of any spasmogen (Bullock *et al.*, 1986). In the present isolated perfused kidney preparation vasoconstriction was not potentiated by the EDRF inhibitors, in agreement with the experiments of Bhardwaj & Moore (1988). One conclusion could be that the basal release of EDRF is subthreshold for vasodilatation in this preparation. However, the possibility that renal EDRF synthesis may have been inhibited by the perfusion conditions employed cannot be ignored.

The validity of using measurements of cyclic GMP synthesis to support the presence of EDRF-mediated vasodilatation relies on the assumption that the properties of EDRF are mediated by this nucleotide. Although evidence from arterial tissues is strong (Murad, 1986), it must be remembered that agonists such as ACh can elevate cyclic GMP in tissues where EDRF has not been proposed as a mediator of its action (Waldman & Murad, 1987) and may also dilate smooth muscle by hyperpolarizing the cell membrane (Taylor *et al.*, 1988). The possibility that EDRF may possess other renal properties besides vasodilatation must also be considered. In a recirculating model of the isolated perfused kidney of the rat, the EDRF inhibitor gossypol reduced the glomerular filtration rate at doses that had no effect on perfusate flow (Pathak *et al.*, 1984). In addition, effluent from the rabbit aorta has been demonstrated to produce endothelium-dependent inhibition of renin release from dog kidney slices (Vidal *et al.*, 1988) although, recent evidence suggests that cyclic GMP may stimulate rather than inhibit renin release (Abu-Kishak *et al.*, 1989).

In conclusion, (a) the presence of EDRF-mediated vasodilatation in the isolated perfused kidney of the rat is supported by the pattern of change in both cyclic GMP release and vascular tone produced by vasodilators in the presence of EDRF inhibitors, (b) neither the increase in cyclic GMP release nor the vasodilator activity of ANF would appear to be dependent on EDRF synthesis, (c) renal EDRF synthesis may contribute approximately 50% to the basal release of cyclic GMP by this preparation.

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Modulation of the reactivity of the guinea-pig isolated trachealis by respiratory epithelium: Effects of cooling

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1 Examination has been made of the effects of epithelium removal on the reactivity of guinea-pig trachealis to methacholine at 37°C and 22°C, and on responses to activation of the Na^+/K^+-pump by abrupt temperature increase from 22°C to 37°C.

2 At 37°C, epithelium removal increased the sensitivity of isolated tracheal strips to methacholine without affecting the maximum isometric contractile response. Epithelium removal resulted at 22°C in a decrease in sensitivity to methacholine, i.e. an effect opposite to that seen at 37°C. While the maximum response of intact strips to methacholine was enhanced at 22°C, the maximum response of denuded preparations was decreased.

3 The increase in sensitivity to methacholine at 37°C after epithelium removal was mimicked in intact preparations by indomethacin (1 μM). Indomethacin did not mimic the decrease in methacholine sensitivity and maximum response caused by epithelium removal at 22°C.

4 Following incubation at 22°C, abrupt increase in temperature to 37°C elicited relaxation in both epithelium-containing and epithelium-denuded tracheal strips. In epithelium-containing preparations the relaxation was more pronounced and followed by contraction. Ouabain (1 μM) converted the relaxation of denuded preparations to contraction, but was ineffective in intact strips. The relaxation of intact strips was, however, inhibited by a greater ouabain concentration (10 μM).

5 These findings indicate that the modulatory effect of the epithelium is temperature-dependent. In cooled preparations, the epithelium enhances reactivity. At 37°C, an epithelium-derived factor reduces reactivity, and this may partially be due to activation of the electrogenic Na^+/K^+-pump.

Introduction

Respiratory epithelial cell damage and loss accompany the late phase airway hyperreactivity response in asthmatics (Laitinen *et al.*, 1985). This finding has prompted investigation into the influence of epithelial cells on airway smooth muscle responsiveness. Mechanical removal of epithelial cells from airway smooth muscle preparations *in vitro* potentiates the response to many bronchoactive agents in several mammalian species (see Fedan *et al.*, 1988 for review). A number of investigators (Tschirhart & Landry, 1986; Ilhan & Sahin, 1986; Hay *et al.*, 1987) have demonstrated the existence of a diffusible, inhibitory factor liberated from the epithelium which modulates smooth muscle responsiveness. The loss of this epithelium-derived relaxing factor (EpDRF) could contribute to hyperreactivity in the asthmatic patient.

Since it occurs for some, but not all bronchoactive agents, the increase in reactivity after epithelium removal could involve the loss of an effect of EpDRF on a physiological process important to the responsiveness of the smooth muscle. Evidence has been obtained that EpDRF may modulate the activity of an electrogenic Na^+/K^+-pump (Raeburn & Fedan, 1989), which is electrogenic and contributes to the resting membrane potential in airway smooth muscle (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981). Alterations in electrogenic Na^+/K^+-pump activity may affect membrane potential and reactivity to excitatory agents (Fleming, 1980).

Several methods may be used to alter electrogenic Na^+/K^+-pump activity. The pump is inhibited when the extracellular fluid is rendered K^+-deficient, and stimulated upon the re-addition of K^+. Stimulation of the pump will relax a contracted smooth muscle, and this relaxation is blocked by ouabain. Likewise, Na^+/K^+-pump activity may be reduced by cooling (Fleming, 1980) and stimulated in a ouabain-sensitive fashion by warming (Taylor *et al.*, 1970; Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981).

In the present study, responses to methacholine of intact and epithelium-denuded guinea-pig tracheal strips at 37°C and 22°C were compared. To evaluate further the possibility that electrogenic Na^+/K^+-pump activity is influenced by the epithelium, responses of intact and denuded preparations to abrupt increase in bath temperature from 22°C to 37°C were examined. A preliminary account of this work has been given (Lamport & Fedan, 1988).

Methods

Preparation of guinea-pig isolated tracheal strips

Male English short hair guinea-pigs (300–700 g; Camm Research Institute, Wayne, NJ, U.S.A.) were killed by cervical dislocation and bled. The trachea was removed and placed in modified Krebs-Henseleit (MKH) solution (37°C) equilibrated with 5% CO_2 in O_2. The trachea was cleaned, cut along the longitudinal axis directly opposite the smooth muscle, and divided into segments two cartilage rings wide. The segments were tied at one end *via* silk suture to holders, placed in 3 ml water-jacketted organ baths containing gassed MKH, and attached at the other end to force displacement transducers for the isometric recording of tension changes. The MKH composition was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.7.

Equilibration conditions

The strips were equilibrated in MKH at 37°C or 22°C for 1 h under an optimum (1 g) resting load and were washed at 15 min intervals. Preparations equilibrated at 37°C were then either held at 37°C during concentration-response determinations (see below), or used to examine the effect of abrupt

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temperature decrease to 22°C (see below). Preparations equilibrated at 22°C remained at 22°C during concentration-response determinations, or were subjected to rapid temperature increase to 37°C. The justification for not first incubating the latter preparations at 37°C was that spontaneous tone was negligibly and transiently affected when preparations which had been incubated at 37°C for 1 h were cooled rapidly to 22°C (see Results).

Epithelium removal

The epithelium was removed by gently rubbing the luminal surface of alternate segments with a cotton-tipped applicator. This procedure has been shown by histological analysis to remove the epithelium without damaging the smooth muscle (Hay *et al.*, 1986). To reduce possible regional differences in muscle reactivity, adjacent tissue segments comprised a test pair (i.e. one intact, one denuded). The region of trachea used was randomized with respect to the particular protocols employed.

Concentration-response curves

Methacholine concentration-response curves were obtained at 37°C and 22°C from responses to cumulative additions. Only one curve was constructed from each tracheal strip. In some experiments, the effect of indomethacin (1 μM) on the methacholine concentration-response curve was examined at 37°C and 22°C. The indomethacin was added at the end of the 1 h equilibration period. Methacholine was applied 40–45 min later, in the presence of indomethacin.

Comparison of force development in the absence and presence of indomethacin

Indomethacin (1 μM) had no effect on spontaneous tone at 22°C, but relaxed both the intact and denuded preparations under resting tension at 37°C, as observed previously (Hay *et al.*, 1986). When methacholine-induced contraction was measured from the indomethacin-lowered baseline, the apparent effect of indomethacin was to increase the magnitude of the tension increment evoked by each concentration step and therefore the total tension change between the foot and the summit of the concentration-response curve (the maximum response). Therefore, when indomethacin was present at 37°C, it was necessary to take this relaxation into account in order to compare methacholine-induced maximum responses with those obtained at 22°, and at 37°C in the absence of indomethacin. We have previously demonstrated (Hay *et al.*, 1986) that the peak tension developed by the preparations in response to methacholine (basal plus methacholine-induced) is not affected by indomethacin, either in the absence or presence of the epithelium. In the present study two parameters are used to report the response to the maximally-effective methacholine concentration. The first will be referred to as 'developed force', which is the tension increment measured from the resting tension prior to the addition of methacholine. Concentration-response curves in the figures are presented in terms of developed force, and Table 1 summarizes maximum developed forces obtained under several conditions. The second parameter will be referred to as 'corrected maximum developed force.' This parameter applies only when indomethacin was present at 37°C; it represents the maximum developed force observed in the presence of indomethacin less the reduction in basal tension induced by indomethacin. Corrected maximum developed force results are given in Table 1.

Abrupt temperature change

Tracheal segments were incubated in MKH for 1 h either at 37°C or at 22°C. The bath temperature was then changed abruptly to the alternate temperature by changing the water-jacket fluid to a second circulator which had been set to the appropriate temperature. The large volume of the water jackets (ca. 46 ml) compared to the volume of the organ chambers (3 ml) allowed rapid temperature changes in the MKH. In some experiments, indomethacin (1 μM) was present during the hour-long incubation at 22°C and subsequent increase to 37°C. In other cases, ouabain (1 or 10 μM) was added 5 min before increasing the temperature to 37°C.

Statistical analysis

The results were quantified as developed force of contraction (g) and % maximum response, and are given as mean ± s.e.mean; n is the number of separate experiments. Geometric mean EC_{50} values (the concentration producing 50% of the maximum response) were determined from nonlinear least square curve fitting analysis (ALLFIT; DeLean *et al.*, 1978). The data were evaluated for differences by analysis of variance or Student's t test (where indicated). The 0.05 level of probability was considered significant. In the figures the s.e.mean is shown by the vertical bars, unless enclosed within the symbol.

Drugs

Methacholine (acetyl-β-methylcholine chloride), indomethacin, and ouabain were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. All drug solutions were prepared freshly each day in 0.9% w/v NaCl solution, with the exception of indomethacin, which was dissolved in 100 mM Na_2CO_3 solution and diluted with water.

Results

Indomethacin absent

The effects of epithelium removal on the reactivity of guinea-pig isolated trachealis to methacholine were temperature-dependent (Figure 1; Table 1). Epithelium removal resulted in a 3.6 fold increase in sensitivity at 37°C. Removal of the epithelium caused an opposite effect at 22°C, i.e. a 6 fold decrease in sensitivity to methacholine. There was, however, no effect of temperature on the sensitivities of intact preparations.

Epithelium removal had no effect at 37°C on the maximum developed force evoked by methacholine. At 22°C, the maximum developed force of intact strips was increased by 60%, but in denuded strips it was decreased by 39%.

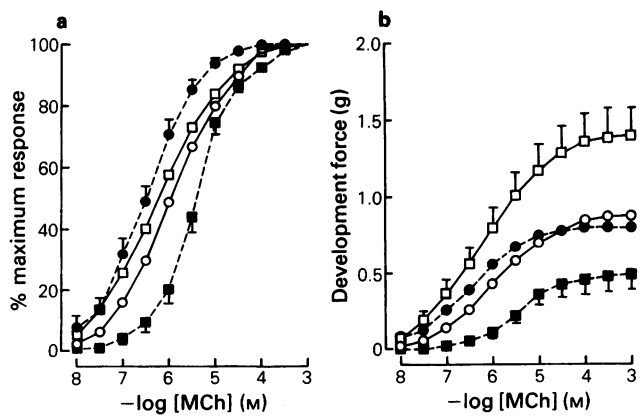


Figure 1 Guinea-pig isolated tracheal strips: effects of epithelium removal on reactivity to methacholine at 37°C and 22°C: (○) epithelium present, 37°C; (●) epithelium absent, 37°C; (□) epithelium present, 22°C; (■) epithelium absent, 22°C. Results are expressed as % of the maximum developed force of contraction to indicate alterations in sensitivity (a) and as g developed force to indicate alterations in maximum developed responses (b). $n = 5$.

Table 1 Effects of epithelium removal, temperature and indomethacin (1 μM) on reactivity to methacholine in guinea-pig isolated trachealis

Temperature	$-\log EC_{50}$ (M) Intact	$-\log EC_{50}$ (M) Denuded	Maximum developed force (g) Intact	Maximum developed force (g) Denuded
		Control ($n = 5$)		
37°C	5.97 ± 0.17	6.53 ± 0.13*†	0.88 ± 0.14	0.80 ± 0.18
22°C	6.22 ± 0.15	5.43 ± 0.09‡	1.40 ± 0.19§	0.49 ± 0.09‡
		Indomethacin ($n = 6$)		
37°C	6.47 ± 0.16¶	6.70 ± 0.11†	1.61 ± 0.30 (0.98 ± 0.14)**	1.24 ± 0.29 (0.85 ± 0.20)**
22°C	6.05 ± 0.18	5.57 ± 0.06‡	1.20 ± 0.23	0.60 ± 0.20‡

Data are means ± s.e.mean. The results were analysed for differences by ANOVA.
* Denuded significantly greater than intact.
† Significantly greater than at 22°C.
‡ Denuded significantly less than intact.
§ Significantly greater than at 37°C.
¶ Significantly greater than control (indomethacin absent) at 37°C.
** Indomethacin caused relaxation of the preparations at 37°C. Values in parentheses give the maximum forces developed from the pre-indomethacin baseline, i.e. the corrected maximum developed forces. These values were not significantly different, nor were they different from respective intact and denuded control values at 37°C.

Indomethacin present

To determine if the effects of epithelium removal at 37°C and 22°C could be due to differences in prostanoid production, concentration-response curves were constructed at both temperatures in the presence of the cyclo-oxygenase inhibitor indomethacin (1 μM) (Figure 2; Table 1). In agreement with earlier findings (Hay *et al.*, 1986), indomethacin reduced spontaneous tone at 37°C: intact strips relaxed by 0.55 ± 0.17 g and denuded preparations relaxed by 0.39 ± 0.12 g ($n = 6$; $P < 0.3$; Student's t test). At 22°C indomethacin had no effect on spontaneous tone.

Indomethacin increased the sensitivity of intact preparations to methacholine at 37°C. Sensitivity to methacholine was not increased further by epithelium removal at 37°C in the presence of indomethacin, i.e. the effect of indomethacin on intact strips mimicked epithelium removal. At 22°C the sensitivities of intact or denuded strips to methacholine were not affected by indomethacin.

At 37°C indomethacin increased the maximum developed force of contraction of both intact and denuded segments to methacholine. However, for both intact and denuded preparations, the corrected maximum developed force was not significantly different from the maximum developed force in the corresponding preparations not exposed to indomethacin. At 22°C indomethacin did not affect the maximum developed force of either control or denuded preparations.

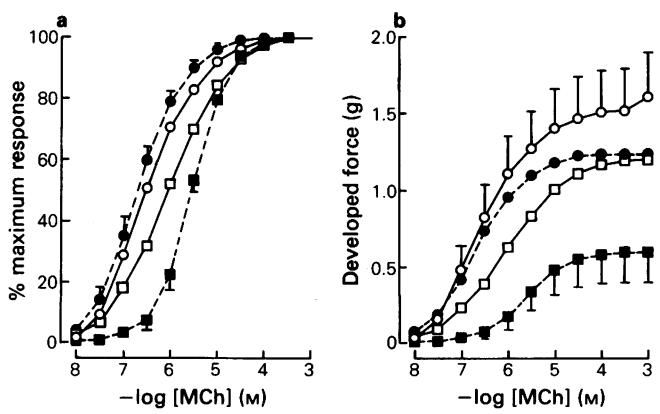


Figure 2 Guinea-pig isolated tracheal strips: effects of indomethacin (1 μM) on reactivity to methacholine in the presence and absence of the epithelium at 37°C and 22°C: (○) epithelium present, 37°C; (●) epithelium absent, 37°C; (□) epithelium present, 22°C; (■) epithelium absent, 22°C. Results are expressed as % of the maximum developed force of contraction (a) and as g developed force (b). $n = 6$.

Responses to abrupt temperature change

Abrupt bath temperature change from 37°C to 22°C did not affect the tone of intact or denuded segments appreciably ($n = 5$; not shown); a slight tension increase was followed by a reduction in tone to the original level. However, in intact segments abrupt increase in temperature from 22°C to 37°C elicited an immediate and pronounced relaxation, followed by a contraction ($n = 17$; Figure 3). A few of the intact segments ($n = 5$) responded to temperature increase with a slow, weak relaxation, followed by a contraction. In some preparations ($n = 17$) tension returned to baseline, while in others ($n = 5$) it did not. In contrast, the denuded preparations relaxed slowly and weakly upon temperature elevation; a spontaneous contraction was not observed ($n = 20$; Figure 3). These experiments were repeated ($n = 20$) in the presence of indomethacin (1 μM) to evaluate whether the relaxation involved the production of an inhibitory prostanoid. The results obtained were not different from those shown in Figure 3. To investigate whether the relaxation responses to temperature increase involved activation of the Na^+/K^+-pump, the effect of ouabain was examined. Ouabain (1 μM) did not inhibit the

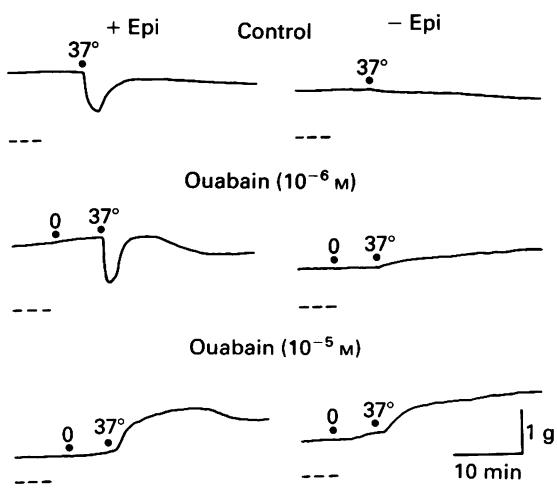


Figure 3 Guinea-pig isolated tracheal strips: representative tracings showing the effects of an abrupt temperature change from 22°C to 37°C in the presence (+Epi; left column of tracings) or absence (−Epi; right column of tracings) of the epithelium. (O) Addition of ouabain; (37°C) abrupt change from 22° to 37°C. The top row illustrates control tracings obtained in the absence of ouabain. Responses obtained in the presence of ouabain are shown in the middle (1 μM ouabain) and bottom (10 μM ouabain) rows. The dashed line below each tracing indicates zero force.

37°C-induced relaxation of intact preparations, but the relaxation of denuded segments was prevented and contraction was seen ($n = 4$; Figure 3). A ten fold higher concentration of ouabain (10 μM) was needed to inhibit the relaxation of intact segments ($n = 4$; Figure 3). Elevated temperature then evoked a contraction, as it had in denuded preparations (Figure 3).

Discussion

The modulatory effect of the epithelium on the sensitivity of guinea-pig trachealis to methacholine was dependent on the temperature of the physiological solution. As observed previously (Hay *et al.*, 1986), sensitivity to methacholine was increased at 37°C by epithelium removal. Although the sensitivity of intact segments at 22°C was the same as at 37°C, the sensitivity of denuded preparations at 22°C was reduced substantially in the absence of the epithelium. The effect of epithelium removal on maximum developed force also was temperature-dependent. There was no effect of the epithelium on the maximum force developed to methacholine at 37°C. However, at 22°C, denuded preparations developed smaller responses to methacholine while intact segments generated greater tension. The present findings indicate that the presence of epithelial cells at 22°C causes an increase in sensitivity and contractility to methacholine, i.e. an excitatory influence on reactivity.

While the reduced responsiveness of denuded segments at 22°C is surprising, potentiation of contractile agents at low temperatures has been observed previously in intact airway preparations of the ox (Souhrada & Souhrada, 1981), guinea-pig (Souhrada & Souhrada, 1981; Kolbeck *et al.*, 1988), man (Black *et al.*, 1984), rat (Ishii & Shimo, 1985) and rabbit (Bratton *et al.*, 1987). As was reported by Kolbeck *et al.* (1988) for guinea-pig airways, but unlike the observations in human airways made by Black *et al.* (1984), in the present study there were no substantial changes in tone upon abrupt temperature reduction from 37°C to 22°C. The alterations in sensitivity and maximum response observed at 22°C in both intact and denuded preparations were not, therefore, likely to be due to sustained change in basal tone.

The possibility that the different reactivities of intact and denuded segments to methacholine at 37°C and 22°C could be due to the influence of a prostanoid was examined. Methacholine concentration-response curves obtained at 22°C were not affected by indomethacin. However, at 37°C the increase in sensitivity observed after epithelium removal was mimicked in intact preparations by indomethacin, as has been reported previously (Hay *et al.*, 1986). At 37°C, the change in reactivity to many bronchoactive substances upon epithelium removal is not attributable solely to the loss of a source of prostanoids, as the cyclo-oxygenase inhibitor does not mimic epithelium removal under all circumstances (Fedan *et al.*, 1988). The failure of indomethacin to alter the reactivity-enhancing effect of the epithelium at 22°C suggests that the effect is entirely independent of prostanoids. The reason for the ineffectiveness of indomethacin at 22°C is of interest but is unexplained at present.

Reduced temperature inhibits electrogenic Na^+/K^+-pumping in guinea-pig trachealis (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981). The present study indicates that the epithelium also modulates the response of the muscle when the Na^+/K^+-pump is activated by raising the temperature rapidly from 22°C to 37°C. The relaxation of denuded preparations was attributable to the stimulation of the electrogenic pump, in so far as the response was inhibited by ouabain. A tenfold greater concentration of ouabain (10 μM) was required to inhibit the relaxation of intact segments. This suggests that EpDRF production is temperature-dependent, and that, once released, it stimulates Na^+/K^+-pump activity. The factor, in a sense, competes functionally with ouabain, and confers a degree of ouabain-resistance. A similar phenomenon was observed at 37°C in the effect of the epithelium on the K^+-induced, ouabain-sensitive relaxation response (Raeburn & Fedan, 1989). EpDRF, by stimulating electrogenic pump activity, may induce membrane hyperpolarization of guinea-pig trachealis and decrease reactivity to depolarizing agents.

Two procedures which activate electrogenic Na^+/K^+-pumping, i.e. the addition of K^+ to K^+-free solution (Raeburn & Fedan, 1989) and rapid warming (present study), induce relaxation responses which are sensitive to ouabain and yet modified by the epithelium. Nevertheless, the effects of ouabain at 37°C and reduced temperature on reactivity to methacholine are not equivalent. At 37°C ouabain increased sensitivity to methacholine but did not affect the maximum response, and mimicked epithelium removal in intact preparations (Raeburn & Fedan, 1989). If the effect of reduced temperature on reactivity to methacholine were to result solely from inhibition of the Na^+/K^+-pump, then sensitivity of the preparations at 22°C also should have been increased, but this was not observed even though evidence was obtained that the pump had been inhibited (Figure 3). A difference between the effect of ouabain at 37°C and reduced temperature is that a reduction in temperature probably interferes with other processes in the smooth muscle cell related to reactivity. It has been suggested that alterations in the release of intracellular Ca^{2+} and Ca^{2+} extrusion mechanisms may be involved in cooling-induced potentiation of responses of (presumably) epithelium-containing tissues (Ishii & Shimo, 1985; 1987). In view of the profound effects of epithelium removal on the sensitivity and maximum responses to methacholine at 22°C, the possible involvement of epithelium-derived factor(s) in these mechanisms might be worthy of investigation.

In summary, the modulatory influence of the epithelium on reactivity of guinea-pig trachealis is changed at reduced temperature, i.e. the epithelium decreases reactivity at 37°C and increases reactivity at 22°C. The epithelium influences the relaxation response of the cooled smooth muscle to abrupt warming, which involves stimulation of electrogenic Na^+/K^+-pumping. The modulation of reactivity at 37°C may involve, at least in part, stimulation of electrogenic Na^+/K^+-pump activity.

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Effects of pinaverium on voltage-activated calcium channel currents of single smooth muscle cells isolated from the longitudinal muscle of the rabbit jejunum

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1 Smooth muscle cells of the longitudinal muscle of the rabbit jejunum were dispersed by enzyme treatment and recordings of membrane current were made in the whole-cell mode by patch clamp technique. The action of pinaverium bromide on the voltage-dependent inward current of single isolated smooth muscle cells was studied in solutions containing normal concentrations of calcium or high concentrations of barium at room temperature.

2 Pinaverium reduced the voltage-dependent inward current with an IC_{50} of 1.5 μM. This IC_{50} is similar to those of verapamil, diltiazem and flunarizine on these cells as described by others. Occasionally evidence of a potentiating action of pinaverium on the inward current was seen.

3 Repetitive stimulation of the cells did not increase blockade of inward current by pinaverium unlike the use-dependent blockade seen with verapamil, methoxyverapamil, and diltiazem in these and in other smooth muscle cells.

4 The inactivation of inward current was studied by holding at various potentials for 2 or 10 s before evoking inward current. The voltage at which current was 50% available was changed very little by pinaverium although other calcium entry blockers, for example the dihydropyridines, have been reported to produce appreciable negative shifts which indicate considerable voltage-dependence of their blockade. This may indicate that pinaverium has similar affinities for the closed available and inactivated calcium channel states so that blockade is not appreciably voltage-dependent.

Introduction

Pinaverium bromide has been used to treat hypermotility disorders of the intestine including irritable bowel syndrome (see Bretaudeau & Foussard-Blanpin, 1980 for references). Pinaverium reduced the contractile activity of canine gastrointestinal muscle and slowed the propagation of the myoelectric complex (Itoh & Takahashi, 1981; Grenier *et al.*, 1983). In experiments on isolated smooth muscle preparations, pinaverium inhibited contractions of intestinal muscle to nerve stimulation, barium chloride and acetylcholine. The inhibition was non-competitive and was antagonized by increasing the calcium concentration of the bathing solution (Bretaudeau & Foussard-Blanpin, 1980; Baumgartner *et al.*, 1985). However, pinaverium has little blocking activity at muscarinic receptors (Bretaudeau *et al.*, 1975) suggesting that its spasmolytic action is exerted in some other way. Pinaverium binds to calmodulin (Ronca-Testoni *et al.*, 1985) but this does not seem to be the basis of its action (Wuytack *et al.*, 1985). Rather it seems to have calcium antagonist (calcium entry blocking) properties although at higher concentrations (10^{-5} M) it may also inhibit intracellular calcium store release. Pinaverium reduced the frequency, amplitude, maximum rate of rise and fall of action potentials in guinea-pig ileum and in uterus without much effect on the resting potential (Droogmans *et al.*, 1983; Mironneau *et al.*, 1984; Wuytack *et al.*, 1985). Contractions of smooth muscle of rabbit ear artery to noradrenaline are much less sensitive to the blocking action of pinaverium than are contractions to high potassium solutions. Resting calcium uptake is not affected by pinaverium, but the calcium uptake in response to high potassium solution and the inward current in myometrial strips are markedly depressed (Droogmans *et al.*, 1983; Mironneau *et al.*, 1984).

The results strongly suggest that pinaverium has calcium antagonist, or calcium-entry blocking, properties. The experiments described in this paper were designed to examine these properties in more detail in single, dispersed, voltage-clamped intestinal smooth muscle cells. A number of calcium antagonists have been studied for their effects on the inward current in these cells: these include dihydropyridines (Droogmans & Callewaert, 1986; Bolton *et al.*, 1987; Terada *et al.*, 1987a,b), flunarizine (Terada *et al.*, 1987c) and methoxyverapamil (D600) or verapamil (Ohya *et al.*, 1987; Terada *et al.*, 1987a). The actions of pinaverium were compared with the known actions of other calcium antagonists on the voltage-dependent inward current in these cells.

Methods

Cells were dispersed from the longitudinal smooth muscle of the rabbit jejunum. Four pieces ($\approx 3 \times 4$ mm) were cut and incubated in a low Ca^{2+} (2 μM added) physiological salt solution (PSS) for 10 min at 37°C. They were subsequently incubated in nominally Ca^{2+}-free PSS containing papain (5 mg ml^{-1}), dithiothreitol (5 mM) and bovine serum albumin (BSA, 5 mg ml^{-1}) for 10–15 min and then collagenase (0.2 mg ml^{-1}, Sigma type XI), trypsin inhibitor (1 mg ml^{-1}) and BSA (5 mg ml^{-1}) for 5–10 min, both at 37°C. After dilution of the enzymes, cells were agitated in the nominally Ca^{2+}-free PSS. The selected suspension of cells was then centrifuged at 100 *g* for 2 min and the pellet re-suspended in $\approx$0.8 mM Ca^{2+} containing PSS. Cells were maintained at 4°C and used within 12 h. All experiments were at room temperature (20–25°C).

The PSS in the bath had the following ionic composition (mM): Na^+ 125, K^+ 6, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 138.4, glucose 10.5, HEPES 10, and was titrated to pH 7.2 with NaOH. The high-barium bath solution had the composition (mM): Ba^{2+} 110, Cl^- 220, HEPES 10, and was titrated to pH 7.2 with NaOH. The pipette (= intracellular) solution had the composition (mM): Na^+ or Cs^+ 130, Mg^{2+} 3.2, TEA^+ 10, Cl^-

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142.4, ATP 2, glucose 11.5, HEPES 5, EGTA 2, and was titrated to pH 7.2 with tetraethylammonium hydroxide.

Cells were voltage-clamped using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). The current-voltage converter was a List EPC-7 and analogue signals were recorded on FM-tape (Racal) at a tape-speed of 7.5 in s^{-1}. Patch pipettes (2–4 MΩ) were made from borosilicate glass. Signals were digitized at 2 kHz using a CED1401 ADC interface in conjunction with a BBC microcomputer and displayed on a graph plotter (Hewlett Packard), or were displayed directly on chart paper (Gould 2400). In both instances, signals were first low-pass filtered at 1 kHz (8-pole Bessel, Barr & Stroud). Functions were fitted by minimizing the unweighted sum of the squares of the deviations using a Marquardt-Levenberg algorithm. Leak current was estimated by applying five different short (0.1 s) and small (<20 mV) hyperpolarizing steps from the holding potential. It was subtracted either by an analogue circuit, which subtracted a scaled linear component directly, or by fitting the mean leakage currents elicited by the hyperpolarizing steps with a straight line and subtracting this function from the currents elicited by depolarizing voltage-steps. Leak subtracted currents are shown in the figures.

Pinaverium bromide (Dicetal) is 4-(6-bromoveratryl)-4-[2-{2-(10 norpinan-2-yl)ethoxy}ethyl] morpholinium bromide and was obtained from Latema (LTM) Paris. It was diluted for use from a 10 mM stock solution in 100% ethanol. Tetraethylammonium (TEA) hydroxide was obtained from Sigma.

Results

In cells held at negative potentials (−60 to −90 mV) under voltage clamp, stepping to potentials positive to −60 mV elicited inward calcium current which was relatively well sustained for smaller depolarizations but which inactivated very substantially for larger depolarizations lasting 0.5 s (Bolton *et al.*, 1987). This current was reduced but not completely abolished by pinaverium (1–100 μM) (Figure 1). The IC_{50} for pinaverium was about 1.5 μM (Figure 2). Peak current was reduced at all potentials (Figure 2a) but the time course of inactivation was not greatly affected (Figure 1).

In cells held under voltage clamp for a period, inward current evoked at various intervals decreased in size although in the best cells this decrease was small. However, in some cells the application of pinaverium (0.01–0.1 μM) occasionally increased the current either close to threshold or by producing a small negative shift in the current-voltage relationship (Figure 3). Although in Figure 3 a −90 mV holding potential was used, potentiation was also seen in another cell held at −70 mV. Since the effect was quite marked on some occasions whereas a slow decline in current size was normally seen in the absence of pinaverium, it seemed possible that the potentiating effect resembles that observed with nifedipine (Aaronson *et al.*, 1988).

The block by pinaverium was not conspicuously use-dependent. If pulses (0.1 s) were applied every 20 s, the reduction of inward current was only slightly greater (57%)

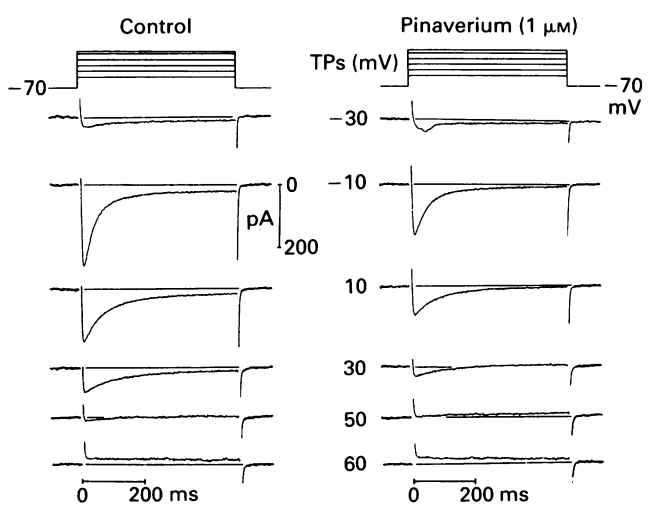


Figure 1 Effect of pinaverium on inward calcium current in a cell bathed in physiological salt solution (PSS) containing 2.5 mM calcium. Leak current was subtracted in all cases (see Methods) and for all subsequent figures. The cell was held at −70 mV in the absence (control) and then in the presence of 1 μM pinaverium and stepped to the indicated test potentials.

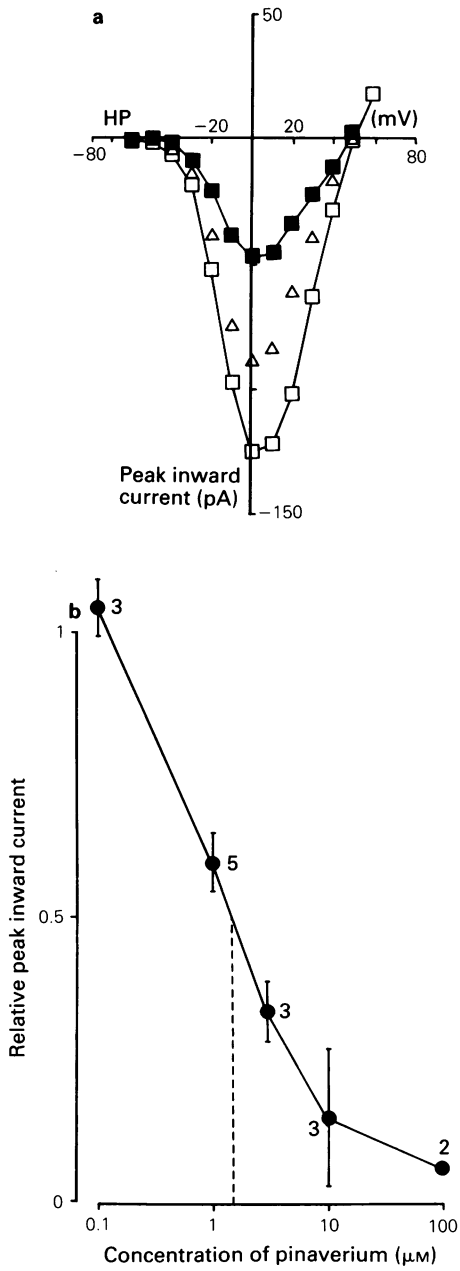


Figure 2 (a) Current-voltage relationship for peak inward calcium current activated from a holding potential (HP) of −70 mV. Control (□), block after 5 min in the presence of 3 μM pinaverium (■) and partial recovery 5 min after wash-out of drug (△) are shown. Ordinate: peak inward current. Abscissa scale: membrane potential. (b) Inhibition by pinaverium of peak inward current evoked in a series of single cells in solution containing 2.5 mM calcium concentration. The dotted line indicates the IC_{50} concentration (1.5 μM) and the numbers of cells involved are indicated by each point (mean with s.e.mean shown by vertical lines). Cells were held at −70 mV and stepped to +10 mV.

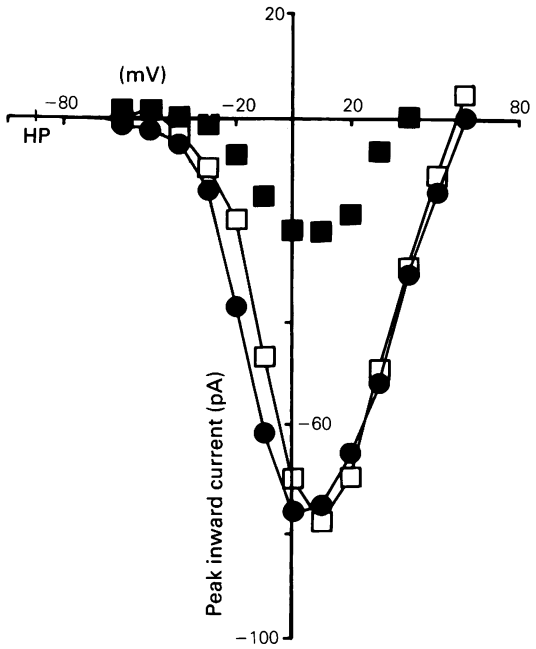


Figure 3 Potentiation of inward current by pinaverium seen in the negative potential range in some cells. Current-voltage relationship for peak inward calcium current activated from a holding potential (HP) of −90 mV. Control (□), increase in the presence of 0.01 μM (●) and block in the presence of 10 μM (■) pinaverium. Ordinate scale: peak inward current. Abscissa scale: membrane potential.

after 15 pulses than it was for the first pulse of a train (51%) (Figure 4). A similar result was obtained when normal solution (2.5 mM) calcium was substituted by a high-barium solution which causes the rate of inactivation of the current to slow (Bolton *et al.*, 1988). Reduction of peak current by 1 μM pinaverium was 16% after 16 pulses at 1 s intervals compared to 14% with the first pulse after a 2 min rest and 69% and 68% at the corresponding pulses in the presence of 10 μM pinaverium (Figure 5a). The smaller effect of 1 μM pinaverium in high-barium solution (Figure 5) compared to normal Ca solution (Figure 4) was not further investigated and may simply reflect experimental variation. The decline of current during a 2 s pulse in a solution containing 110 mM barium was also

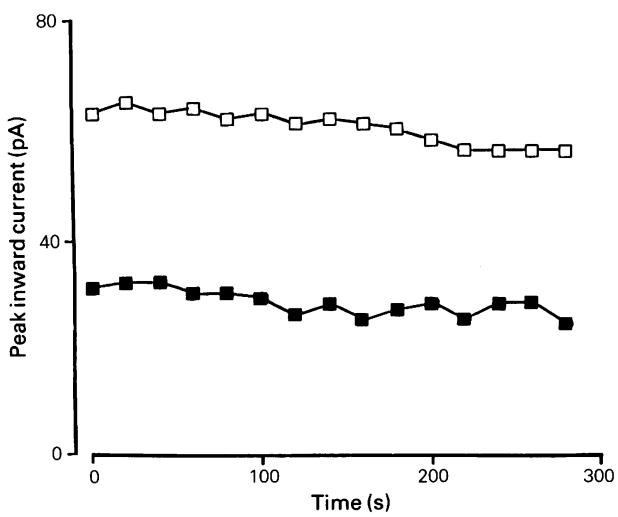


Figure 4 Block of calcium current by pinaverium is not use-dependent. The cell was stepped to −10 mV for 0.1 s every 20 s from a holding potential of −70 mV when bathed in PSS containing 2.5 mM calcium concentration. Peak inward current during a train of pulses is plotted against time for the control (□) and after 2 min at the holding potential in 1 μM pinaverium (■).

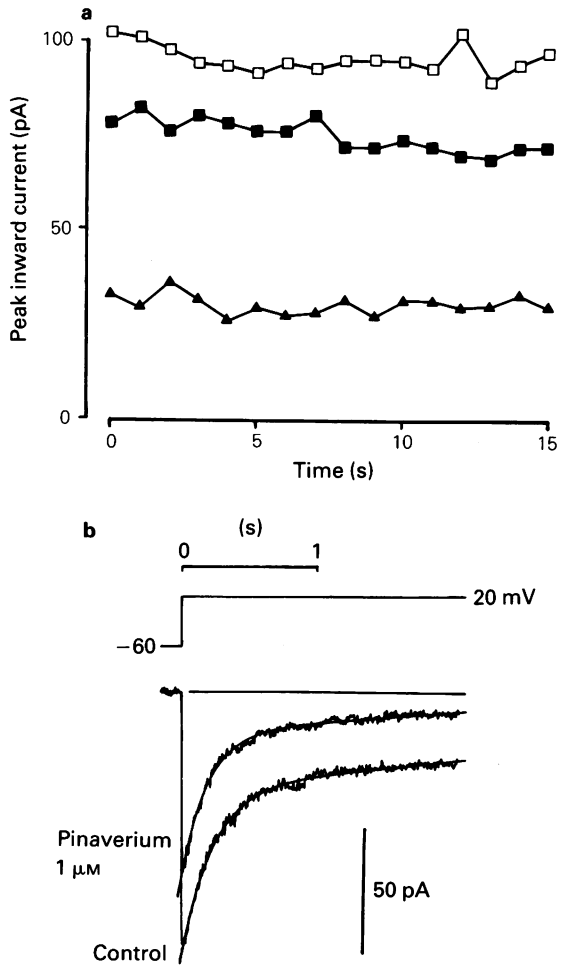


Figure 5 Action of pinaverium on inward current in a high-Ba solution. In (a) the cell was stepped to +20 mV for 0.1 s every 1 s from a holding potential of −60 mV when bathed in 110 mM barium-solution. Peak inward current is plotted against time for the control (□), and after 2 min at the holding potential in 1 μM (■) and then in 10 μM pinaverium (▲). (b) In the same cell, the effect of pinaverium on the rate of decay of inward current over 2 s at +20 mV is shown. The currents are each the average of 3 records. Bi-exponential functions with a constant were fitted (shown) and these had the time constants (τ) 0.22 and 9.1 s for the control, and 0.18 and 13.7 s in 1 μM pinaverium.

slightly affected; two exponentials could be fitted to the time course of the current in the control; the first was slightly accelerated and the second of these was moderately slowed in pinaverium (Figure 5b).

The availability of the inward current in 2.5 mM calcium was tested by measuring the absolute size of peak current evoked by a test potential of +20 mV after holding for 2 s at various conditioning potentials. The holding potential was −90 mV. More positive conditioning potentials than −90 mV reduced the availability of inward current, especially positive to −60 mV. The current was half available at $V_h = -39$ mV, and the slope, V_s, was 7.5 mV, when a Boltzmann equation of the form $I/I_{max} = [1 + \exp\{(V - V_h)/V_s\}]^{-1}$ was fitted to the inactivating component of the current. V is the conditioning membrane potential and I/I_{max} the fraction of current available relative to that available from −90 mV. Pinaverium (1 μM) reduced the peak current evoked at +20 mV from all potentials with little change in V_h (−40 mV) or V_s (8.5 mV) (Figure 6) i.e. the fractional reduction in current was similar at all conditioning potentials.

Similar results were obtained in solutions where 2.5 mM Ca was replaced by 110 mM barium. Under these conditions the availability was shifted positively ($V_h = -7$ and −13 mV for 2 s and 10 s conditioning pulses) by about 30 mV (see also

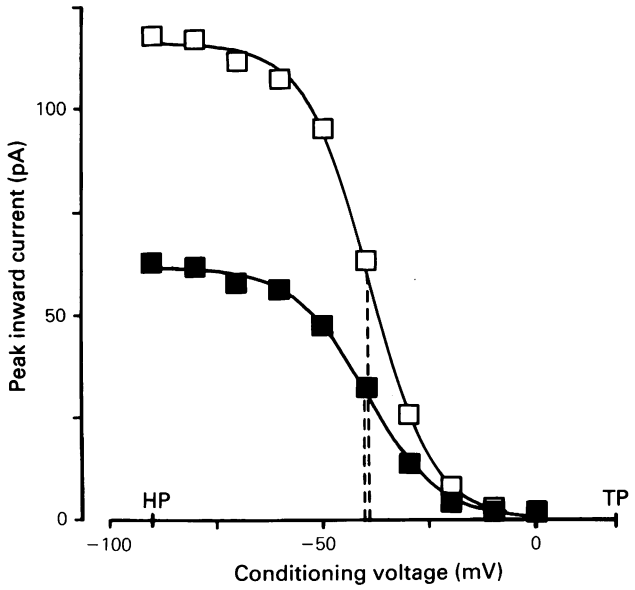


Figure 6 The effect of pinaverium on the inactivation curves for the inward current (2.5 mM calcium in bathing solution). Inward current was elicted by stepping to +20 mV for 0.5 s. Cells were held at the conditioning voltage for 2 s, and 12.5 s was allowed for recovery from inactivation at the holding potential (HP) (−90 mV). Points represent peak inward current and they were fitted by the Boltzman equation (see text). Inward current was 50% inactivated at −39 mV (control, □) and at −40 mV in 1 μM pinaverium (■).

Aaronson *et al.*, 1988). Pinaverium (1 μM) had a small effect on V_h (−19 mV) if a 10 s conditioning pulse was used (Figure 7b) although only a small change in V_h was seen (V_h = −9.6 mV) if a 2 s conditioning pulse was used (Figure 7a). Slope factors (V_s) were 16.7 and 10 mV (2 s and 10 s conditioning pulses) in controls and 10.9 and 12 mV in pinaverium.

Discussion

The results described in this paper indicate that pinaverium blocks the calcium currents in single isolated intestinal smooth muscle cells with a potency very similar to that of verapamil (Terada *et al.*, 1987a), diltiazem (Terada *et al.*, 1987a) and flunarizine (Terada *et al.*, 1987c) although it is much less potent than dihydropyridines such as nicardipine (Terada *et al.*, 1987a). Dihydropyridines such as KW-3049 (Terada *et al.*, 1987b) and CV-4093 (Okabe *et al.*, 1987) are also more potent in single vascular smooth muscle cells. When negative holding potentials (e.g. −80 mV) and/or low concentrations are used, dihydropyridine calcium entry blockers increase rather than decrease inward calcium or barium current (Okabe *et al.*, 1987; Terada *et al.*, 1987a; Aaronson *et al.*, 1988). Pinaverium also sometimes potentiated the inward current, although it is not a dihydropyridine and this result may indicate that it acts at the same binding site on the calcium channels as dihydropyridines or that potentiation is a more general feature of the mechanism of action of calcium entry blockers on the calcium channel. Potentiation could be masked in the case of certain drugs normally considered to be antagonists, by their much stronger blocking action on the channel.

Very little negative shift in the availability curve was observed with pinaverium even when 10 s conditioning pulses were used. Under these conditions dihydropyridines cause a distinct negative shift (Terada *et al.*, 1987a,b; Okabe *et al.*, 1987) as do diltiazem and verapamil (Terada *et al.*, 1987a). The lack of shift with pinaverium suggests that the drug has similar affinities for the closed available state and the inactivated state of the calcium channel; thus holding at a depolarized potential, which would substantially increase the

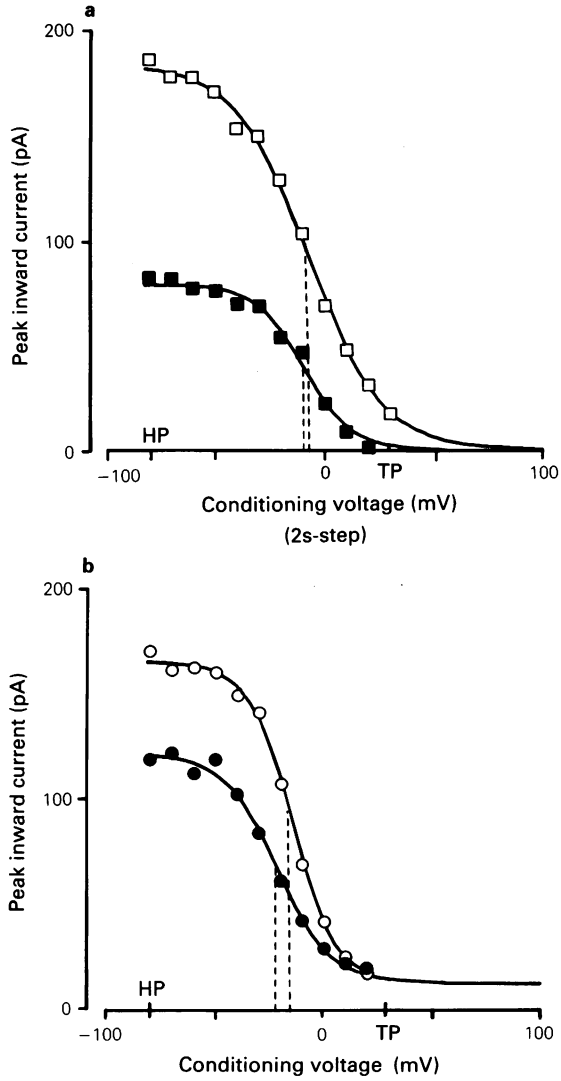


Figure 7 The effect of pinaverium on the inactivation of inward current (110 mM barium in bathing solution). Inward current was elicted by stepping to +30 mV for 0.5 s. Conditioning potential step-length was 2 s (a) and 10 s (b), and 7.5 s (a) and 4.5 s (b) were allowed for recovery from inactivation at the holding potential (−80 mV). Points are for peak inward current and the fitted Boltzmann equations indicated that 50% inactivation occurred at −7 mV (a) and −13 mV (b) for the controls (a, □: b, □), and −9.6 mV (a) and −19 mV (b) when in 1 μM pinaverium (a, ■: b, ●).

fraction of channels in the inactivated state with a corresponding reduction in the fraction in the resting (closed) state, apparently did not increase affinity for the channel. In the case of other calcium entry blockers, this increasing affinity is seen as an increasing reduction of current as the holding potential is made less negative, a phenomenon which has led to the postulation of the 'modulated receptor hypothesis' (Bean, 1984; Bean *et al.*, 1986) to explain the phenomenon. Essentially this hypothesis invokes a much higher affinity of the drug for the inactivated as compared to the resting (closed) state of the calcium channel. However, in the case of D600, verapamil and possibly diltiazem, the negative shift in the availability curve may indicate entry into and block of the open channel as an alternative mechanism of blockade (Hering *et al.*, 1989).

D600, verapamil and diltiazem (Ganitkevich *et al.*, 1986; Terada *et al.*, 1987c; Hering *et al.*, 1989) differed from pinaverium in that blockade was increased by repetitive stimulation. D600 blockade is dramatically reversed even at 10^{-4} M by a 3 min rest period (Hering *et al.*, 1989) but no such rest recovery was seen with pinaverium. Nifedipine also showed no

use-dependent blockade if first equilibrated (Hering *et al.*, 1988) and blockade by nicardipine is increased little if at all by stimulation (Terada *et al.*, 1987a). This also seems to be true of other dihydropyridines in smooth muscle (Okabe *et al.*, 1987; Terada *et al.*, 1987b).

The results suggest that pinaverium blockade of calcium channels in single isolated cells of small intestine occurs by a mechanism which differs from those of other calcium entry blockers so far studied in detail on these cells. Pinaverium blockade was neither appreciably use-dependent nor voltage-dependent, which implies that it probably does not enter the channel in its open state to produce block as D600 or verapamil may do, and may combine with closed and inactivated channel states with similar affinities.

Two or more types of voltage-dependent calcium channel have been distinguished on the basis of their different unitary conductances and pattern of channel openings (e.g. Worley *et al.*, 1986; Benham *et al.*, 1987; Yatani *et al.*, 1987). In some smooth muscles it has been inferred that these separate populations contribute components to the voltage-dependent whole-cell calcium current which can be identified by various voltage protocols. In the case of longitudinal muscle of rabbit small intestine, a more rapidly inactivating component of inward current becomes available if more negative holding potentials are used. However, it is uncertain whether this component represents current through a separate population of calcium channels (Bolton *et al.*, 1988). In the present experiments, no evidence for more than one component of voltage-dependent inward current was found on the basis of the sensitivity to pinaverium.

These results indicate that pinaverium is a calcium entry blocking drug which is active in blocking the voltage-dependent calcium current responsible for the action potential in intestinal smooth muscle. Its action at this site *in vivo* would be expected to reduce the velocity of action potential upstroke and attenuate action potential discharge and contraction in the intestine. This property might be expected to be useful in conditions such as irritable bowel syndrome which are characterized by hypermotility of the smooth muscle.

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The role of the endothelium in mediating the actions of ATP, adenosine and acetylcholine on flow through blood vessels in the rabbit knee joint

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1 An *in vitro* preparation of the rabbit knee joint, perfused with oxygenated Locke solution, was used to investigate the presence of purinoceptors and the role of endothelium within articular blood vessels.

2 The basal tone of the blood vessels was not affected by adenosine or acetylcholine. Adenosine 5'-triphosphate (ATP) injection produced vasoconstriction which was unaffected by removal of the endothelial layer, but diminished by α, β methylene ATP, a compound which desensitizes P_2-purinoceptors.

3 When knee joint blood vessel tone was raised by perfusion with vasopressin (10^{-8} M) or 5-hydroxytryptamine (10^{-5} M), acetylcholine, ATP and adenosine were all found to induce concentration-dependent relaxation of these vessels. ATP was found to have a dual effect of transient constriction followed by longer-lasting dilatation.

4 3-Methylxanthine, a P_1-purinoceptor antagonist significantly reduced the relaxation response to adenosine but had no effect on the vasodilator effect of ATP.

5 Removal of the endothelial layer virtually abolished the vasodilator effects of acetylcholine and ATP but not adenosine.

6 These results demonstrate that articular blood vessels supplying the rabbit knee contain P_1-purinoceptors located on the vascular smooth muscle which mediate vasodilatation. P_2-purinoceptors mediating a constrictor effect are also present on this smooth muscle. It is likely that the vasodilator effect of ATP is mediated via P_2-purinoceptors located on the endothelial layer.

Introduction

Despite the prevalence of inflammatory joint disease, relatively little is known about the factors which influence blood flow to joints. Although there have been numerous *in vitro* and *in vivo* studies of blood vessels in a variety of tissues which have characterized their responses to different agents, little is known about articular blood vessels.

Cobbold & Lewis (1956) found that close intra-arterial injection of adrenaline and noradrenaline both produced vasoconstriction of blood vessels in the canine knee joint. This finding has recently been confirmed in a more extensive and quantitative investigation of rabbit knee joint blood vessels using different α- and β-adrenoceptor agonists and antagonists (Ferrell & Khoshbaten, 1989). β-Adrenoceptors were found to be absent and the vasoconstrictor effects of the catecholamines were mediated by α_1-adrenoceptors.

In recent years attention has been focussed on purine nucleotides and nucleosides which have been found to have widespread and potent vascular actions (Haddy & Scott, 1968; Burnstock, 1980; Su, 1981; Burnstock *et al.*, 1984). Some of these actions appear to be mediated via the endothelium as Furchgott (1981) found that removal of the endothelial layer of dog arteries abolished the vasodilator action of acetylcholine (ACh), adenosine 5'-triphosphate (ATP), ADP, substance P and bradykinin. Subsequently, it was shown that ATP, but not adenosine, induced vasodilatation via a purinoceptor located on the endothelium in many different blood vessels (De Mey & Vanhoutte, 1981; 1982; 1983; Vanhoutte & Rimele, 1983; Burnstock & Kennedy, 1986). However, other studies have found that the vasodilator action of adenosine can be mediated via the endothelium (Gordon & Martin, 1983; Frank & Bevan, 1983). Kennedy & Burnstock (1985) and Burnstock & Kennedy (1986) have shown that ATP can produce vasoconstriction via P_2-purinoceptors located on vascular smooth muscle cells.

The aim of these experiments was to assess the types of purinoceptors present on articular blood vessels and whether or not any effects of ATP, adenosine and acetylcholine are mediated via the endothelium.

Methods

White New Zealand rabbits of either sex weighing between 2–2.3 kg, were killed by a blow to the skull followed by exsanguination. Immediately thereafter, the posterior aspect of the knee joint was exposed, the popliteal artery cannulated and its muscular branches ligated as described by Ferrell & Khoshbaten (1989). The tissue was perfused with warmed and oxygenated Locke solution by means of a peristaltic pump (Gilson Minipuls). The knee was separated from the animal by sawing through the femur above the point of cannulation. The lower part of the limb was also removed by sawing mid-way through the tibia. The isolated knee joint preparation was then transferred to a thermostatically controlled bath (37 ± 1°C) containing oxygenated Locke solution. At the start of the experiment the pump was set to provide a perfusion rate of 0.5–0.8 ml min^{-1} which resulted in a perfusion pressure of about 40–50 mmHg as measured by a pressure transducer connected 'downstream' from the pump. After a 30 min equilibration period a steady resting pressure was achieved and subsequent changes in perfusion pressure provided an indirect means of assessing articular blood vessel calibre. Peak response was compared to control (pre-injection) value and expressed as percentage change from control (or baseline). 'Control' injections of Locke solution were administered periodically and were found to produce little change in pressure, apart from a transient rise during the injection phase.

Vascular tone was increased by the addition of vasopressin 10^{-8} M or 5-hydroxytryptamine (5-HT) 10^{-5} M or 10^{-6} M to the perfusate. These resulted in perfusion pressure increases of about 160–200% of control (100%) values.

Removal of the endothelial layer in these preparations was achieved by perfusion with 1–3 mg ml^{-1} solution of sodium

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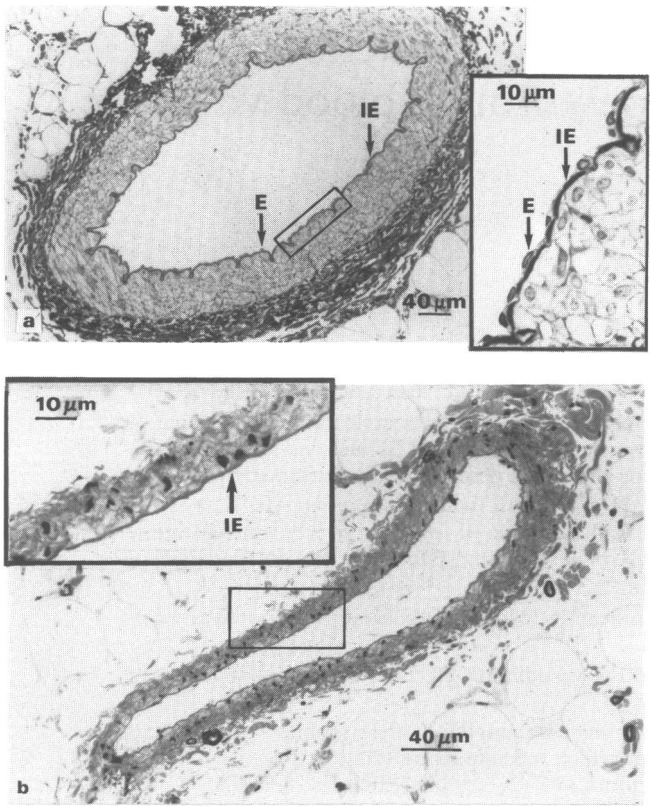


Figure 1 Photomicrographs of blood vessels from the rabbit knee joint. (a) Normal appearance of a muscular artery; inset shows a higher power view of the area outlined, with endothelial cells (E) lining the internal elastic lamina (IE). Haemalum and eosin. (b) After perfusion with deoxycholate, endothelial cells no longer line the blood vessel. Inset shows the internal elastic lamina (IE) intact but an absence of endothelial cells. Toluidine blue.

deoxycholate, in Locke solution for 30 s on two separate occasions 1 min apart (Byfield *et al.*, 1986). As illustrated in Figure 1, this procedure was effective in disrupting the endothelial layer in five preparations tested.

The composition of Locke solution was (mM): NaCl 115, KCl 4.7, $NaHCO_3$ 24.1, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2 and glucose 5.6. $CaCl_2$ was added after oxygenating the solution with 95% O_2 and 5% CO_2 mixture. Compounds at different concentrations were administered as a bolus (0.2 ml) injection into tubing beyond the pump. In the case of antagonists, these were added to Locke solution which was then perfused continuously. The following agents were administered: [Arg^8]-vasopressin acetate salt, adenosine, adenosine 5'-triphosphate (sodium salt), α, β methyleneadenosine 5'-triphosphate (lithium salt), 5-hydroxytryptamine HCl (5-HT), deoxycholic acid (sodium salt), acetylcholine HCl, 3-methylxanthine, and atropine sulphate, all from Sigma. All drugs were dissolved in Locke solution.

Statistical data analysis was by either paired or unpaired *t* test. An *F* test was used to test the assumption of homogeneity of variances. Where this exceeded tabled *F* values, modified *t* values were generated using the formula described by Phillips (1978). All responses have been expressed as the means ± s.e.mean. Differences between means were considered significant if the *P* values were 5% or less (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). In all graphs data are presented as mean ± s.e.mean.

Results

Basal vascular tone

Intra-arterial injection of ATP in doses ranging from 0.02 to 2 μM produced vasoconstriction of articular blood vessels

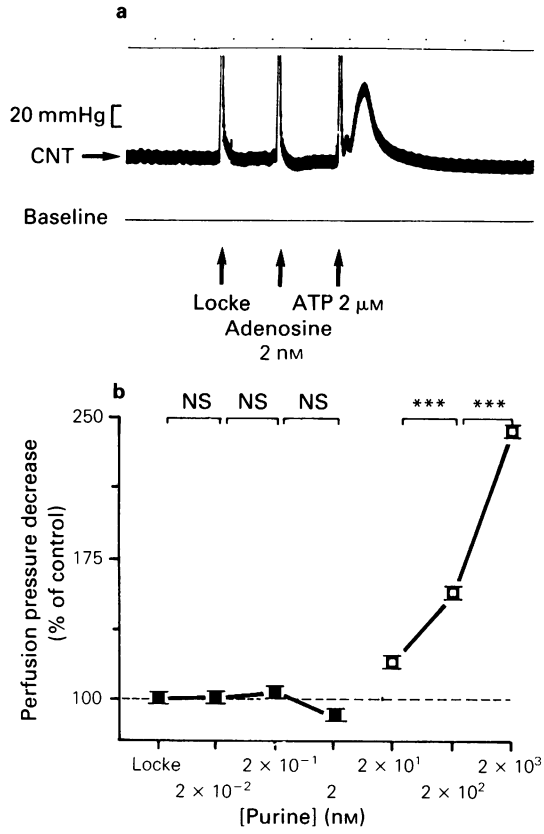


Figure 2 (a) Trace showing the effect of injection of Locke solution, adenosine and ATP on the perfusion pressure. CNT indicates the control pressure prior to injection. The interval between each dot on the timescale indicates 1 min. (b) Dose-response relationship for adenosine (■) and ATP (□). Locke indicates response to injection of Locke solution. *** $P < 0.001$. In both (a) and (b) the responses were obtained with basal vascular tone. For adenosine $n = 7$ at each dose, and for ATP $n = 8$–11.

(Figure 2a) which was dose-dependent (Figure 2b). Maximum vasoconstriction was usually achieved within a minute, but was not maintained (Figure 2a). Almost no desensitization was observed, even when the drugs were re-administered after a short interval.

Exogenous adenosine (0.02 to 2 nM) had no vasoconstrictor effect on knee joint blood vessels (Figure 2a,b) but a slight and transient dilator effect was observed at 2 nM. In a few preparations higher doses of adenosine were tried but no further vasodilatation occurred. Acetylcholine (ACh, 0.2 to 20 nM) also failed to constrict these vessels except in two preparations where ACh did elevate perfusion pressure when administered as a single dose. Perfusion with α, β methylene ATP transiently increased the perfusion pressure but this was not sustained. None of the above constrictor responses was reduced by removal of endothelium.

Raised vascular tone

Vascular tone was raised by the addition of either vasopressin 10^{-8} M (before ACh injection) or 5-HT 10^{-5} M or 10^{-6} M (before purine injections) to the perfusate. Adenosine in doses from 0.02 to 2 nM and ATP (0.02 to 2 μM) were then found to produce concentration-dependent relaxation of articular blood vessels (Figure 3b). The dual effect of ATP is illustrated (Figure 3a), consisting of a transient constriction followed by a long-lasting dilatation. Only rarely was the initial constrictor response absent.

Perfusion of the preparation with 3-methylxanthine (10^{-5} M), a P_1-purinoceptor antagonist (Burnstock, 1978), did not affect the vasodilator action of ATP but abolished the response to injected adenosine (Figure 3b). The dilator action

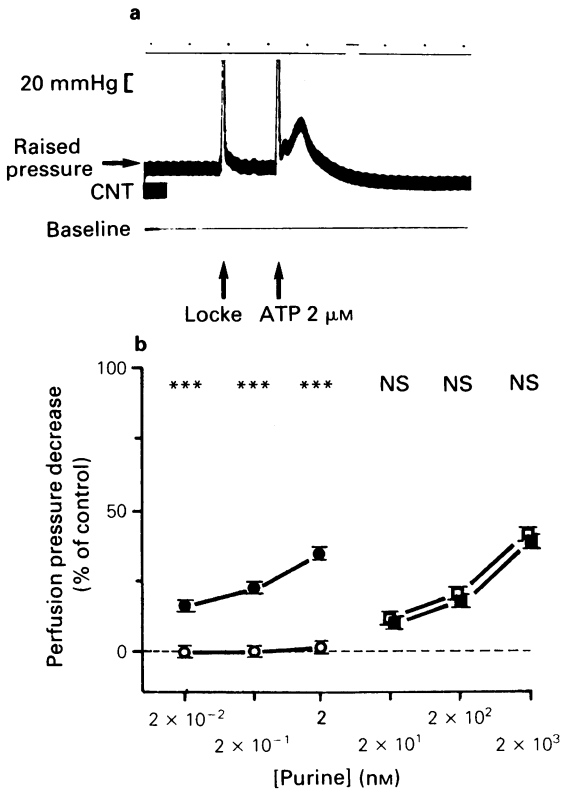


Figure 3 (a) Trace showing the dual effect of ATP on joint blood vessels after elevation of vascular tone by perfusion with 5-hydroxytryptamine (10^{-6} M). CNT represents the perfusion pressure prior to raising blood vessel tone. Small dots represent 1 min intervals. (b) Vasodilator effect of adenosine (●) and ATP (■) on blood vessels with raised tone. During perfusion with the P_1-purinoceptor antagonist 3-methylxanthine (10^{-5} M), the response to adenosine (○) was abolished ($n = 7$) but the response to ATP (□) was unaffected ($n = 6$).

of adenosine was unaffected by removal of the endothelial layer suggesting that this is mediated via P_1-purinoceptors on the smooth muscle cells of the knee joint blood vessels. The vasoconstrictor effect of ATP was mediated via P_2-purinoceptors as perfusion of articular blood vessels with

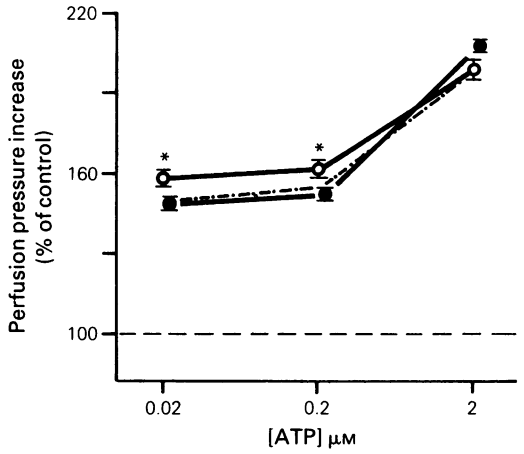


Figure 5 The constrictor response to injected ATP before (●) and after removal of the endothelial layer by sodium deoxycholate pretreatment (○). The dot-dash line shows the response to ATP during perfusion with 3-methylxanthine (10^{-5} M). In both cases vascular tone was elevated by perfusion with 5-hydroxytryptamine (10^{-5} M). $n = 6$–7.

α,β methylene ATP, a potent P_2-purinoceptor desensitizing agent, abolished the vasoconstrictor effect of ATP (Figure 4a). This vasoconstriction was unaffected by perfusion with 3-methylxanthine but was slightly increased after removal of the endothelial layer (Figure 5).

The vasodilator action of ATP was mediated via the endothelium as pretreatment with deoxycholic acid (DCA) virtually abolished this response (Figure 4b). As the dilator response was not mediated via P_1-purinoceptors (Figure 3), ATP may be acting via P_2-purinoceptors on the endothelial layer.

ACh was found to have inconsistent effects on blood vessel calibre prior to elevation of vascular tone. In two preparations constriction was obtained, in one relaxation occurred and in a further three no effect was observed. However, when tone was elevated by perfusing with vasopressin (10^{-8} M), ACh then produced dose-dependent (0.2 to 20 nM) vasodilatation which was mediated via muscarinic receptors as it could be blocked by atropine. The receptors mediating this effect were located on the vascular endothelial layer as its removal virtually abolished the actions of ACh (Figure 6).

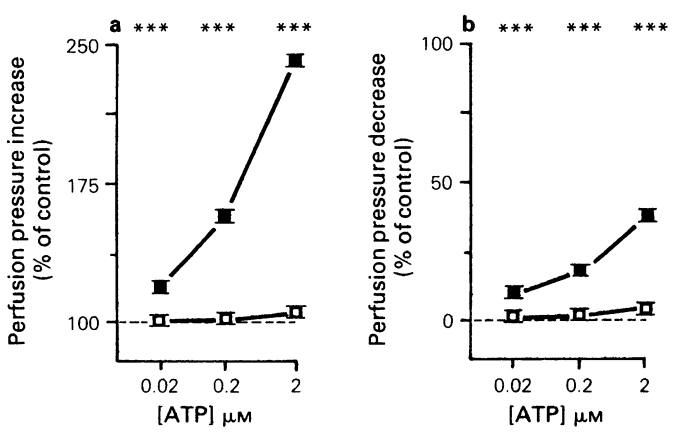


Figure 4 (a) Constrictor response to injected ATP before (■) and during perfusion (□) with the P_2-purinoceptor desensitizer α,β methylene ATP (10^{-6} M). Responses in blood vessels prior to elevation of vascular tone ($n = 7$). (b) Dilator response to injected ATP before (■) and after removal of the endothelial layer (□) by sodium deoxycholate pretreatment. Vascular tone was elevated by perfusion with 5-hydroxytryptamine (10^{-5} M). $n = 6$ at each dose.

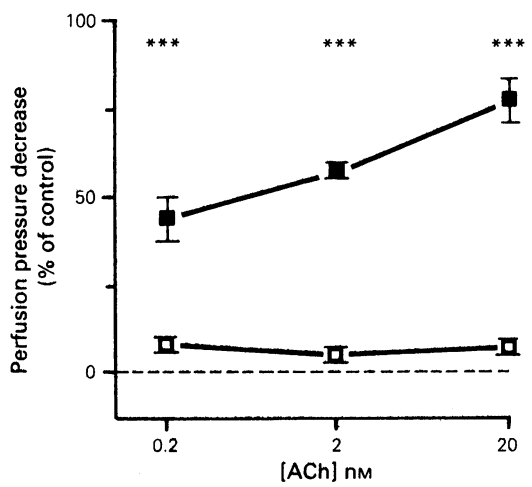


Figure 6 The dilator effect of acetylcholine (ACh) ($n = 9$–12) before (■) and after removal of the endothelial layer (□) by sodium deoxycholate pretreatment ($n = 6$–8). Vascular tone was raised by vasopressin (10^{-8} M).

Discussion

The purpose of the present study was twofold. Firstly, to establish whether purinoceptors are present in rabbit knee joint blood vessels and determine the effects they mediate. Secondly, to investigate whether any actions of purinoceptors are mediated via the endothelial layer of articular blood vessels.

Burnstock (1978) proposed that there were two subtypes of purinoceptors: P_1-purinoceptors, the interaction of which with adenosine can be antagonized by methylxanthines and P_2-purinoceptors which are more sensitive to ATP and can be antagonized by α,β methylene ATP (Burnstock, 1982; Kasakov & Burnstock, 1983). Research on various tissues in different species indicates that purine nucleotides act via P_1-purinoceptors to induce relaxation of vascular smooth muscle cells (Burnstock, 1982; Burnstock *et al.*, 1986) but that actions via P_2-purinoceptors produce contraction in some tissues and relaxation in others (Burnstock & Brown, 1981; Kennedy & Burnstock, 1985). The relaxation of smooth muscle cells can either be direct via receptors on smooth muscle cells or indirectly via receptors on the endothelial layer (Furchgott, 1981).

The results of the present experiments demonstrate the dual effect of ATP on articular blood vessels in the rabbit. ATP can produce vasoconstriction via P_2-purinoceptors on vascular smooth muscle. The desensitizing action of α,β methylene ATP which has been described previously (Kasakov & Burnstock, 1983; Hedlund *et al.*, 1983; Sneddon & Burnstock, 1984; Kennedy & Burnstock, 1985; Kennedy *et al.*, 1985) was confirmed here. Removal of the endothelium resulted in a slight increase in the vasoconstrictor action of ATP suggesting that the responses prior to DCA treatment were reduced by continuous release of endothelium derived relaxing factor (EDRF).

In contrast to ATP, adenosine did not produce any constrictor effect. However, after increasing the tone of joint blood vessels it caused concentration-dependent relaxation. This response was mediated via P_1-purinoceptors on vascular smooth muscle as it was antagonized by 3-methylxanthine, but unaffected after removal of the endothelium. The weak effect of adenosine prior to elevation of vascular tone was probably due to this agent acting on blood vessels which were already vasodilated in the absence of tonic sympathetic constrictor discharge.

ACh was also found to exert a dilator effect which was consistently observed after increasing the vascular tone. However, this was virtually abolished by removal of the endothelium, which is similar to the results obtained by Furchgott & Zawadski (1980) on rabbit thoracic aorta.

Contrary to an earlier report based on rabbit ear artery by Kennedy & Burnstock (1985), ATP appears to produce vasodilatation via P_2-purinoceptors, as 3-methylxanthine fails to block this effect whereas removal of the endothelium does. This finding is in agreement with another study on rat femoral artery carried out by Kennedy *et al.* (1985). Whether the dilatation is due to a direct action of ATP, or whether it is mediated by the breakdown products of ATP such as AMP and adenosine (Burnstock, 1978), remains to be answered. Previous reports have suggested that P_2-purinoceptors appear to play little or no role in ATP-elicited vasodilatation (Su, 1981; Kennedy & Burnstock, 1985). However, the results of the present experiments suggest that ATP acting presumably via P_2-purinoceptors on the endothelial cells of articular blood vessels does produce vasodilatation.

The physiological significance of the different distribution of P_1- and P_2-purinoceptors in different vascular beds remains unclear (Kennedy & Burnstock, 1985). However, it is likely that these purinoceptors and the endothelium may prove to have an important role in the regulation of flow through articular blood vessels.

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Inhibition of elevated arginine vasopressin secretion in response to osmotic stimulation and acute haemorrhage by U-62066E, a κ-opioid receptor agonist

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1 The effect of kappa (κ) opioid receptor activation on the increase in arginine vasopressin (AVP) secretion evoked by two acute and quite different stimuli (i.e., haemorrhage and osmotic stimulus due to hypertonic saline infusion) were evaluated in conscious Long-Evans rats, by use of U-62066E, a highly selective κ-opioid receptor agonist, and MR2266, an opioid receptor antagonist with some selectivity for κ-receptors.

2 An acute haemorrhage, which reduced the mean blood pressure by approximately 50%, resulted in a large increase in the plasma AVP (pAVP) levels of control rats. However, the administration of U-62066E ($0.2\,mg\,kg^{-1}$ or $2.0\,mg\,kg^{-1}$) reduced the increase due to haemorrhage in a dose-dependent manner. In contrast, concomitant administration of $2.0\,mg\,kg^{-1}$ of MR2266 with U-62066E significantly attenuated the inhibition of pAVP levels produced by U-62066E $2.0\,mg\,kg^{-1}$.

3 Hypertonic saline infusion (5% hypertonic saline solution at a rate of $0.24\,ml\,kg^{-1}\,min^{-1}$ for 10 min) caused the elevation of plasma osmolality (pOsm) from $294.0 \pm 1.6\,mosmol\,kg^{-1}$ to $304.4 \pm 1.9\,mosmol\,kg^{-1}$, simultaneously resulting in a significant increase in pAVP levels from $2.34 \pm 0.28\,pg\,ml^{-1}$ to $4.54 \pm 0.51\,pg\,ml^{-1}$. However, the administration of U-62066E ($0.05\,mg\,kg^{-1}$ or $0.2\,mg\,kg^{-1}$) reduced the osmotically induced increase in pAVP in a dose-dependent manner although pOsm showed the same degree of increase as in controls. In contrast, concomitant administration of $0.2\,mg\,kg^{-1}$ of MR2266 with U-62066E significantly attenuated the inhibition of pAVP levels produced by U-62066E $0.2\,mg\,kg^{-1}$, whereas pOsm showed the same degree of increase as in controls. No significant changes in the mean blood pressure of the respective groups were observed during this experiment.

4 It is suggested that the κ-opioid receptor activation reduces the increase in AVP secretion evoked by these two different stimuli and that the inhibitory involvement occurs in the neural lobe in the process of AVP secretion.

Introduction

It is well known that the release of arginine vasopressin (AVP) is stimulated not only by osmotic stimulus but also by volume depletion i.e. baroreceptor and volume-receptor mediated responses (Aisenbrey *et al.*, 1981; Paller & Linas, 1984). Indeed, acute haemorrhage stimulates the secretion of AVP (Ginsburg & Heller, 1953; Laycock *et al.*, 1979). Baroreflex-mediated changes in sympathetic and cardiovascular function are strongly affected by μ- and δ-opioid receptor activation (Gordon, 1986; Faden & Feuerstein, 1983). However, little has been reported as to whether the κ-opioid receptor can affect the increase in AVP secretion in response to the acute baroreflex. κ-Opioid receptors have been found to be abundant in neurosecretosome preparations (Falke & Martin, 1985; Pesce *et al.*, 1987). An autoradiographic analysis has shown that κ-receptors are concentrated around the neural lobe perimeter (Herkenham *et al.*, 1986). However, specific binding activity of κ-agonists was also observed in the rat hypothalamus, preoptic area, central nucleus of the amygdala, and rostral nucleus of the solitary tract. Carter & Lightman (1985) reported that a κ-receptor agonist, U-50488H, microinjected into the nucleus tractus solitarius, generally accepted as the vasomotor centre for control of AVP secretion by peripheral baroreceptors (Share, 1974) and the site of visceroreceptive afferent termination (Torvitz, 1956), markedly increased plasma AVP. Thus, the evidence strongly suggests that central κ-opioid receptor activation stimulates AVP secretion. On the other hand, it has been reported that the administration of κ-opioid receptor agonists decreases plasma AVP levels in water-deprived rats (Slizgi & Ludens, 1982; Leander, 1983; Leander *et al.*, 1987), and that the suppression of plasma AVP levels might be responsible for the increase in urinary output (Yamada *et al.*, 1989a).

Therefore, the present study was designed to determine whether the effect of a κ-opioid receptor agonist on the response of AVP to two acute and different stimuli (i.e. osmotic stimulus due to hypertonic saline infusion and haemorrhage) which activate the reflex arc through the nucleus tractus solitarius, takes place somewhere in the final common pathway or in the previous separate ones in the process of AVP neurosecretion. A highly selective κ-opioid receptor agonist, U-62066E (Lahti *et al.*, 1985; Mickelson & Lahti, 1984), and an opioid antagonist with some selectivity for the κ-opioid receptor, MR2266 (Jacob & Ramabadram, 1977), were used.

Methods

Long-Evans rats weighing 300–350 g were used. Animals were anaesthetized with sodium pentobarbitone ($50\,mg\,kg^{-1}$, i.p.) and polyethylene catheters were placed in both femoral arteries for measurement of arterial pressure, infusion of hypertonic saline infusion, blood samplings and induction of haemorrhage. Rats were housed with free access to water but without chow in a temperature-controlled room (22 ± 2°C). Experiments were carried out on the following day.

Protocol

On the day following the surgery, blood pressure was monitored by a transducer (Nihon Koden type 101T) coupled to a polygraph (Nihon Koden RM-600) in conscious rats. Blood was sampled over 30 s (B_0 and B_1, each 0.5 ml as EDTA-

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treated whole blood). An equal volume of normal saline was replaced each time.

Haemorrhage After withdrawing blood (B_0), the κ-opioid agonist (U-62066E, 0.2 mg kg^{-1} or 2.0 mg kg^{-1}) or vehicle was administered i.p. The κ-opioid antagonist MR2266 (0.2 mg kg^{-1} or 2.0 mg kg^{-1}) was administered i.p. 5 min after U-62066E or the vehicle. The second sampling of blood (B_1) was performed 20 min after U-62066E or vehicle administration. Thereafter, blood was drawn continuously through a catheter for 5 min at a rate of 1 ml min^{-1} per 300 g rat with a pump (Mastertilex, Cole-Palmer Instrument Co.). The rats were killed by decapitation immediately when mean blood pressure became half of the value just prior to haemorrhage, and the blood was collected for subsequent determination of plasma AVP (pAVP) (B_2) and osmolality (pOsm).

Hypertonic saline infusion Infusion of 5% hypertonic saline solution at a rate of 0.24 ml kg^{-1} min^{-1} for 10 min with a Harvard pump (Harvard Apparatus 973C) was carried out. As a preliminary experiment, the time course of the plasma AVP response to the above hypertonic saline infusion was examined in blood before and at 10, 20, and 30 min after the start of the infusion. As shown in Figure 1, the maximal level of pAVP was observed at 10 min after the start of the infusion. Therefore, for measuring the change in pAVP level in response to hypertonic saline infusion, we chose the 10 min value in the following experiments.

After withdrawing blood (B_0), the κ-opioid agonist (U-62066E, 0.05 mg kg^{-1} or 0.2 mg kg^{-1}) or the vehicle was administered i.p. The κ-opioid antagonist MR2266 (0.05 mg kg^{-1} or 0.2 mg kg^{-1}) was administered i.p. 5 min after U-62066E or vehicle administration. The second sampling of blood (B_1) was performed 20 min after U-62066E or vehicle administration, after which, the hypertonic saline solution was infused. The rats were killed by decapitation just 10 min after the start of infusion and the blood was collected for determinations of pAVP and pOsm.

Plasma AVP was determined by the method previously described (Yamada *et al.*, 1989b). AVP in EDTA-treated plasma was measured by radioimmunoassay (RIA). In brief, after acidifying the plasma to pH 3.5 with 0.1 N HCl, a sample was passed through a column of octadecylsilyl silica (Seppak C18 cartridges) which had been prewashed with methanol (8 ml) and then with water (12 ml); the column was then washed with 4% acetic acid (10 ml) and AVP was eluted with 2.0 ml of methanol. The eluate was dried under nitrogen gas and the residue was reconstituted with RIA buffer. AVP was determined by radioimmunoassay. The lower limit of detection of the assay was 0.05 pg per tube (B/B_0 = 95%). B/B_0 of 50% was 0.85 pg per tube. The antibody used did not cross-react with oxytocin, bradykinin, α-human atrial natriuretic polypeptide, angiotensin I, or angiotensin II. Inhibitory potencies of these peptides were 1% or less, judged by their ability to achieve half maximal inhibition (B/B_0 = 50%). The recovery of AVP was 83.0 ± 2.1% (mean ± s.e.) when unlabelled AVP was added to plasma (0.5 to 8.0 pg ml^{-1}) before extraction. The intra-assay and interassay variabilities were 9.5 ± 0.6% and 10.6 ± 0.9%, respectively. In AVP assay with small sample volumes, the volume factor was checked by taking 0.2 ml, 0.3 ml, 0.5 ml or 1.0 ml of the pooled plasma sample (2.0 pg ml^{-1}) extracting and assaying AVP by the same procedure. Plasma AVP could be assayed linearly down to a sample volume of 0.2 ml. Sodium and potassium concentrations were determined by flame photometry. The osmolality of plasma was determined by freezing point depression.

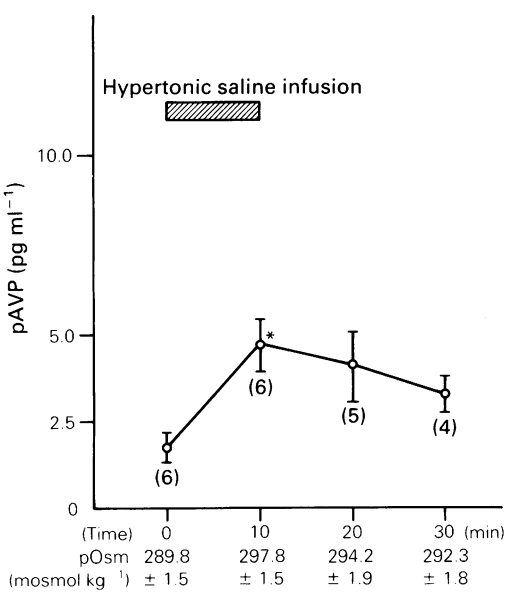


Figure 1 Time course of plasma arginine vasopressin (AVP) concentration changes in response to the infusion of hypertonic saline solution. Numerals in parentheses indicate the number of rats. Plasma osmolality is indicated below each time point. *$P < 0.01$.

Materials

[^{125}I]-AVP was purchased from New England Nuclear (Boston, MA, U.S.A.). Anti-AVP rabbit serum was donated by Mitsubishi Yuka (Tokyo, Japan). Seppak C18 cartridges were obtained from Waters Associates, Millipore (Milford, MA, U.S.A.). MR2266 ((−)-2-(3-furylmethyl)-noretazocine) was donated by Boehringer-Ingelheim (West Germany). AVP, bradykinin, angiotensin I, angiotensin II, α-human ANP and oxytocin were purchased from Protein Research Institute (Osaka). U-62066E ((5α,7α,8β)-(±)-3,4-dichloro-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide methanesulphonate) was obtained from Upjohn Pharmaceuticals. Other reagents used were of analytical grade.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical analysis was performed with the Student's paired or unpaired *t* test.

Results

Haemorrhage

The changes in blood pressure during the experiment are shown in Figure 2. No significant change in blood pressure or in plasma AVP levels (B_1) was observed in these conscious rats followed the administration of U-62066E or the vehicle, and/or MR2266, as compared to the control (Figure 2 and Table 1). In addition, no significant difference in mean blood pressure or in plasma AVP levels (B_1) just before the start of haemorrhage among these groups was observed (Figure 2 and Table 1).

Table 1 Effect of i.p. administration of U-62066E and/or MR2266 on plasma arginine vasopressin (AVP)

	Plasma AVP (pg ml^{-1})	
Drugs	B_0 (0 min)	B_1 (20 min)
Control (saline) (n = 6)	1.78 ± 0.38	1.09 ± 0.21
U-62066E (0.2 mg kg^{-1}) (n = 9)	2.11 ± 0.34	1.75 ± 0.37
U-62066E (2.0 mg kg^{-1}) (n = 12)	1.94 ± 0.35	1.47 ± 0.26
U-62066E (2.0 mg kg^{-1}) + MR2266 (0.2 mg kg^{-1}) (n = 6)	2.48 ± 0.41	1.44 ± 0.31
U-62066E (2.0 mg kg^{-1}) + MR2266 (2.0 mg kg^{-1}) (n = 6)	1.94 ± 0.62	1.60 ± 0.35
MR2266 (2.0 mg kg^{-1}) (n = 4)	1.61 ± 0.33	1.72 ± 0.38

Abbreviations: n: number of rats; B_0: plasma before drug administration; B_1: plasma at 20 min after U-62066E or saline administration.

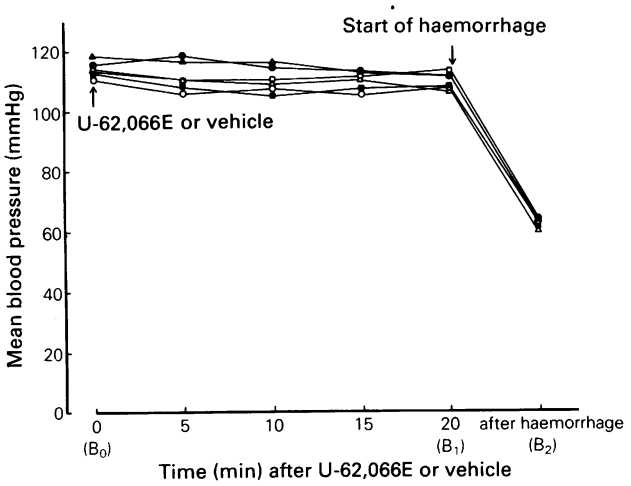


Figure 2 Effect of i.p. administration of U-62066E and/or MR2266, and haemorrhage on mean blood pressure in Long-Evans rats: (○) vehicle (saline) [number of rats (n) = 6]; (△) 2.0 mg kg^{-1} U-62066E (n = 9); (□) 2.0 mg kg^{-1} U-62066E (n = 12); (▲) 2.0 mg kg^{-1} U-62066E + 0.2 mg kg^{-1} MR2266 (n = 6); (●) 2.0 mg kg^{-1} U-62066E + 2.0 mg kg^{-1} MR2266 (n = 6); (■) vehicle (saline) + 2.0 mg kg^{-1} MR2266 (n = 4).

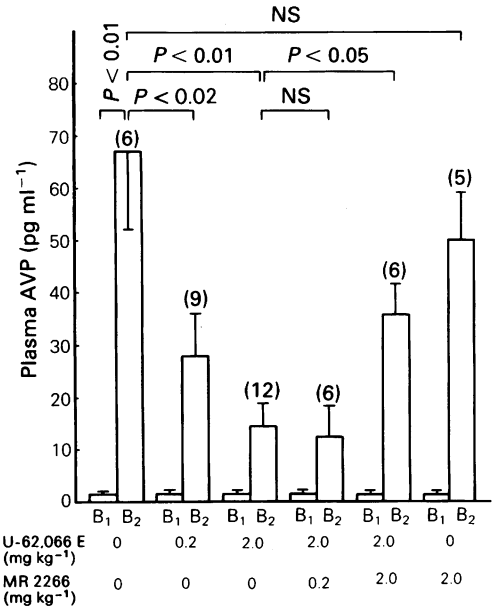


Figure 3 Effects of U-62066E and/or MR2266 on haemorrhage-induced plasma arginine vasopressin (AVP) concentrations. NS: not significant. Numerals in parentheses indicate the number of experiments. B_1: plasma just before haemorrhage; B_2: plasma after haemorrhage.

An acute haemorrhage reduced the mean blood pressure by approximately 50% without showing any difference in decrement among different drug-treated groups. Acute haemorrhage also resulted in a huge increase in the plasma AVP levels of control rats (Figure 3). However, the administration of U-62066E reduced the increase due to haemorrhage in a dose-dependent manner (Figure 3). In contrast, concomitant administration of 2.0 mg kg^{-1} of MR2266 with U-62066E partially prevented the inhibitory effect of 2.0 mg kg^{-1} of U-62066E on plasma AVP. On the other hand, the administration of 2.0 mg kg^{-1} of MR2266 alone did not significantly affect the increase in plasma AVP levels following haemorrhage.

In spite of the marked changes in the plasma AVP levels, plasma osmolality did not show any significant differences among the different groups, indicating that plasma osmolality was not a determinant for the changes in plasma AVP under these experimental conditions.

Hypertonic saline infusion

As shown in Figure 4, hypertonic saline infusion caused an elevation of plasma osmolality in the control rats, simultaneously resulting in a significant increase of pAVP levels. However, the administration of U-62066E reduced the osmotically induced increase in pAVP in a dose-dependent manner (Figure 4). At the same time, plasma osmolality

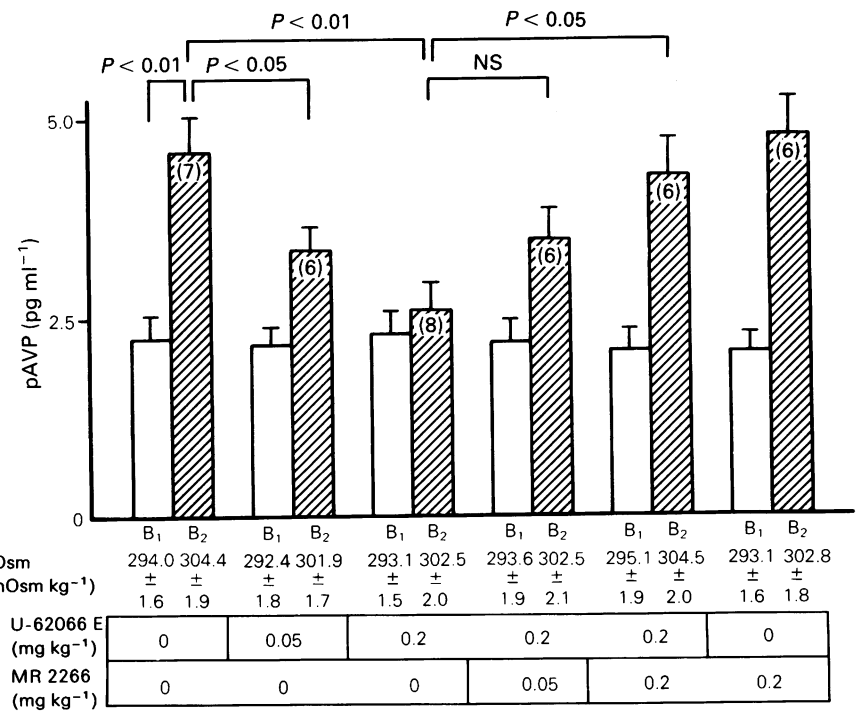


Figure 4 Effect of U-62066E and/or MR2266 on plasma arginine vasopressin (AVP) levels when hypertonic saline solution was infused into Long-Evans rats. NS: not significant. Numerals in parentheses indicate the number of experiments. B_1: plasma just before the infusion of hypertonic saline; B_2: plasma at 10 min after the start of the infusion. Plasma osmolality is indicated below each plasma sample.

showed the same degree of increase as that of control. In contrast, concomitant administration of MR2266 with U-62066E partially prevented the inhibitory effect of U-62066E, whilst showing the same degree of increase in plasma osmolality as that of control (Figure 4). No significant changes in the mean blood pressure of any group were observed during this experiment.

Discussion

The secretion of AVP is stimulated both by osmotic and by non-osmotic stimuli (Schrier *et al.*, 1979) such as hypotension, hypovolaemia and hypoglycaemia. Osmotic stimulation is mediated by hypothalamic osmoreceptors (Verney, 1947), which connect to the neurohypophysis through the supraoptic nucleus (SON) and paraventricular nucleus (PVN). On the other hand, non-osmotic, baroreceptor and volume-receptor mediated AVP responses appear to be mediated through many of the safe afferent pathways, including the vasomotor centre in the brain stem, that regulate sympathetic activity (Share, 1976; Schrier *et al.*, 1977).

Although the haemorrhage examined in the present study is thought to be a complex cardiovascular stimulus, it seems, at least in part, to cause AVP secretion by the activation of the baroreceptor-reflex arc (Ginsburg & Heller, 1953; Laycock *et al.*, 1979). Therefore, the impulses evoked by the two stimuli used (i.e. osmotic stimulus and acute haemorrhage) run along separate afferent pathways to the SON and PVN to stimulate AVP secretion. Thereafter a common pathway is involved (Zerbe *et al.*, 1983).

It is well known that neuropeptide secretion is modulated by opioids (Clarke *et al.*, 1979). The present study demonstrates that the administration of this highly selective κ-opioid receptor agonist, U-62066E, dose-dependently inhibited the acute AVP response to two quite different stimuli and that the inhibition was blocked by a κ-opioid receptor antagonist, MR2266. This strongly suggests that the κ-opioid receptor mechanism is mediated at the level of AVP secretion. κ-Opioid receptors have been found to be abundant in neurosecretosome preparations (Falke & Martin, 1985; Pesce *et al.*, 1987). An autoradiographic analysis has shown that pituitary κ-opioid receptor-binding was confined to the neural lobe where it was densest in the external rim (Herkenham *et al.*, 1986). Accordingly, it is suggested, from the present study, that the increase in the secretion of AVP with two different stimuli was inhibited somewhere during the final common pathway in the neural lobe, and that probably the activation of κ-opioid receptors might inhibit the release of AVP from nerve terminals and/or processes of pituicytes (Herkenham *et al.*, 1986).

Substantial amounts of dynorphin peptides (dynorphin A (1–17) and its related peptide dynorphin A (1–8)), which are thought to be endogenous κ-opioid receptor agonists (Chavkin & Goldstein, 1981), are found in the rat neurohypophysical system localized within AVP-containing magnocellular perikarya and terminals (Watson *et al.*, 1982). At a subcellular level they co-localized with AVP in common neurosecretory vesicles (Whitnall *et al.*, 1983). Furthermore, neurohypophysial dynorphin levels have been found to vary in parallel with AVP levels during physiological stimulation (Zamir *et al.*, 1985). Therefore, it might be speculated that endogenous κ-opioids, especially dynorphins, are involved in controlling the release of AVP from the pituitary in the final common pathway in the process of AVP secretion from an acute haemorrhage or hypertonic saline infusion.

The presence of κ-opioid binding activity in the rat hypothalamus, preoptic area, central nucleus of the amygdala, and rostral nucleus of the solitary tract, where they outnumber μ-receptors, has been reported (Herkenham *et al.*, 1986). Moreover, Carter & Lightman (1985) reported that a κ-opioid receptor agonist, U-50488H, markedly increased plasma AVP when it was microinjected into the nucleus tractus solitarius of rats. The reason for this discrepancy remains to be determined. Perhaps it may be due to the differences in experimental conditions. It may also be possible that the reported stimulation of AVP secretion is mediated by a separate mechanism occurring at the level of the nucleus tractus solitarius.

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Enhancement by neuropeptide Y (NPY) of the dihydropyridine-sensitive component of the response to α_1-adrenoceptor stimulation in rat isolated mesenteric arterioles

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1 The mechanism by which neuropeptide Y (NPY) potentiates the vasoconstriction induced by α_1-adrenoceptor agonists was investigated in 3rd generation mesenteric arterioles of the rat.

2 At a maximally active concentration, nitrendipine (10^{-6} M) displaced to the right the concentration-response curves to noradrenaline (pD_2 decreased from 6.2 ± 0.06 to 5.7 ± 0.03) and phenylephrine (pD_2 decreased from 5.6 ± 0.03 to 5.3 ± 0.03). Diltiazem (10^{-5} M) also shifted to the right the concentration-response curve to phenylephrine (pD_2 decreased from 6.0 ± 0.06 to 5.5 ± 0.04). In addition, the maximal response to phenylephrine was significantly decreased in the presence of either nitrendipine or diltiazem.

3 In the absence of a calcium channel blocking agent, NPY (100 nM) produced a leftward shift of the concentration-response curves to noradrenaline (pD_2 increased from 6.2 ± 0.06 to 6.5 ± 0.05) and phenylephrine (pD_2 increased from 5.6 ± 0.03 to 6.0 ± 0.06 and from 6.0 ± 0.06 to 6.3 ± 0.11). In the presence of either nitrendipine (10^{-6} M) or diltiazem (10^{-5} M), NPY (100 nM) did not alter the concentration-response curves to either noradrenaline or phenylephrine.

4 NPY was added to arterioles brought to the same level of tension (40% of the maximal contraction) either by phenylephrine alone (1.5×10^{-6} M) or by a higher concentration of phenylephrine (3×10^{-6} M) followed by the addition of prazosin (1.3×10^{-9} M; a concentration at which it partially blocks α_1-adrenoceptors). In these conditions, the response to phenylephrine was completely abolished by nitrendipine (10^{-6} M) or by diltiazem (10^{-5} M). Furthermore, NPY (10^{-10} to 10^{-7} M) increased the arteriolar tension up to the maximal contractile capacity of the vessels with pD_2 values of 8.6 ± 0.02 and 8.7 ± 0.01, in the absence and presence of prazosin, respectively.

5 Prazosin was replaced in the above protocol by other vasodilator agents acting through different mechanisms. Whether in the presence of 2×10^{-7} M forskolin, 6×10^{-7} M sodium nitroprusside (which stimulate adenylate cyclase or guanylate cyclase, respectively) or 2×10^{-7} M diltiazem (a concentration at which calcium entry is partially blocked), NPY enhanced phenylephrine-induced contraction to the maximum level with an identical potency (pD_2 values of the peptide ranged from 8.3 to 8.7).

6 The results show that, in rat mesenteric arterioles, NPY potentiates only the calcium entry blocker-sensitive component of contraction induced by stimulation of α_1-adrenoceptors. In addition, they provide evidence that the peptide counteracts with an equal potency the inhibitory effect of partial block of α_1-adrenoceptors and of relaxing agents acting through different mechanisms. It is suggested that NPY enhances calcium entry induced by stimulation of α_1-adrenoceptors in this tissue.

Introduction

Neuropeptide Y (NPY) is a potent vasoconstrictor peptide which is co-stored with catecholamines in sympathetic nerve endings. It is also co-released with noradrenaline upon sympathetic nerve stimulation (see reviews, O'Donohue *et al.*, 1985; Allen & Bloom, 1986; Gray & Morley, 1986; Sundler *et al.*, 1986; Edvinsson *et al.*, 1987; Lundberg *et al.*, 1987; Potter, 1988). Systemic administration of NPY to conscious rats increase blood pressure and this is associated with an increase in total peripheral resistance (Dählof *et al.*, 1985; Zukowska-Grojec *et al.*, 1986; Pegram & Hunter, 1988).

The effects of NPY on the various vascular beds appear to be diverse. It possesses a direct vasoconstrictor action in some but not all isolated vessels and it reduces prejunctional release of noradrenaline. However, at the postjunctional level, the main property of this peptide is its ability to potentiate responses caused either by nerve stimulation or by a variety of vasoconstrictor agents, including noradrenaline (Lundberg & Tatemoto, 1982; Lundberg *et al.*, 1982; Edvinsson *et al.*, 1984a,b; Ekblad *et al.*, 1984; Edvinsson, 1985; Glover, 1985; Wahlestedt *et al.*, 1985; 1986; Zukowska *et al.*, 1987; Han & Abel, 1987; Neild, 1987; 1988; Juan *et al.*, 1988).

In a previous study on rat isolated mesenteric resistance arterioles, NPY enhanced contraction elicited by a number of agonists or by KCl-depolarization, at a concentration which produced no effect by itself. It was suggested that these effects could be accounted for by partial depolarization and subsequent calcium influx through voltage-dependent calcium channels (Andriantsitohaina & Stoclet, 1988a). Abel & Han (1989) reached similar conclusions on rabbit cerebral arteries. However, two other mechanisms have been proposed to explain the potentiating effect of NPY on vasoconstriction induced by noradrenaline: inhibition of adenylate cyclase activity (Fredholm *et al.*, 1985; Häggblad & Fredholm, 1987; Lundberg *et al.*, 1988) and enhanced accumulation of inositol trisphosphate (Häggblad & Fredholm, 1987).

In the present work, the action of NPY was further investigated in rat mesenteric arterioles. Experiments have been carried out in order to examine the ability of the peptide to amplify the dihydropyridine-sensitive and -insensitive components of the responses to two α_1-adrenoceptor agonists, noradrenaline and phenylephrine; these two components of the response are produced through different pathways and

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might even be due to stimulation of different α_1-adrenoceptor subtypes (Han *et al.*, 1987). In addition, the capacity of the peptide to enhance contraction in the presence of various vasodilator agents acting through different mechanisms was also studied. The results show that the peptide did not potentiate the dihydropyridine-insensitive component of the agonist-induced contractions, but was able to potentiate the component which is sensitive to calcium entry blockers. For this latter component, NPY was also able to counteract the inhibitory effect of all vasodilator agents tested. It is suggested that NPY enhances the efficiency of coupling between receptor occupancy and calcium entry through a mechanism sensitive to dihydropyridines and diltiazem.

Part of this work was presented at the December 1988 meeting of the British Pharmacological Society (Andriantsitohaina & Stoclet, 1988b).

Methods

Arterial preparation

In all experiments, third generation branches of the superior mesenteric artery were dissected from 12–14 week old female Wistar rats bred in our institute. Rats were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.). Arterial segments (length = 2 mm, internal diameter = 100 μm) were prepared and mounted in a myograph (Mulvany & Halpern, 1977; Julou & Freslon, 1986). Briefly, two tungsten wires (30 μm diameter) were inserted through the lumen of the vessel. Mechanical activity was recorded isometrically by a force transducer (DSG BE4, Kistler–Morse), connected to one of the two tungsten wires, the other being attached to a support carried by a micromanipulator (MR 50, Micro–Contrôle).

After being set up, vessels were equilibrated for 1 h in a physiological salt solution (PSS) (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 0.4, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, $CaCl_2$ 2.5, glucose 5.5) kept at 37°C and continuously gassed with a 95% O_2, 5% CO_2 mixture (pH 7.4). The resting tension of the preparation was adjusted to about 200 mg. Following equilibration, the contractile capacity was tested by exposing the arterial segment to 10^{-5} M noradrenaline, a concentration which elicited maximum contractions (Mulvany *et al.*, 1982). The presence of functional endothelium was checked in all preparations by the ability of acetylcholine (10^{-6} M) to induce more than 50% relaxation of vessels precontracted with noradrenaline (10^{-5} M).

Effects of NPY on noradrenaline- and phenylephrine-induced contractile responses in the absence and in the presence of a calcium entry blocker

Sixty min after the contractile capacity of the vessels had been tested with noradrenaline, concentration-response curves were constructed to either noradrenaline or phenylephrine. After a washout period of 45 min, NPY (100 nM) was applied 5 min before the cumulative addition of the agonist was repeated. When a calcium channel blocker (10^{-6} M nitrendipine or 10^{-5} M diltiazem) was used, it was added 20 min before addition of the agonist. During experiments with noradrenaline, cocaine (3×10^{-6} M) was present in the bath to inhibit neuronal uptake. In control experiments in the absence of NPY, two concentration-response curves obtained by successive cumulative addition of the agonists were not different from each other.

Effect of NPY on vessels precontracted with phenylephrine

Sixty min after the contractile capacity of the vessels had been tested with noradrenaline, concentration-response curves to NPY (10^{-10} to 10^{-7} M) were constructed non-cumulatively by the addition of a single concentration of the neuropeptide to an arteriole precontracted with 1.5×10^{-6} M phenylephrine. When the contraction to NPY had reached equilibrium, both the phenylephrine and the neuropeptide were washed out and the preparation left 30 min before the addition of the same concentration of phenylephrine and a different concentration of the neuropeptide.

Effects of prazosin, forskolin, sodium nitroprusside and diltiazem on vessels precontracted with phenylephrine

Sixty min after the test addition of noradrenaline, the arterioles were precontracted with 3×10^{-6} M phenylephrine. When the response was stable, the arterioles were relaxed by addition of either prazosin(10^{-10} to 10^{-7} M), forskolin (10^{-8} to 10^{-5} M) sodium nitroprusside (10^{-8} to 10^{-5} M) or diltiazem (10^{-8} to 10^{-4} M). Concentration-response curves to each agent were constructed non-cumulatively by the addition of a single concentration of the chosen vasodilator to an arteriole precontracted with 3×10^{-6} M phenylephrine (except nitrendipine). When the relaxation had reached equilibrium, both the phenylephrine and the vasodilator were washed out and the preparation left 30 min before the addition of the same concentration of phenylephrine and a different concentration of the vasorelaxant. In control experiments, it was found that the responses induced by 3×10^{-6} M phenylephrine were consistent over the period of the experiment and did not differ significantly between preparations.

Effects of NPY on relaxation induced by prazosin, forskolin, sodium nitroprusside and diltiazem on vessels precontracted with phenylephrine

Sixty min after the contractile capacity of the vessels had been tested by exposure to 10^{-5} M noradrenaline, responses to NPY were elicited on arterioles which had first been precontracted with 3×10^{-6} M phenylephrine and then relaxed by different vasodilator agents (prazosin, forskolin, sodium nitroprusside or diltiazem), at the concentrations at which they produced half-maximal relaxation before addition of NPY. Concentration-response curves to NPY (10^{-10} to 10^{-7} M) were then constructed non-cumulatively by the addition of a single concentration of NPY to an arteriole precontracted with 3×10^{-6} M phenylephrine and relaxed by the chosen agent. When the response to NPY had reached equilibrium, phenylephrine, the vasodilator and NPY were washed out and the preparation left 30 min before the addition of the same concentrations of phenylephrine and the vasorelaxant but a different concentration of NPY. Control experiments were run in parallel; the relaxations induced by the vasodilator agents were not significantly different from each other and were consistent over the period of the experiments in the absence of NPY.

Expression of results and statistical analysis

Sensitivities to the agonists (noradrenaline, phenylephrine and NPY) and to the relaxant agents (forskolin, sodium nitroprusside, prazosin, diltiazem and nitrendipine) were expressed as pD_2 values, where $pD_2 = -\log EC_{50}$, EC_{50} being the concentration of agonist or relaxant agent required to give half-maximal response of the vessels. EC_{50} values were obtained by logit/log regression analysis.

All results are expressed as mean ± s.e.mean of n experiments. The differences between the pD_2 values, obtained in the same artery rings, were tested for significance by use of Student's t test for paired observations. One-way analysis of variance was used to compare the effect of different treatments studied in separate experiments.

Drugs

Noradrenaline bitartrate (Sigma) was dissolved in deionized water, containing 34 mM HCl and 7.9 mM Na_2SO_3, to give a

stock solution of 10^{-2} M. Acetylcholine hydrochloride (Sigma) was prepared as a 10^{-2} M stock solution by dissolving it in deionized water containing NaH_2PO_4 (0.1 M). Phenylephrine hydrochloride (Sigma), diltiazem hydrochloride (L.E.R.S.Synthelabo) and cocaine hydrochloride (Cooperation Pharmaceutique Française) were prepared daily as 10 mM solutions in deionized water. Forskolin (Sigma) and sodium nitroprusside (Merck) were diluted in absolute ethanol as 10 mM solutions. Porcine NPY (Sigma) was dissolved in deionized water containing 9 g l^{-1} NaCl and 1 g l^{-1} bovine serum albumin (Sigma) to give a stock solution of 10^{-4} M, this was subsequently stored frozen until use. Nitrendipine was dissolved in absolute ethanol to give a 1 mM stock solution which was kept frozen until use. Nitrendipine was kept in the dark and experiments with this drug were performed in light-proof apparatus to minimize light-induced degradation.

Results

The influence of nitrendipine and diltiazem on the responses to cumulative additions of noradrenaline and phenylephrine

As illustrated in Figure 1, the responses of the arterioles to cumulative additions of noradrenaline and phenylephrine were altered in the presence of the calcium entry blockers nitrendipine, at a maximally active concentration (10^{-6} M), and diltiazem (10^{-5} M). In the absence of a calcium entry blocker, contractions reached a sustained plateau after each addition of agonist, whereas in their presence, contractions reached a maximum within the first 30 s and then decreased progressively with time. In these conditions, nitrendipine (10^{-6} M) shifted the concentration-response curves for both noradrenaline and phenylephrine to the right (Figure 2a and

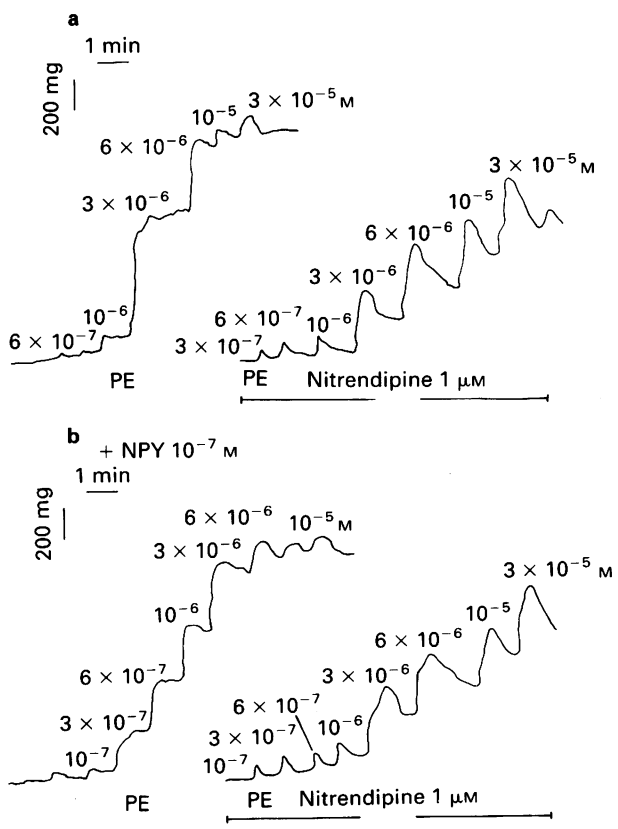


Figure 1 The influence of nitrendipine (10^{-6} M) on the contractile response of rat isolated mesenteric arterioles to cumulative addition of phenylephrine (PE) in the absence (a) and in the presence (b) of neuropeptide Y (NPY, 10^{-7} M). Similar traces were obtained in the presence of diltiazem (10^{-5} M).

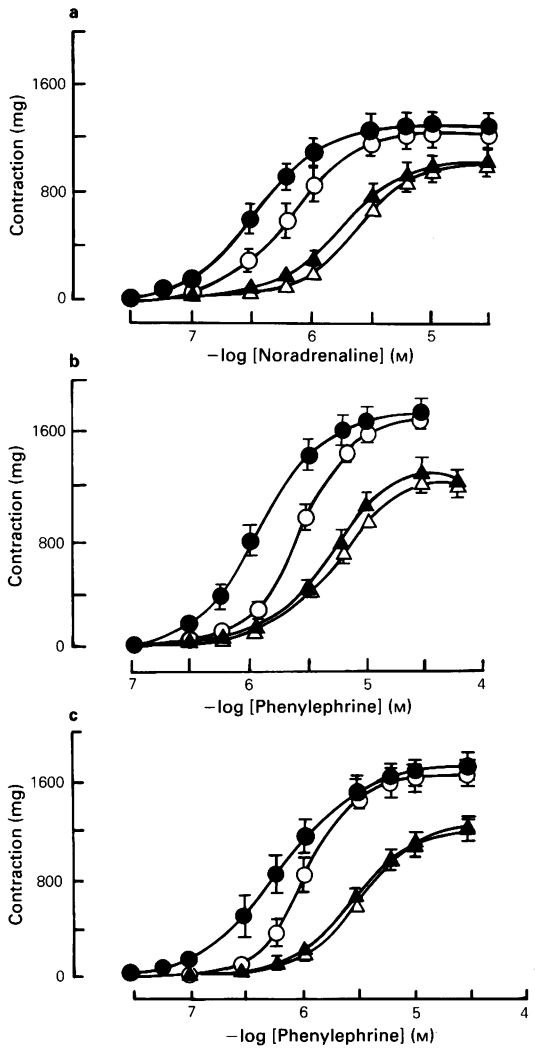


Figure 2 Effect of neuropeptide Y (NPY, 10^{-7} M) on contraction elicited by noradrenaline in the absence and presence of nitrendipine (10^{-6} M; a, $n = 10$), and by phenylephrine in the absence and presence of either nitrendipine (10^{-6} M; b, $n = 5$) or diltiazem (10^{-5} M; c, $n = 6$). Control curves (○), in the presence of NPY (●), in the presence of the calcium antagonist (△) and in the presence of NPY plus the calcium antagonist (▲). The values are the mean with the error bars representing s.e.mean.

b), diltiazem (10^{-5} M) also shifted the concentration-response curves for phenylephrine to the right (Figure 2c). The pD_2 values were decreased in the presence of nitrendipine from 6.2 ± 0.06 to 5.7 ± 0.03 (noradrenaline, $P < 0.001$) and from 5.6 ± 0.03 to 5.3 ± 0.03 M (phenylephrine, $P < 0.001$). The pD_2 values of phenylephrine were also decreased in the presence of diltiazem from 6.0 ± 0.06 to 5.5 ± 0.04 ($P < 0.001$). Nitrendipine (10^{-6} M) did not significantly affect the maximal response to noradrenaline but it decreased the maximal responses induced by phenylephrine ($P < 0.01$). Diltiazem (10^{-5} M) significantly decreased ($P < 0.01$) the maximal response induced by phenylephrine to the same extent as nitrendipine (10^{-6} M).

In the absence of a calcium entry blocker, NPY (10^{-7} M; a concentration at which it had no contractile effect by itself), produced significant leftward shifts of the noradrenaline and phenylephrine concentration-response curves, without changing the maximal responses (Figure 2). The pD_2 values were significantly enhanced by NPY from 6.2 ± 0.06 to 6.5 ± 0.05 ($P < 0.01$) for noradrenaline (Figure 2a), from 5.6 ± 0.03 to 6.0 ± 0.06 ($P < 0.001$) for phenylephrine (Figure 2b) and from 6.0 ± 0.06 to 6.3 ± 0.11 ($P < 0.05$) also for phenylephrine (Figure 2c). In contrast, in the presence of nitrendipine or diltiazem NPY did not potentiate the responses induced by

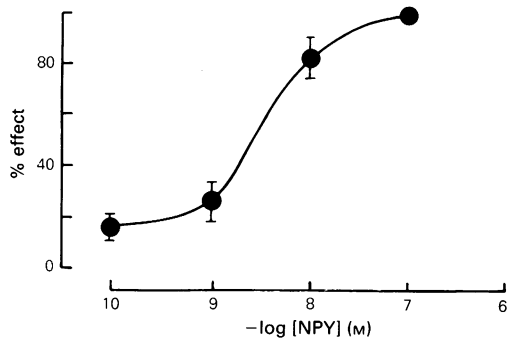


Figure 3 Concentration-response curve to neuropeptide Y (NPY, 10^{-10} to 10^{-7} M) in rat isolated mesenteric arterioles precontracted with 1.5×10^{-6} M phenylephrine ($n = 8$). Contraction is expressed as % of maximal contractile response of the vessels and the points show the mean with the bars representing s.e.mean.

either noradrenaline or phenylephrine. The pD_2 values of noradrenaline (5.8 ± 0.04, Figure 2a) and phenylephrine (5.3 ± 0.05, Figure 2b and 5.5 ± 0.04, Figure 2c) were not altered by NPY in the presence of either nitrendipine or diltiazem. Thus the components of the contractile responses of the vessels to the α-adrenoceptor agonists which were insensitive to nitrendipine or diltiazem were not affected by NPY.

Effect of NPY on vessels precontracted with phenylephrine

In order to study the relationship between the potentiation of the response to stimulation of α_1-adrenoceptors and the concentration of NPY, the peptide was added to vessels precontracted with 1.5×10^{-6} M phenylephrine. At this concentration, phenylephrine alone produced $30 \pm 6\%$ of the maximal contraction of the vessels. Further addition of NPY (10^{-10} to 10^{-7} M) increased tension in a concentration-dependent manner with a pD_2 value of 8.7 ± 0.1 ($n = 8$) as seen in Figure 3. The maximal tension reached in the presence of NPY was identical to the maximal tension induced by 10^{-5} M noradrenaline alone on the same arterioles.

Effects of NPY on relaxation induced by prazosin, forskolin, sodium nitroprusside or diltiazem in vessels precontracted with phenylephrine

The ability of NPY to reverse relaxation induced through different mechanisms was investigated on arterioles precontracted with phenylephrine and then relaxed by different drugs. The agonist was added at a single concentration, 3×10^{-6} M, which produced $78 \pm 6\%$ of the maximal response. In these conditions, further addition of 10^{-6} M nitrendipine or 10^{-5} M diltiazem completely relaxed the vessels, showing that only the calcium entry blocker-sensitive component of the response to phenylephrine was being studied. As shown in Figure 4, prazosin, forskolin and diltiazem relaxed the arterioles to the baseline tension with mean pD_2 values of 8.9 ± 0.1 ($n = 10$), 6.7 ± 0.1 ($n = 6$) and 6.7 ± 0.1 ($n = 6$), respectively. Sodium nitroprusside produced a maximal relaxation of $83 \pm 6\%$ with a pD_2 of 6.3 ± 0.1 ($n = 6$). For the following experiments, concentrations equal to the EC_{50} values were used to relax vessels precontracted with phenylephrine. The level of tension which was reached in these conditions was not significantly different from that obtained with 1.5×10^{-6} M phenylephrine alone. In addition, the level of tension did not differ significantly between preparations and was consistent over the period of the experiment.

Figure 5 shows the concentration-response curves to NPY on vessels precontracted with phenylephrine in the presence of the selected concentration of relaxing agent. NPY (10^{-10} to 10^{-7} M) increased tension up to the same maximum as

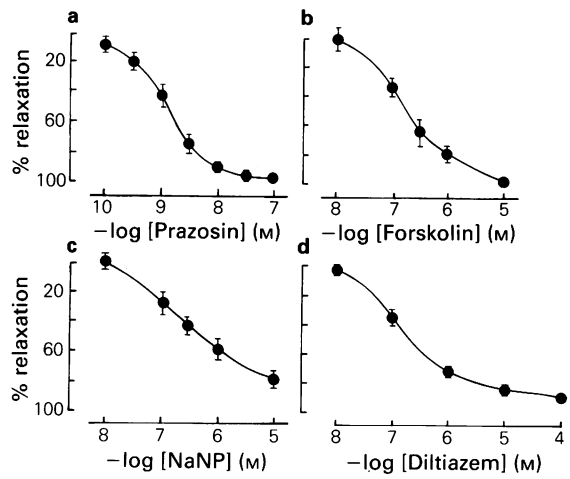


Figure 4 Relaxation induced by prazosin (a, $n = 10$), forskolin (b, $n = 6$), sodium nitroprusside (NaNP) (c, $n = 6$) and diltiazem (d, $n = 6$) in rat isolated mesenteric arterioles precontracted with 3×10^{-6} M phenylephrine. Relaxation is expressed as % of maximal relaxing response of the vessels to the baseline tension. Values are mean and vertical lines indicate s.e.mean.

obtained with 10^{-5} M noradrenaline, which represented the maximum contractile capacity of the vessels. The maximum contractions induced by NPY were approximately 20% higher than the precontractions induced by phenylephrine (3×10^{-6} M). The pD_2 values for NPY were respectively 8.4 ± 0.1 in the presence of prazosin ($n = 6$), 8.6 ± 0.2 in the presence of forskolin ($n = 6$), 8.7 ± 0.1 in the presence of sodium nitroprusside ($n = 6$), and 8.3 ± 0.1 in the presence of diltiazem ($n = 5$). These values, when compared by analysis of variance, were not significantly different from each other or from the pD_2 value of NPY obtained in the presence of 1.5×10^{-6} M phenylephrine alone.

Interaction between NPY and nitrendipine and between NPY and diltiazem on arterioles precontracted with phenylephrine

The concentration-response curve to nitrendipine was obtained cumulatively on vessels precontracted with 3×10^{-6} M phenylephrine since, unlike the other vasodilator agents, the dihydropyridine could not be removed between

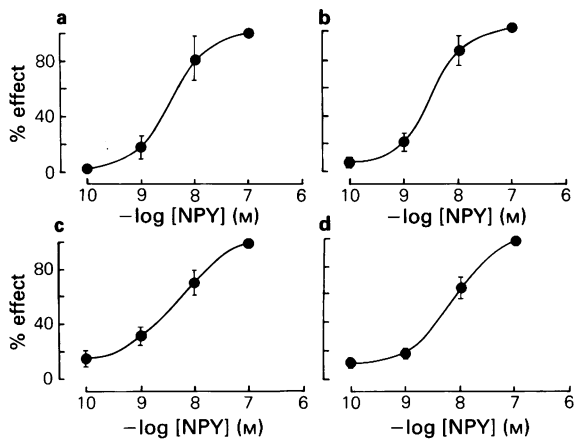


Figure 5 Effect of neuropeptide Y (NPY) on vessels precontracted with phenylephrine (3×10^{-6} M) in the presence of selected relaxing agents at a concentration equal to their respective EC_{50} values. Concentration-response curves to NPY in the presence of prazosin (a, $n = 6$), forskolin (b, $n = 6$), sodium nitroprusside (c, $n = 6$) and diltiazem (d, $n = 5$). The contraction is expressed as % of maximal contractile response of the vessels. The points are the mean with the error bars showing s.e.mean.

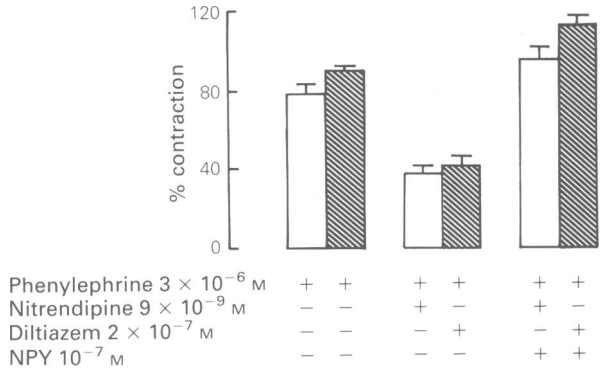


Figure 6 The effect of calcium antagonists, nitrendipine (9×10^{-9} M, open columns) and diltiazem (2×10^{-7} M, hatched columns) on contractions induced by 3×10^{-6} M phenylephrine and by subsequent addition of neuropeptide Y (NPY, 10^{-7} M) in rat mesenteric arterioles. Contractions are expressed as % of the contractile responses induced by 10^{-5} M noradrenaline which were 1331 ± 96 mg ($n = 7$) and 1662 ± 182 mg ($n = 5$), respectively with the experiments using nitrendipine or diltiazem as vasorelaxant agent. Each column represents the mean with the bars representing s.e.mean.

doses by washing. With this protocol, nitrendipine relaxed the arterioles to the baseline tension with mean pD_2 values of 9.0 ± 0.1 ($n = 6$).

For the following experiments, the protocol described in the Methods section was followed in order to compare the interaction between NPY and nitrendipine and between NPY and diltiazem. Only the maximal concentration of NPY (100 nM) was used in this experiment, since the concentration-response curve to NPY constructed non-cumulatively could not be obtained in the presence of nitrendipine due to the irreversibility of the action of dihydropyridine. Figure 6 shows that, when the contractile response of each vessel was expressed as % of maximal contraction with 10^{-5} M noradrenaline, the same level of contraction was obtained with 3×10^{-6} M phenylephrine plus 9×10^{-9} M nitrendipine and with 3×10^{-6} M phenylephrine plus 2×10^{-7} M diltiazem, which were $38 \pm 4\%$ ($n = 7$) and $42 \pm 5\%$ ($n = 5$), respectively. Subsequent addition of NPY (100 nM) increased tension up to the same maximum obtained with 10^{-5} M noradrenaline, both in the presence of phenylephrine plus 9×10^{-9} M nitrendipine ($95 \pm 7\%$) and in the presence of phenylephrine plus 2×10^{-7} M diltiazem ($113 \pm 5\%$), at a concentration at which the calcium entry blockers partially inhibited the influx of calcium induced by the agonist. Thus, no difference was observed in the interaction between NPY and nitrendipine and between NPY and diltiazem.

Discussion

The aim of the present work was to investigate the mechanism or mechanisms that might be implicated in the enhancement by NPY of the responses to α_1-adrenoceptor agonists in rat mesenteric arterioles. In these resistance arteries, NPY potentiates contractions elicited not only by the sympathetic neurotransmitter, noradrenaline, but also by other agonists and by addition of calcium to depolarized vessels (Andriantsitohaina & Stoclet, 1988a). Thus, the enhancement by NPY of the contractile response is not a receptor-specific mechanism and must involve a more general alteration of the pathway leading from smooth muscle excitation to contraction. Indeed, binding studies have not shown any change in the number or affinity of α-adrenoceptors in rat blood vessels (Pernow *et al.*, 1986), and only a slight increase in the number of α_2- (but not α_1) binding sites in rat medulla oblongata (Agnati *et al.*, 1983), under the influence of NPY.

The α-adrenoceptors of rat mesenteric arterioles have been described as being predominantly, if not exclusively, of the α_1 type (Cauvin & Malik, 1984). In accordance with this conclusion, we have found that the α_2-adrenoceptor agonists, clonidine and UK 14304, were unable to induce any contraction of the vessels. In addition, propranolol (3×10^{-6} M) did not alter the concentration-effect curves of noradrenaline, whether in the absence or in the presence of NPY (Andriantsitohaina & Stoclet, unpublished observations). Thus, the effects of noradrenaline and phenylephrine obtained in the present study did not involve any participation of β-adrenceptors.

The mechanisms thought to be implicated in the coupling between α_1-adrenoceptor activation and vascular smooth muscle cell contraction are the production of inositol 1,4,5-trisphosphate ($InsP_3$) and the influx of extracellular calcium through dihydropyridine-sensitive channels (see reviews: Exton, 1988; Minneman, 1988; Nichols & Ruffolo, 1988). The second pathway can be entirely inhibited in the presence of a dihydropyridine calcium entry blocker at a concentration which does not affect the production of $InsP_3$ (Han *et al.*, 1987). In the present study, we performed experiments with either nitrendipine at a maximally active concentration (10^{-6} M) or diltiazem (10^{-5} M), which belongs to another class of calcium entry blocker, at a concentration at which it blocked the dihydropyridine-sensitive component of the response to phenylephrine. Higher concentrations of diltiazem (10^{-4} M) decreased the maximal response to phenylephrine by about 80%, but this effect is probably due to an additional action, which depresses phenylephrine-contraction dependent on intracellular calcium release (Cauvin *et al.*, 1983; Saida & Van Breemen, 1983; Dacquet *et al.*, 1987). The results of the present work clearly demonstrate that, on rat mesenteric arterioles, NPY enhances the dihydropyridine-sensitive component of the response to α-adrenoceptor agonists, but does not affect the dihydropyridine-insensitive one.

An increase in α_1-adrenoceptor-induced accumulation of $InsP_3$ under the influence of NPY has been demonstrated in different regions of the rat brain and in rat vas deferens (Häggblad & Fredholm 1987; Hinson *et al.*, 1988), but was not found in rabbit cultured pulmonary artery cells (Reynolds & Yokota, 1988). In the latter cells, NPY did not elevate cytosolic calcium concentration. These results and those of the present work suggest that the mechanism by which NPY potentiates vasoconstriction induced by α_1-adrenoceptor stimulation might not involve the production of $InsP_3$ and subsequent release of intracellular calcium, even though NPY may act on the $InsP_3$ pathway in other tissues.

Another series of experiments was carried out to study further the potentiating effect of NPY on the calcium entry blocker-sensitive component of the response to stimulation of α_1-adrenoceptors. The experimental protocol was designed in order to obtain concentration-effect curves of NPY in various conditions. The peptide was added to arterioles brought to the same level of tension either by phenylephrine alone or by successive additions of a higher concentration of phenylephrine and of a relaxing agent at an appropriate concentration. Prazosin was used in order to displace partially the binding of phenylephrine from its sites, forskolin and sodium nitroprusside were used to increase adenylate cyclase (Seamon & Daly, 1986) or guanylate cyclase (Trembley *et al.*, 1988) activity respectively. Diltiazem and nitrendipine were used to block partially the influx of calcium. The contractile response induced by phenylephrine in these conditions was completely dependent on the entry of calcium through dihydropyridine- and diltiazem-sensitive channels, since it could be abolished by the addition of either of these agents. In all experimental conditions, NPY increased the arteriolar tension to that obtained with 10^{-5} M noradrenaline, which corresponds to the maximal contractile capacity of the vessels (further addition of other agonists or KCl did not produce any further increase in tension). It was found that the sensitivity of the vessels to NPY was not significantly different in the absence or in the presence of any of the relaxing agents and it was similar to sensitivities obtained for other isolated blood vessels (Edvinsson *et al.*, 1984a; Hanko *et al.*, 1986; Brayden & Conway, 1988; Suzuki *et al.*, 1988).

The extent of the potentiating effect of NPY seems to depend on the experimental protocol used. In the first series of

experiments, shown in Figure 2, 10^{-7} M NPY was added 5 min before the construction of a cumulative dose-response curve to the α_1-adrenoceptor agonist, whereas in subsequent experiments NPY was added when the response to phenylephrine alone or phenylephrine plus a vasorelaxant agent had reached equilibrium. In the latter case, the maximal response elicited by NPY was obtained rapidly (about 1 min), then the response decreased with time and returned to the level of precontraction in about 10 min. Thus, after 5 min preincubation, the effect of NPY may already be below its maximum and probably this time-dependent effect of NPY could explain why NPY was apparently less efficient in the first series of experiments.

As mentioned above, it has been shown in previous work that, in rat mesenteric arterioles, the potentiating effect of NPY is not receptor-specific and must involve a pathway which is common to coupling between stimulation of various receptors or depolarization and contraction (Andriantsitohaina & Stoclet, 1988a). In agreement with the view that NPY increases the efficiency of coupling between stimulation of α_1-adrenoceptors and contraction, the present results show no difference in the effect of NPY whether α_1-adrenoceptor stimulation was partial (phenylephrine alone or phenylephrine plus prazosin) or the response to submaximal stimulation of α_1-adrenoceptors was attenuated by forskolin, sodium nitroprusside, nitrendipine or diltiazem (the two latter at a concentration which was not sufficient to block completely the influx of calcium elicited by phenylephrine).

In rat mesenteric arterioles, a quantitative relationship has recently been demonstrated between the degree of stimulation of α_1-adrenoceptors and the intracellular concentration of free calcium ions (Bukoski *et al.*, 1989). In different vascular preparations, vasorelaxant agents which induce increases in cyclic AMP levels inhibit force production without reducing intracellular free calcium and those which increase cyclic GMP levels not only uncouple the calcium concentration-force relations but also decrease basal and vasoconstrictor-elevated cytosolic free calcium (Morgan & Morgan, 1984; Hassid, 1986; Paglin *et al.*, 1988). The results presented here do not support the contention that NPY affects the relationship between calcium concentration and active tension in rat mesenteric arterioles, since the peptide did not alter the dihydropyridine-insensitive component of the response to α_1-adrenoceptor agonists. They rather support the view that NPY increased calcium entry, in accordance with the fact that its effect was abolished in the presence of nitrendipine and diltiazem at concentrations which are able to block calcium influx entirely. This hypothesis is also supported by previous results showing that NPY potentiated contractions elicited by addition of $CaCl_2$ to KCl-depolarized rat isolated mesenteric arterioles and increased the sensitivity of these vessels to the calcium channel agonist Bay K 8644 (Andriantsitohaina & Stoclet, 1988a). Such a mechanism could also explain why NPY counteracted the relaxing effects of partial inhibition of calcium entry by nitrendipine and diltiazem at low concentrations, as well as those of drugs which decrease cytosolic free calcium concentration or calcium concentration and force relation.

Noradrenaline itself produces partial depolarization of rat mesenteric arterioles (Mulvany *et al.*, 1982). NPY might potentiate the neurotransmitter-induced vasoconstriction of these resistance vessels either by increasing depolarization and subsequent opening of voltage-dependent calcium channels, or by modulating calcium channel function through interaction with a coupling protein and/or the production of a second messenger. The evidence presented above does not support the hypothesis that increased $InsP_3$ is responsible for the potentiation by NPY of noradrenaline-induced vasoconstriction in rat mesenteric arterioles. However, it has to be noted that the analysis of data obtained by use of a mixture of three drugs in *in vitro* studies is complex. Nevertheless, the interpretations of the results given above open a new pathway for exploration of the mechanism of action of NPY. The intervention of a G protein and/or second messenger, subsequent to stimulation of NPY receptors, seems to be the more likely hypothesis.

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Prevention of non-specific airway hyperreactivity after allergen challenge in guinea-pigs by the PAF receptor antagonist SDZ 64-412

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1 Allergen challenge by aerosol in sensitized guinea-pigs elicited non-specific airway hyperreactivity assessed by reactivity to i.v. histamine or acetylcholine. Airway hyperreactivity to histamine persisted for at least 48 h and was accompanied by pulmonary eosinophilia as determined by bronchoalveolar lavage cell analysis.

2 Airway hyperreactivity was independent of vagal reflex mechanisms since it was not abrogated by bilateral vagotomy.

3 The novel platelet-activating factor (PAF) receptor antagonist SDZ 64-412 inhibited the development of airway hyperreactivity, as measured 24 h after aerosol allergen challenge, when given as a single treatment orally 2 h before allergen challenge. The PAF receptor antagonist WEB 2086 as well as methylprednisolone and ketotifen also showed efficacy in preventing development of airway hyperreactivity.

4 Neither the two PAF antagonists nor ketotifen had any effect on bronchoalveolar lavage (BAL) eosinophil numbers. Methylprednisolone was the only substance which readily prevented eosinophil recruitment in addition to airway hyperreactivity.

5 We conclude that allergen-induced airway hyperreactivity in guinea-pigs is inhibited by prophylactic anti-asthma drugs and specific PAF receptor antagonists, thus demonstrating a pivotal role of PAF in this response. There was a lack of correlation between airway hyperreactivity and the presence of BAL eosinophils.

Introduction

Among the functional impairments associated with allergic asthma are acute antigen-induced bronchoconstriction and increased non-specific airway hyperreactivity. Although many inflammatory mediators have been implicated in the pathogenesis of this disease by virtue of their bronchoconstrictive properties, platelet-activating factor (PAF) has recently attracted attention due to its ability to induce not only acute bronchoconstriction (Rubin *et al.*, 1987) but also sustained, non-specific airway hyperreactivity (Cuss *et al.*, 1986) in man. Airway hyperreactivity is a hallmark feature of asthmatics, and PAF is the only mediator to date which induces this response in a sustained manner in experimental animals and man.

Pathological features of asthma include pronounced pulmonary eosinophilia associated with epithelial cell damage (Laitinen *et al.*, 1985). Attention has focused on the role of the eosinophil in inducing much of the epithelial damage seen in asthma because it is a major source of PAF (Lee *et al.*, 1984). Furthermore, PAF is the most potent chemoattractant for human eosinophils yet described (Wardlaw *et al.*, 1986).

The purpose of the present studies was to examine the potential therapeutic benefit of a PAF antagonist in an animal model of allergic asthma, as determined by its ability to inhibit the development of non-specific airway hyperreactivity induced by aerosol allergen exposure in sensitized guinea-pigs. Furthermore, we also attempted to correlate the presence or absence of eosinophils in bronchoalveolar lavage fluid with airway hyperreactivity.

Methods

Sensitization procedure

Male Hartley strain guinea-pigs were used throughout the study. Animals were barrier bred and initially weighed 250 to 280 g. Animals were sensitized to ovalbumin (OA) by a series of three i.p. injections of ovalbumin precipitated with aluminum hydroxide ($Al(OH)_3$) in saline. Each animal received 10 μg OA and 10 mg $Al(OH)_3$ in a volume of 0.5 ml. The first injection was done 7 to 14 days after arrival of the animals. The second and third booster injections were performed 14 days apart. Animals were used between 14 and 21 days after the last injection. This procedure has previously been shown to result in the development of IgE-type antibody (Andersson & Bergstrand, 1981) and was verified by its ability to produce passive cutaneous anaphylaxis in naïve guinea-pigs.

Antigen challenge procedure

All animals were pretreated with 10 mg kg^{-1} diphenhydramine (DPH) i.p. 1 h before aerosol antigen exposure. This treatment was necessary to prevent acute, fatal anaphylaxis. Negative control animals (see below) were treated with DPH at a corresponding time. Those animals receiving additional drugs were dosed as described for each group below. Animals were then placed in a clear plastic chamber (approximate volume 40 l) which was connected to the output of a DeVilbiss ultrasonic nebulizer (Model 100 HD). The nebulizer chamber was filled with an OA solution (0.1% in saline). Nebulizer output was approximately 1.5 ml min^{-1}. The duration of the antigen challenge was 60 min.

Treatment groups

A negative control group consisted of animals ($n = 25$) which were sensitised to OA but not exposed to aerosol antigen. The positive control group consisted of animals ($n = 35$) which were sensitized and subsequently exposed to aerosol antigen without prior drug treatment. The above two groups were run concurrently with all subsequent drug treatment groups, which accounts for the higher number of animals in these two groups compared to the others.

The drug treatment groups consisted of animals that were sensitized and exposed to antigen in addition to drug treatment. 2,3-Dihydro-5-[4-[2-(3,4,5-trimethoxyphenyl)ethyl] phenyl] imidazo[2,1-α] isoquinoline hydrochloride (SDZ

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64-412)-treated animals received the PAF antagonist SDZ 64-412 at either 10 ($n = 8$) or 20 mg kg^{-1} ($n = 10$). The drug, dissolved in water, was given orally 2 h before antigen challenge. 4-[3-[6-(2-Chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4, 3-a] thieno [3,2-f]-1,4-diazepin-8-yl]-1-oxopropyl-morpholine (WEB 2086)-treated animals received the structurally dissimilar PAF antagonist WEB 2086, 10 mg kg^{-1} ($n = 6$), orally 2 h before challenge. WEB 2086 was synthesized by the Medical Chemistry Department at Sandoz, E. Hanover, and its chemical purity and pharmacological activity assured as described previously (Handley *et al.*, 1988). Methylprednisolone (MP)-treated animals ($n = 9$) received MP, 37.5 mg kg^{-1} i.p., at 24 and 2 h before antigen challenge. Ketotifen-treated animals ($n = 7$) were given ketotifen (1 mg kg^{-1} orally) 2 h before antigen exposure. Since ketotifen has H_1 receptor antagonist properties, a drug control group ($n = 5$) was also included which received the same dose of ketotifen but without antigen challenge to test for residual histamine antagonist properties.

In all groups, oral dosing of the guinea-pigs was accomplished by inducing light CO_2 narcosis by exposure of the animals to dry-ice vapour in a closed container followed by gavage through a short length of polyethylene tubing. Animals fully recovered from this exposure within 15 s. The negative and positive control groups received distilled water as vehicle controls.

Bronchoprovocation testing

Except as noted, bronchoprovocation testing was performed 24 h after antigen challenge. Animals were anaesthetised with 45 mg kg^{-1} sodium pentobarbitone i.p. A tracheostomy was made and the animals ventilated with a Harvard rodent ventilator (tidal volume = 4 ml, rate = 50 breaths min^{-1}) through a heated Fleisch pneumotachograph (size 0000) to measure tracheal flow rate. A small incision was made in the right thorax and a side-holed polyethylene catheter was inserted into the pleural space. Transpulmonary pressure was measured as the difference between tracheal pressure and intrapleural pressure by a Validyne DP45 transducer. Flow and pressure signals were fed to a Buxco Model 6 respiratory analyser the output of which was in turn fed to a DL-16 data logger and an 80286-based microcomputer for visual display and data storage. The right jugular vein was cannulated for administration of acetylcholine and histamine. Gallamine (1 mg kg^{-1} i.p.) was given to prevent spontaneous respiratory efforts. In those animals in the vagal section group, a bilateral vagal section was performed just before baseline resistance measurements.

Baseline pulmonary resistance measurements were made after the lung had been inflated with 3 tidal volumes. There were no differences in baseline resistances between any of the groups. Animals first received an ascending i.v. acetylcholine (ACh) challenge consisting of 2, 5, 10, 20 and 40 μg kg^{-1} acetylcholine chloride in saline. Three minutes were allotted between doses and the lungs were inflated with 3 tidal volumes before each challenge. Ten minutes after the last ACh challenge baseline resistance was again measured and was within 10% of the starting value. The animals then received an ascending i.v. histamine challenge consisting of 2, 5, 10, 20 and 40 μg kg^{-1} histamine diphosphate in saline. In some groups, as noted below, the ACh bronchoprovocation was omitted. The maximum value of pulmonary resistance after each dose of spasmogen was recorded and expressed as a % of the respective baseline value to construct ACh and histamine dose-response curves.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed after the bronchoprovocation testing. The lungs were lavaged with 5 aliquots of 10 ml each of a modified Tris-Tyrode buffer solution. Samples were centrifuged and resuspended in 1 ml of buffer. Total white blood cell (WBC) counts were performed by an electronic cell counter (Sysmex model CC-180) and differential counts were done on slides stained with a modified Wright stain (Diff-Quik, American Scientific Products). A total of 200 cells was counted to determine the percentage of BAL cells that were eosinophils. Total BAL eosinophils were expressed as 10^6 cells recovered.

Analysis of data

Data are presented as mean ± s.e.mean. Resistance values are expressed as the % increase from the baseline value. To compare differences between treatments, we performed an analysis of variance. When an overall significant F value was obtained we performed a t test for unpaired data. Statistical significance was assumed when $P \leqslant 0.05$.

Results

The time course of airway hyperreactivity in antigen exposed guinea-pigs was examined at 24, 48 and 96 h after antigen exposure (Figure 1). At 24 h after antigen challenge, guinea-pigs were markedly hyperreactive to histamine. At 48 h after antigen challenge, animals were still hyperreactive to histamine. Although there is a suggestion that the magnitude of the response had diminished, there was no significant difference between the response at 24 and 48 h. By 96 h after antigen exposure histamine reactivity had returned to control. Therefore, this antigen challenge procedure elicited sustained airway hyperreactivity to histamine, lasting at least 48 h. Since the airway reactivity was maximal at 24 h post antigen challenge, this time point was used in all further experiments to examine drug effects.

The involvement of vagal reflexes in the development of airway hyperreactivity in experimental animals and man has been proposed (Barnes, 1986). To determine the role of vagal reflexes in this model, a set of experiments was performed in guinea-pigs which were subjected to bilateral vagal section before histamine bronchoprovocation. The results of these experiments are shown in Figure 2. In the negative control animals (sensitized but not antigen challenged), bilateral vagotomy resulted in a diminution of the baseline histamine response, as has previously been demonstrated (Mills & Widdicombe, 1970). However, vagotomy did not attenuate the development of airway hyperreactivity to histamine after antigen exposure in the positive control group, effectively ruling out a contribution of vagal reflexes in this model.

Figure 3 shows the effect of the various drug treatments on histamine reactivity as evaluated at 24 h after antigen chal-

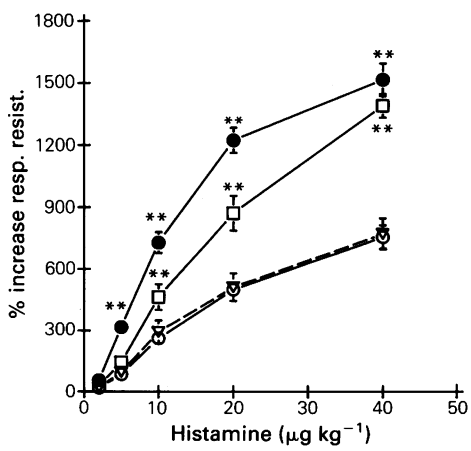


Figure 1 Airway reactivity to histamine at varying times after allergen challenge. (○) Negative control; (● positive control 24 h after allergen; (□) positive control 48 h after allergen; (▽) positive control 96 h after allergen. **$P < 0.05$ vs negative control group. In this and subsequent figures each point represents the mean and vertical lines show s.e.mean.

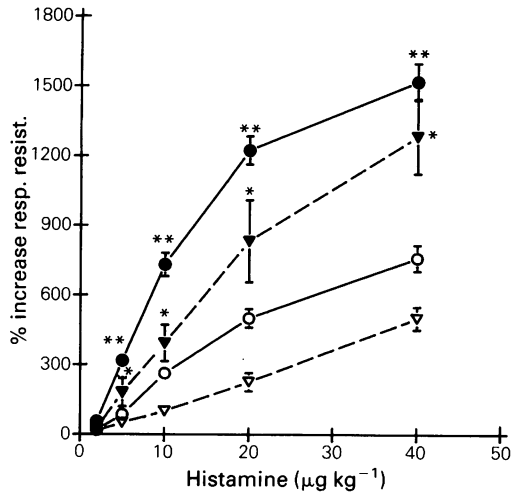


Figure 2 Effect of vagal reflexes on histamine hyperreactivity. Bilateral vagotomy was performed before histamine bronchoprovocation (24 h after allergen challenge). (○) Negative intact controls; (▽) negative vagotomized controls; (●) positive intact control group; (▼) positive vagotomized control group. $^{**}P < 0.05$ vs negative intact group; $^{*}P < 0.05$ vs negative vagotomized group.

lenge. The PAF antagonist SDZ 64-412 showed a modest effect at 10 mg kg^{-1}. However, at 20 mg kg^{-1} SDZ 64-412, histamine reactivity was significantly reduced at the three higher doses of histamine. A structurally dissimilar PAF receptor antagonist, WEB 2086 (Casals-Stenzel, 1986) was also used. At a dose of 10 mg kg^{-1}, this compound showed a variable response, with a statistically significant effect obtained at only one point on the histamine dose-response curve. Methylprednisolone and ketotifen also inhibited the airway hyperreactivity to histamine, with ketotifen being the only treatment that was able to abolish the response completely. Since the pharmacology of ketotifen includes H_1 antagonist properties (Phillips *et al.*, 1983), a seperate set of experiments was undertaken to eliminate the possibility that the results obtained with histamine as the bronchoconstrictor were merely the result of a sustained antihistamine action of ketotifen. The baseline histamine responses 24 h after H_2O vehicle or ketotifen (1 mg kg^{-1} p.o.) were identical (data not shown), eliminating the possibility that the histamine antagonist effects of ketotifen were responsible for the decreased reactivity to histamine.

Figure 4 shows the effects of these drugs on ACh reactivity. Similar results to those shown for histamine were obtained with this bronchoconstrictor. Acetylcholine reactivity was not done in the WEB 2086 group.

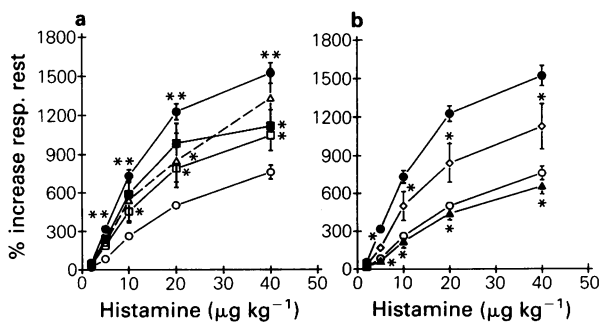


Figure 3 Effect of drug treatment on airway hyperreactivity assessed by histamine bronchoprovocation 24 h after allergen challenge. In (a), (○) negative control group; (●) positive control group; (■) SDZ 64-412 (10 mg kg^{-1}); (□) SDZ 64-412 (20 mg kg^{-1}); (△) WEB 2086 (10 mg kg^{-1}). In (b) negative and positive control groups are the same as in (a); (▲) ketotifen (1 mg kg^{-1}); (◇) methylprednisolone (75 mg kg^{-1} in 2 doses). $^{**}P < 0.05$ vs negative control group; $^{*}P < 0.05$ vs positive control group.

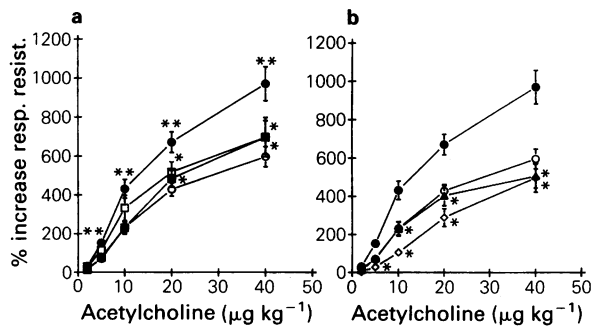


Figure 4 Effect of drug treatment on airway hyperreactivity assessed by acetylcholine bronchoprovocation 24 h after allergen challenge. Symbols are the same as in Figure 3.

Table 1 shows the number of eosinophils present in bronchoalveolar lavage from representative animals from the above experiments. There were no differences in recovery of BAL fluid between the groups (45 to 48 ml). At 24 h after antigen challenge there was a marked increase in the number of BAL eosinophils, which remained elevated at least until 96 h, the last time point studied. Neither of the PAF antagonists, SDZ 64-412 or WEB 2086 has any effect on eosinophil accumulation. Ketotifen also had no effect on BAL eosinophil accumulation. Methylprednisolone, the only treatment given twice, at 24 and 2 h before antigen, was unique in showing efficacy in preventing eosinophil accumulation.

Discussion

In these studies we have shown that the PAF receptor antagonist SDZ 64-412 inhibits the development of sustained airway hyperreactivity to histamine and acetylcholine which occurs in sensitized guinea-pigs after aerosol antigen challenge. SDZ 64-412 shares this property with two other clinically used drugs, methylprednisolone and ketotifen. Previously, Coyle and coworkers (1988) have shown inhibition of airway hyperreactivity after antigen challenge in guinea-pigs after treatment with the PAF receptor antagonist BN 52021. Effects on non-specific airway hyperreactivity have been proposed as the mechanism of action of prophylactic asthma drugs, especially steroids and disodium cromoglycate (Cockcroft & Murdock, 1987). Also, a central role for PAF in the exacerbation of asthma has been proposed (Page & Morley, 1986; Barnes & Chung, 1987); thus strengthening the concept that a PAF receptor antagonist may have therapeutic benefit as a prophylactic asthma drug.

The mechanism of action of ketotifen in inhibiting antigen-induced airway hyperreactivity in this animal model is not completely understood. Although it has been proposed that the clinical benefit of ketotifen derives from its ability to antagonize the action of PAF functionally (Morley, 1987),

Table 1 Eosinophils recovered from bronchoalveolar lavage fluid

Treatment	*Eosinophils* ($\times 10^6$ cells)
Negative control	2.7 ± 0.3
Positive control (24 h)	12.3 ± 3.5
Positive control (96 h)	13.3 ± 3.9
SDZ 64-412 (20 mg kg^{-1})	13.5 ± 1.5
WEB 2086 (10 mg kg^{-1})	15.0 ± 2.9
Ketotifen	14.8 ± 5.5
Methylprednisolone	2.1 ± 0.5*

Data shown are means ± s.e.mean. $^{*}P < 0.05$.

ketotifen is a relatively weak inhibitor of PAF-induced platelet aggregation (Criscuoli *et al.*, 1986; Casals-Stenzel *et al.*, 1987). Also, it is unlikely that it functions directly as a PAF receptor antagonist in the dose used here. Possibly, ketotifen acts proximally to the end organ effects of PAF by preventing the biosynthesis of PAF by inflammatory cells, as suggested by Joly *et al.* (1987), or distally to interfere with as yet unknown secondary mediators.

We are unaware of any studies that indicate that steroids are receptor antagonists to PAF. The effectiveness of steroids in inhibiting PAF-dependent responses may depend on the nature of the response studied. For example, at high doses steroids do not inhibit PAF-induced haemoconcentration in the guinea-pig (Handley *et al.*, 1985), but do protect against PAF-induced lethality in mice (Myers *et al.*, 1983). In the present experiments, steroid pretreatment may have inhibited the development of airway hyperreactivity by actions on cells that synthesize PAF in response to antigen exposure. Methylprednisolone was the only drug which we administered more than once in these studies (at 24 and 2 h before allergen challenge). It is well known that corticosteroids act through synthesis of secondary mediators, therefore sufficient time for protein synthesis must be allowed for steroids to exert an effect. We do not know what the effect of chronic treatment with a PAF antagonist would be. Perhaps repeated treatment would allow penetration of the drugs into compartments which are not accessible when given only 2 h before the allergen challenge.

Although PAF is a potent bronchoconstrictor in several species including man (Cuss *et al.*, 1986; Rubin *et al.*, 1987), the potential clinical utility of a PAF receptor antagonist may lie primarily in the prophylactic treatment of asthma, rather than the reversal of acute bronchoconstriction. Indeed, several studies have shown little effect of PAF antagonists in acute anaphylaxis. Pretolani *et al.* (1987) and Casals-Stenzel (1987) showed that WEB 2086 was not effective in blocking the bronchopulmonary responses to intravenous antigen in actively sensitized guinea-pigs, although there were modest effects in animals that had been passively sensitized. Also, WEB 2086 had no effect on the increased airway microvascular permeability in guinea-pigs after intravenous allergen challenge (Evans *et al.*, 1988). Danko *et al.* (1988) used the PAF receptor antagonists CV-3988 and L-652,731 and similarly showed no effects against intravenous antigen challenge in guinea-pigs, and concluded that PAF is not an important mediator of acute anaphylaxis in the guinea-pig. This is in accord with the work of Daffonchio *et al.* (1987) who demonstrated that bronchoconstriction after antigen challenge in the guinea-pig is due primarily to histamine and leukotrienes.

However, some studies have demonstrated an effect of PAF receptor antagonists in acute anaphylaxis. Cirino *et al.* (1986) showed that, in guinea-pigs passively sensitized with IgE-type antibody, the PAF antagonist BN 52021 inhibited the bronchoconstriction provoked by an aerosol of allergen. The PAF antagonist Ro 19-3704 was shown to be ineffective in blocking bronchoconstriction induced by intravenous antigen challenge, but showed effectiveness when tested against aerosol antigen exposure.

These findings suggest that the route of allergen presentation (i.e. inhaled vs intravenous) is an important determinant of the relative contribution of PAF to the acute response (Lagente *et al.*, 1988). Furthermore, we would also suggest that reversal or prevention of acute allergen-induced bronchospasm is not necessarily indicative of the therapeutic potential of prophylactic asthma therapy.

A prominent role for the eosinophil in the pathogenesis of asthma has been proposed (Durham & Kay, 1985; DeMonchy *et al.*, 1985). PAF has the potential for being important in eosinophil-mediated pathology for at least two reasons. Firstly, PAF is a potent chemoattractant for eosinophils (Wardlaw *et al.*, 1986), and secondly, eosinophils are a potential source of PAF (Lee *et al.*, 1984). Therefore, an interaction between PAF and eosinophils could include the possibility that PAF is one of the major signals responsible for pulmonary eosinophil recruitment, with subsequent tissue injury resulting from release of eosinophil granule proteins. Alternatively, eosinophils may enter the lung due to the presence of other chemoattractants and subsequently release PAF, resulting in tissue injury, airway inflammation and airway hyperreactivity.

The present study shows that three compounds that inhibited the development of airway hyperreactivity (SDZ 64-412, WEB 2086 and ketotifen) had no effect on BAL eosinophil recruitment (Table 1). These data argue that the presence of eosinophils is not sufficient in itself to cause airway hyperreactivity. Eosinophils may be required for the induction of airway hyperreactivity, but recruitment and activation may both be necessary before they exert their role. In this case, a PAF antagonist may exert its beneficial effect by blocking the end-organ effects of PAF released by activated eosinophils.

Inhibition of the functional manifestations of allergen exposure (airway hyperreactivity) did not correlate with the presence or absence of BAL eosinophils in our experiments. Similarly, other investigators have shown that the late-phase pulmonary obstruction, but not the accompanying pulmonary eosinophilia, following allergen challenge in guinea-pigs can be prevented by prior treatment with nedocromil sodium (Hutson *et al.*, 1988). Therefore, one final possibility which must be considered is that the eosinophil may not play a causative role in the development of airway hyperreactivity, but is recruited to the lung at a later time in response to tissue injury. Recent observations serve to support this hypothesis. Daffonchio *et al.* (1987) found that guinea-pigs were hyperreactive to 5-hydroxytryptamine (5-HT) as early as 1 h after aerosol antigen challenge, at a time when the prominent BAL cell is the neutrophil, not the eosinophil. Therefore, after antigen exposure, the early cellular response is a neutrophil infiltration which occurs during the time period consistent with development of airway hyperreactivity. Eosinophil recruitment is a relatively late occurrence (Dunn *et al.*, 1988) and may occur after airway hyperreactivity has already been established.

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Muscarinic receptor stimulation and cyclic AMP-dependent effects in guinea-pig ventricular myocardium

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1 The effect of carbachol on force of contraction, contraction duration, intracellular Na^+ activity and cyclic AMP content was studied in papillary muscles of the guinea-pig exposed to isoprenaline or the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX). The preparations were obtained from reserpine-pretreated animals and were electrically driven at a frequency of 0.2 Hz.

2 Isoprenaline (10 nM) and IBMX (100 μM) produced comparable positive inotropic effects of 9.8 and 9.7 mN, respectively. Carbachol (3 μM) attenuated the inotropic effects by 82% (isoprenaline) and by 79% (IBMX). The shortening of contraction duration which accompanied the positive inotropic effect of isoprenaline (by 14.9%) and of IBMX (by 22.4%) was not significantly affected by 3 μM carbachol.

3 The positive inotropic effect of 10 nM isoprenaline and of 100 μM IBMX was accompanied by an increase in cellular cyclic AMP content of 58 and 114%, respectively. Carbachol (3 μM) failed to reduce significantly the elevated cyclic AMP content of muscles exposed to either isoprenaline or IBMX.

4 In the quiescent papillary muscle, isoprenaline (10 nM) and IBMX (100 μM) reduced the intracellular Na^+ activity by 28 and 17%, respectively. This decline was not influenced by the additional application of 3 μM carbachol.

5 The results demonstrate that muscarinic antagonism in guinea-pig ventricular myocardium exposed to cyclic AMP-elevating drugs is restricted to force of contraction. The underlying mechanism does not apparently involve the cytosolic signal molecule cyclic AMP.

Introduction

The parasympathetic and sympathetic divisions of the autonomic nervous system control cardiac function by their opposing effects (for review see Levy, 1971; Higgins *et al.*, 1973). The parasympathetic modulation of sympathetic effects on the heart occurs at both the presynaptic and postsynaptic level. In the presynaptic interaction, neuronally released acetylcholine inhibits, via stimulation of muscarinic cholinoceptors, noradrenaline release from sympathetic nerve terminals. In the postsynaptic interaction, activation of atrial muscarinic cholinoceptors results in a negative inotropic effect by a mechanism that induces conductance changes of K^+ and Ca^{2+} channels. In ventricular myocardium, acetylcholine has been demonstrated to have no direct negative inotropic effect but to attenuate force of contraction elevated by enhanced sympathetic activity (Levy *et al.*, 1981). In isolated ventricular tissues, acetylcholine antagonizes not only the positive inotropic effect of β-adrenoceptor agonists, but also that of phosphodiesterase inhibitors and of forskolin, indicating that intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) plays an important role in postsynaptic muscarinic inhibition (for review see Löffelholz & Pappano, 1985). However, the biochemical mechanisms by which activation of muscarinic cholinoceptors interferes with the cyclic AMP-modulated cell function is still a matter of controversy. Depending on the relationship between acetylcholine-induced changes in cellular cyclic AMP content and force of contraction, inhibition of adenylate cyclase and/or inhibition of some reaction beyond cyclic AMP accumulation has been proposed as the underlying mechanism (for review see Löffelholz & Pappano, 1985). In a recent study, acetylcholine was shown to reduce catecholamine- and forskolin-induced increase in Ca^{2+} inward current (I_{Ca}) in guinea-pig isolated ventricular myocytes, but to be ineffective when the cell was dialysed directly with cyclic AMP or the catalytic subunit of protein kinase (Hescheler *et al.*, 1986). Although this observation strongly indicates that inhibition of adenylate cyclase is the underlying mechanism, the inability of acetylcholine to decrease intracellular cyclic AMP content in guinea-pig heart is not in line with this conclusion (Watanabe & Besch, 1975).

With the exception of force of contraction and I_{Ca}, surprisingly little is known of how muscarinic receptor agonists influence other cell functions altered by an elevated level of cyclic AMP. It was the aim of the present study to investigate whether activation of muscarinic receptors by carbachol antagonizes cyclic AMP-dependent shortening of contraction duration and Na^+–K^+ pump stimulation in guinea-pig papillary muscle. The latter was of particular interest since the Na^+–K^+ pump, besides voltage-dependent Ca^{2+} channels, constitutes another cyclic AMP-modulated transsarcolemmal ion transfer system (Pecker *et al.*, 1986). In order to complement the functional experiments, a possible effect on adenylate cyclase was also directly assessed by measuring the influence of carbachol on tissue cyclic AMP levels elevated by phosphodiesterase inhibition and by β-adrenoceptor activation. Some results of this study have been presented in preliminary form (Schmied & Korth, 1988).

Methods

Preparations

Guinea-pigs of either sex weighing 250–350 g were killed by cervical dislocation. If not stated otherwise, the animals were pretreated with reserpine (5 mg kg^{-1} body weight, injected intraperitoneally 24 h before the experiment) to avoid the release of endogeneous noradrenaline. Right ventricular papillary muscles (diameter 0.5–0.8 mm) were rapidly excised from the isolated heart and mounted in a two-chambered organ bath with internal circulation of the bath solution (volume 50 ml) as described by Reiter (1967). The bath solution was constantly gassed and kept in circulation by 95% O_2 and 5% CO_2; the temperature was maintained at 35°C, pH 7.5. The composition of the bath solution was (in mM): NaCl 115, KCl

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4.7, $MgSO_4$ 1.2, $CaCl_2$ 3.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10.

Isometric contraction recording

The muscles were stimulated at their base through two punctate platinum electrodes with square wave pulses of 1 ms in duration and an intensity slightly above threshold. Force of contraction was recorded isometrically by means of an inductive force transducer (Q-11, 10p; Hottinger Baldwin Meßtechnik, Darmstadt, F.R.G.) connected to an oscilloscope and a pen recorder. The resting force was kept constant at 4 mN throughout the experiment. An equilibration period of at least 1 h at a stimulation frequency of 1 Hz preceded each experiment. Subsequently, the frequency of stimulation was lowered to 0.2 Hz and the drug intervention was started as soon as force of contraction had reached a steady state. The following parameters of the isometric contraction were evaluated: peak force of contraction (F_c); positive inotropic effect (ΔF_c), i.e., increase in force over its basal value; contraction duration determined at 10% of F_c.

Cyclic AMP assay

Catecholamine-depleted papillary muscles were obtained and mounted as described above. The muscles were exposed to isoprenaline (10 nM) for 5 or 10 min and to 3-isobutyl, 1-methyl xanthine (100 μM) for 20 min. In some experiments carbachol (3 μM) was added in the presence of the catecholamine or IBMX for 5 min. Thereafter, the muscles were removed from the organ bath and immediately frozen in liquid nitrogen. Cyclic AMP was measured by radioimmunoassay by the methods of Harper & Brooker (1975), as described by Korth *et al.* (1987). Recoveries, run with each experiment, were found to be 100% and were not altered by the substances under investigation. All measurable material was destroyed by treatment with phosphodiesterase.

Electrophysiological measurements

In order to measure intracellular Na^+ activity (a^i_{Na}), papillary muscles were mounted horizontally in a perfusion chamber (volume 2 ml) perfused at a constant rate of 10 ml min^{-1}. The voltage recording electrodes had tip resistances of 20 MΩ and small tip potentials when filled with 3 M KCl acidified to a pH of 2 with HCl. The construction and calibration of the Na^+-sensitive microelectrodes with the neutral ion exchange resin ETH 227 (Steiner *et al.*, 1979) has been described in detail elsewhere (Sheu & Fozzard, 1982). The muscles were impaled with a conventional electrode and a Na^+-sensitive microelectrode so that the impalements were as close as possible. From the potentials measured with the two microelectrodes, the a^i_{Na} of the cell was calculated by means of the following equation

$$E^i_{Na} - E_m = E_0 + S \log (a^i_{Na} + k_{Na,K}\, a^i_K), \qquad (1)$$

where E^i_{Na} is the transmembrane potential measured with the Na^+-sensitive microelectrode with respect to the reference electrode in the bath, E_m the transmembrane potential measured with the conventional microelectrode, E_0 a constant potential of the Na^+-sensitive microelectrode, and S the slope of the Na^+-sensitive microelectrode (ranging from 50 to 61 mV per decade) as determined in NaCl solutions containing 0.1 mM EGTA. The $k_{Na,K}$ is the selectivity coefficient of the Na^+-sensitive microelectrode which ranged from 0.01 to 0.02 for $K^+ : Na^+$ in a mixture of 10 mM NaCl/140 mM KCl. The a^i_K is the intracellular K^+ activity which was 110 mM, as determined with K^+-sensitive microelectrodes in 3 papillary muscles. Before and after each experiment the electrodes were calibrated at 35°C with pure solutions of NaCl and with mixtures of NaCl and KCl, with the sum ($Na^+ + K^+$) kept constant at 150 mM. Any change in calibration meant that the experiments were discarded. Conventional and Na^+-sensitive microelectrodes were connected to a dual channel high impedance electrometer (model 773, WP Instruments, New Haven, Conn., U.S.A.). The signals were displayed separately and electronically subtracted on a pen recorder and on digital panel meters. The panel meter readings were used for calculating a^i_{Na}.

Materials

The drugs used were: carbamylcholine chloride (carbachol), 3-isobutyl, 1-methyl xanthine, (±)-propranolol hydrochloride, reserpine (dissolved in 5% ascorbic acid) from Sigma (München, F.R.G.); (±)-isoprenaline hydrochloride from Serva (Heidelberg, F.R.G.); sodium ionophore I (ETH 227), N,N-dimethyltrimethylsilylamine from Fluka (Buchs, Switzerland); cyclic AMP radioimmunoassay (^{125}I) from Becton Dickinson (Heidelberg, F.R.G.).

Statistics

The data are presented as arithmetic means ± s.e.mean. Significance tests were performed by Student's *t* test for paired or unpaired observations. Differences between means were regarded as statistically significant when $P < 0.05$.

Results

Inotropic effects

Figure 1a and b shows superimposed isometric contractions of a catecholamine-depleted papillary muscle and the effect of isoprenaline (a) or the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX; b) in the absence and presence of 3 μM carbachol. As expected, 10 nM isoprenaline and 100 μM IBMX produced a positive inotropic effect accompanied by a reduction in contraction duration. Additional application of 3 μM carbachol strongly attenuated the positive inotropic effect but did not affect the shortening of contraction duration. In contrast to its postsynaptic action, 3 μM carbachol almost completely abolished the positive inotropic effect and shortening of contraction duration evoked by noradrenaline, released neuronally by means of field stimulation (Figure 1c). Figure 2 summarizes the concentration-dependent effects of carbachol on the isometric contraction in the presence of isoprenaline or IBMX. Positive inotropic effects of six muscles, respectively, were plotted versus contraction dura-

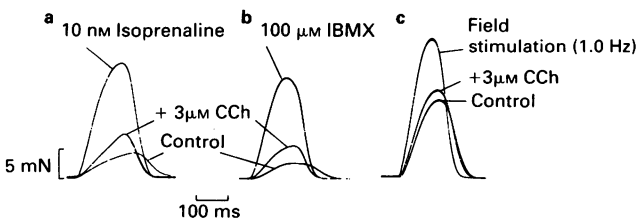


Figure 1 Superimposed isometric contraction curves of the guinea-pig papillary muscle showing the effects of isoprenaline (a) and 3-isobutyl, 1-methyl xanthine (IBMX; b) and field stimulation (c) in the absence and presence of 3 μM carbachol (CCh). In (a) and (b) the same noradrenaline-depleted papillary muscle was used. Records were taken when steady-state effects were achieved. Contraction frequency 0.2 Hz in (a) and (b), 1.0 Hz in (c).

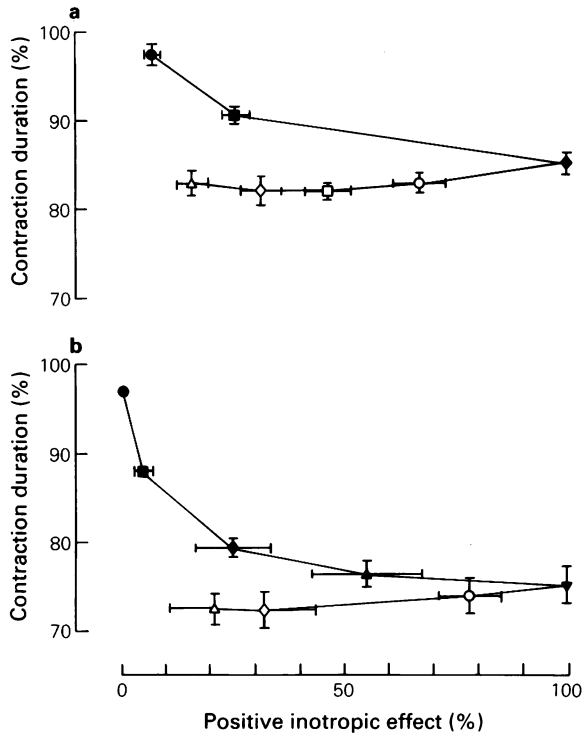


Figure 2 The effects of different concentrations of carbachol (open symbols) on the relationship between contraction duration and positive inotropic effect in the presence of (a) isoprenaline and (b) the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX). Values of contraction duration are correlated at identical concentrations with the corresponding values of the positive inotropic effect. Symbols represent the arithmetic means, with s.e.mean shown as horizontal and vertical bars, of 6 muscles. Cumulative drug application: isoprenaline (●) 1, (■) 3 and (◆) 10 nM; IBMX (●) 3, (■) 20, (◆) 60, (▲) 80, and (▼) 100 μM; carbachol (○) 0.1, (□) 0.3, (◇) 1 and (△) 3 μM. Ordinate scales: contraction duration, expressed as % of the control value (100% = 240.6 ± 10.7 ms in (a) and 231 ± 9.8 ms in (b)). Abscissa scales: positive inotropic effect, expressed as % of the value observed with 10 nM isoprenaline (100% = 9.8 ± 0.4 mN) or with 100 μM IBMX (100% = 9.7 ± 0.4 mN). Pre-drug control contraction force was 3.4 ± 0.7 mN and 3.1 ± 0.5 mN in the isoprenaline and IBMX group, respectively. Reserpine-pretreated guinea-pigs were used in all experiments depicted in (a) and (b). Contraction frequency 0.2 Hz.

tion. The data are presented before (closed symbols) and after the cumulative addition of various carbachol concentrations. In order to allow comparison of data, only those muscles which developed nearly identical positive inotropic effects at 10 nM isoprenaline (ΔF_c 100% = 9.80 ± 0.35 mN) and 100 μM IBMX (ΔF_c 100% = 9.72 ± 0.42 mN), were selected for normalization. As can be seen from Figure 2, isoprenaline (a) and IBMX (b) shortened contraction duration in a concentration-dependent fashion. However, at a given positive inotropic effect, there was a stronger effect on contraction duration in the presence of IBMX. For example, contraction was shortened with 20 μM IBMX by nearly 15% while force of contraction had barely increased. In contrast, with the same percentage of shortening, isoprenaline produced a positive inotropic effect which was 10 times stronger than that induced by 20 μM IBMX. As further shown in Figure 2, carbachol (3 μM) did not significantly affect shortening of contraction duration, although, at the same time, it attenuated the positive inotropic effect of 10 nM isoprenaline by 82 ± 3.5% ($n = 6$) and that of 100 μM IBMX by 79 ± 10.2% ($n = 6$).

The effect of carbachol on cellular cyclic AMP content

In order to determine whether a decrease in cellular cyclic AMP content may be involved in the negative inotropic action of carbachol, the effect of 3 μM carbachol on force of contraction and cyclic AMP level was investigated in the presence of 10 nM isoprenaline and 100 μM IBMX. Cyclic AMP content and force of contraction were always determined in the same muscle at the same time. From Table 1 it is evident that the positive inotropic effect of isoprenaline (10 nM) 5 and 10 min after drug addition was accompanied by a significant increase in cyclic AMP content (by 58 ± 4 and 55 ± 5%, respectively; $n = 7$). Additional application of carbachol (3 μM) for 5 min, reduced the positive inotropic effect of isoprenaline by 85.4 ± 6% (catecholamine present for 10 min). However, the cyclic AMP content remained elevated, i.e., the cyclic AMP content in the presence of carbachol did not differ significantly from the corresponding values obtained with isoprenaline alone. Control experiments revealed that carbachol (3 μM) alone produced a small positive inotropic effect (see also Korth & Kühlkamp, 1985) which was not accompanied by a change in cyclic AMP content. Results similar to those observed with isoprenaline were also obtained with carbachol in the presence of 100 μM IBMX. Carbachol (3 μM) reduced the

Table 1 Effects of carbachol on isoprenaline- and 3-isobutyl, 1-methyl xanthine (IBMX)-induced increase in cyclic AMP content and force of contraction

	Cyclic AMP (pmol mg^{-1} wet weight)	*Positive inotropic effect* (mN)
Control	0.62 ± 0.03 (23)	—
Carbachol (3 μM)	0.61 ± 0.04 (6)	0.53 ± 0.1 (6)
Isoprenaline (10 nM) 5 min	0.98 ± 0.03 (7)	10.3 ± 0.8 (7)
Isoprenaline (10 nM) 10 min	0.96 ± 0.05 (7)	9.6 ± 0.6 (7)
Isoprenaline (10 nM) + carbachol (3 μM) 10 min[a]	0.95 ± 0.04 (8)[NS]	1.8 ± 0.2 (8)*
Isoprenaline (10 nM) + carbachol (3 μM) 5 min[b]	0.93 ± 0.04 (6)[NS]	1.5 ± 0.3 (6)*
IBMX (100 μM) 20 min	1.33 ± 0.06 (7)	11.2 ± 0.9 (7)
IBMX (100 μM) + carbachol (3 μM) 20 min[a]	1.31 ± 0.05 (6)[NS]	1.7 ± 0.3 (6)*

Results are expressed as means ± s.e.mean. Numbers in parentheses denote numbers of experiments.
[a] Carbachol present during the last 5 min.
[b] Simultaneous addition of carbachol and isoprenaline.
* $P < 0.01$ vs isoprenaline or IBMX alone.
[NS] Not significant vs isoprenaline or IBMX alone.

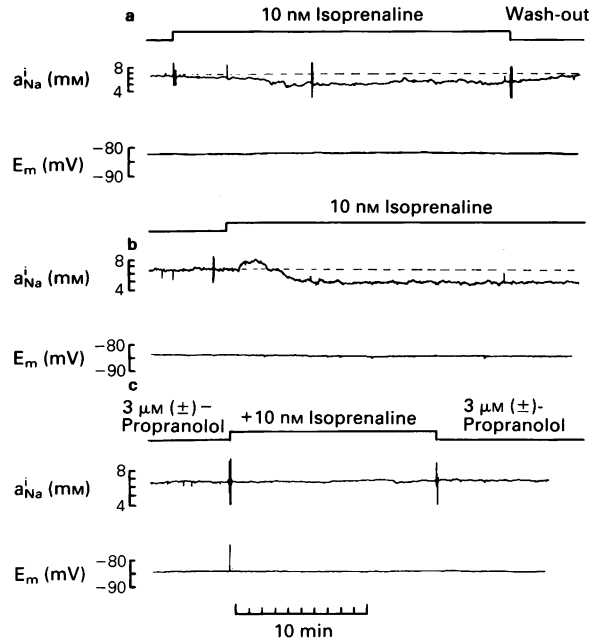


Figure 3 (a) Isoprenaline-induced decrease in intracellular Na^+ activity (a^i_{Na}) and its reversal by washing with drug-free solution. (b) Transient increase in a^i_{Na} upon superfusion of a muscle with 10 nM isoprenaline. Note that this effect was only occasionally seen. (c) Abolition of the isoprenaline-induced decrease in a^i_{Na} in the presence of 3 μM (±)-propranolol. Propranolol was added 60 min before the superfusion with the catecholamine. Resting papillary muscles from reserpine-pretreated guinea-pigs were used in the experiments depicted in (a), (b) and (c).

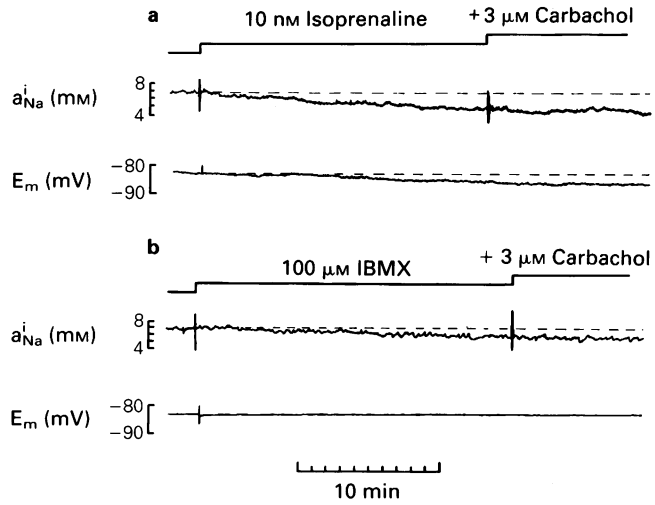


Figure 4 Effect of the cyclic AMP-elevating drugs isoprenaline and 3-isobutyl, 1-methyl xanthine (IBMX) on intracellular Na^+ activity (a^i_{Na}) and resting membrane potential (E_m) in the absence and presence of carbachol. (a) Decrease in a^i_{Na} and hyperpolarization of E_m upon superfusion of 10 nM isoprenaline. Failure of 3 μM carbachol to attenuate both isoprenaline effects. Note that hyperpolarization of E_m was very pronounced in this particular experiment. (b) Decrease in a^i_{Na} during superfusion with 100 μM IBMX. This effect remains unchanged in the presence of 3 μM carbachol. Resting papillary muscles from reserpine-pretreated guinea-pigs were used in the experiments depicted in (a) and (b).

positive inotropic effect of the phosphodiesterase inhibitor by 84.8 ± 8% ($n = 7$) but failed to decrease significantly the elevated cyclic AMP content. Interestingly, IBMX evoked a positive inotropic effect within 20 min which was comparable to that of 10 nM isoprenaline, but at the same time induced an elevation of the cyclic AMP level which exceeded that of the catecholamine by 36%. The possibility that the sequence of drug addition might have influenced the effects was considered. Isoprenaline was therefore added simultaneously with carbachol instead of consecutively. As can be seen from Table 1, force of contraction rose within 5 min to 15% of the effect obtained with 10 nM isoprenaline alone, while the cyclic AMP level was increased to about the same amount as with the catecholamine alone. Thus, the results obtained with the simultaneous application of isoprenaline and carbachol were similar to those obtained after the sequential addition of the drugs.

The effect of carbachol on Na^+ activity

The effect of 10 nM isoprenaline on a^i_{Na} and resting membrane potential in a quiescent papillary muscle is shown in Figure 3. Application of isoprenaline to the superfusing solution caused a^i_{Na} to decline from 6.5 mM to 5.0 mM within 12 min. After a^i_{Na} was constant for 15 min, the muscle was again superfused with catecholamine-free solution, and a^i_{Na} returned to its pre-drug control level within 6 min. In a total of 10 papillary muscles, exposure to isoprenaline (10 nM) caused a decrease in a^i_{Na} (Table 2) with an average change of 1.9 ± 0.2 mM (equivalent to a decrease of 30%). In the experiment presented in Figure 3a, membrane resting potential remained stable at −82 mV. This, however, was not a regular finding, in 7 out of 10 experiments, isoprenaline evoked a 1 to 3 mV hyperpolarization which paralleled the decrease in a^i_{Na} (see Figure 4a and Table 2). As can be seen from Figure 3c, the decline in a^i_{Na} induced by isoprenaline was due to stimulation of β-adrenoceptors, for it was completely antagonized by 3 μM (±)-propranolol. In 3 out of 10 experiments, isoprenaline (10 nM) caused a transient

Table 2 Effects of isoprenaline on intracellular Na^+ activity (a^i_{Na}) and resting membrane potential (E_m) before and after the addition of carbachol or in the presence of (±)-propranolol

Condition	E_m (mV)	ΔE_m (mV)	a^i_{Na} (mM)	Δa^i_{Na} (mM)	n
Control	−82.8 ± 0.4		6.8 ± 0.2		10
Isoprenaline (10 nM)	−84.5 ± 0.5	−1.7 ± 0.5**	4.9 ± 0.2	−1.9 ± 0.2***	
Isoprenaline (10 nM) + carbachol (3 μM)	−84.4 ± 0.4		4.9 ± 0.2		
After wash-out	−82.5 ± 1.0		6.6 ± 0.9		
(±)-Propranolol (3 μM)	−83.8 ± 0.6		6.9 ± 0.3		4
(±)-Propranolol (3 μM) + isoprenaline (10 nM)	−83.7 ± 0.6		6.9 ± 0.3		

Results are expressed as means ± s.e.mean. Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg^{-1} body weight reserpine injected i.p. 24 h before the experiment).
n = number of muscles.
**$P < 0.01$, ***$P < 0.001$; Student's paired t test.

Table 3 Influence of carbachol on 3-isobutyl, 1-methyl xanthine (IBMX)-induced decrease in intracellular Na^+-activity (a^i_{Na})

Condition	*Resting potential* (mV)	a^i_{Na} (mM)	Δa^i_{Na} (mM)	n
Control	−82.7 ± 0.4	7.1 ± 0.3		3
IBMX (100 μM)	−83.1 ± 1.1	6.0 ± 0.3	−1.1 ± 0.3*	
IBMX (100 μM) + carbachol (3 μM)	−83.2 ± 1.0	6.0 ± 0.3		

Results are expressed as means ± s.e.mean. Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg^{-1} body weight reserpine injected i.p. 24 h before the experiment).
n = number of muscles.
*$P < 0.05$; Student's paired t test.

increase in a^i_{Na} which was then followed by a sustained decrease. Figure 3b shows such an experiment in which a^i_{Na} rose within 2 min from 6.7 mM to 8.2 mM and then declined over the following 5 min to a steady-state level of 4.9 mM. Since a temperature effect upon switching to the catecholamine-containing solution could be ruled out, the reason for the transient increase in a^i_{Na} is not apparent. Figure 4a shows a typical experiment in which the effect of carbachol (3 μM) on isoprenaline-induced decrease in a^i_{Na} was investigated. Isoprenaline (10 nM) caused a^i_{Na} to decline from 6.8 mM to 4.1 mM and membrane resting potential to hyperpolarize from −83.0 to −85.5 mV. Superfusion of the preparation with isoprenaline plus carbachol for 10 min was without further effect on a^i_{Na} and membrane resting potential. On switching to drug-free solution, both a^i_{Na} and resting potential returned towards control levels. Table 2 summarizes the effects of isoprenaline, isoprenaline plus carbachol and of washout on a^i_{Na} and resting membrane potential, determined in 10 catecholamine-depleted papillary muscles. Figure 4b shows that IBMX (100 μM) resembled the catecholamine by causing a^i_{Na} to decrease from 6.8 mM to 5.3 mM within 20 min. This effect was not influenced by additional application of 3 μM carbachol. As summarized in Table 3, exposure of 2 other preparations to 100 μM IBMX in the absence and in the presence of carbachol produced changes in a^i_{Na} which were identical to those shown in Figure 4b.

Discussion

The present study shows that force of contraction when enhanced by isoprenaline or the phosphodiesterase inhibitor IBMX, was strongly reduced by carbachol in a concentration-dependent manner. Such an antagonism has been previously described in ventricular preparations exposed to drugs that either stimulate adenylate cyclase or inhibit phosphodiesterase activity (Meester & Hardman, 1967; Endoh, 1980; Inui *et al.*, 1982). Muscarinic receptor agonists have been demonstrated to decrease the transsarcolemmal Ca^{2+} current, I_{Ca}, in guinea-pig ventricular myocytes exposed to catecholamines or phosphodiesterase inhibitors (Hescheler *et al.*, 1986) and consequently to reduce the myoplasmic Ca^{2+} transient recorded in aequorin-injected contracting papillary muscles (McIvor *et al.*, 1988). Thus, reduction of cyclic AMP-enhanced I_{Ca} seems to be directly responsible for the negative inotropic effect of muscarinic receptor agonists.

Because most if not all cardiac effects of elevated cyclic AMP are mediated by the cyclic AMP-dependent protein kinase (Robinson-Steiner & Corbin, 1986; Kameyama *et al.*, 1986; Solaro, 1986), it has been postulated that muscarinic receptor agonists interfere at some point in this system. The discovery that cardiac muscarinic receptors are coupled to adenylate cyclase via an inhibitory transducer protein (G_i), which couples the inhibitory signals from the receptors to the catalytic subunit of the cyclase (Gilman, 1984; Rodbell, 1980), led to the notion that the mechanism of muscarinic inhibition is due to reductions in cyclic AMP levels as a result of inhibition of adenylate cyclase. Indeed, muscarinic inhibition of catecholamine-induced elevation of cyclic AMP levels has been demonstrated in cardiac tissues. This has led some investigators to suggest that this is the major mechanism of action of muscarinic receptor agonists on ventricular tissues (Gardner & Allen, 1976; Keely *et al.*, 1978; Endoh, 1980; Inui *et al.*, 1982; Linden *et al.*, 1985). The present results, however, demonstrate that the negative inotropic effect of carbachol was not accompanied by a reduction in isoprenaline- or IBMX-enhanced cyclic AMP content. From this it must be concluded that inhibition of adenylate cyclase cannot be the mechanism for the decrease in I_{Ca} and hence for the negative inotropic action of carbachol in guinea-pig papillary muscle. A similar conclusion was reached by Watanabe & Besch (1975) who showed, in the whole heart preparation of the guinea-pig, that acetylcholine did not influence the cyclic AMP content elevated by isoprenaline. However, in a more recent study Lindemann & Watanabe (1985b) found a small but significant inhibition of cyclic AMP accumulation when acetylcholine was given simultaneously with isoprenaline or IBMX to guinea-pig hearts perfused by the Langendorff technique. In the present study, neither the simultaneous nor the sequential addition of carbachol had any influence on the increase in cyclic AMP induced by 10 nM isoprenaline or 100 μM IBMX.

Besides their positive inotropic- and cyclic AMP-elevating effects, isoprenaline as well as IBMX shortened contraction duration of the guinea-pig papillary muscle. At low and moderately effective concentrations, this effect was due to a shortening of time to peak force, while at high concentrations shortening of relaxation time became the prevailing effect (see also Korth & Schmied, 1988). In accordance with the cyclic AMP measurements, carbachol did not abolish the shortening of contraction duration induced by either the catecholamine or the phosphodiesterase inhibitor. It appears, therefore, as if contraction duration is a more reliable parameter for estimating changes in cellular cyclic AMP content than force of contraction or I_{Ca}. In this context, it is interesting to note that isoprenaline and IBMX differed with respect to the relationship between positive inotropic effect and shortening of contraction duration (see Figure 2). This difference was no longer apparent when contraction duration was correlated with the respective cyclic AMP levels, as determined in an earlier study under otherwise identical conditions (Korth *et al.*, 1987). The present results are in accord with the recent finding that acetylcholine, despite its strong negative inotropic effect, failed to alter the isoprenaline-induced shortening of relaxation time in ferret papillary muscle (McIvor *et al.*, 1988). In the same study, it was shown that acetylcholine, by a cyclic AMP-independent mechanism, reversed the isoprenaline-induced decrease of the affinity of troponin for Ca^{2+}. Both observations combined seem to exclude the possibility that the myocardial relaxation is modulated by changes in myofibrillar Ca^{2+} sensitivity. The finding that cyclic AMP-dependent phosphorylation of phospholamban is associated with increases in the rate of Ca^{2+} accumulation by sarcoplasmic reticulum (Tada *et al.*, 1975; Hicks *et al.*, 1979) and shortening of relaxation time (Lindemann *et al.*, 1983; Lindemann & Watanabe, 1985a) is consistent with the hypothesis that the rate of Ca^{2+} pumping out of the myoplasm determines the relaxation time of ventricular myocardium. The present findings, as well as those of McIvor *et al.* (1988), are in contrast to

studies on guinea-pig hearts perfused by the Langendorff technique (Lindemann *et al.*, 1983; Lindemann & Watanabe, 1985b). By use of cyclic AMP-elevating drugs such as isoprenaline, IBMX and forskolin, which activates directly the catalytic subunit of adenylate cyclase (Daly, 1984), these authors found that additional stimulation of muscarinic receptors attenuated phospholamban phosphorylation, Ca uptake into the sarcoplasmic reticulum and shortening of relaxation time. Since the present study and the experiments of Lindemann *et al.* (1983) and of Lindemann & Watanabe (1985b) were carried out on guinea-pig ventricular myocardium, species differences can hardly account for the discrepancy in results. Moreover, the failure of carbachol to antagonize cyclic AMP-dependent shortening of contraction duration was also apparent when the muscle was exposed to isoprenaline or IBMX initially, carbachol initially, or to both drugs simultaneously. This indicates that the experimental protocol can hardly account for the disparity in findings.

In cardiac muscle cells, catecholamines increase Na^+ efflux and K^+ influx, and, under certain conditions, hyperpolarize resting membrane potential (Glitsch *et al.*, 1965; Vasalle & Barnabei, 1971; Hougen *et al.*, 1981). More recently, direct measurements of the effects of catecholamines on intracellular Na^+ activity were made on multicellular heart preparations (Wasserstrom *et al.*, 1982; Lee & Vasalle, 1983) and on isolated cardiac myocytes (Désilets & Baumgarten, 1986). In all three studies, catecholamines were found to decrease a^i_{Na} by directly stimulating Na^+–K^+ pump activity in heart muscle. Since cyclic AMP derivatives (Pecker *et al.*, 1986) and forskolin (Zalups & Sheu, 1987) mimicked the catecholamine effect on a^i_{Na}, it can be concluded that stimulation of the Na^+–K^+ pump is a cyclic AMP-dependent effect. However, the nature, and role of proteins that are presumedly phosphorylated as part of the molecular mechanism causing Na^+–K^+ pump stimulation are presently unknown. The present results are in agreement with the aforementioned studies, demonstrating a persistent decrease in a^i_{Na} (by 30%) when the papillary muscle was superfused with 10 nM isoprenaline. Hyperpolarization of membrane resting potential, which accompanied the decline in a^i_{Na} in most of the preparations, was probably due to the electrogenicity of the stimulated Na^+–K^+ pump (Gadsby, 1984). As with shortening of contraction duration and elevated cyclic AMP level, additional application of carbachol failed to attenuate the decline in a^i_{Na} and the hyperpolarization of membrane resting potential. The phosphodiesterase inhibitor IBMX mimicked the catecholamine effect by producing a decline in a^i_{Na} which was also resistant to stimulation of muscarinic receptors.

In conclusion, the present results demonstrate that the antagonism of cardiac muscarinic receptor stimulation in the presence of cyclic AMP-elevating drugs is restricted to a negative inotropic effect, which presumedly depends on inhibition of I_{Ca}. Elevated cyclic AMP levels, shortening of contraction duration and Na^+–K^+ pump stimulation remains unaffected by muscarinic receptor agonists. The results are compatible with the hypothesis that stimulation of muscarinic receptors leads to inhibition of voltage-dependent Ca^{2+} channels by a mechanism that does not involve directly the cytosolic signal molecule cyclic AMP.

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L-N^G-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*

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1 The effect of L-N^G-nitro arginine (L-NOARG) was compared with that of L-N^G-monomethyl arginine (L-NMMA) on vasodilatation of the isolated aorta of the rabbit and perfused mesentery of the rat in response to acetylcholine (ACh) and sodium nitroprusside (NP).

2 L-NOARG (1.5–100 μM) and L-NMMA (3–100 μM) produced concentration-related contraction of the rabbit aorta precontracted with phenylephrine (700–900 nM). Similarly, L-NOARG (10–200 μM) and L-NMMA (30–100 μM) elevated perfusion pressure of the noradrenaline (NA, 0.6–2.5 mM)-preconstricted rat mesentery preparation.

3 L-NOARG (1.5–100 μM) and L-NMMA (3–100 μM) caused concentration-related inhibition of the vasodilator effect of ACh (0.01–1.0 μM) on the rabbit aorta without influencing responses to NP (0.03–0.5 μM). L-NOARG methyl ester (30 μM) also inhibited ACh-induced vasorelaxation with similar potency to NOARG. L-arginine (30–150 μM) but not D-arginine (100 μM) caused graded reversal of the inhibitory effect of both L-NOARG (15 μM) and L-NMMA (30 μM). Complete reversal of the effect of both inhibitors was achieved with 150 μM L-arginine. L-Alanine (50 μM), L-arginosuccinic acid (5 μM), L-citrulline (50 μM), L-methionine (50 μM) and L-ornithine (50 μM) failed to reverse the inhibitory effect of L-NOARG (15 μM).

4 L-NOARG (10–200 μM) and L-NMMA (30–100 μM) inhibited the vasodilator effect of ACh (0.006–18.0 nmol) in the rat mesentery without affecting vasodilatation due to NP (1.1–11.1 nmol). L-Arginine (100 μM) but not D-arginine (100 μM) produced partial reversal of the effect of L-NOARG (30 μM) and L-NMMA (30 μM).

5 L- and D-N^α-butyloxycarbonyl N^G-nitro arginine (100 μM) produced modest (approximately 20%) inhibition of the effect of ACh on the rabbit aorta; this effect was not reversible with L-arginine (100 μM). L-N^α-monocarbobenzoxy arginine (L-NMCA, 50 μM), L-N^α-N^G-dicarbobenzoxy arginine (L-NDCA, 5 μM) and L-N^G-tosyl arginine (50 μM) were inactive.

6 These results identify L-NOARG as a potent, L-arginine reversible inhibitor of endothelium-dependent vasodilatation. The available data suggests that L-NOARG, like L-NMMA, inhibits endothelial nitric oxide (NO) biosynthesis.

Introduction

Endothelium-derived relaxing factor (EDRF) mediates the vasodilator effect of acetylcholine (ACh) and a number of other substances on isolated blood vessels (Furchgott, 1984) and exhibits potent platelet anti-aggregatory activity both *in vitro* (Azuma *et al.*, 1986) and *in vivo* (Bhardwaj *et al.*, 1988; Hogan *et al.*, 1988). Evidence has been presented in the literature that EDRF is identical with nitric oxide (NO) (Palmer *et al.*, 1987) and is formed from L-arginine (Palmer *et al.*, 1988a). Recently, L-N^G-monomethyl arginine (L-NMMA) was found to inhibit NO biosynthesis by vascular endothelial cells (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988). We show here that a second guanidino-substituted L-arginine analogue, L-N^G-nitro arginine (L-NOARG), produces a similar L-arginine-reversible inhibition of the vasodilator effect of ACh in rabbit aortic rings and the rat perfused mesentery. Some of these results have been reported in preliminary form to the British Pharmacological Society (Moore *et al.*, 1989).

Methods

Rabbit isolated aorta

Endothelium-dependent relaxation of rabbit isolated aortic rings was studied as described by Rees *et al.*, (1989a). Male New Zealand White rabbits (2.5–3.5 kg) were killed by injection of pentobarbitone (60 mg kg^{-1}, i.v.). The thoracic aorta was removed, cleared of extraneous connective tissue and cut into rings of approximate 3 mm diameter. In some experiments endothelial cells were removed by rubbing the intimal surface with moistened cotton wool. Such preparations failed to relax in response to a maximal concentration of ACh (1 μM) but responded normally to a maximal concentration of NP (0.5 μM).

Aortic rings were mounted using stainless steel clips under a tension of 2 g in 15 ml organ baths containing warmed (37°C), oxygenated (95% O_2 : 5% CO_2) Krebs solution (composition, mM: NaCl 118, $NaHCO_3$ 25, KCl, 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2$ 2.5, glucose 5.6, pH 7.3). Indomethacin (7 μM) was added to inhibit vascular prostacyclin (PGI_2) biosynthesis. After an initial equilibration period of 1 h, preparations were contracted with a submaximal concentration of phenylephrine (700–900 nM) and thereafter relaxed by cumulative addition of either ACh (0.01–1.0 μM) or sodium nitroprusside (NP) (0.03–0.5 μM). Potential inhibitors of NO biosynthesis i.e. L-NOARG, L-NOARG methyl ester, L-NMMA, L-N^α-monocarbobenzoxy arginine (L-NMCA), L-N^α-N^G-dicarbobenzoxy arginine (L-NDCA), L- and D-N^α-butyloxycarbonyl N^G-nitro arginine and L-N^G-tosyl arginine were preincubated in the organ bath for 12 min before construction of a second cumulative concentration-response curve to ACh or NP. In experiments to determine the reversibility of the inhibitory effect of guanidino-substituted arginine analogues, selected amino acids, L-citrulline, L-ornithine or L-arginosuccinic acid were added to the organ bath containing inhibitor 6 min before further addition of ACh or NP. Each preparation was exposed to a single concentration of one inhibitor.

Rat isolated perfused mesentery

Rat mesentery preparations were perfused as described by Bhardwaj & Moore (1988). Briefly, male Sprague-Dawley rats

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(250–350 g) were killed by a blow to the head and exsanguinated. The mesenteric vascular bed was perfused (7 ml min^{-1}) with warmed (37°C), oxygenated (95% O_2 : 5% CO_2) Krebs solution via a cannula inserted in the superior mesenteric artery. Perfusion pressure was monitored continuously by means of a Bell & Howell pressure transducer connected to a Devices pen recorder. Indomethacin (7 μM) was added to the perfusing Krebs solution.

The vasodilator effect of drugs was assessed in so-called 'high tone' preparations in which noradrenaline (0.06–2.5 mM) was added to the Krebs reservoir at a concentration sufficient to increase perfusion pressure by 70–90 mmHg representing approximately 60–75% of the maximum response. No significant loss of vasoconstrictor tone or change in mesentery weight was detected over a 4 h perfusion period which was thus the maximum length of each experiment. ACh (0.01–10 nmol) or NP (0.1–20 nmol) were injected in a small volume (< 10 μl) in order to prevent changes in perfusion pressure due to an injection artefact. In some experiments, mesentery preparations were perfused (30 s) with 4.7 mg ml^{-1} 3-(3-cholamidopropyl) 1-propanesulphonate (CHAPS) to remove endothelial cells lining resistance blood vessels. Treatment in this way reduced (> 90%) the vasodilator activity of a maximally effective dose of ACh (10 nmol) without influencing the response to NP (20 nmol). Putative inhibitors of NO biosynthesis were added to the Krebs solution and allowed to perfuse 'high tone' preparations for 10 min before challenge with either ACh or NP. Thereafter, L-arginine or D-arginine was added to the perfusate and the effect of ACh re-evaluated 10–60 min later in order to assess the reversibility of any inhibition detected.

Chemicals

L-NMMA was the generous gift of Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent. L-NOARG, guanidino- and N$^\alpha$-substituted arginine derivatives, L-acetylcholine chloride, L-alanine, L-arginine, D-arginine, L-arginosuccinic acid, L-citrulline, CHAPS, L-methionine, L-ornithine, (−)-phenylephrine hydrochloride, sodium nitroprusside, and (−)-noradrenaline bitartrate were purchased from Sigma Ltd. All drugs were dissolved in saline (0.9% w/v NaCl) with the exception of L-NDCA which was prepared in saline acidified to pH 4.0 with 4 N HCl. Drugs were kept on ice throughout the experiment. Stock solutions of L-NMMA were prepared fresh each day. L-NOARG was soluble in saline following sonication.

Statistics

Results indicate mean ± s.e.mean with the number of observations shown in parentheses. Statistical analysis of differences between groups was determined by Student's unpaired *t* test. A *P* value of 0.05 or less was taken as statistically significant.

Results

Effect of L-NOARG on endothelium-dependent relaxation of the rabbit aorta

ACh (−log EC_{50}, 7.1 ± 0.2 M, $n = 39$) and NP (−log EC_{50}, 7.4 ± 0.4 M, $n = 8$) produced dose-related relaxation of the phenylephrine-precontracted rabbit aorta. At concentrations in excess of 1 μM, ACh caused further contraction.

Incubation of phenylephrine-precontracted aortic rings with L-NOARG (1.5–100 μM) or L-NMMA (3–100 μM) produced a concentration-related contraction which gradually declined over the next 12 min. For example, L-NOARG (15 μM) or L-NMMA (30 μM) administration caused a further increase in tension of 16.4 ± 1.1% ($n = 8$) and 27.3 ± 2.9% ($n = 8$) of the response to phenylephrine. In contrast, neither inhibitor (up to 100 μM) influenced the tone of phenylephrine precontracted aortic rings which had been rubbed to remove endothelial cells or caused contraction of intact preparations in the absence of phenylephrine. Continued exposure of aortic rings to either L-NOARG (Figure 1) or L-NMMA (Figure 2) resulted in concentration-related inhibition of the vasorelaxant effect of ACh without affecting the response to NP.

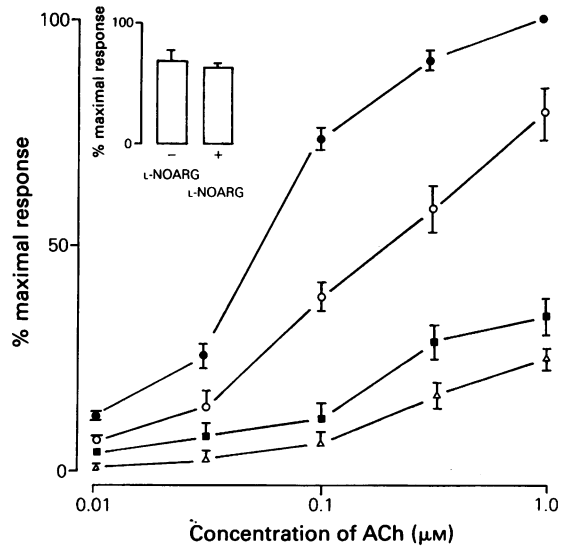


Figure 1 Relaxant effect of acetylcholine (ACh) on the phenylephrine-precontracted rabbit aorta in the absence (●) and presence of L-N^G-nitroarginine (L-NOARG) 1.5 μM (○), 15 μM (■) and 100 μM (△). Inset shows the effect of L-NOARG (30 μM) on the relaxant response to an approximate EC_{70} of sodium-nitroprusside (0.05 μM). Results show mean; s.e.mean indicated by vertical bars, $n = 8$. Where no error bar is shown standard error lies within the dimensions of the symbol.

Of the other guanidino-substituted L-arginine analogues investigated, L-N^G-nitro arginine methylester (30 μM) produced an inhibition of ACh vasodilatation which was similar to that of an identical concentration of L-NOARG (e.g. 0.33 μM ACh, 62.3 ± 4.6% inhibition, $n = 6$, cf. 64.8 ± 1.9% inhibition, $n = 6$, $P > 0.05$). L-N$^\alpha$-butyloxycarbonyl N^G-nitro arginine

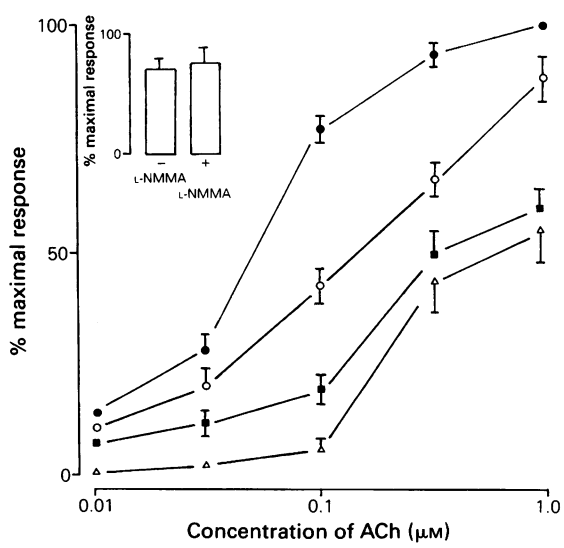


Figure 2 Relaxant effect of acetylcholine (ACh) on the phenylephrine-precontracted rabbit aorta in the absence (●) and presence of L-N^G-monomethyl arginine (L-NMMA), 3 μM (○), 30 μM (■) and 100 μM (△). Inset show the effect of L-NMMA (30 μM) on the relaxant response to an approximate EC_{70} of sodium-nitroprusside (0.05 μM). Results show mean, with s.e.mean indicated by vertical bars, $n = 8$. Where no error bar is shown standard error lies within the dimensions of the symbol.

(100 μM) produced a modest but significant inhibition of responses to ACh (e.g. 1 μM ACh, 17.6 ± 5.4%, $P < 0.05$, $n = 6$) without affecting the response to NP (data not shown). This effect was mimicked by the same concentration of D-N^{α}-butyloxycarbonyl N^{G}-nitro arginine (e.g. 1 μM ACh, 21.2 ± 4.4%, $P < 0.05$, $n = 6$). L-Arginine (100 μM) did not reverse the inhibitory effect of either isomer. In separate experiments on 3–6 preparations, L-NMCA (50 μM), L-NDCA (5 μM), and L-N^{G}-tosyl arginine (50 μM) did not influence the relaxant effect of either ACh or NP. A higher concentration of L-NDCA (50 μM) produced small but consistent relaxation of the rabbit aorta and thus could not be employed. This effect may be pH-related since administration of a similar volume of saline adjusted to pH 4.0 with 4N HCl also relaxed the phenylephrine-precontracted rabbit aorta.

Inhibition of endothelium-dependent responses to ACh following exposure of aortic rings to high concentrations of L-NOARG (15 μM) or L-NMMA (30 μM) persisted despite repeated washouts for up to 60 min. Addition of L-arginine (30–150 μM) to the bathing medium produced a concentration-related reversal of the effect of both L-NOARG (15 μM) and L-NMMA (30 μM) (Figure 3). Complete reversal of the inhibitory effect of both analogues was achieved at the highest concentration of L-arginine employed. In contrast, D-arginine (100 μM) did not reverse the inhibitory effect of either L-NOARG or L-NMMA at these concentrations.

The L-arginine precursors, L-arginosuccinic acid (50 μM) and L-citrulline as well as L-ornithine and the amino acids L-alanine and L-methionine (all 100 μM) failed to reverse inhibition due to L-NOARG (15 μM) (Figure 3). Of the above substances, L-arginine (100 μM) and L-arginosuccinic acid (50 μM) produced relaxations of the rabbit aorta in their own right (12.0 ± 8.4% and 13.5 ± 14.1 of the maximal phenylephrine response, $n = 6$). However, these effects were small and inconsistent between tissues.

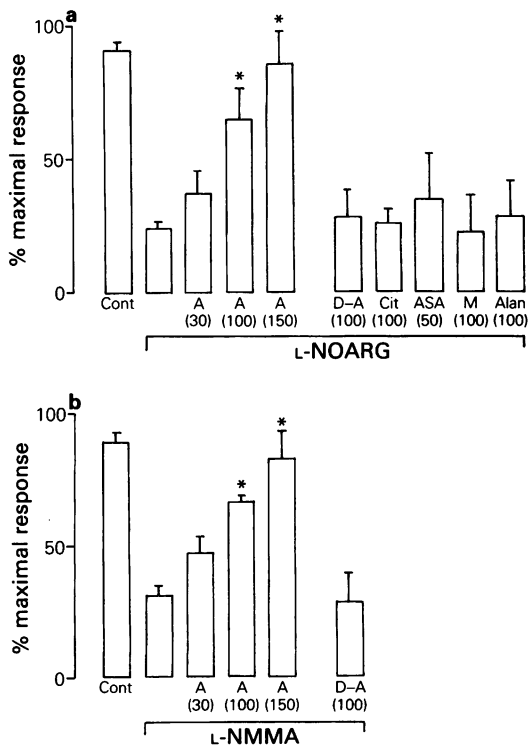


Figure 3 Inhibition of the relaxant effect of acetylcholine (ACh) (0.33 μM) on the phenylephrine-precontracted rabbit aorta by L-N^{G}-nitro arginine (L-NOARG) (30 μM, a) and L-N^{G}-monomethyl arginine (L-NMMA) (30 μM, b). Effect of L-arginine (A), D-arginine (D-A), L-citrulline (Cit), L-arginosuccinic acid (ASA), L-methionine (M) and L-alanine (Alan) in reversing blockade. Figures in parentheses indicate concentration in μM. Results show mean, with s.e.mean shown by vertical bars, $n = 6$, * $P < 0.05$ cf. response to ACh in the presence of inhibitor alone (Cont).

Effect of L-NOARG on endothelium-dependent vasodilatation of the perfused rat mesentery

Bolus injection of ACh (ED_{50}, 0.07 ± 0.02 nmol, $n = 22$) or NP (ED_{50}, 5.2 ± 0.6 nmol, $n = 5$) resulted in dose-related and transient falls in perfusion pressure of the isolated perfused 'high tone' rat mesentery preparation. Addition of either L-NOARG (10–200 μM) or L-NMMA (30–100 μM) to the perfusing Krebs solution resulted in a rapid and graded increase in the perfusion pressure of noradrenaline-preconstricted preparations. For example, L-NOARG (30 μM) and L-NMMA (30 μM) increased perfusion pressure by 54.5 ± 6.7 and 33.9 ± 7.7 mmHg respectively ($n = 8$). Vasoconstriction was not maintained in the continued presence of inhibitor, declining to the previous level within 5–10 min. Under these conditions L-NOARG and L-NMMA inhibited responses to ACh without affecting vasodilatation due to NP. For example, the response to a maximally effective dose of ACh (18 nmol) was inhibited by 25.3 ± 5.6%, 56.5 ± 2.1%, 68.0 ± 5.2% and 98.7 ± 2.3% (all $P < 0.05$, $n = 4$–8) by L-NOARG concentrations of 10 μM, 30 μM, 50 μM and 200 μM respectively. L-NMMA was less potent (16.7 ± 3.0% inhibition at a concentration of 30 μM, $P < 0.05$, $n = 6$). Concentration-response curves to show the effect of L-NOARG and L-NMMA on ACh-induced vasodilatation in the rat mesentery are included in Figure 4 and 5.

Removal of L-NOARG or L-NMMA from the Krebs perfusate did not restore vasodilatation due to ACh at least over the 1 h recovery period of these experiments. However, addition of L-arginine (100 μM) to the Krebs perfusate partially reversed the inhibitory effect of both L-NOARG (30 μM) and L-NMMA (30 μM) following perfusion for 1 h (Figure 6). No reversal was detected within 10 min of L-arginine addition. D-Arginine (100 μM) did not reverse inhibition of responses to ACh by either analogue.

L-NDCA (40 μM), L-NMCA (100 μM) and L-N^{α}-butyloxycarbonyl N^{G}-nitro arginine (100 μM) did not influence per-

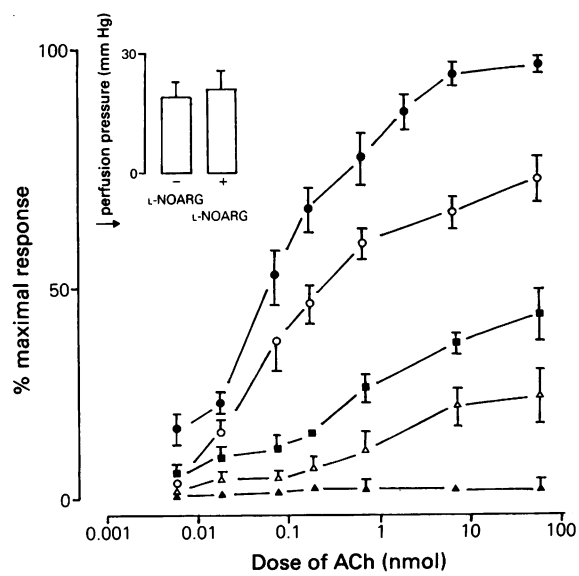


Figure 4 Vasodilator effect of acetylcholine (ACh) on the noradrenaline-preconstricted rat mesentery in the absence (●) and presence of L-N^{G}-nitroarginine (L-NOARG) 10 μM (○), 30 μM (■), 50 μM (△) and 200 μM (▲). Inset shows the effect of L-NOARG (30 μM) on the vasodilator response to an approximate ED_{70} of sodium nitroprusside (3.7 nmol). Results show mean with s.e.mean indicated by vertical bars, $n = 4$–8.

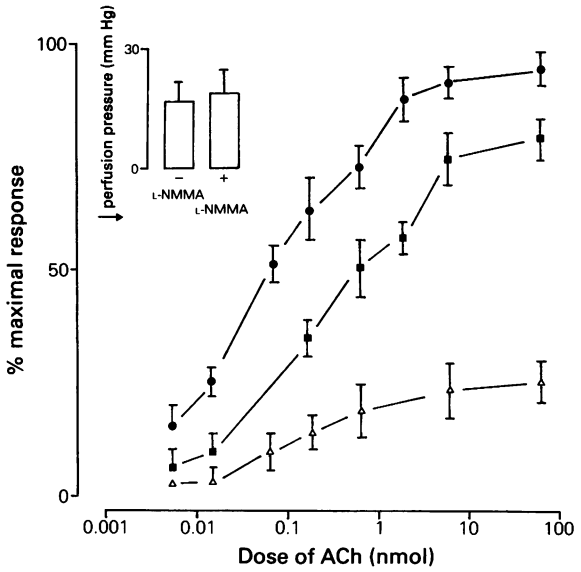


Figure 5 Vasodilator effect of acetylcholine (ACh) on the noradrenaline-preconstricted rat mesentery in the absence (●) and presence of L-N^G-monomethyl arginine (L-NMMA), 30 μM (■) and 100 μM (△). Inset shows the effect of L-NMMA (30 μM) on the vasodilator response to an approximate ED_{70} of sodium nitroprusside (3.7 nmol). Results show mean with s.e.mean indicated by vertical bars, $n = 6$.

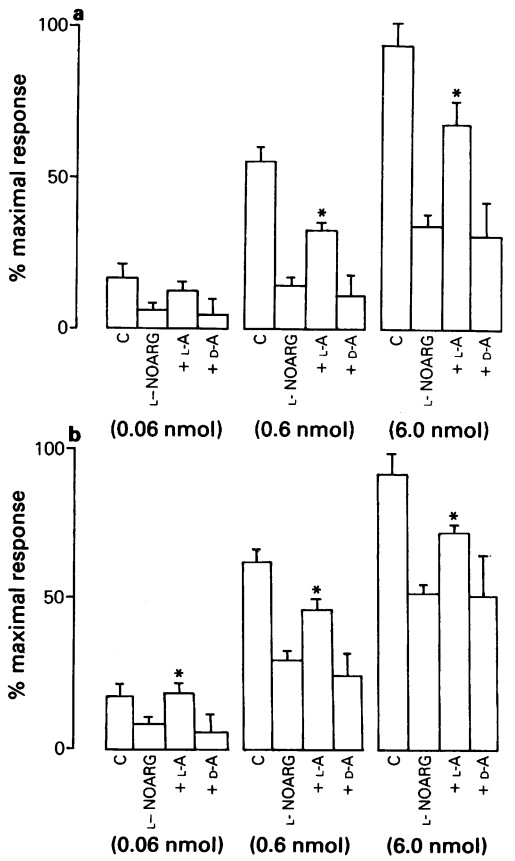


Figure 6 Inhibition of the vasodilator effect of acetylcholine (ACh) in the noradrenaline-preconstricted rat mesentery by L-N^G-nitro arginine (L-NOARG) (30 μM, a) and L-N^G-monomethyl arginine (L-NMMA) (30 μM, b). Reversal of inhibition by L-arginine (L-A, 100 μM) and lack of reversal by D-arginine (D-A, 100 μM). Control responses to ACh are shown in the first column labelled C. Figures in parentheses represent dose of ACh administered in nmol. Results show mean with s.e.mean indicated by vertical bars, $n = 6$, * $P < 0.05$ cf. response to ACh in the presence of inhibitor.

fusion pressure of 'high tone' preparations directly or affect vasodilatation following ACh or NP injection.

Discussion

The biosynthesis of NO occurs in vascular endothelial cells in culture (Palmer *et al.*, 1987; Kelm *et al.*, 1989), isolated blood vessels (Ignarro *et al.*, 1987), rat peritoneal leucocytes (Rimele *et al.*, 1988) and rat brain (Garthwaite *et al.*, 1989). Evidence has been presented that NO is synthesized within vascular endothelial cells from the guanidino terminus of L-arginine (reviewed in Moncada *et al.*, 1989).

The present study demonstrates that L-NOARG and L-NMMA exhibit similar pharmacological effects on isolated blood vessels. Thus, both L-NOARG and L-NMMA contracted the rabbit aorta and caused vasoconstriction of the rat perfused mesentery. These effects may be explained by the loss of a tonic vasodilator influence following inhibition of endogenous NO biosynthesis by endothelial cells since neither L-NOARG nor L-NMMA caused contraction of rabbit aortic rings which had been rubbed to remove the endothelium. Additionally, L-NOARG and L-NMMA inhibited the vasodilator effect of ACh (but not NP) in both preparations. L-NOARG was approximately 6 × and 4 × more potent than L-NMMA as an inhibitor of endothelium-dependent vasodilatation in the rabbit aorta and rat mesentery respectively. In each case, the effect of L-NOARG and L-NMMA was long-lasting. Exposure to either inhibitor resulted in diminished responsiveness of the rabbit aorta and rat mesenteric blood vessels to ACh for up to 1 h thereafter. L-Arginine (but not D-arginine) produced graded reversal of the effect of both L-NOARG and L-NMMA on the rabbit aorta preparation; 50% reversal of inhibition required a 3.3 fold excess of L-arginine whilst a 5 fold excess resulted in complete reversal. A similar 3.3 fold molar excess of L-arginine also produced approximately 50% reversal of the effect of L-NOARG and L-NMMA in the perfused rat mesentery. These results are consistent with those of Amezcua *et al.*, (1989) who reported that excess L-arginine only partly reversed the inhibitory effect of L-NMMA on ACh-induced vasodilatation in the rabbit heart.

Finally, precursors of mammalian L-arginine biosynthesis such as L-citrulline and L-arginosuccinic acid did not reverse L-NOARG or L-NMMA inhibition in the rabbit aorta. Similarly, L-amino acids such as L-alanine and L-methionine, which elicit endothelium-dependent vasodilatation in the perfused rat mesentery (al Swayeh & Moore, 1989), failed to reverse inhibition due to L-NOARG or L-NMMA. L-Ornithine was also without effect. Taken together, these results suggest that L-NOARG, like L-NMMA, prevents endothelium-dependent vasodilatation by inhibition of NO biosynthesis.

Preliminary attempts have also been made to characterize the chemical features of L-arginine analogues necessary for inhibition of vascular NO formation. Modification of the guanidino (but not the N$^\alpha$) terminus of L-arginine appears to be a pre-requisite for prevention of endothelium dependent relaxation since L-NMCA was inactive whilst insertion of either a nitro (as in L-NOARG) or a methyl group (as in L-NMMA) into the guanidino terminus of L-arginine produces potent inhibitors. However, the nature of the chemical substituent in this region of the molecule is important since the introduction of an N^G-tosyl moiety did not produce an effective inhibitor. Similarly, altering the structure of L-NOARG to include a bulky substituent attached to the N$^\alpha$ region of the amino acid (i.e. D- and L-N$^\alpha$-butyloxycarbonyl N^G-nitro arginine) resulted in weak inhibitors of endothelium-dependent vasodilatation which were not reversed by L-arginine and are therefore unlikely to inhibit NO formation. These analogues may interact chemically with released NO although this possibility was not addressed in the present experiments. In contrast, addition

of a methyl group to the α-carboxyl moiety of L-NOARG (as in L-NOARG methyl ester) did not affect the ability to inhibit endothelium-dependent inhibition. More detailed analysis of the chemical features of guanidino-substituted L-arginine derivatives necessary for inhibition of endothelium-dependent vasodilatation is desirable and may provide important information about the nature of the enzyme responsible for NO biosynthesis.

Whether L-NOARG inhibits the endothelial enzyme responsible for the conversion of L-arginine to NO, reportedly the mechanism of action of L-NMMA, or inhibits NO formation by a separate mechanism, is not known. In addition, it remains to be seen whether L-NOARG mimics other pharmacological effects of L-NMMA such as the increase in rabbit blood pressure following intravenous administration (Rees *et al.*, 1989b) and inhibition of neutrophil NO formation (Marletta *et al.*, 1988). Whatever its precise mechanism of action and pharmacological activity it is clear that L-NOARG is a more potent and considerably less expensive inhibitor of vascular NO biosynthesis than L-NMMA and thus may provide a useful alternative with which to study the physiological significance of endogenous nitrates. For example, the use of L-NOARG in this paper has uncovered a role for NO in the control of mesenteric vascular tone and reactivity. In addition, we have recently reported that L-NOARG is some 30× more potent than L-NMMA as an inhibitor of non-adrenergic, non-cholinergic (NANC) transmission in the mouse anococcygeus (Gibson *et al.*, 1989). In this particular case, L-NOARG may prove to be of assistance in probing the identity of the substance responsible for NANC transmission in this preparation and the role of endogenous nitrates in general (see Garthwaite *et al.*, 1989).

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Phaclofen-insensitive presynaptic inhibitory action of (±)-baclofen in neonatal rat motoneurones *in vitro*

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1 Intracellular recordings were made from antidromically identified motoneurones in transverse spinal cord slices from neonatal (12–16 day) rats.

2 Superfusion of (±)-baclofen (0.5–50 μM) reduced the excitatory postsynaptic potentials (e.p.s.ps) and inhibitory postsynaptic potentials (i.p.s.ps) evoked by dorsal root or dorsal root entry zone stimulation in a concentration-dependent manner; the calculated EC_{50} was 2.4 μM. Baclofen in comparable concentrations also reversibly eliminated spontaneously occurring e.p.s.ps and i.p.s.ps.

3 (−)-Baclofen was more effective as compared to baclofen in reducing the synaptic responses, whereas (+)-baclofen at concentrations as high as 50 μM was ineffective.

4 Baclofen (<5 μM) attenuated the synaptic responses without causing a significant change of passive membrane properties and depolarizations induced by exogenously applied glutamate. In addition to synaptic depression, baclofen (>5 μM) caused a hyperpolarization associated with decreased membrane resistance in some of the motoneurones; the glutamate responses were also attenuated.

5 Baclofen reversibly depressed the spike after-hyperpolarization of the motoneurones.

6 GABA (1–10 mM) depressed synaptic transmission and depolarized or hyperpolarized motoneurones. While potentiated by the uptake inhibitor nipecotic acid, the synaptic depressant effect of GABA was not antagonized by bicuculline.

7 The synaptic depressant effect of baclofen was neither blocked by $GABA_A$ antagonists bicuculline and picrotoxin (10–50 μM) nor by the $GABA_B$ antagonist phaclofen (0.1–1 mM).

8 It is suggested that baclofen depresses excitatory and inhibitory transmission in rat motoneurones by primarily a presynaptic mechanism in reducing the liberation of chemical transmitters from nerve endings via a phaclofen-insensitive $GABA_B$ receptor.

Introduction

Baclofen, a lipophilic analogue of γ-aminobutyric acid (GABA) that interacts with a bicuculline-insensitive GABA receptor, known as $GABA_B$ receptor (Bowery *et al.*, 1980; Hill & Bowery, 1981), is an effective antispastic drug (Bein, 1972; Fehr & Bein, 1974). The finding that baclofen inhibits spinal neurotransmission at concentrations (Pierau & Zimmerman, 1973; Curtis *et al.*, 1974; Davidoff & Sears, 1974; Fehr & Bein, 1974; Fukuda *et al.*, 1977; Fox *et al.*, 1978; Curtis *et al.*, 1981; Davies, 1981) much lower than that producing skeletal neuromyal transmission blockade (Glavinovic, 1979) indicates that an important site of its antispastic action resides in the spinal cord.

A number of questions with respect to the spinal action of baclofen remain to be firmly resolved. For example, where is the primary inhibitory site of action in the spinal cord? Results from several studies showed that baclofen inhibits synaptic transmission without causing a significant change of the membrane potential and input resistance of motoneurones, suggesting that baclofen attenuates synaptic transmission by a presynaptic mechanism in reducing transmitter output (Pierau & Zimmerman, 1973; Fox *et al.*, 1978; Curtis *et al.*, 1981; Davies, 1981). Conversely, baclofen has been shown to hyperpolarize motoneurones and to attenuate the membrane excitability; this may contribute to its synaptic depressant action (Fox *et al.*, 1978; Davies, 1981).

The second question relates to the selectivity of baclofen in depressing synaptic transmission. Earlier experiments indicate that baclofen selectively inhibits excitatory transmission to the motoneurones, leaving the inhibitory transmission intact (Pierau & Zimmerman, 1973; Fukuda *et al.*, 1977; Kato *et al.*, 1978; Davies, 1981). On the other hand, baclofen reduces the excitatory and the inhibitory potentials evoked in neurones of the hippocampal and cortical slices (Scholfield, 1983; Blaxter & Carlen, 1985; Inoue *et al.*, 1985; Howe *et al.*, 1987) as well as the inhibitory potential in cultured hippocampal neurones (Harrison *et al.*, 1988). Hence, the suggestion that baclofen affects only excitatory synapses in the spinal cord is intriguing (Pierau & Zimmerman, 1973; Davies, 1981).

Another question concerns the type of $GABA_B$ receptors mediating the inhibitory action of baclofen. Recent studies indicate the presence of two pharmacologically distinct types of $GABA_B$ receptors in the rat hippocampus. The postsynaptic $GABA_B$ receptors mediating membrane hyperpolarization, are coupled to K^+ channels and blocked by the $GABA_B$ antagonist phaclofen, whereas the presynaptic $GABA_B$ receptors responsible for the depression of synaptic potentials, appear to be coupled to different ionic channels and are insensitive to phaclofen (Dutar & Nicoll, 1988). It remains to be established whether the $GABA_B$ receptor mediating the inhibitory action of baclofen in the spinal cord is phaclofen-sensitive or insensitive.

Methods

Neonatal (12–16 day) Sprague-Dawley rats were used in this study. The procedures used in obtaining thin (500 μm) transverse thoracolumbar spinal cord slices have been described in detail before (Dun & Mo, 1989). Slices with ventral and dorsal rootlets removed from the neonatal rats were incubated in Krebs solution at room temperature (21 ± 1°C) until used. The slices were transferred to the recording chamber (<0.5 ml) and superfused with a Krebs solution of the following composition (mM): NaCl 127, KCl 1.9, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10. The solution was saturated with 95% O_2 and 5% CO_2, and the temperature of the solution reaching the slice was maintained at 34 ± 1°C.

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Intracellular recordings were made from neurones in the ventral horn of the thoracolumbar spinal cord slices by means of glass microelectrodes filled with 3 M potassium acetate; their impedance varied between 60 and 100 MΩ. Electrical stimulation of the ventral and dorsal roots was accomplished via a concentric bipolar electrode positioned close to the respective rootlets. Signals were amplified via an Axoclamp 2A, using the bridge mode and displayed on a Gould Digital Oscilloscope and on a Gould pen recorder. Pharmacological agents, with the exception of glutamate, were dissolved in Krebs solution and applied to the slices in known concentrations. Glutamate (100 mM) dissolved in Krebs solution was applied from a micropipette positioned above the recording motoneurone by a puff of nitrogen (Picospritzer, General Valve Co.) at 350 kPa and a duration of 10 to 20 ms. E.p.s.ps and i.p.s.ps illustrated in the figures usually represent averages of four to eight signals. Unless stated otherwise, (±)-baclofen was used.

The following compounds were used: (±)-baclofen (Research Biochemicals Inc.), phaclofen (Research Biochemicals Inc.), (−)-bicuculline methobromide (Sigma), γ-amino-n-butyric acid (Sigma), picrotoxin (Sigma), sodium L-glutamic acid (Sigma), (±)-nipecotic acid (Research Biochemicals Inc.), (+)-baclofen and (−)-baclofen (gift from Ciba-Geigy).

Results

Data were collected from 108 motoneurones from 78 slices. Neurones in the ventral horn were identified as motoneurones by the appearance of an antidromic spike potential following stimulation of the ventral rootlets. We have previously characterized two types of motoneurones, type I and type II, in the neonatal rat spinal cord slices, according to their axonal conduction velocity and passive membrane properties (Jiang & Dun, 1986, 1987). Type I motoneurones have mean conduction velocity, resting membrane potential, input resistance and time constant of 41 m s^{-1}, −66 mV, 21 MΩ and 3 ms, whereas the corresponding values for type II motoneurones are 12 m s^{-1}, −63 mV; 109 MΩ and 7 ms, respectively. Type I and type II motoneurones may correspond to the α- and γ-motoneurones described in *in vivo* cat spinal cord (Eccles, 1964). Here, as the effects of baclofen on synaptic responses were similar irrespective of the types of rat motoneurones, the results were pooled.

Spontaneous synaptic activities

Spontaneously occurring excitatory (depolarizing) postsynaptic potentials (e.p.s.ps) were recorded in six motoneurones. When recorded at the membrane potential of between −60 and −70 mV, the amplitude of spontaneous e.p.s.ps varied from less than 1 to over 10 mV (Figure 1). Baclofen (1 or 5 μM) reversibly eliminated the spontaneous e.p.s.ps in all 4 cells tested (Figure 1).

GABA at higher concentrations, i.e. 1 to 10 mM, abolished spontaneous activities as well. The inhibitory action of GABA could be potentiated by pretreating the slices with the GABA uptake inhibitor, nipecotic acid (0.1–1 mM; Krogsgaard-Larsen & Johnston, 1975). Figure 1 illustrates a typical experiment in which GABA (0.1 mM) in the presence of nipecotic acid (0.1 mM) reversibly abolished the spontaneous activities in the same motoneurone where the inhibitory effect of baclofen (1 μM) was first determined. Similar results were obtained in three other motoneurones. Nipecotic acid caused a transient reduction of the frequency and amplitude of spontaneous e.p.s.ps in this but not in three other motoneurones. This might be due to a potentiating effect of nipecotic acid on endogenously released GABA from the slice.

Spontaneously occurring inhibitory (hyperpolarizing) postsynaptic potentials (i.p.s.ps) constituted a second type of spontaneous activities that were detected in four motoneurones (Figure 2a). The amplitude of spontaneous i.p.s.ps ranged from less than 1 to 10 mV. Baclofen (1 or 5 μM) eliminated the spontaneous i.p.s.ps in all four motoneurones tested and the effect was fully reversible after washing with drug-free solution (Figure 2a).

Evoked i.p.s.ps

It has been reported previously that stimulation of ventral or dorsal roots elicits a strychnine-sensitive i.p.s.p. in rat

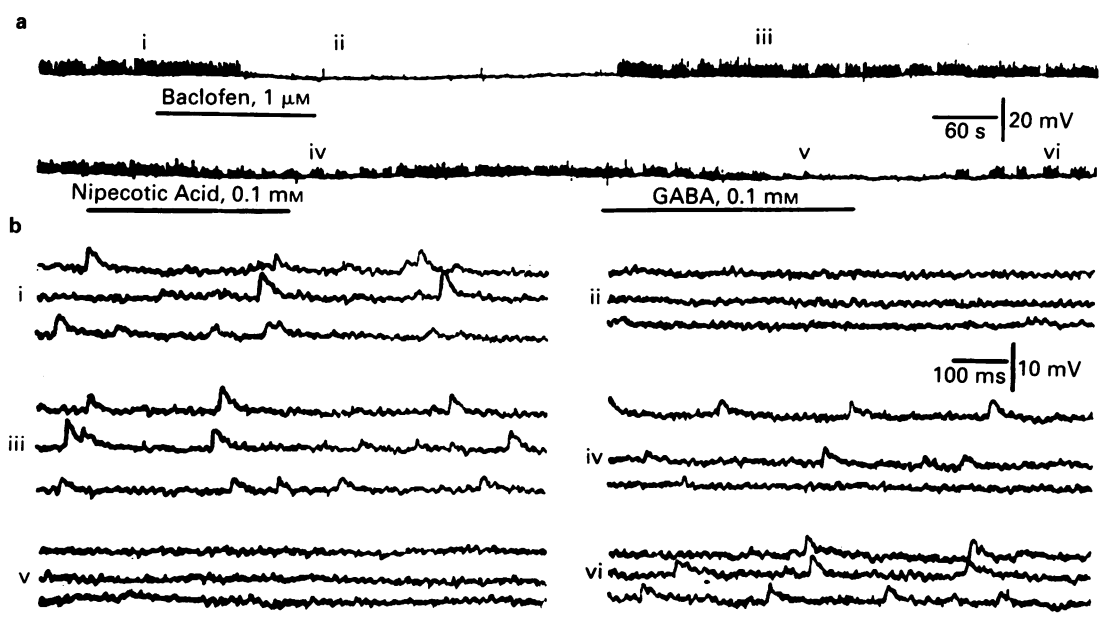


Figure 1 Reduction of spontaneous e.p.s.ps by baclofen and GABA in the presence of nipecotic acid in a rat motoneurone. Spontaneous e.p.s.ps occurred at a high frequency in an otherwise quiescent motoneurone. Superfusion of baclofen (1 μM) as indicated by a solid bar eliminated the spontaneous activities and the effect was fully reversible after 4 minutes of wash. Superfusion of the slice with the GABA uptake inhibitor nipecotic acid (0.1 mM) transiently reduced the frequency as well as the amplitude of spontaneous e.p.s.ps. After superfusing the slice with nipecotic acid, GABA (0.1 mM) reversibly eliminated the spontaneous activities. Representative traces of spontaneous e.p.s.ps taken at times (i)–(vi) marked on the top two traces are shown in the lower panel: (a) are pen recorder traces and (b) are oscilloscope tracings plotted by a Gould Colorwriter. The resting membrane potential was −61 mV.

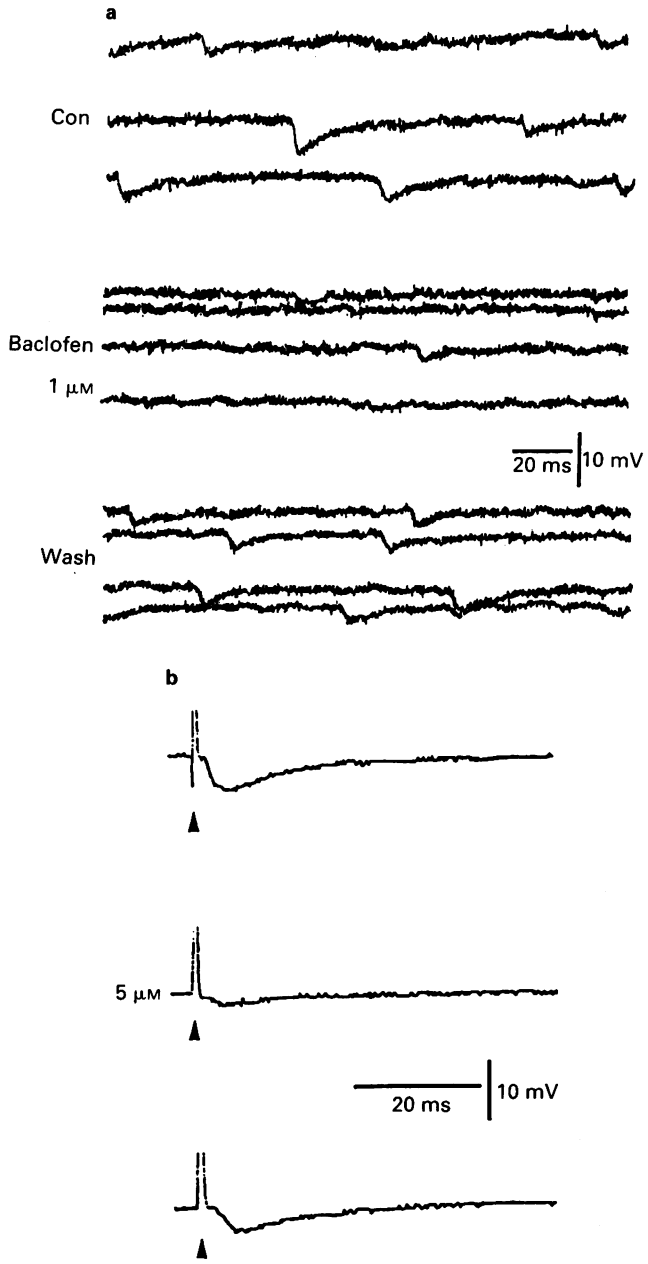


Figure 2 Spontaneous and evoked i.p.s.ps blocked by baclofen in two rat motoneurones. (a) Spontaneously occurring i.p.s.ps before, during and after superfusion of the slice with baclofen (1 μM); (b) i.p.s.ps evoked by dorsal root stimulation as indicated by an arrowhead (upper trace). After superfusing the slice with baclofen (5 μM) for 5 min, the i.p.s.p. was reduced by 65%. The effect was fully reversible. The fast transient preceding the i.p.s.p. was a stimulus artifact. Recordings in (a) and (b) were taken from two different motoneurones which had a resting membrane potential of −74 and −68 mV, respectively.

motoneurones (Dun & Jiang, 1986). In the present study, dorsal root stimulation evoked an i.p.s.p. in five motoneurones (Figure 2b) and a mixed response consisting of an initial e.p.s.p. followed by an i.p.s.p. in another four motoneurones (Figure 3).

In contrast to the earlier report that the inhibitory potential in cat motoneurones was resistant to baclofen (Pierau & Zimmermann, 1973), the i.p.s.ps were sensitive to baclofen in all three motoneurones tested; a representative experiment is illustrated in Figure 2b. Moreover, baclofen preferentially blocked the i.p.s.p. in two motoneurones that displayed a mixed response, resulting in an initial augmentation of the e.p.s.p. amplitude; the latter was eventually eliminated by the continuous superfusion of baclofen (Figure 3). The recovery

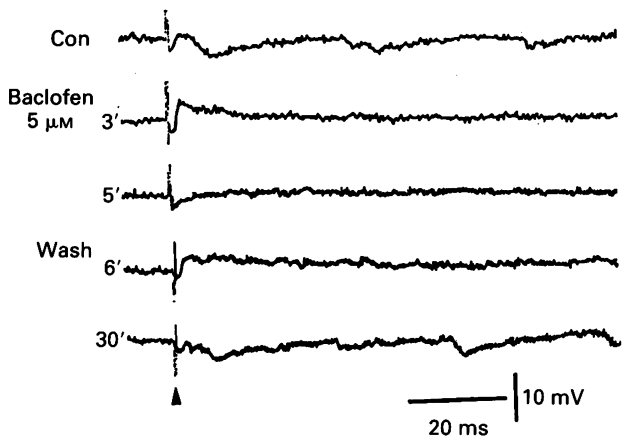


Figure 3 A mixed response of e.p.s.p. and i.p.s.p. blocked by baclofen in a rat motoneurone. Con: dorsal root stimulation as indicated by an arrowhead evoked a small e.p.s.p. followed by an i.p.s.p. with several spontaneous i.p.s.ps superimposed on the tail-end of the trace. Superfusion of baclofen (5 μM) for 3 min eliminated the i.p.s.p., consequently increasing the initial e.p.s.p.; both the e.p.s.p. and i.p.s.p. were eliminated after 5 min. The recovery of the synaptic responses followed a reversed order, that is, e.p.s.p. first returned after 6 min of washing. After 30 min of washing, dorsal root stimulation elicited an i.p.s.p. which was not preceded by a detectable e.p.s.p. This cell had a resting membrane potential of −67 mV.

from the effect of baclofen followed a reversed order, i.e., the e.p.s.p. first returned and then the i.p.s.p. (Figure 3).

Evoked e.p.s.ps

Stimulation of dorsal rootlets evoked a monophasic e.p.s.p. (e.g. Figure 4) or a composite response of two or more e.p.s.ps (Figures 6, 7, 9, 10). The synaptic latency of e.p.s.ps varied in different motoneurones, ranging from 1 to a few milliseconds (Figures 4–10). The short-latency e.p.s.ps probably represent monosynaptic connections from the primary afferents, whereas e.p.s.ps of longer latencies constitute di- or polysynaptic responses. Again, as the effects of baclofen on short and long-latency e.p.s.ps were similar, data were pooled here.

When applied to motoneurones, baclofen consistently reduced the e.p.s.p. amplitude in a concentration-dependent manner; the concentrations used in this study ranged from 0.5 to 50 μM. The dose-effect relationship of baclofen on the amplitude and duration of e.p.s.ps is shown in Table 1; the EC_{50} calculated by the Hill plot was 2.4 μM. Figure 4 shows the effects of three concentrations of baclofen on a motoneurone. The amplitude of e.p.s.ps was depressed in a concentration-dependent manner, whereas only at the highest concentration (50 μM) did baclofen cause a small and transient hyperpolarization.

The depressant effect of lower concentrations (≤10 μM) of baclofen was readily reversible several minutes after washing the slices with drug-free Krebs solution (Figures 4, 6, 7). At the higher concentrations (10–50 μM), a wash period of 30 min or more was needed for the response to return to control level (for example, Figure 10).

Table 1 Reduction of e.p.s.p. amplitude and duration by baclofen

	% *reduction* (mean ± s.d.)				
	0.5 μM ($n = 6$)	1 μM ($n = 14$)	5 μM ($n = 19$)	10 μM ($n = 9$)	50 μM ($n = 6$)
Amplitude[a]	29 ± 18[b]	33 ± 15[c]	58 ± 21[c]	63 ± 16[c]	90 ± 18[c]
Duration[a]	20 ± 14[b]	27 ± 10[c]	40 ± 20[c]	45 ± 25[c]	87 ± 20[c]

[a] One-way variance analysis: $P < 0.01$.
[b] Paired t test: $P < 0.05$.
[c] Paired t test: $P < 0.01$.

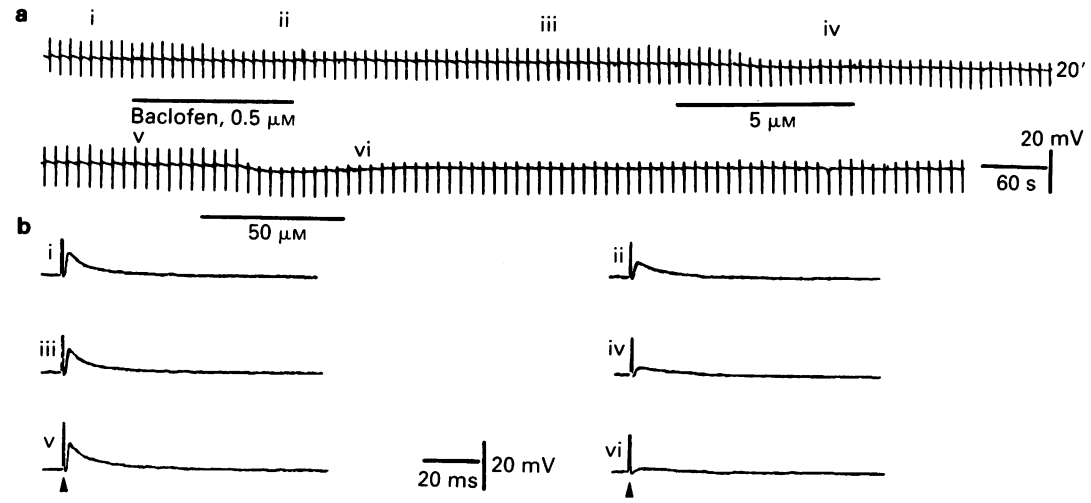


Figure 4 Concentration-dependent depression of evoked e.p.s.ps by baclofen. The upper two traces in (a) represent continuous chart recording interrupted by 20 min of wash at the end of the upper trace. Upward deflections represent e.p.s.ps evoked by dorsal root stimulation and downward deflections are hyperpolarizing electrotonic potentials induced by constant current pulses (not shown). Baclofen was applied in successively higher concentrations as indicated by solid bars and reduced the e.p.s.p. amplitude in a concentration-dependent manner. At concentrations of 0.5 and 5 μM, baclofen decreased the amplitude of e.p.s.ps without causing a detectable change of membrane potential or input resistance as indicated by no apparent change of the amplitude of hyperpolarizing electrotonic potentials. At the concentration of 50 μM, baclofen reduced the e.p.s.ps and caused a small and transient hyperpolarization. Traces in (b) are signal averagings of 4 e.p.s.ps taken at times marked on the upper two traces. The fast transient preceding each e.p.s.p. was a stimulus artifact. The resting membrane potential was -73 mV.

The stereospecificity of the action of baclofen on motoneurones was evaluated by use of (+)- and (−)-baclofen. At concentrations up to 50 μM, (+)-baclofen caused little or no depression of the synaptic responses nor a hyperpolarization in any of the 8 motoneurones tested (Figure 5). On the other hand, (−)-baclofen was consistently effective in reducing the e.p.s.ps in all 10 motoneurones tested (Figure 5); the average reduction caused by (−)-baclofen (2 μM) was 80.8 ± 13.5% (mean ± s.d.). When tested in the same motoneurones, the average reduction of e.p.s.ps caused by baclofen (2 μM) and by the same concentration of (−)-baclofen was 38.2 ± 12.8% and 73.8 ± 8.3%, respectively ($n = 4$).

GABA also depressed the amplitude of e.p.s.ps, but at much higher concentrations (1–10 mM). Even when tested at such high concentrations, the magnitude of reduction was moderate and variable among different motoneurones tested. A more consistent and longer-lasting effect of GABA could be demonstrated by pretreating the slices with the GABA uptake inhibitor nipecotic acid; a typical experiment is illustrated in Figure 6. It should be noted that when compared in the same motoneurones, baclofen at the concentrations that reduced synaptic potentials caused little or no change of membrane potential or input resistance, whereas GABA at the concentrations that produced a reduction of e.p.s.ps invariably caused

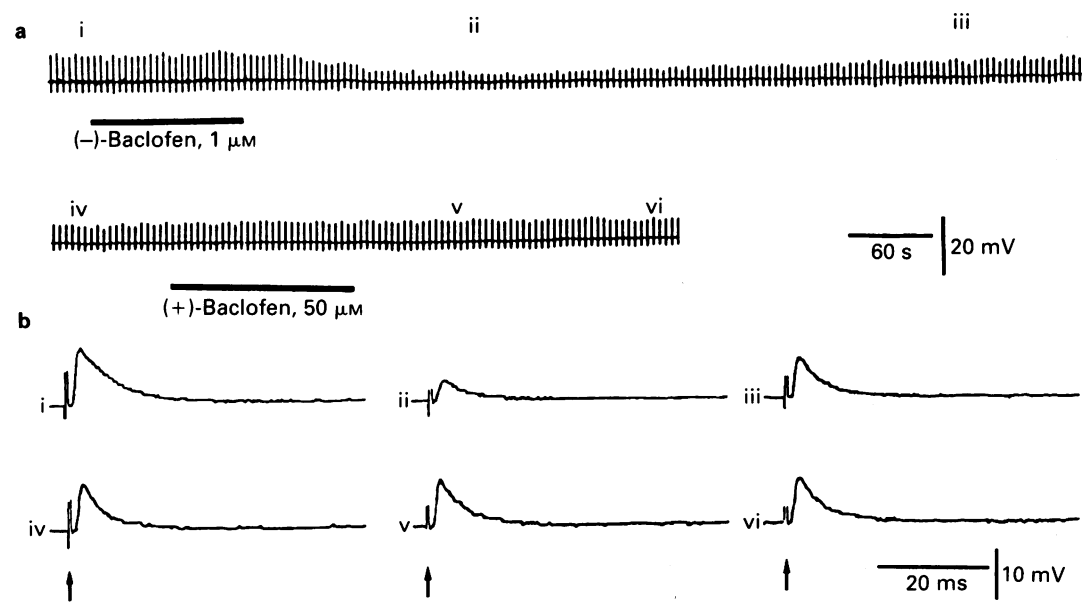


Figure 5 Effects of (−)- and (+)-baclofen on the e.p.s.ps in a rat motoneurone. Traces in (a) are continuous chart recordings. Upward and downward deflections represent e.p.s.ps and hyperpolarizing electrotonic potentials used to monitor membrane resistance change, respectively. Superfusion of (−)-baclofen (1 μM) as indicated by the solid bar reduced the amplitude of e.p.s.ps by 70% without causing a significant change of the resting membrane potential and input resistance; the effect was reversible after washing. Superfusion of (+)-baclofen (50 μM) was without effect. E.p.s.ps taken at times indicated by numbers on upper two traces are shown in panel (b). Recordings were taken from the same motoneurone which had a resting membrane potential of -72 mV.

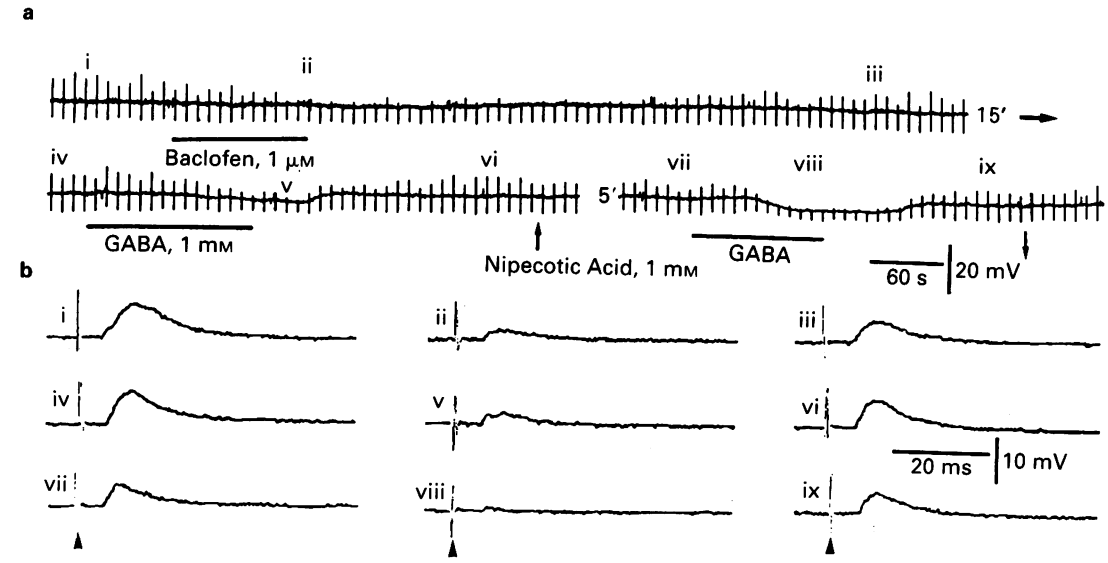


Figure 6 Comparison of the effects of baclofen and GABA in a rat motoneurone. Upper two traces represent continuous chart recordings interrupted by numerals indicating minutes. Upward and downward deflections are evoked e.p.s.ps and hyperpolarizing electrotonic potentials used to monitor membrane input resistance change, respectively. Baclofen (1 μM) reduced the e.p.s.ps by 65%, without causing a significant change of membrane potential and input resistance, and the effect was fully reversible. GABA (1 mM) reduced the e.p.s.ps to about 30% of control level and caused a small hyperpolarization associated with a decrease of membrane resistance, as indicated by a reduction of the hyperpolarizing electrotonic potentials; the effect was rapidly reversed. Nipecotic acid (1 mM) was added to the perfusing solution between two arrows. GABA in the presence of nipecotic acid caused complete abolition of e.p.s.ps and a marked but transient hyperpolarization. E.p.s.ps taken at times indicated by numbers on upper two traces are shown in panel (b). Recordings were taken from the same motoneurone which had a resting membrane potential of −61 mV.

either a depolarization or hyperpolarization; both types of response were associated with a decrease of apparent input resistance (Figures 6 and 10b). The synaptic depressant action of GABA was not antagonized by bicuculline (50 to 100 μM) in any of the 5 motoneurones tested (Figure 7).

Membrane hyperpolarization by baclofen

At the concentration of 5 μM or below, baclofen depressed the e.p.s.ps without causing a detectable change of membrane potentials and input resistances (Figures 4, 6, 7, 10). At higher concentrations, baclofen blocked synaptic transmission and hyperpolarized some but not all the neurones examined. Even at the highest concentration (50 μM), baclofen hyperpolarized 6 of the 10 motoneurones tested. The magnitude of response varied considerably in different cells, ranging from a few (Figure 4) to about 10 mV (Figure 10). The hyperpolarization was associated with a small to moderate (5–30%) decrease in membrane input resistance (Figures 4 and 10). The magnitude of reduction of e.p.s.ps was significantly greater than that of decrease of membrane resistance in a given motoneurone tested.

Further, the time course of depression of e.p.s.ps generally outlasted the hyperpolarization by many minutes. In the experiment illustrated in Figure 10c, baclofen (50 μM) blocked the e.p.s.p. and hyperpolarized the cell in question; the membrane potential returned to the resting level at a time when the e.p.s.p. amplitude showed only a marginal recovery.

Effects on spike after-hyperpolarization

The after-hyperpolarization following the spike elicited by direct intracellular stimulation was reversibly reduced by baclofen (10 μM) in 3 of the 5 motoneurones investigated (Figure 8); the mean reduction was 61%. On the other hand, the peak amplitude and duration of the spike was not affected.

Effects of GABA antagonists

Pretreating the slices with two known $GABA_A$ receptor antagonists bicuculline and picrotoxin (10–50 μM) did not nullify the inhibitory effects of baclofen in any of the 9 motoneurones tested.

Phaclofen, a $GABA_B$ receptor antagonist (Kerr *et al.*, 1987), in concentrations of 0.1 to 1 mM was not effective in antagonizing the inhibitory action of baclofen on e.p.s.ps in any of the 5 cells tested. Phaclofen (0.1 mM) did not cause a significant change of the e.p.s.p. amplitude ($n = 2$), whereas at 1 mM, phaclofen reduced the e.p.s.ps by an average of 36% in three neurones tested; one such experiment is shown in Figure 9. The effect was reversible upon washing. Moreover, the magnitude of reduction caused by baclofen in the presence of phaclofen was greater than that produced by baclofen alone (Figure 9).

Effects on glutamate-induced depolarizations

To learn more about the site of inhibitory action, the effect of baclofen on synaptic responses and on glutamate-induced depolarizations were evaluated in the same motoneurones ($n = 5$). Glutamate or a related excitatory amino acid is thought to be the most likely candidate for a transmitter role in the spinal cord (Fagg & Foster, 1983). In these experiments, glutamate (100 mM) was applied to the motoneurones by pressure, resulting in a rapid membrane depolarization (Figure 10). Baclofen at lower concentrations ($\leqslant 5$ μM) depressed the e.p.s.p. without causing a significant change of the glutamate-induced depolarization (Figure 10a). At higher concentrations, e.g. 50 μM, baclofen blocked the e.p.s.p., hyperpolarized the neurone and attenuated the glutamate-induced depolarization (Figure 10c). However, returning the membrane potential to the original level by injection of depolarizing currents did not restore the e.p.s.p. and the glutamate depolarization to the control level (Figure 10c). The recovery of glutamate-induced depolarization preceded the return of e.p.s.ps (Figure 10c). Finally, when tested in the same cell, GABA (10 mM) caused a depolarization and a reduction of the amplitude of e.p.s.ps as well as the glutamate-induced depolarizations (Figure 10b). The effects of GABA were shorter-lasting compared to those caused by baclofen.

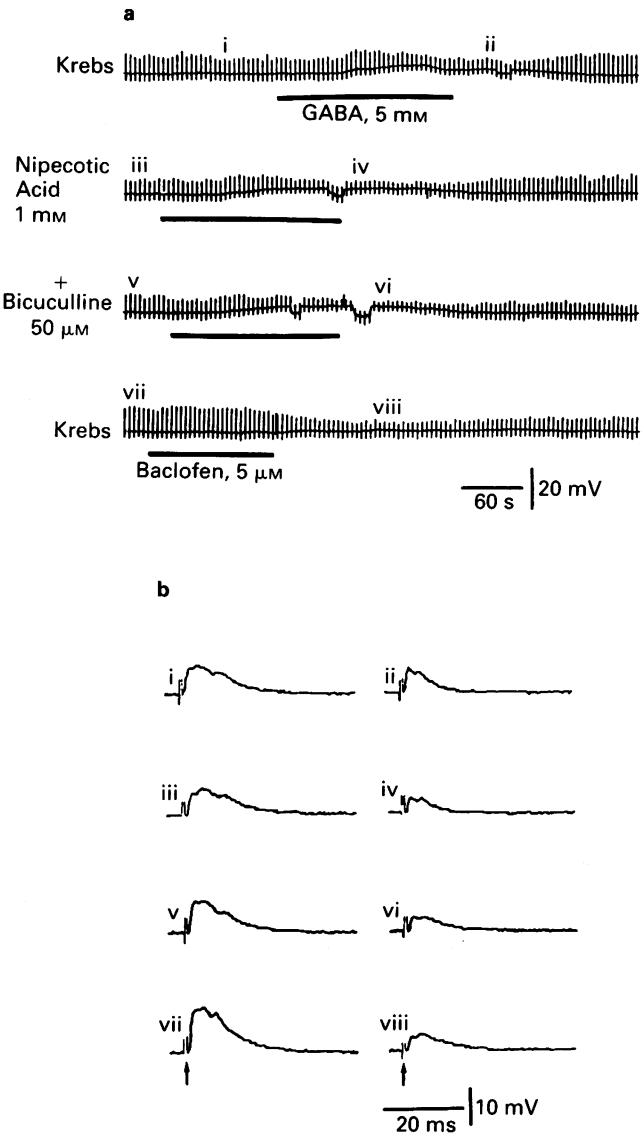


Figure 7 Effects of GABA in the presence of nipecotic and bicuculline in a rat motoneurone. Traces in (a) represent continuous chart recordings interrupted by washing periods (not shown). Upward and downward deflections represent evoked e.p.s.ps and hyperpolarizing electrotonic potentials. In the upper trace, superfusion of GABA (5 mM) as indicated by a solid bar caused a small depolarization, reduction of the amplitude of electrotonic potentials and of e.p.s.ps; the effects were fully reversible. Second trace: in the presence of nipecotic acid (1 mM), GABA caused a larger depolarization and reduction of the e.p.s.ps. At the peak of depolarization, returning the membrane potential to the resting level by current injection did not restore the e.p.s.ps to the control level. Third trace: in the presence of nipecotic acid and bicuculline (50 μM), the effects of GABA were essentially the same as those shown in the second trace. Last trace: superfusion of baclofen (5 μM) reduced the e.p.s.ps but did not cause a noticeable change of membrane potential. E.p.s.ps taken at times indicated by numbers in (a) are shown in (b). Arrows indicate the stimulus artifacts. Recordings were taken from the same motoneurone which had a resting membrane potential of −64 mV.

Discussion and conclusions

The present experiments were designed to address three questions concerning the actions of baclofen in the spinal cord that may underlie its effectiveness as an antispastic agent. In this respect, the findings that (−)-baclofen was more effective than baclofen in reducing the amplitude of e.p.s.ps and that (+)-baclofen was ineffective at concentrations as high as 50 μM indicate that the inhibitory action of baclofen on spinal transmission was stereospecific.

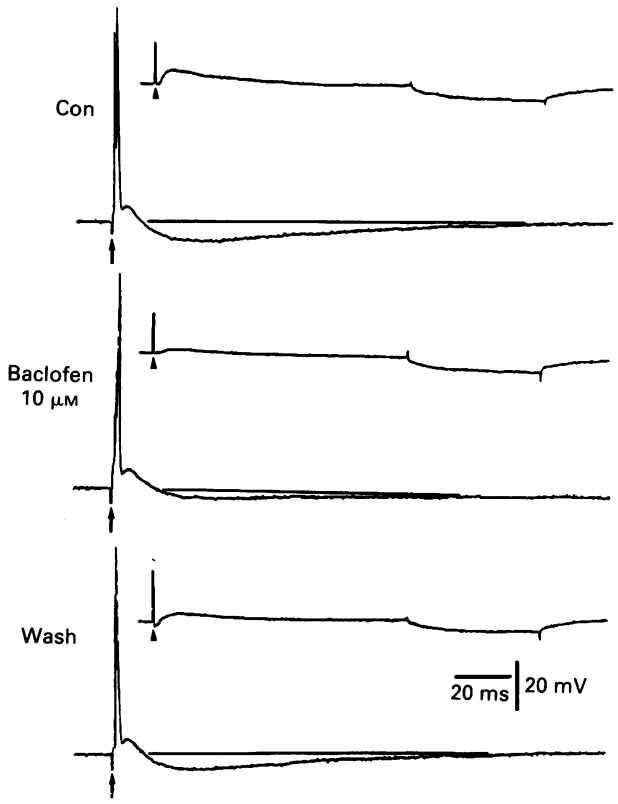


Figure 8 Reduction of spike after-hyperpolarization and e.p.s.p. by baclofen in a rat motoneurone. Action potential elicited by depolarizing current pulse (arrows) was followed by a prominent after-hyperpolarization. Inset: e.p.s.p elicited by dorsal root stimulation was followed by a hyperpolarizing electrotonic potential. Baclofen (10 μM) reduced the amplitude of after-hyperpolarization by over 50% and nearly blocked the e.p.s.p., the action potential and electrotonic potential were not changed. The effect was reversible after wash. The membrane potential of this motoneurone was −68 mV.

First, the selectivity of baclofen with respect to excitatory and inhibitory spinal transmission was examined. That baclofen inhibits central excitatory transmission has been amply demonstrated (Pierau & Zimmerman, 1973; Davidoff & Sears, 1974; Davies & Watkins, 1974; Fehr & Bein, 1974; Fukudo *et al.*, 1977; Fox *et al.*, 1978; Kato *et al.*, 1978; Curtis *et al.*, 1981; Davies, 1981; Lanthorn & Cotman, 1981; Scholfield, 1983; Blaxter & Carlen, 1985; Inoue *et al.*, 1985; Howe *et al.*, 1987; Dutar & Nicoll, 1988; Allerton *et al.*, 1989). On the other hand, the action of baclofen on inhibitory transmission appears to be less consistent; positive and negative results have been reported (Pierau & Zimmerman, 1973; Kato *et al.*, 1978; Davies, 1981; Scholfield, 1983; Blaxter & Carlen, 1985; Inoue *et al.*, 1985; Howe *et al.*, 1987; Harrison *et al.*, 1988).

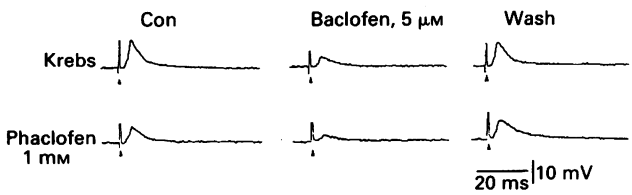


Figure 9 Effect of baclofen not antagonized by phaclofen in a rat motoneurone. Con: e.p.s.p. evoked by dorsal root stimulation. Baclofen (5 μM) reduced the e.p.s.ps to about 35% of control, and the effect was reversible after washing. Phaclofen (1 mM) reduced the e.p.s.ps to 70% of control value. Baclofen in the presence of phaclofen further depressed the e.p.s.ps to 20% of control level. The effect of baclofen and phaclofen was reversible after wash. The fast transient preceding each e.p.s.p. was a stimulus artifact. This motoneurone had a resting membrane potential of −75 mV.

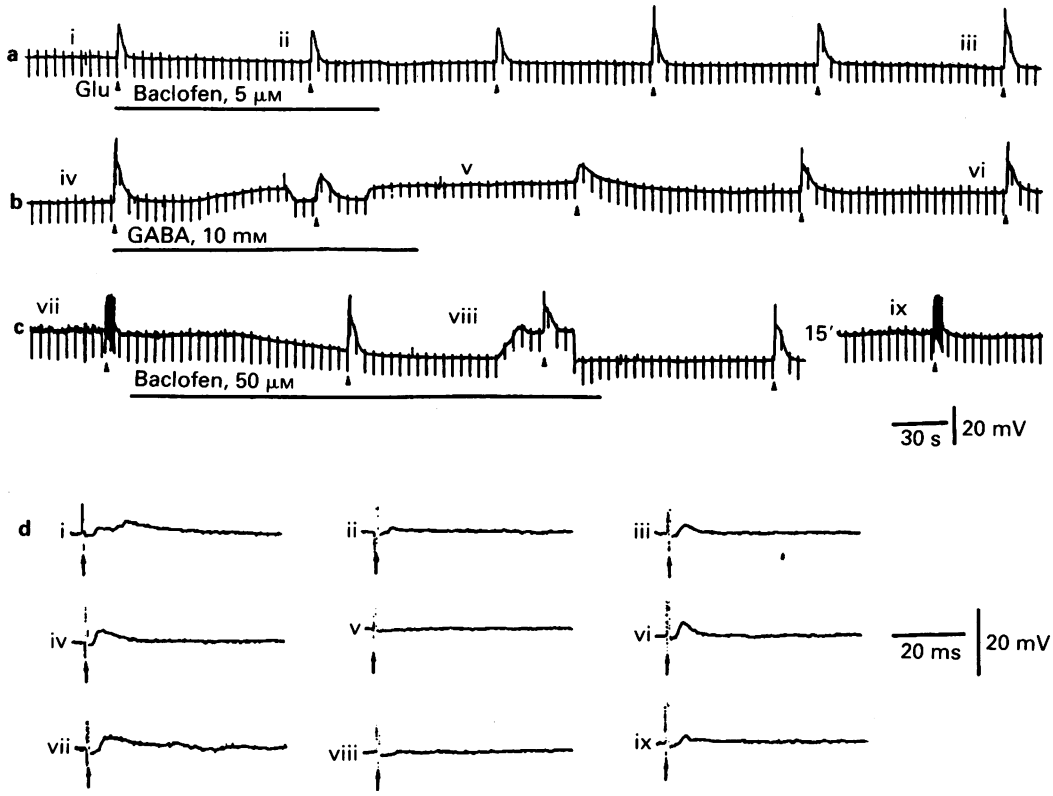


Figure 10 Effects of baclofen and GABA on e.p.s.ps and depolarizations induced by glutamate in a rat motoneurone. A micropipette containing glutamate (Glu, 100 mM) was positioned above the recording motoneurone. Glutamate was applied (indicated by arrowheads) to the vicinity of the motoneurone by a brief pulse of 20 ms and evoked a brisk depolarization and spike discharge, which was truncated by the limited frequency response of the pen recorder. (a) Small upward deflections represent evoked e.p.s.ps the amplitude of which was rather small even during control period. Baclofen (5 μM) eliminated the e.p.s.ps, whereas the glutamate-induced depolarizations were not affected. (b) Superfusion of GABA (10 mM) caused a sustained depolarization and a blockade of e.p.s.ps. Returning the membrane potential to the original level by current injection did not result in a restoration of e.p.s.ps; glutamate was still capable of eliciting a response, though attenuated. A substantial recovery of the e.p.s.p. and glutamate-induced depolarization was seen a few minutes after discontinuation of GABA superfusion. (c) Baclofen at a high concentration (50 μM) blocked the e.p.s.p. and hyperpolarized the neurone, whereas the glutamate-induced depolarization was only moderately reduced. The e.p.s.ps were not restored when the membrane potential was returned to the original level by current injection. After a period of 15 min wash, the glutamate-induced depolarization but not the e.p.s.ps returned to control level. This cell had a resting membrane potential of −72 mV.

Our results show that in concentrations comparable to those suppressing the excitatory responses, baclofen blocked the spontaneous as well as evoked i.p.s.ps in the rat motoneurones. In the two motoneurones where dorsal root stimulation evoked a mixed response of e.p.s.p. and i.p.s.p., baclofen first suppressed the i.p.s.p. and augmented the e.p.s.p. The reason for a preferential blockade of inhibitory transmission by baclofen is not known. One explanation might be that baclofen is more effective in suppressing the polysynaptic than the monosynaptic event; the longer synaptic latency of i.p.s.ps would support this contention.

However, the possibility that abolition of i.p.s.ps by baclofen as observed in the present study is secondary to the blockade of release of excitatory transmitters acting on inhibitory interneurones cannot be entirely excluded. In this respect, paired recordings made from rat hippocampal neurones in culture clearly demonstrated that baclofen inhibits directly the release of GABA from GABAergic neurones (Harrison *et al.*, 1988). This finding in conjunction with earlier results that baclofen reduces the release of a number of putative transmitters including noradrenaline and acetylcholine, in addition to excitatory amino acids (Nistri, 1975; Potashner, 1979; Bowery *et al.*, 1980; Johnston *et al.*, 1980; Collins *et al.*, 1982; Kato *et al.*, 1982) is not in favour of the contention that baclofen selectively inhibits the release of excitatory amino acids, hence excitatory transmission in the central nervous system.

A second question the present study attempted to address is the site of inhibitory action of baclofen in the spinal cord. In aggreement with the majority of earlier reports, our results suggest that baclofen inhibits synaptic transmission by diminishing transmitter release from nerve fibres presynaptic with respect to motoneurones.

Firstly, baclofen at concentrations that depressed synaptic transmission caused little or no change of the electrical properties of motoneurones. Only at higher concentrations did baclofen cause a membrane hyperpolarization and conductance increase as well as synaptic blockade. In the latter cases, the synaptic depression and hyperpolarization exhibited different time courses, and the e.p.s.ps were not restored to the control level upon returning the membrane potential to the resting level by depolarizing currents. These findings indicate that hyperpolarization and synaptic depression are two separate events. Similarly, the magnitude of reduction of synaptic potentials produced by a given concentration of baclofen was much greater than could be accounted for by an increase of membrane conductance. Hence, the site responsible for membrane hyperpolarization on one hand and synaptic depression on the other appears to be distinct.

Secondly, baclofen at the concentrations that depressed synaptic transmission, did not significantly change the postsynaptic chemosensitivity to the putative excitatory transmitter glutamate (Fagg & Foster, 1983). An attenuation of the glutamate-induced responses occurred only in those neurones where higher concentrations of baclofen caused a concomittant membrane hyperpolarization and decreased input resistance. The reduction of glutamate response could

be explained by a short-circuiting effect of baclofen on postsynaptic membrane and is not related to the diminished sensitivity of postsynaptic receptors to glutamate. In this regard, a small reduction of glutamate response as observed here was consistent with a relatively small change of membrane resistance.

Collectively, these findings provide strong evidence that the primary site of inhibitory action of baclofen is presynaptic, although at higher concentrations, the postsynaptic short-circuiting effect may accentuate the presynaptic inhibitory action of baclofen. As baclofen caused a relatively small or no detectable hyperpolarization in the majority of motoneurones investigated, its postsynaptic action was not studied in any detail. Other studies indicate that balcofen hyperpolarizes the membrane by increasing K^+ conductance (Dutar & Nicoll, 1988). On the other hand, GABA in concentrations that diminished e.p.s.ps invariably depolarized or hyperpolarized the neurones. The synaptic depressant effect of GABA was not antagonized by bicuculline, suggesting the involvement of $GABA_B$ receptors in the inhibitory action of GABA.

The last question concerned the type of $GABA_B$ receptors with which baclofen interacts to produce synaptic inhibition in the spinal cord. Recent studies show that baclofen interacts with post- and pre-synaptic $GABA_B$ receptors in the hippocampus, resulting in a hyperpolarization and depression of synaptic transmission, respectively (Dutar & Nicoll, 1988). In addition, the postsynaptic $GABA_B$ receptors are coupled to potassium channels and are blocked by phaclofen, a $GABA_B$ receptor antagonist (Kerr *et al.*, 1987), whereas the presynaptic $GABA_B$ receptors are phaclofen-insensitive (Dutar & Nicoll, 1988). A negative result with respect to phaclofen on the presynaptic inhibitory action of baclofen in cultured hippocampal neurones has also been reported (Harrison, 1988). Our finding that the presynaptic inhibitory action of baclofen persisted in the presence of phaclofen in concentrations that have been shown to be effective in suppressing the $GABA_B$ receptor-mediated hyperpolarization (Dutar & Nicoll, 1988; Hauso & Gallagher, 1988), raises the possibility that the pharmacological characteristics of presynaptic $GABA_B$ receptors in the spinal cord are similar to those described in the hippocampus. It is pertinent to mention that the e.p.s.p. amplitude was consistently and reversibly depressed by phaclofen at high concentrations, suggesting that the latter behaves like a weak agonist in the spinal cord. Phaclofen has been shown to antagonize the inhibitory action of baclofen on cat spinal transmission (Kerr *et al.*, 1987). The reason for the discrepancy between our results and those reported by Kerr *et al.* (1987) is not immediately clear. Species difference notwithstanding, differences in the methods of administration, i.e., bath application vs. microiontophoresis, and the concentrations of phaclofen might explain the discrepancy.

The mechanism by which baclofen reduces the output of transmitters from presynaptic nerve fibres in the spinal cord is of interest and remains to be investigated. Results from the hippocampus indicate that the phaclofen-sensitive $GABA_B$ receptors are coupled to K^+ channels (Dutar & Nicholl, 1988). Studies on somata of sensory neurones show that activation of $GABA_B$ receptors by baclofen reduced the duration of calcium-dependent action potentials and voltage-dependent Ca^{2+} currents (Dunlap, 1981; Robertson & Taylor, 1986; Dolphin & Scott, 1987). If the results from the hippocampus and sensory ganglia can be extrapolated to the spinal neurones, it may be speculated that the phaclofen-insensitive $GABA_B$ receptors are coupled to voltage-dependent Ca^{2+} channels. In this respect, the observation that baclofen reduced the spike after-hyperpolarization, which is thought to be caused by an influx of Ca ions (Barrett & Barrett, 1976) is consistent with the hypothesis that activation of presynaptic $GABA_B$ receptors may reduce Ca^{2+} influx across the terminal membrane, thereby attenuating synaptic transmission (Robertson & Taylor, 1986; Allerton *et al.*, 1989). There is however an apparent disparity between the concentrations of baclofen necessary to cause an attenuation of e.p.s.p. amplitude and inhibition of calcium currents. For example, 100 μM (−)-baclofen produces an average reduction of 28% and 70% calcium currents in cat and rat dorsal root ganglion cells, respectively (Robertson & Taylor, 1986; Dolphin & Scott, 1987), whereas, 50 μM baclofen causes 90% inhibition of e.p.s.p. amplitude in the present study. Thus, a validation of this hypothesis will need a direct testing of the effect of baclofen on nerve terminal membrane calcium currents.

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Antagonism of $GABA_B$-receptor-mediated responses in the guinea-pig isolated ileum and vas deferens by phosphono-analogues of GABA

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1 The phosphono-analogues of γ-aminobutyric acid (GABA), 4-amino-butylphosphonic acid (4-ABPA), 3-amino-2-(4-chlorophenyl)-propylphosphonic acid (phaclofen) and 3-amino-2-cyclohexylpropyl-phosphonic acid, each antagonized the GABA- and baclofen-induced $GABA_B$-receptor-mediated depression of twitch responses to transmural stimulation in the guinea-pig isolated ileum, in a concentration-dependent, reversible and surmountable manner (apparent $pA_2 = 4.0 \pm 0.1$, 4 ± 0.2 and 3.7 ± 0.2 respectively, compared with 3.9 ± 0.1 for δ-aminovaleric acid). No such activity was found in a variety of related analogues.

2 By contrast, 3-amino-propylphosphonic acid (3-APPA) behaved as a partial agonist, itself partly depressing ileal twitch contractions in a manner sensitive to 4-ABPA and phaclofen, as well as antagonizing the depression of the ileal twitch by GABA and baclofen (apparent $pA_2 = 4.0 \pm 0.2$).

3 Both 4-ABPA and phaclofen also antagonized the baclofen-induced depression of the twitch in the guinea-pig isolated vas deferens (apparent $pA_2 = 4.0 \pm 0.1$ for each), whilst 3-APPA behaved as a partial agonist, slightly depressing the vas twitch, and antagonised the baclofen-induced depression of the twitch (apparent $pA_2 = 3.9 \pm 0.1$).

4 None of these phosphono-analogues exhibited any action at ileal $GABA_A$-receptors, nor influenced the ileal twitch depression with morphine, adenosine or noradrenaline, suggesting their selectivity as antagonists at $GABA_B$-receptors.

Introduction

It is now recognised that γ-aminobutyric acid (GABA) acts at two distinct classes of receptor, the classical bicuculline-sensitive $GABA_A$-receptor and the bicuculline-insensitive $GABA_B$-receptor, for which baclofen is a specific agonist and phaclofen a specific antagonist (Hill & Bowery, 1981; Kerr *et al.*, 1986, 1987; Dutar & Nicoll, 1988). $GABA_B$-receptors are widely distributed in the periphery (Kerr & Ong, 1986) as well as in the central nervous system (CNS) (Bowery *et al.*, 1987). In the latter, phaclofen both antagonizes hyperpolarizations to baclofen and blocks late inhibitory postsynaptic potentials (i.p.s.ps) linked to an increase of potassium conductance (Dutar & Nicoll, 1988), whilst central and peripheral presynaptic depressant actions of GABA and baclofen are also antagonized by phaclofen (Kerr *et al.*, 1987; Baumfalk & Albus, 1988; Bonanno *et al.*, 1988; Olpe *et al.*, 1988). However, there are suggestions that $GABA_B$-receptors may be heterogeneous, based on divergent biochemical functional responses (Scherer *et al.*, 1988), and on regional differences in their sensitivity to phaclofen (Kerr *et al.*, 1987; Dutar & Nicholl, 1988). In this regard, phosphono-analogues of GABA are of particular interest since, although both 3-amino-propyl- and 4-amino-butyl-phosphonic acids (3-APPA; 4-ABPA) displace [^{3}H]-baclofen binding with comparable potency, the butyl-analogue is inactive in $GABA_B$ receptor-mediated modulation of second messenger production, in contrast to the propyl-analogue (Scherer *et al.*, 1988). However, both analogues antagonize baclofen actions in rat cortical slices (Kerr *et al.*, 1989a), and the further analysis of such differences requires a wider range of ligands for these receptors.

Phaclofen, 3-amino-2-(4-chlorophenyl)-propylphosphonic acid, is the phosphono-analogue of baclofen and was developed by inserting the 2-(4-chlorophenyl)-substituent into 3-APPA (Chiefari *et al.*, 1987) which itself exhibits activity at $GABA_B$-receptors. As a background to the development of phaclofen, we examined the actions of a number of phosphono-analogues of GABA at $GABA_B$-receptors, there being two considerations that prompted our search for antagonists amongst such phosphono-analogues. Firstly, although previously found inactive in the periphery (Muhyaddin *et al.*, 1982), 3-APPA depresses central neuronal activity in a bicuculline-insensitive manner (Curtis & Watkins, 1965; Bioulac *et al.*, 1979), and shows affinity for $GABA_B$-receptors (Cates *et al.*, 1984) suggesting that 3-APPA might be a $GABA_B$-receptor agonist; preliminary investigations showed 3-APPA in fact to be a partial agonist at these receptors (Kerr *et al.*, 1986; see also Luzzi *et al.*, 1986 which appeared contemporaneously). Secondly, antagonism is conferred upon glutamate analogues by ω-phosphono-substitution (Watkins, 1981), which in turn originally suggested to us that similar analogues of GABA might be antagonists at $GABA_B$-receptors. Since δ-aminovaleric acid (DAVA), the higher homologue of GABA, is an antagonist at peripheral $GABA_B$-receptors (Muhyaddin *et al.*, 1982), we prepared the corresponding higher phosphono-analogue, 4-ABPA, in the expectation that this would be a $GABA_B$-receptor antagonist. In addition, as part of a survey of the structural requirements for this antagonism, we have prepared a series of phosphono-GABA analogues and related 2-phenyl derivatives including phaclofen (Figure 1), and here describe their actions at peripheral $GABA_B$ receptors. A preliminary account of this work was presented at the 10th IUPHAR Satellite Symposium on Amino Acid Transmitters, Canberra, Australia, August 1987.

Methods

Guinea-pigs of either sex, weighing between 200–400 g, were stunned by a blow on the head and bled. Segments of the distal ileum, 2–3 cm in length, taken 3–4 cm from the ileo-caecal valve, were quickly removed and mounted in a 10 ml

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organ bath containing normal Krebs-bicarbonate solution of the following composition (mM): Na^+ 151.0, K^+ 4.6, Mg^{2+} 0.6, Ca^{2+} 2.8, Cl^- 134.9, HCO_3^- 24.9, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6 and glucose 7.7 (pH 7.4 at 37°C). The Krebs solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2. Effects of drug treatments were examined on the resting tissue or on electrically evoked twitch contractions of the ileum elicited through parallel platinum electrodes positioned around the segments of the intestine, using pulses (duration 0.1 ms, 0.2 Hz, with a just submaximal voltage) delivered from a Grass S48 stimulator to give transmural stimulation of cholinergic neurones. Mechanical activity of the longitudinal muscle was recorded isometrically at a resting tension of 10 mN, using a Grass Model FT03 force transducer, and changes in tension were displayed on a Grass polygraph. In a further series, prostatic segments of guinea-pig vas deferens were similarly mounted and tension recorded in response to stimulation with a train duration of 125 ms and 0.1 Hz repetition rate, at 25 Hz, 0.5 ms pulse duration with a just submaximal voltage.

Tissues were allowed to equilibrate for 1 h before drug application or electrical stimulation. Drugs were applied within 15–20 min intervals, depending on the recovery of the tissue responses to baseline level. Drug volumes used were never more than 1% of the bath volume. Student's t test for paired and unpaired samples was used to assess the significance ($P < 0.05$) of differences between mean values of the concentration-response effects. All experiments were repeated in duplicate on tissues from at least 4 different animals. Where limited amounts of compound were available, apparent pA_2 values were obtained by the 'single dose' method of Kosterlitz & Watt (1968); otherwise, concentration-response curves were constructed and apparent pA_2 values determined using the relationship $pA_2 = \log (CR - 1) - \log (B)$, the concentration ratio (CR) being obtained by log probit estimates of the EC_{50} from the displacements of the curve in the presence of the given concentrations (B) of the antagonist, the concentration ranges (less than 10 fold) of antagonists obtainable precluding construction of Schild plots to provide true pA_2 values.

Sources of drugs and chemicals

Acetylcholine perchlorate, 2-amino-ethylphosphonic acid (2-AEPA), 3-amino-propylphosphonic acid (3-APPA), 3-amino-propylsulphonic acid (3-APS), gamma-aminobutyric acid (GABA), carbachol, muscimol, noradrenaline hydrochloride and picrotoxinin were all obtained from Sigma; baclofen was from Ciba-Geigy; adenosine was from Calbiochem; bicuculline methochloride was from Pierce, and morphine sulphate was from D.H.A. The phosphono-analogues of GABA (Figure 1) were synthesized by R.H.P., including 4-amino-butylphosphonic acid (4-ABPA, latterly available from Sigma), phaclofen and its des-chloro derivative (BP-3-APPA), by methods described elsewhere (Chiefari *et al.*, 1987); phaclofen is now available through Tocris-Neuramin. The 3-amino-2-cyclohexylpropylphosphonic acid (BC-3-APPA) was prepared by Dr K.N. Mewett, Department of Pharmacology, The University of Sydney.

Results

There was no evidence of bicuculline-sensitive $GABA_A$-receptor-mediated contractions induced by any of the phosphono-compounds listed in Figure 1, although 4-ABPA, the phosphono-analogue of DAVA occasionally elicited with variable delay a slight transient contraction of the ileum at concentrations of 1 mM and above, the contraction declining back to the baseline within 1–2 min; such contractions were, however, insensitive to bicuculline methochloride (5–10 μM) or picrotoxinin (10 μM). Furthermore, none of the phosphono-analogues when tested at concentrations between 0.1–1 mM, showed any influence on GABA-induced contractile responses of the unstimulated ileum, or on such responses to other

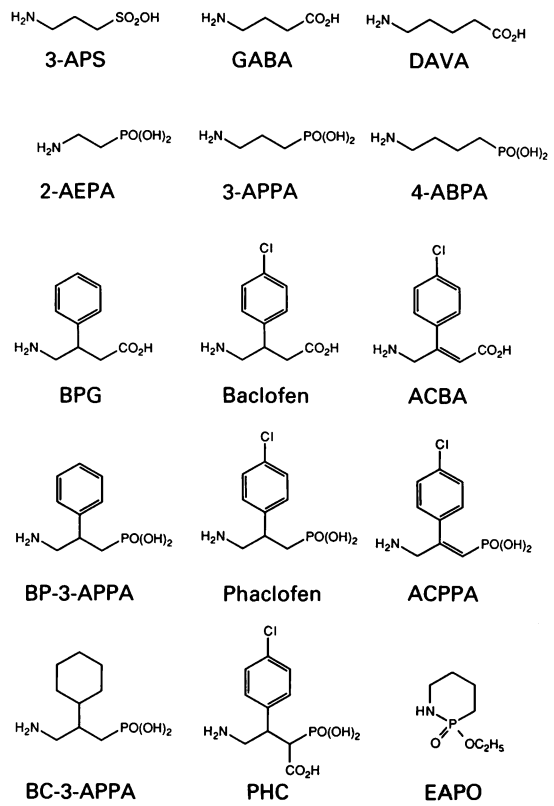


Figure 1 Structures of γ-aminobutyric acid (GABA)-analogues, including phosphono-derivatives investigated for activity at $GABA_B$-receptors in the guinea-pig isolated ileum and vas deferens: (From top left) 3-APS*, 3-amino-propylsulphonic acid; GABA; DAVA*, δ-aminovaleric acid; 2-AEPA, 2-amino-ethylphosphonic acid; 3-APPA**, 3-amino-propylphosphonic acid; 4-ABPA*, 4-amino-butylphosphonic acid; BPG**, β-phenyl-GABA; baclofen, 4-amino-3-(4-chlorophenyl)-butanoic acid; ACBA, 4-amino-3-(4-chlorophenyl)-2-butenoic acid; BP-3-APPA, 3-amino-2-phenyl-1-propylphosphonic acid (des-chloro-phaclofen); phaclofen*, 3-amino-2-(4-chlorophenyl)-1-propylphosphonic acid; ACPPA, 3-amino-2-(4-chlorophenyl)-prop-1-enylphosphonic acid; BC-3-APPA*, 3-amino-2-cyclohexyl-1-propylphosphonic acid; PHC, phaclofen-1-carboxylate; EAPO, 2-ethoxy-hexahydro-1,2-azaphosphorine-2-oxide (ethyl ester of cyclic 4-ABPA). Among the carboxylate analogues, baclofen is a full agonist, whilst BPG is a partial agonist at $GABA_B$-receptors. * antagonists, ** partial agonist.

$GABA_A$-receptor agonists such as muscimol (1–10 μM) or 3-amino-propylsulphonic acid (3-APS; 1–10 μM); neither did they affect ileal contractions to acetylcholine (10 nM) or carbachol (10 nM), $n = 8$ for each.

In confirmation of Luzzi *et al.* (1986), 3-APPA (0.1–3 mM) itself depressed the ileal twitch response to transmural stimulation, the extent of the depression of the twitch by 3-APPA being less than that achievable with GABA or baclofen, both of which are full agonists at $GABA_B$-receptor sites. Here the maximum depression by 3-APPA never exceeded 30% of that seen with baclofen, indicative of partial agonist properties, but was not tested beyond 3 mM. Such depression of ileal twitch responses by 3-APPA was sensitive to phaclofen (0.5 mM). None of the other phosphono-analogues exhibited any such phaclofen-sensitive depression of ileal twitch responses. Again confirming Luzzi *et al.* (1986), 3-APPA (0.1–3 mM) also reversibly antagonized the twitch depression induced by baclofen, as well as that induced by GABA. This surmountable antagonism behaved in a manner consistent with 3-APPA being a partial agonist at ileal $GABA_B$-receptor sites; thus, application of 3-APPA (1 mM) itself depressed the twitch (15%) for 3–4 min, yet application of baclofen or GABA (20 μM; IC_{50}) during this period did not cause a fully additive depression of the twitch, but rather the depression by these latter agents was reduced or abolished (not shown). This effect

occurred too rapidly for desensitization to have set in, even within 2 min of the application of 3-APPA, the initial depression due to 3-APPA still being present. Generally, however, a longer period (5–10 min) was allowed between the application of 3-APPA and the test agonist, by which time the small response to 3-APPA had faded, but the antagonist action was still present.

By contrast, 4-ABPA (0.1–5 mM) showed no $GABA_B$-receptor-mediated depression of ileal twitch responses to transmural stimulation, but rather, at higher concentrations (0.5–5 mM) slightly augmented (2–5%) the twitch height; generally this augmentation was short-lived, the responses reverting to the control height in 1–2 min. Prior application of 4-ABPA (0.1–5 mM) once the control twitch height was restored, antagonized in a concentration-dependent manner the depression of the twitch induced by GABA or baclofen (Figure 2a); likewise, it antagonized the depression of the twitch by 3-APPA (0.1–3 mM). Such antagonism was surmountable, and control responses to baclofen or GABA were restored upon wash-out. Of the compounds investigated, only 3-APPA, 4-ABPA, phaclofen and 3-amino-2-cychlohexyl-1-propylphosphonic acid (BC-3APPA, Figure 1) exhibited any antagonism of the $GABA_B$-receptor-mediated depression of ileal twitch responses, using GABA or baclofen as $GABA_B$-receptor agonists. With each of these phosphono-analogues, the antagonism was readily reversible on repeated washing of the tissue with Krebs solution. The remaining phosphono-compounds of Figure 1 were inactive at ileal $GABA_A$- or $GABA_B$-receptors, using concentrations up to 1 mM. In particular, the unsaturated propenyl compound (ACPPA) was inactive, as was the corresponding carboxylate (ACBA). Furthermore, the mono- and di-methyl- or ethyl-esters of 4-ABPA and phaclofen were inactive at 0.5 mM. None of the phosphono-analogues had any influence on the depression of the twitch induced by adenosine (3 μM), morphine (0.05 μM), or noradrenaline (1.5 μM), $n = 8$ for each.

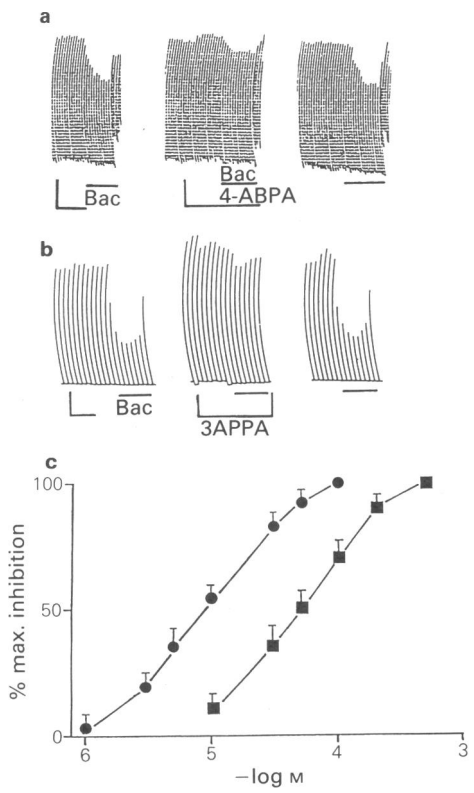


Figure 2 $GABA_B$-receptor antagonism by phosphono-analogues of γ-aminobutyric acid (GABA) in guinea-pig isolated ileum and vas deferens. (a) Baclofen (Bac, 20 μM) depressed the ileal twitch responses to transmural stimulation (0.2 Hz, 0.1 ms, just submaximal voltage), and this effect was antagonized by 4-amino-butylphosphonic acid (4-ABPA, 0.2 mM), without restoration of the baclofen-induced depression following washout. (b) Baclofen (30 μM) depressed the twitch response of the guinea-pig isolated vas deferens (0.1 Hz, 125 ms trains at 25 Hz, 0.5 ms, just submaximal voltage), antagonized by 3-amino-propylphosphonic acid (3-APPA, 100 μM) which itself slightly depressed the twitch, with recovery of the baclofen effect upon washout. In (a) and (b), the vertical bar represents 10 mN; also, 20 min elapsed between records in each. (c) Concentration-response curves for baclofen in the guinea-pig isolated ileum: (●) baclofen alone and (■) in the presence of 4-amino-butylphosphonic acid (0.4 mM), showing the dextral shift of the baclofen concentration-response curve with this antagonist ($n = 6$); the antagonism was competitive, surmountable and reversible.

There was a concentration-dependent dextral shift of the concentration-response curves for the depression of the ileal twitch by baclofen or GABA in the presence of 3-APPA, 4-ABPA or phaclofen (4-ABPA, Figure 2c). Although surmountable in each case, a true Schild plot for this antagonism could not be constructed since the concentration range was too small, and for 3-APPA the shifts in the concentration-response curves were not parallel (see Luzzi *et al.*, 1986). Instead, an apparent pA_2 was calculated for each, using the Kosterlitz & Watt (1968) single dose method for the partial agonist 3-APPA or from the dose-ratios, assuming competitive antagonism as described in the method. This yielded an apparent $pA_2 = 4.0 \pm 0.2$ for both 3-APPA and 4-ABPA ($n = 6$ for each). In the same way, an apparent pA_2 of 4.0 ± 0.1 was also found with phaclofen ($n = 8$), the dextral shift of the curves being parallel over the concentration-range employed (0.1–0.4 mM), which suggests competitive antagonism, whereas the apparent pA_2 for BC-3APPA was only 3.7 as estimated from two concentration-ratios. A comparable apparent pA_2 of 3.9 ± 0.2 was found for DAVA ($n = 8$), which also slightly (< 5%) augment the twitch height.

In the transmurally stimulated guinea-pig isolated vas deferens, baclofen depressed the vas twitches in a concentration-dependent manner, the depression not exhibiting fade as in the ileum, thus allowing cumulative responses to be elicited (Bowery *et al.*, 1981). In the same way, 3-APPA (0.1–1 mM) depressed the vas twitch, but even at 1 mM this depression was only $20 \pm 4\%$ of that seen with baclofen which reached a maximum at this concentration. 3-APPA at 100 μM depressed the twitch by only $12 \pm 2\%$ of the baclofen maximum, and antagonized the response to baclofen (30 μM), again behaving as a partial agonist, with restoration of the baclofen response upon washout (Figure 2b). By use of the Kosterlitz & Watt (1968) method, an apparent pA_2 of 3.9 ± 1 was obtained for 3-APPA in this preparation ($n = 6$). Both 4-ABPA and phaclofen antagonized the baclofen-induced depression of the vas deferens twitch response in a reversible and surmountable manner (apparent $pA_2 = 4.0 \pm 0.2$; $n = 8$ for both agents, based on two dose-ratios for each).

Discussion

As anticipated, 4-ABPA was devoid of $GABA_B$-receptor agonist activity in the guinea-pig isolated ileum or vas deferens. 4-ABPA also prevented the depressant action of baclofen on the twitch, in a surmountable manner, with an apparent $pA_2 = 4.0$ in each tissue. This pA_2 is the same as that found previously, and confirmed here, for the lower phosphono homologue 3-APPA, which however shows a marked deviation from a parallel shift in the baclofen concentration-response curves (Luzzi *et al.*, 1986; Kerr *et al.*, 1987) in keeping with its partial agonist properties. In the ileum, with phaclofen, the shift in the concentration-response curves was parallel suggesting competitive antagonism over the narrow concentration-range employed. The likely specificity of this antagonism by 4-ABPA and phaclofen is indicated by their lack of action on the depression of the ileal twitch induced by adenosine, morphine or noradrenaline, nor did they show any influence on ileal contractile responses to GABA, 3-APS, muscimol, ACh or carbachol. Consequently both 4-ABPA and phaclofen are potentially useful as antagonists against

$GABA_B$-receptor-mediated depression of transmitter release in the periphery, as well as the CNS (Kerr *et al.*, 1989a). However, in view of its behaviour in biochemical studies, where it was inactive on $GABA_B$-receptor-mediated modulation of second messenger production (Scherer *et al.*, 1988), 4-ABPA clearly should be examined further in a wider range of CNS preparations, where it possibly may distinguish $GABA_B$-receptor subtypes.

Luzzi *et al.* (1986) have previously described the non-parallel shift of the GABA and baclofen concentration-response curves in the presence of 3-APPA which behaves as a partial agonist at the $GABA_B$-receptor (Kerr *et al.*, 1986; 1987; Luzzi *et al.*, 1986). Even so, 3-APPA can also be used as an antagonist against $GABA_B$-receptor-mediated depression of cholinergic transmission in the isolated ileum, or in the vas deferens, with the caveat that it is an agonist in the CNS (Curtis & Watkins, 1965; Bioulac *et al.*, 1979), although it behaved as a partial agonist in our study on the isolated cortex (Kerr *et al.*, 1989a). There is the possibility that the antagonism in the ileum arises from desensitization to 3-APPA rather than its partial agonist property, but Luzzi *et al.* (1986) have dispelled this by demonstrating that 3-APPA immediately reverses a pre-existing GABA-induced depression of ileal twitch responses before desensitization could have occurred. Here we confirm this, where a prompt antagonism of the baclofen-induced depression of the twitch was seen if the baclofen was added within 1–2 min of the prior addition of 3-APPA, i.e. during the small depression of the twitch engendered by the latter, and prior to the onset of desensitization. Nevertheless, such desensitization could contribute to the antagonism of $GABA_B$-receptor-mediated actions of 3-APPA, following longer contact times with the tissue prior to agonist application.

With 4-ABPA and phaclofen the question of desensitization does not arise, since they showed only antagonism; instead, each of these tended to augment the twitch height, generally by not more than 5%. It is possible that this augmenting action of the phosphono-analogues contributes to their antagonism of the depression induced by GABA or baclofen, but we are inclined to discount such a possibility since this augmentation declines over a period of time and yet the antagonism of baclofen remains unchanged. One possibility, then, is that the twitch response of the electrically stimulated isolated ileum is normally somewhat depressed due to a simultaneous release of endogenous GABA by the stimulus, antagonism of this depression could then account for the slight twitch augmentation observed in the presence of these $GABA_B$-receptor antagonists. In keeping with this, a similar transient augmentation of the twitch height was seen with DAVA which in turn is a $GABA_B$-receptor antagonist (Ong & Kerr, 1983).

Some indications of the structural requirements for antagonism at presynaptic $GABA_B$-receptor sites in the ileum are provided by the present results, where the apparent pA_2 values give a guide to the relative potencies of the phosphono analogues. It is already known that modification of the carboxyl function in GABA markedly alters its properties toward $GABA_A$- and $GABA_B$-receptors. Thus the sulphonic analogue 3-APS is a potent $GABA_A$-receptor agonist, but is a weak $GABA_B$-receptor antagonist (Giotti *et al.*, 1983), whilst 2-hydroxy-saclofen is an effective $GABA_B$-receptor antagonist (Curtis *et al.*, 1988; Kerr *et al.*, 1988). Also, as confirmed in the present study, the corresponding phosphonic analogue, 3-APPA, lacks $GABA_A$ agonist activity but is a weak partial agonist at $GABA_B$-receptors where it can function as an antagonist (Kerr *et al.*, 1986, 1987; Luzzi *et al.*, 1986). By contrast, although lacking such an action in the study by Scherer *et al.* (1988), the higher analogue 4-ABPA here showed antagonism of the $GABA_B$-receptor-mediated actions of GABA and baclofen in the ileum, but without any agonist effect at either receptor type, whereas the lower analogue 2-amino-ethylphosphonic acid was devoid of any actions at either $GABA_A$- or $GABA_B$-receptors. However, 4-ABPA was not more powerful than 3-APPA as an antagonist at $GABA_B$-receptors (each with an apparent pA_2 of 4.0), despite being the phosphono-analogue of DAVA which itself is an antagonist (Muhyaddin *et al.*, 1982), for which we estimate an apparent pA_2 of 3.9. None of these phosphono-analogues was more potent than phaclofen, with an apparent $pA_2 = 4 \pm 0.1$. This pA_2 of 4 ± 0.1 for phaclofen is comparable to the K_B value of 120 μM found by Hasuo & Gallagher (1988) using phaclofen against $GABA_B$ receptor-mediated actions in the rat isolated septum.

Other than 4-ABPA, 3-APPA, phaclofen and BC-3APPA, none of the remaining phosphono-GABA analogues or derivatives listed in Figure 1 showed any activity at $GABA_A$- or $GABA_B$-receptors, using concentrations up to 1 mM. Not even the des-chloro derivative of phaclofen (3-amino-2-phenyl-propylphosphonic acid) was active as an antagonist at ileal $GABA_B$-receptors, despite the corresponding carboxylic derivative (β-phenyl GABA) possessing partial agonist properties at these receptors (Ong *et al.*, 1987). The antagonist action of the cyclohexyl analogue BC-3APPA was unexpected in the face of this inactivity of des-chloro-phaclofen; the corresponding β-cyclohexyl GABA seems not to have been investigated at $GABA_B$-receptors. Whilst this cyclohexylphosphono-analogue was weaker (apparent $pA_2 = 3.7$) than phaclofen as an antagonist, nevertheless similar β-substituted GABA analogues may provide further examples of antagonists at $GABA_B$-receptors, as for instance the benzofuran analogues recently described by Berthelot *et al.* (1987), which are antagonists at these receptors (Beattie *et al.*, 1989; Kerr *et al.*, 1989b). Here, the higher potency of phaclofen, and lack of activity in the des-chloro derivative recalls the similar structural requirements for agonist activity of baclofen analogues at the $GABA_B$-receptor (Bowery *et al.*, 1981). Unfortunately, none of the phosphono-analogues has proved at all potent as an antagonist at $GABA_B$-receptors; indeed, sulphonic analogues are more potent in this regard, as we have shown elsewhere (Kerr *et al.*, 1988).

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Simultaneous perfusion of rat isolated superior mesenteric arterial and venous beds: comparison of their vasoconstrictor and vasodilator responses to agonists

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1 A new isolated perfused preparation is described that allows a direct comparison to be made of the responses of the perfused arterial and retrogradely perfused venous circulations of the rat superior mesenteric vascular bed.

2 In experiments comparing the responses of the intact arterially perfused mesentery and small intestine to those of the same preparation following removal of the intestine and division of the circulations, the increases in perfusion pressure produced by arginine-vasopressin (30 pmol) and noradrenaline (1 nmol) were retained by the arterial circulation and those induced by angiotensin II (30 pmol) by the venous circulation. Endothelin-1 (30 pmol) constricted both portions of the vasculature but the prolonged nature of its response was associated with only the venous vessels.

3 In the simultaneously perfused arterial and venous preparation arginine vasopressin (3–100 pmol) was a selective constrictor of the arterial circulation and angiotensin II (3–100 pmol) of the venous circulation. In addition, noradrenaline (0.3–10 nmol), 5-hydroxytryptamine (0.3–10 nmol) and KCl (1–60 μmol) were more active as constrictors of the arterial than the venous vessels, and U46619 (10–300 pmol) a more active constrictor of the venous than the arterial vessels. Endothelin-1 (3–100 pmol) constricted both the arterial and venous portions of the vasculature but was significantly longer acting as a venoconstrictor than an arterioconstrictor.

4 Angiotensin I (300 pmol) caused constrictions of the venous circulation which were dependent upon the presence of angiotensin converting enzyme for captopril (10 μM) abolished constrictions caused by angiotensin I but not by angiotensin II.

5 In preparations preconstricted by U46619 (0.3–3 μM), acetylcholine (0.01–100 nmol), bradykinin (0.001–1 nmol), sodium nitroprusside (0.01–10 nmol) or isoprenaline (1–100 pmol) produced dose-related dilatations of both the arterial and the venous vasculatures, whereas adenosine diphosphate (ADP, 0.01–100 nmol) caused dose-dependent dilatations of the arterial circulation but principally constrictions of the venous circulation. The dilatations caused by acetylcholine and bradykinin in both portions of the circulation, and by ADP in the arterial circulation, were endothelium-dependent as they were inhibited by gossypol (3 μM), whereas dilatations to sodium nitroprusside were not.

6 This preparation allows the responses of the arteries and veins of a single perfused mesenteric bed to be compared. In addition, with this preparation it is possible to demonstrate that veins, as well as arteries, show significant endothelium-dependent relaxations. It is concluded that the venous portion of the vasculature is significantly involved in the responses of the intact circulation.

Introduction

The venous compartment of the vasculature is crucial to the functioning of the circulation as a whole as it can affect both cardiac preload and capillary filtration (Folkow & Mellander, 1964). However, the study of venous function has always been second to that of arterial function. This is in part due to the assumption that the responses of arteries are more important than those of veins. However, it must also be because of the methodological problems involved in the study of the venous circulation. The veins are by their nature more difficult for isolated tissue studies than arteries, while *in vivo* the responses of the veins have to be interpreted indirectly from other measures. In this paper a retrogradely perfused venous preparation is described that allows direct comparisons to be made between the responses of the veins and arteries within the isolated perfused superior mesenteric vascular bed of the rat.

Methods

Double perfused mesentery

Male albino Wistar rats (200–350 g) were killed by a blow to the head and exsanguinated. The abdomen was opened and the ileocolic and colic branches of the superior mesenteric artery were tied. The portal and superior mesenteric veins were separated from the connecting tissue in the region of the portal vein and a plastic cannula (Portex, size 3FG, external diameter 1.02 mm, internal diameter 0.61 mm) inserted retrogradely into the portal vein and passed into the superior mesenteric vein such that the tip of the cannula was 1.0–1.3 cm distal from the junction of the superior mesenteric and portal veins. The superior mesenteric artery was cannulated, as described before (McGregor, 1965), with an identical cannula and the superior mesenteric vascular bed perfused via the artery for 5 min at 2 ml min^{-1} with Krebs buffer (at room temperature) containing heparin (100 u ml^{-1}). During this time the effluent from the bed passed out from the venous cannula. At the end of this period the intestine was separated from the mesentery by cutting close to the intestinal border of the mesentery and the preparation supported on a Petri dish while the arteries and veins were perfused independently at 2 ml min^{-1}, with warmed (37.5°C) and gassed (95% O_2:5% CO_2) Krebs buffer containing indomethacin (5 μM). Changes in perfusion pressures were measured by means of pressure transducers approximately 15 cm from the tips of the arterial and venous cannulae.

Vasoconstrictor responses

After an equilibration period of 45 min vasoconstrictor responses of the two sides of the preparation were tested by

injection of bolus doses (1–15 μl) of agents into both parts of the circulation, with a time delay between the injections into each side of less than 5 s. Individual drugs were administered at intervals of more than 30 min to minimize desensitization.

Vasodilator responses

In some preparations U46619 (9,11-dideoxy-9α-methano epoxyprostaglandin $F_{2\alpha}$, 0.3–3 μM) was infused intra-arterially and intravenously to produce an initial peak (24.5 ± 3.5 mmHg, $n = 8$) and then a sustained (17.8 ± 2.5 mmHg, $n = 8$) increase of perfusion pressure in the arterial vasculature that were not significantly different; and a peak (10.5 ± 1 mmHg) and sustained increase (6.3 ± 1 mmHg, $n = 8$) in the perfusion pressure of the venous vasculature, which were significantly different ($P < 0.01$). Thus, the venous vessels did not maintain tone as reliably as the arterial vessels and, although higher concentrations of U46619 did cause larger peak increases in perfusion pressure (data not shown), they did not increase the sustained perfusion pressure. Vasodilatations in response to bolus doses of drugs, administered into both sides of the preparation, were then recorded. Drugs were injected in volumes of 1 μl to minimize vascular effects due to an injection artefact. In order to discriminate between endothelium-dependent and independent vasodilatations in some preparations gossypol (3 μM), an inhibitor of EDRF-dependent responses (Förstermann *et al.*, 1986), was infused throughout the experiment.

In all experiments in which the vasodilator responses of the arterial and venous vessels were examined, the arterial portion of the vasculature was perfused throughout at 5 ml min^{-1}. This increased flow rate was found to decrease greatly the spontaneous activity of the arterial vessels which occurred when a contractile agent was infused in preparations perfused at 2 ml min^{-1}.

Intact perfused mesentery and intestine

In a further series of experiments, the pressure responses of the entire perfused mesentery and intestine were recorded to assess the relative contribution of the arterial and venous portions of the mesentery to the responses of the whole vascular bed. The preparation was perfused through the artery with Krebs buffer (2 ml min^{-1}) containing indomethacin (5 μM) and bovine serum albumin (0.5% w/v) and the effluent allowed to pass to waste out of the venous cannula. In order to avoid the build up of fluid within the lumen of the small intestine, and an increase in the basal perfusion pressure, the intestinal lumen was flushed through for 10 min at 2 ml min^{-1} with Krebs buffer, at the beginning of the experiment, and holes made with a 19 gauge needle at approx. 1 cm intervals along the length of the intestine. After control responses had been obtained the mesentery was dissected clear of the intestine and the responses of the independently perfused arterial and venous circulations tested.

Materials

The Krebs buffer had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Acetylcholine chloride, adenosine diphosphate di(monocyclohexylammonium) salt (ADP), albumin (bovine fraction V), angiotensin I acetate, angiotensin II acetate, arginine-vasopressin acetate, ascorbic acid sodium salt, bradykinin acetate, gossypol, 5-hydroxytryptamine creatinine sulphate complex, indomethacin, isoprenaline hydrochloride, noradrenaline bitartrate and sodium nitroprusside were purchased from Sigma Chemical Co., Poole, Dorset. Heparin sodium was obtained from Evans Medical Ltd, Greenford, Middlesex. Captopril was obtained from Squibb. U46619 was a generous gift of J. Pike (Upjohn, Kalamazoo, MI, U.S.A.). Endothelin-1 was obtained from Scientific Marketing Associates, London.

All drugs were dissolved in either saline or distilled water except for noradrenaline and 5-hydroxytryptamine, which were dissolved in ascorbic acid (5 mM); indomethacin, which was prepared as a stock solution of 14 mM in $NaHCO_3$ (5% w/v); and gossypol, which was prepared as a stock solution of 2 mM in ethanol.

Statistics

Results are shown as mean values ± s.e.mean for n experiments. Student's unpaired t test was used to determine the significance of differences between means and a P value of less than 0.05 was taken as significant.

Results

Perfusion pressure

At a flow of 2 ml min^{-1}, there was a significant difference between the basal perfusion pressures of the arterial (11.5 ± 1 mmHg, $n = 8$) and venous vasculatures (5.5 ± 1 mmHg, $n = 8$). When the arterial portion was perfused at 5 ml min^{-1} the basal perfusion pressure of the arterial vasculature was significantly raised (17.5 ± 1.5 mmHg, $n = 13$) whereas the venous perfusion pressure was not different from control (4.0 ± 0.5 mmHg, $n = 13$).

The intact perfused mesentery and intestine had a basal perfusion pressure of 22 ± 3.5 mmHg ($n = 7$). When the preparation was separated and the arterial and venous vasculatures perfused independently the arterial and venous basal perfusion pressures were 13 ± 3 mmHg ($n = 7$) and 10 ± 2 mmHg ($n = 7$), respectively.

Intact perfused mesentery and intestine

In the intact perfused mesentery and intestine angiotensin II (30 pmol), arginine vasopression (30 pmol), noradrenaline (1 nmol) and endothelin-1 (30 pmol) all caused vasoconstrictions ($n = 5$–6, Figure 1). When the intestine was removed and the arterial and venous portions of the mesentery perfused individually the responses to arginine vasopressin and noradrenaline were associated with the arterial portion of the vasculature and those to angiotensin II with the venous vasculature. Endothelin-1 constricted both parts of the circu-

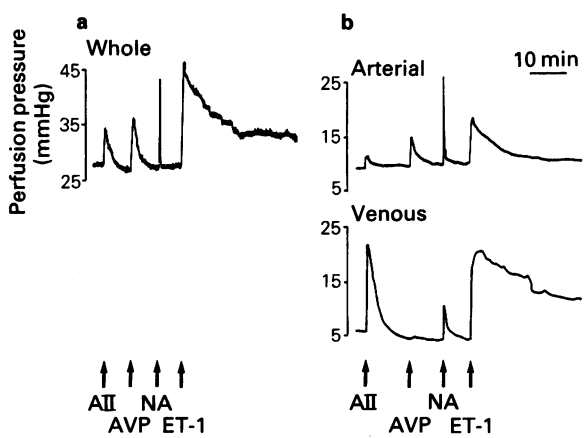


Figure 1 The role of the arterial and venous vessels in the responses of the intact perfused mesentery. (a) The responses of the whole mesentery. (b) The responses of the separated mesentery, with the responses of the arterial vessels at the top and the venous vessels at the bottom. The vasoconstrictions of the intact mesentery to noradrenaline (NA, 1 nmol) and arginine-vasopressin (AVP, 30 pmol) were matched by the vasoconstrictions of the arterial portion and those to angiotensin II (AII, 30 pmol) by the venous portion. Endothelin-1 (ET-1, 30 pmol) constricted both the arterial and the venous vessels.

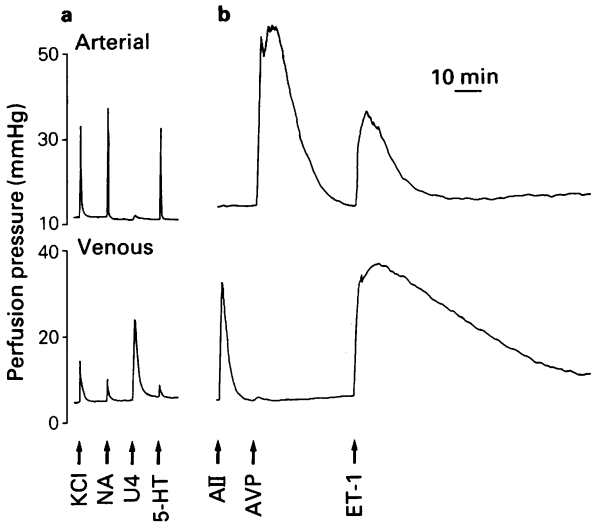


Figure 2 Comparison of the contractile responses of the arterial and venous vessels of the rat perfused mesentery. Traces from two experiments, (a) and (b), are shown. The responses of the arterial vessels are shown in the upper panel and those of the venous vessels in the lower panel. KCl (60 μmol), noradrenaline (NA, 3 nmol), and 5-hydroxytryptamine (5-HT, 3 nmol) caused principally vasoconstrictions of the arterial circulation whereas U46619 (U4, 300 pmol) principally constricted the venous circulation. Angiotensin II (AII, 100 pmol) caused only constriction of the venous vessels and arginine vasopressin (AVP, 100 pmol) only the arterial vessels. Endothelin-1 (ET-1, 100 pmol) constricted both sides of the circulation but produced a markedly more prolonged effect on the venous than the arterial vessels.

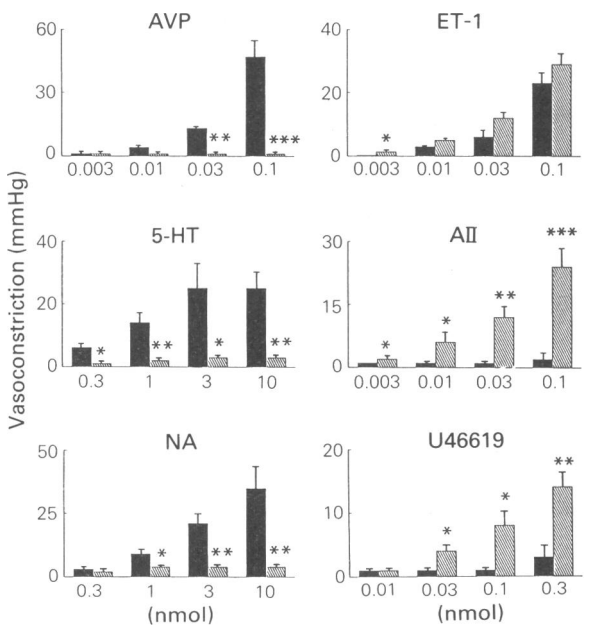


Figure 3 Comparison of constrictors within the arterial and venous portions of the rat mesenteric vascular bed. Arterial responses (solid columns) and venous responses (hatched columns) are shown. Arginine vasopressin (AVP, 0.003–0.1 nmol), 5-hydroxytryptamine (5-HT, 0.3–10 nmol) and noradrenaline (NA, 0.3–10 nmol) were arterioselective vasoconstrictors. Conversely, angiotensin II (AII, 0.003–0.1 nmol) and U46619 (0.01–0.3 nmol) were selective venoconstrictors. Endothelin-1 (ET-1, 0.003–0.1 nmol) constricted both portions of the circulation but the threshold dose for constriction was lower on the venous than the arterial vessels. Mean data from 4–5 experiments. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$; significant differences between the responses of the arterial and venous vessels.

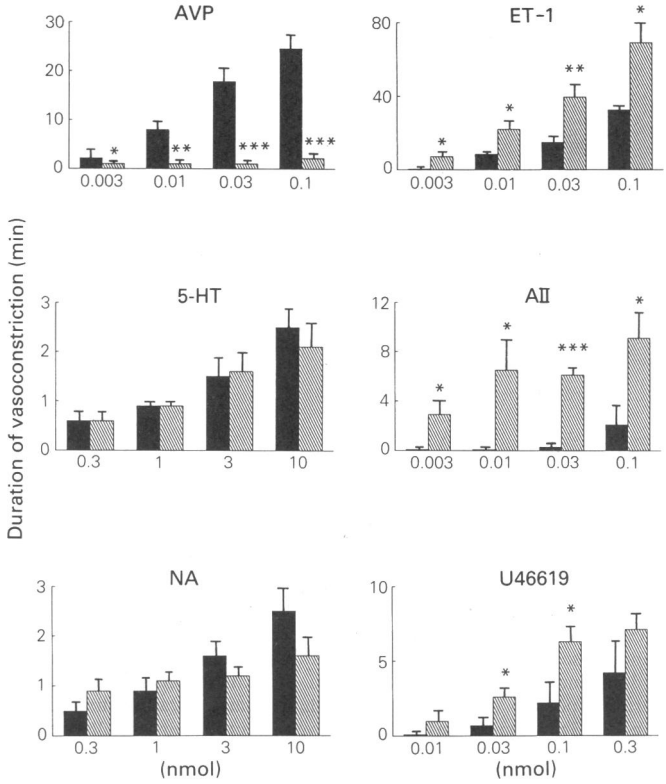


Figure 4 Comparison of the time course of response to constrictors within the arterial and venous portions of the rat mesenteric vascular bed. Arterial (solid columns) and venous responses (hatched columns) are shown. The bars represent the duration of the responses, i.e. the time for the response to return to baseline. Arginine vasopressin (AVP, 0.003–0.1 nmol), 5-hydroxytryptamine (5-HT, 0.3–10 nmol) and noradrenaline (NA, 0.3–10 nmol) were arterioselective vasoconstrictors, but only arginine vasopressin caused significantly longer vasoconstrictions of the arterial than venous vessels. Angiotensin II (AII, 0.003–0.1 nmol), U46619 (0.01–0.3 nmol) and endothelin-1 (ET-1, 0.003–0.1 nmol) all caused significantly more prolonged vasoconstrictions of the venous than arterial vessels. Mean data from 4–5 experiments. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$; significant differences between the time course of response of the arterial and venous vessels.

lation, although the venous response was maintained for a longer period (Figure 1).

Vasoconstrictor responses

The arterial and venous portions of the mesenteric bed demonstrated distinct profiles of sensitivity to a range of contractile agents. Noradrenaline (0.3–10 nmol), 5-hydroxytryptamine (0.3–10 nmol) and KCl (1–60 μmol) were significantly more active as constrictors of the arterial portion of the vasculature than the venous portion, whereas U46619 (10–300 pmol) was more active as a constrictor of the venous vasculature (Figures 2 and 3). Interestingly, however, the time courses of the responses of the arterial and venous vessels to noradrenaline and 5-hydroxytryptamine were not significantly different, although the vasoconstrictions produced by U46619 were more prolonged on the venous than arterial vessels (Figure 4). Arginine vasopressin (3–100 pmol) was found to be a selective constrictor of the arterial vessels and angiotensin II (3–100 pmol) a selective venoconstrictor, estimated either as the peak (Figure 3) or duration (Figure 4) of the vasoconstrictor response. Endothelin-1 (3–100 pmol) constricted both sides of the vasculature (Figures 2 and 3), but its effect was significantly prolonged on the venous as compared to the arterial portion at all doses tested (Figures 2 and 4).

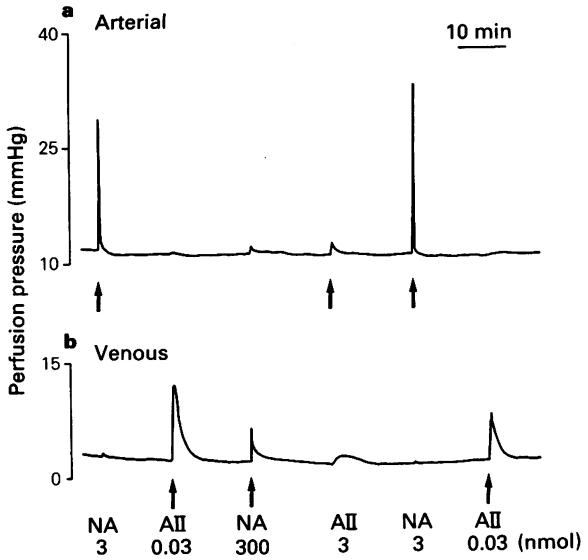


Figure 5 Bioassay of the exchange of perfusate between the arterial and venous portions of the rat isolated perfused mesentery. The responses of the arterial portion of the circulation are shown in (a) and those of the venous circulation in (b). Three hundred nmol noradrenaline (NA) injected i.v. caused a smaller constriction of the arterial portion of the vasculature than 3 nmol noradrenaline given i.a. and 3 nmol angiotensin II (AII) given i.a. produced a smaller venoconstriction than 0.03 nmol angiotensin II injected i.v.

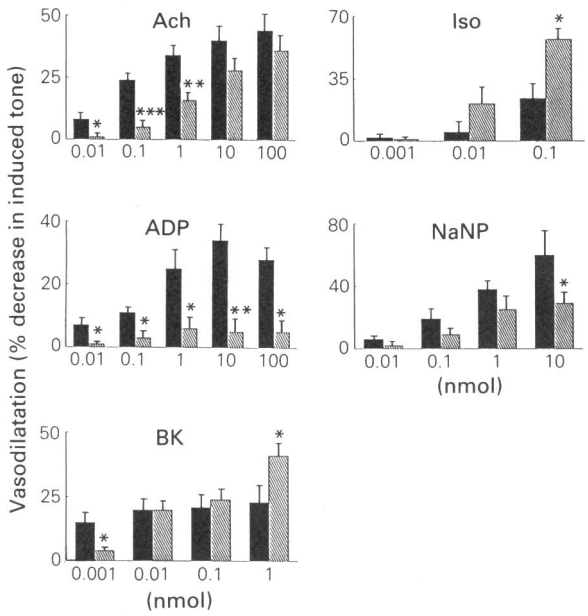


Figure 7 Comparison of dilators within the arterial and venous vessels of the rat mesentery. Dilatations are expressed as % reduction in induced tone. Responses of arterial vessels (solid columns) and of venous vessels (hatched columns) are shown. Acetylcholine (ACh, 0.01–100 nmol), ADP (0.01–100 nmol) and sodium nitroprusside (NaNP, 0.01–10 nmol) were significantly more active as dilators of the arterial than the venous circulations. Bradykinin (BK, 0.001–1 nmol) produced a poor dose-related dilatation of the arterial vessels but at a dose of 1 nmol was a significantly more potent dilator of the venous than the arterial vessels. Isoprenaline (Iso, 0.001–0.1 nmol) was more active as dilator of venous than arterial vessels. Mean data from 3–9 experiments. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.005$; significant differences between the responses of the arterial and venous vessels.

Role of angiotensin converting enzyme in the responses of the isolated perfused veins

Angiotensin I (0.3 nmol) produced vasoconstrictions of the venous vasculature (14.5 ± 2.3 mmHg, $n = 4$) that were abolished by infusion of captopril (10 μM) for 20 min (1 ± 0.5 mmHg, $n = 4$, $P < 0.005$). Vasoconstrictions in response to angiotensin II (0.01 nmol; 6.5 ± 2 mmHg, $n = 4$) were unaffected by captopril (8 ± 1.5 mmHg, $n = 4$).

Independence of the arterial and venous circulations

The exchange of perfusate from one side of the vasculature to the other was estimated, by bioassay, to be less than 1%; 300 nmol noradrenaline injected intravenously caused a vasoconstriction of the arterial portion less than that to 3 nmol noradrenaline injected intra-arterially ($n = 4$, Figure 5). Conversely, 3 nmol angiotensin II given intra-arterially caused a venoconstriction less than that produced by 0.03 nmol angiotensin II administered intravenously ($n = 4$, Figure 5).

Vasodilator responses

In preconstricted preparations, injections of acetylcholine (0.01–100 nmol), bradykinin (0.001–1 nmol), sodium nitroprusside (0.01–10 nmol) or isoprenaline (1–100 pmol) produced dose-related vasodilatations of both the arterial and venous sides of the vasculature (Figures 6 and 7). In contrast, ADP (0.01–100 nmol) while producing vasodilatations of the arterial

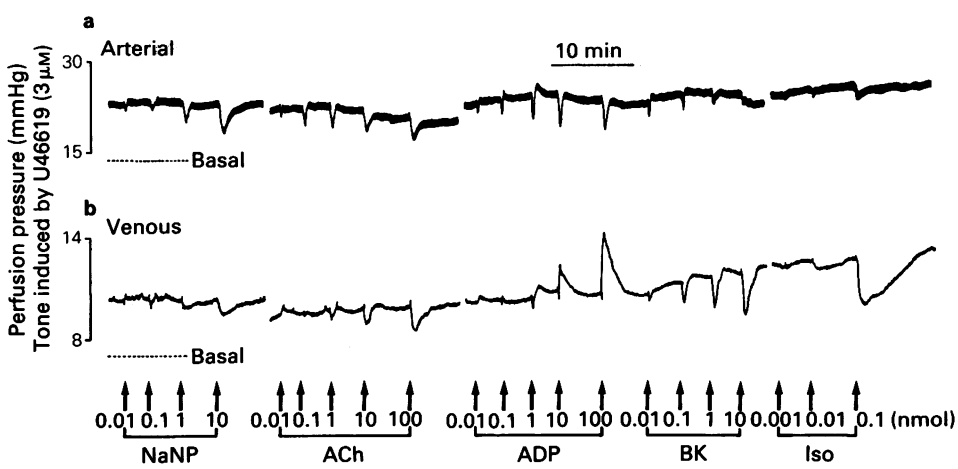


Figure 6 Vasodilator responses of the arterial and venous vessels of the rat mesentery. The responses of the arterial vessels are shown in (a) and those of the venous vessels in (b). Sodium nitroprusside (NaNP, 0.01–10 nmol), acetylcholine (ACh, 0.01–100 nmol) and bradykinin (BK, 0.001–1 nmol) caused dilatations of both circulations. ADP (0.01–100 nmol) caused dilatations of the arterial vessels but principally constrictions of the venous vessels. Isoprenaline (Iso, 0.001–0.1 nmol) caused dilatations of both sides of the circulation but was particularly potent as a venodilator.

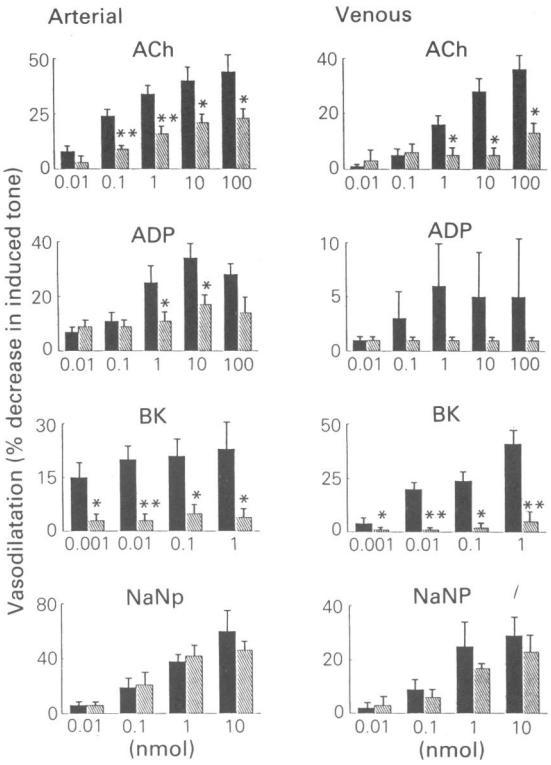


Figure 8 The effects of gossypol (3 μM) on dilatations in the arterial and venous vessels of the rat isolated perfused mesentery. Dilatations are expressed as % of induced tone. (a) Arterial responses and (b) venous responses. Control responses are shown as solid columns and those in the presence of gossypol as hatched columns. Gossypol inhibited dilatations of both the arterial and venous vessels to acetylcholine (ACh, 0.01–100 nmol), ADP (0.01–100 nmol) and bradykinin (BK, 0.001–1 nmol). Dilatations to sodium nitroprusside (NaNP, 0.01–10 nmol) were not affected. Mean values from 3–9 experiments. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.005$; significant differences between the responses of the arterial and venous vessels.

portion of the bed (Figures 6 and 7) was a poor dilator of the venous portion of the vasculature, where it caused principally vasoconstriction (Figure 6).

Effects of gossypol

In the presence of gossypol (3 μM) infusion of U46619 (0.3 μM) caused an increase in perfusion pressure of the arterial vasculature (13 $\pm$ 2 mmHg, $n = 6$) that was not significantly different from control. In contrast, in the venous vasculature U46619 produced a significant increase in perfusion pressure (3 $\pm$ 0.5 mmHg) in only 3 out of 6 experiments. Relaxations in response to acetylcholine and bradykinin, in both sides of the vasculature, and to ADP, in the arterial vasculature, were inhibited with respect to control, whereas those to sodium nitroprusside were not (Figure 8).

Discussion

This paper describes a new method that allows the simple measurement of the perfusion pressures at constant flow within the arterial and venous circulations of the isolated perfused mesentery of the rat. The results show that there is a clear distinction between the effects on the two sides of the circulation of a number of agents. For instance, angiotensin II constricts only the venous portion of the vascular bed and arginine vasopressin only the arterial portion. ADP, for its part, causes dilatation of the arterial vessels but constriction of the venous ones. Thus, these vasoactive agents demonstrate pronounced arterial-venous selectivity.

The finding that the venous portion of the mesentery is strongly constricted by angiotensin II is supported by an earlier observation that, *in vivo*, angiotensin II causes an initial increase in the venous return associated with the hepatosplanchnic bed (Emerson, 1966) which is suggestive of venoconstriction in this circulation. Conversely, and as the results presented here suggest, arginine vasopressin does not cause this initial increase (Emerson, 1966). However, as would be expected of such a potent constrictor of the arterial vessels (Bhardwaj & Moore, 1988; Randall *et al.*, 1988), it does cause a sustained increase in the mesenteric perfusion pressure *in vivo* (Dresel & Wallentin, 1966). Endothelin-1 is a constrictor of the arterial vessels of the rat mesenteric vascular bed (de Nucci *et al.*, 1988). The results presented here confirm these findings but also demonstrate that endothelin-1 is an even more potent and prolonged constrictor of the venous portion of the vasculature, as has been found in previous comparisons of arterial and venous vessels (de Nucci *et al.*, 1988). Indeed, even though there was no difference between the peak responses of the arterial and venous vessels to the maximum dose of endothelin-1 used (100 pmol), the duration of the response of the venous vessels was more than twice that of the arterial vessels. This may well explain why endothelin-1 causes such a pronounced and prolonged increase in resistance in the mesentery and intestine *in vivo* (Fortes *et al.*, 1989; Hiley *et al.*, 1989; Walder *et al.*, 1989).

Interestingly, the venous portion of the vasculature did not constrict to noradrenaline, although either stimulation of the sympathetic supply to the mesentery or administration of noradrenaline into this circulation is associated with pronounced and maintained constriction of the mesenteric capacitance vessels (Mellander, 1960; Folkow *et al.*, 1964; Oberg, 1967; Henrich, 1985). This lack of response may reflect the low basal venous perfusion pressure, for the mesenteric capacitance vessels do not constrict well to nerve stimulation when the intraluminal pressure is lower than about 10 mmHg (Oberg, 1967).

As shown previously, acetylcholine (Byfield *et al.*, 1986; Furchgott *et al.*, 1987), ADP (Byfield *et al.*, 1986; Ralevic & Burnstock, 1988) and bradykinin caused endothelium-dependent dilatations of the arterial portion of the rat mesentery, as confirmed by their inhibition by gossypol (Förstermann *et al.*, 1986), which inhibits EDRF-mediated vasodilatations in this preparation (Bhardwaj & Moore, 1988). The venous circulation also dilated in an endothelium-dependent manner in response to acetylcholine and bradykinin, but primarily constricted in response to ADP. It has been found previously that acetylcholine causes endothelium-dependent relaxation of isolated arteries but principally constriction of isolated veins (De Mey & Vanhoutte, 1982; Vedernikov *et al.*, 1988), whereas *in vivo* it may cause venodilatation (Emerson, 1968). The results presented here support those found *in vivo* and show that the principal response of the capacitance vessels to acetylcholine is indeed dilatation. In the organ bath the indirect dilator effects of acetylcholine, mediated by the endothelium, may be over-ridden by constrictor effects, mediated directly by the smooth muscle, for acetylcholine has greater access to the smooth muscle than in a perfused system. As previously found for ATP (De Mey & Vanhoutte, 1982), ADP caused only transient dilatations of the venous circulation followed by more prominent vasoconstrictions. Interestingly, bradykinin also causes primarily constriction of isolated mesenteric veins (Toda *et al.*, 1987) and is relatively poor at releasing EDRF from mesenteric arteries (Cherry *et al.*, 1982). Here, in a perfused system, bradykinin was a potent dilator of the capacitance vessels through the release of EDRF and, as previously shown, a relatively poor releaser of EDRF from the arterial vessels. Once again this difference in the activity of bradykinin in venous vessels may be explained by the different experimental systems used. In contrast to the results presented here, bradykinin constricts the venous portion of the mesenteric circulation *in vivo* (Emerson, 1967). However, this can be explained as a secondary effect due to bradykinin-induced constriction of the intestinal smooth muscle. Interestingly, although it is generally

accepted that nitrates have a stronger effect on veins than arteries (Ablad & Mellander, 1963) such a difference in activity was not seen in this preparation. Indeed, as described earlier the veins were particularly sensitive to the dilator effects of isoprenaline (Folkow *et al.*, 1963; De Mey & Vanhoutte, 1982).

The finding that the venous portion of the vasculature converts angiotensin I to angiotensin II demonstrates the presence of active angiotensin converting enzyme in these vessels. As bradykinin is a potent dilator of the capacitance vessels and since it is inactivated by angiotensin converting enzyme, this shows that the venous vessels act to increase the amounts of the venoconstrictor, angiotensin II, while decreasing the levels of the venodilator, bradykinin. Thus, angiotensin converting enzyme inhibitors, such as captopril, will tend to cause a reduction in the tone of these venous vessels by two complementary mechanisms.

Clearly, although this preparation lacks the majority of the resistance vessels of the intact mesentery and intestine (Richardson & Zweifach, 1970; Singbartl & Henrich, 1974; Bohlen & Gore, 1977), it reflects the responses of the intact mesenteric circulation, for there is a direct correlation between systemic arterial pressure and the pressure throughout the microvascular arterial tree (Bohlen & Gore, 1977). Furthermore, although *in vivo* intravenous administration of noradrenaline does not cause constriction of the arterial vessels within the mesentery, but does constrict arterioles within the intestine (Hebert & Marshall, 1985), these former vessels are sensitive to the contractile effects of noradrenaline (McGregor, 1965). This apparent discrepancy is explained by studies that have shown that, within the arterial vessels of the frog mesentery, the maximum constrictor response to noradrenaline is achieved when the tangential stress of these vessels is within an optimum range (Gore, 1972). Thus, *in vivo*, the increase in wall stress of the large arteries, caused by constriction of arterioles further downstream, may prevent any further decrease in diameter of these vessels. In other words, under these conditions the larger arterial vessels constrict isometrically rather than isotonically (Hebert & Marshall, 1985). Thus, this preparation will indeed give information about the responses of the entire bed.

Another question is whether or not the responses of the veins have a significant role in the responses of the entire vascular bed. The results presented here suggest that they do, for the responses of the intact mesentery and small intestine to angiotensin II, and in some respects to endothelin-1, were associated with the venous part of the vasculature. Indeed, it has been demonstrated previously that 60–80% of an increase in venous pressure within the mesenteric circulation is directly reflected as an increase in arterial resistance (Selkurt & Johnson, 1958; Folkow *et al.*, 1963; Richardson & Zweifach, 1970), possibly because the venous vessels do not contain many venular valve-like structures (Gaehtgens & Uekermann, 1971).

Finally, what is the relevance of this to the whole circulation? As the mesenteric vascular bed may be the destination for 10% of the cardiac output, obviously changes in this can have important effects on the whole cardiovascular system. For instance, removal of the hepatosplanchnic vasculature *in vivo* abolishes both the increase in venous return caused by acetylcholine (Emerson, 1968) and the initial increase in venous return caused by angiotensin II (Emerson, 1966). It is concluded that this preparation can supply useful information about the behaviour of the mesenteric circulation *in vivo* as well as providing a simple system in which many of the difficult to study responses of the venous portion of the vasculature may be examined.

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