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SPECIAL REPORT

Evidence that agonist and antagonist enantiomers of the dihydropyridine PN 202-791 act at different sites on the voltage-dependent calcium channel of vascular muscle

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S(+)-PN 202-791, a calcium channel agonist, and its optical isomer R(−)-PN 202-791, a calcium channel antagonist, respectively increased and decreased inward current carried by barium through voltage-dependent calcium channels in isolated ear artery cells of the rabbit in a concentration-dependent manner. The EC_{50} or IC_{50} derived from the concentration-response relationship was unaltered in the presence of the other enantiomer indicating that no competitive antagonism exists between these enantiomers and suggesting that the actions of these two enantiomers involve two separate binding sites.

Introduction Dihydropyridine drugs exert actions on voltage-operated calcium channels (VOCs). Some dihydropyridines, such as PN 202-791, exist as two different enantiomers: S(+)-PN 202-791 is an agonist but its enantiomer, R(−)-PN 202-791, acts as a calcium antagonist (Hamilton *et al.*, 1987). Generally, both agonist and antagonist dihydropyridines are believed to act by binding to a single site associated with the VOCs (Triggle & Rampe, 1989). If this site is responsible for both actions of dihydropyridines then agonist and antagonist dihydropyridines should compete for binding to this site and hence display competitive antagonism. We have studied this issue in single voltage-clamped vascular smooth muscle cells.

Methods Single ear artery cells of the rabbit were prepared as previously described (Benham & Bolton, 1986). Whole cell voltage clamp technique (Hamill *et al.*, 1981) was used with an extracellular solution of the following composition (mM): $BaCl_2$ 110, tetraethylammonium Cl 10, HEPES 10, pH 7.2 (with NaOH), and intracellular pipette solution of composition (mM): NaCl 126, Mg_2SO_4 3.2, NaH_2PO_4 1, EGTA 2, HEPES 5, tetraethylammonium Cl 10, adenosine 5'-triphosphate 2, pH 7.2 (with NaOH). These solutions optimised recording of inward current through VOCs (Aaronson *et al.*, 1988). Pipette resistance was 3–6 MΩ. S(+)- and R(−)-PN 202-791 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate, gifts from Sandoz) were prepared as 1 mM stock solutions in ethanol and diluted appropriately. The maximum concentration of ethanol used (0.3%) had no effect on currents. Inward currents were evoked every 1 s by a 20 ms step to +10 mV from a holding potential of −60 mV. With this protocol there was negligible 'run down' of current. Concentration-response data were analysed by fitting to a logistic function ($E/E_{max} = C/(EC_{50} + C)$), where E/E_{max} is the relative effect of drug (agonist or antagonist) on the inward current and C is the molar concentration of drug, using non-linear regression analysis. Data are presented as mean $-\log(EC_{50})$ or $-\log(IC_{50})$ (i.e. pEC_{50} or pIC_{50}) ± s.e.mean of *n* observations.

Results S(+)-PN 202-791 increased and R(−)-PN 202-791 decreased inward barium current in a concentration-dependent fashion (Figures 1 and 2). However, the pEC_{50} for S(+)-PN 202-791 (6.91 ± 0.08; $n = 6$) was not significantly different from its pEC_{50} in the presence of 3 μM R(−)-PN 202-791 (7.02 ± 0.12; $n = 4$). Likewise, the pIC_{50} for R(−)-PN 202-791 (6.34 ± 0.03; $n = 5$) was not significantly different from its pIC_{50} in the presence of 1 μM S(+)-PN 202-791 (6.42 ± 0.08; $n = 5$). However when 3 μM R(−)-PN 202-791 was present, the currents when S(+)-PN 202-791 was added were smaller than when R(−)-PN 202-791 was absent, although the relative increases produced by various concentrations of S(+)-PN 202-791 were similar (Figure 1a,c and 2a,c). R(−)-PN 202-791 also had similar relative effects in the presence of 1 μM S(+)-PN 202-791 as in its absence (Figure 1b,d and 2b,d).

Discussion The concentration-response relationships of S(+)- and R(−)-PN 202-791 were not appreciably shifted by the presence of the other enantiomer in concentrations which would be anticipated to cause approximately a ten fold increase in the concentration necessary to produce half maximal effects from considerations of classical competitive antagonism. As can be seen from Figure 2 both enantiomers acted as non-competitive antagonists of the action of the other enantiomer. The failure of R(−)-PN 202-791 to block responses to S(+)-PN 202-791 has also recently been reported in cardiac myocytes (Kamp *et al.*, 1989).

These electrophysiological results contrast with most, but not all (Kokubun *et al.*, 1986; Lee *et al.*, 1987), ligand binding studies which show a single binding site for ^{3}H-labelled dihydropyridines (Triggle & Rampe, 1989). The reason for this apparent discrepancy is unclear. Others (Brown *et al.*, 1986; Kokubun *et al.*, 1986) have proposed the existence of two dihydropyridine binding sites on the basis of whole cell and unitary calcium current studies in cardiac cells. Kokubun *et al.* (1986) concluded that co-operative interactions could occur between enantiomers and Brown & Lacerda (1989) have also recently proposed a model involving co-operative interactions between dihydropyridine binding sites regulating VOCs from single channel data. Our data do not provide support for co-operative interactions between enantiomers of PN 202-791, as none of the Hill slopes of the concentration-response curves was found to differ significantly from unity. A simple interpretation of our data is that the action of agonist and antagonist enantiomers is at two independent sites, one to which the agonist, the other to which the antagonist, bind. These two sites respectively increase and decrease current through VOCs in vascular smooth muscle. If so, this finding has important implications for understanding the mechanisms of action of dihydropyridine drugs and for the development of new modulators of voltage-dependent calcium channels.

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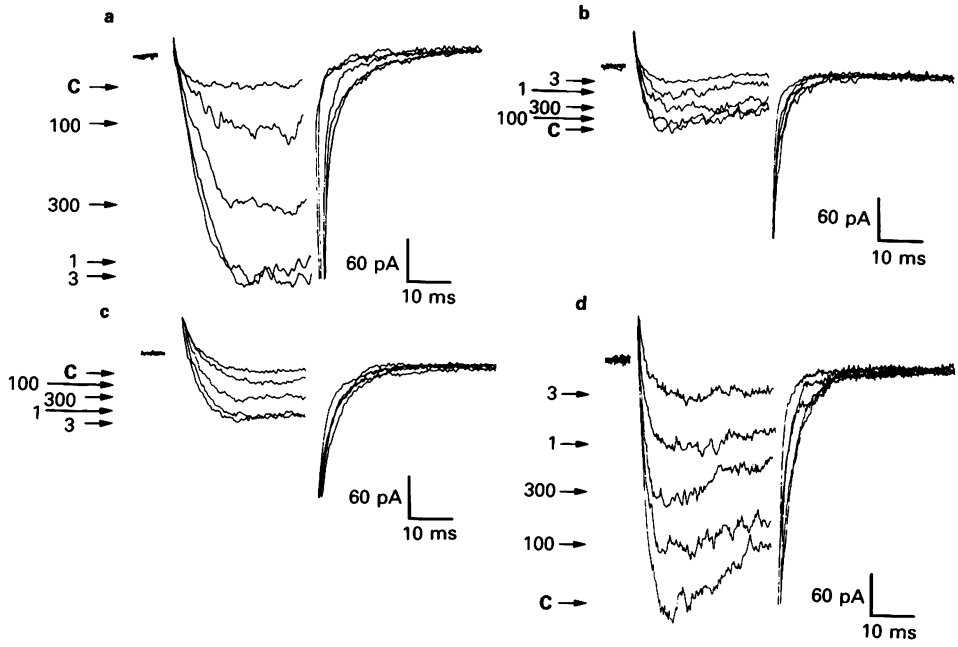

Figure 1 The effect of the enantiomers of PN 202-791 on calcium channel current, with 110 mM Ba^{2+} as charge carrier. Current was evoked from a holding potential of −60 mV by a 20 ms step to +10 mV every 1 s in single ear artery cells. (a) S(+)-PN 202-791 (100 nM–3 μM). (b) R(−)-PN 202-791 (100 nM–3 μM). (c) S(+)-PN 202-791 (100 nM–3 μM) in the presence of R(−)-PN 202-791 (3 μM). (d) R(−)-PN 202-791 (100 nM–3 μM) in the presence of S(+)-PN 202-791 (1 μM). C is the current before drug application (a,b) or in the presence of the opposite enantiomer (c,d).

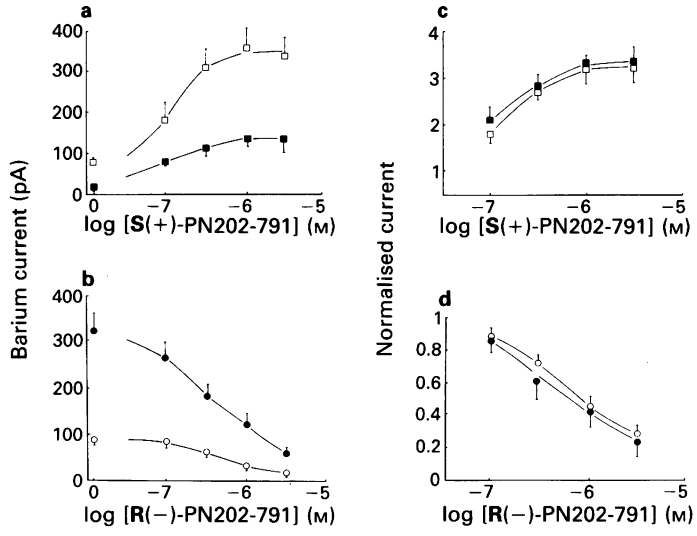

Figure 2 Concentration-response relationships for enantiomers of PN 202-791. (□) S(+)-PN 202-791 ($n = 6$); (○) R(−)-PN 202-791 ($n = 5$); (■) S(+)-PN 202-791 in the presence of R(−)-PN 202-791 (3 μM) ($n = 4$); (●) R(−)-PN 202-791 in the presence of S(+)-PN 202-791 (1 μM) ($n = 5$). The effect of S(+)-PN 202-791 in the absence and presence of R(−)-PN 202-791 is shown in (a) and after normalization in (c). The effect of R(−)-PN 202-791 in the absence and presence of S(+)-PN 202-791 is shown in (b) and after normalization in (d). Points represent means and vertical bars s.e.mean of n observations.

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SPECIAL REPORT

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Interactions of spironolactone with (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil binding sites in vascular smooth muscle

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Spironolactone partially inhibited the specific binding of (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil to vascular smooth muscle membranes. It is suggested that spironolactone interacts at a binding site of the calcium channel complex and allosterically modulates ligand binding at receptor sites in the channel.

Introduction A series of drugs, termed calcium entry blockers, form an important class of therapeutic agents which has been particularly useful in the study of the physiological, biochemical and biophysical properties of the L-type calcium channels (Godfraind *et al.*, 1986). Calcium entry blockers are a chemically heterogeneous group of compounds, but they can be classified into three major subgroups: dihydropyridines (nifedipine and isradipine), phenylalkylamines (desmethoxyverapamil) and benzothiazepines (diltiazem). Characterization of the receptor sites for these drugs through binding protocols (De Pover *et al.*, 1982) is useful in predicting the site of action of new, previously uncharacterized, calcium channel modulators. In this paper we investigated the interactions of spironolactone, a synthetic steroid (Ramsay, 1982), with the calcium channel complex of vascular membranes.

Methods Membranes were prepared from equine portal vein (Dacquet *et al.*, 1989). Protein concentration was determined according to Bradford (1976).

Binding assays (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil interactions with vascular membranes (both equilibrium binding and association and dissociation kinetics) were measured as previously described (Dacquet *et al.*, 1989; Loirand *et al.*, 1989). Scatchard plots were analyzed by a non linear least-square ligand programme (Munson & Rodbard, 1980).

The experimental results are expressed as mean ± s.e.mean and significance was tested by Student's *t* test. The drugs used (with their sources) were as follows: (+)-[^{3}H]-isradipine (84 Ci mmol^{-1}) and (−)-[^{3}H]-desmethoxyverapamil (79 Ci mmol^{-1}) (Amersham), (+)-isradipine (Sandoz), nifedipine (Bayer), (−)-desmethoxyverapamil and methoxyverapamil (Knoll), cromakalim (Rhône-Poulenc), spironolactone (Searle), aldosterone and progesterone (Sigma).

Results (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil bind with high affinity to distinct receptors in portal vein smooth muscle membranes (Dacquet *et al.*, 1989; Loirand *et al.*, 1989). Increasing concentrations of spironolactone gradually inhibited isradipine and desmethoxyverapamil binding (Figure 1a–b) with similar IC_{50}s of 2.0 ± 0.2 and 2.2 ± 0.3 μM ($n = 4$), respectively, but maximal inhibition was not complete. In contrast, the same concentrations of progesterone and aldosterone had no significant effect on isradipine and desmethoxyverapamil binding (5 ± 2%, $n = 4$, $P > 0.05$). To elucidate the mechanism by which spironolactone inhibited binding of calcium entry blockers, Scatchard

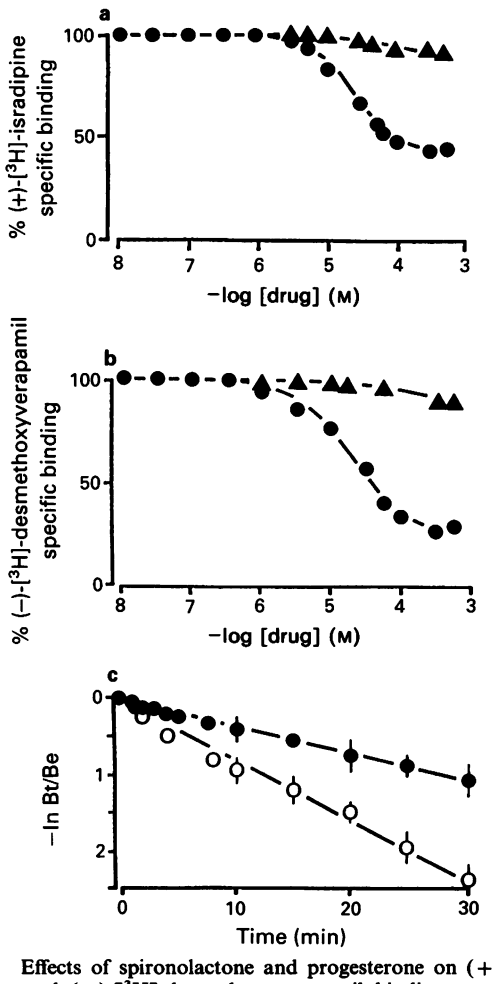

Figure 1 Effects of spironolactone and progesterone on (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil binding to vascular smooth muscle membranes. (a–b) Specific binding of isradipine (a, 0.1 nM) or desmethoxyverapamil (b, 0.3 nM) was measured at 25°C under equilibrium conditions with vascular membranes (0.1 mg ml^{-1}) in the presence of increasing concentrations of spironolactone (●) or progesterone (▲). Results are expressed as a percentage of specific binding obtained in the absence of unlabelled drugs, and are means of 4 experiments. The vertical lines representing s.e.mean do not exceed the size of the symbols. (c) Dissociation kinetics of (−)-[^{3}H]-desmethoxyverapamil. Dissociation of ligand was initiated by addition of 2 μM unlabelled methoxyverapamil in the absence (●) or presence of 100 μM spironolactone (○). First-order representation of binding showing that k_{-1} increased from 0.046 ± 0.002 min^{-1} (control) to 0.076 ± 0.006 min^{-1} (spironolactone, $P < 0.05$). Each point represents the mean of 4 experiments with s.e.mean shown by vertical lines. Bt = ligand bound at a given time; B_o = ligand bound at time zero.

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Table 1 Parameters of (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil binding to vascular membranes in the presence of spironolactone

	(+)-[^{3}H]-*isradipine*			(−)-[^{3}H]-*desmethoxyverapamil*		
	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n
Control	0.093 ± 0.007	69 ± 3	6	0.516 ± 0.047	104 ± 4	10
Spironolactone						
10 μM	0.081 ± 0.008	56* ± 2	3	0.536 ± 0.067	72* ± 5	4
50 μM	0.110 ± 0.006	46* ± 2	3	0.875 ± 0.110	61* ± 9	3
100 μM	0.117 ± 0.012	38* ± 2	4	0.728 ± 0.214	39* ± 9	3

* Significant change ($P < 0.05$). Each result is mean ± s.e.mean. n, number of determinations.

analysis was carried out in the presence of different concentrations of spironolactone (Table 1). A reduction in B_{max} with no significant change in apparent K_D was observed when spironolactone (10–100 μM) was added. This was a typical result showing that inhibition of (+)-[^{3}H]-isradipine and (−)-[^{3}H]-desmethoxyverapamil binding was not of the competitive type. As temperature had no important effect on the (+)-[^{3}H]-isradipine binding (Maan & Hosey, 1987; Dacquet *et al.*, 1989) it was of interest to investigate the action of spironolactone on (−)-[^{3}H]-desmethoxyverapamil binding at 25° and 37°C. Although the extent of inhibition of desmethoxyverapamil was similar at both temperatures, the potency of spirolactone in affecting ligand interaction was increased approximately 3 fold at the higher temperature (not shown). As one way of ascertaining the site in the calcium entry blocker receptor complex at which spironolactone interacts, association and dissociation kinetics of (−)-[^{3}H]-desmethoxyverapamil were monitored. The dissociation rate constant (k_{-1}) was approximately doubled in the presence of 100 μM spironolactone (Figure 1c) while the association rate constant (k_1) was not significantly affected (3.2 ± 2.4%, $n = 4$, $P > 0.05$).

Discussion The experiments presented in this paper suggest that spironolactone interacts at a binding site of the calcium entry blocker receptor complex in vascular membranes and allosterically modulates the binding of calcium entry blockers to this complex. Several lines of evidence support this conclusion. (1) Spironolactone exerts incomplete inhibition of isradipine and desmethoxyverapamil binding and this effect is due to a decrease in site density. (2) Studies of the kinetics of desmethoxyverapamil association and dissociation demonstrate that spironolactone clearly accelerates the rate of dissociation of the ligand from its binding site. (3) Ability of spironolactone to modify receptor-ligand interactions of desmethoxyverapamil is temperature-dependent. These observations are in good agreement with electrophysiological studies demonstrating inhibition of L-type calcium channel current by spironolactone in vascular smooth muscle cells (Dacquet *et al.*, 1987).

In conclusion, these findings suggest that spironolactone interacts with the calcium entry blocker receptor complex and provide a rational explanation for the spironolactone-induced inhibition of vascular contractility.

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SPECIAL REPORT

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Potassium-stimulated release of nitric oxide from cerebellar slices

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This study shows that nitric oxide release can be evoked from intact neuronal tissue. Stimulation of rat cerebellar slices for 4 min with 50 mM potassium significantly increased the release of nitric oxide and glutamate and was associated with a rise in the guanosine 3':5'-cyclic monophosphate (cyclic GMP) content of the tissue. Nitric oxide levels in the perfusate increased from 160 to 262 parts per billion. It is suggested that an increased release of endogenous glutamate in the slice, induced by exposure to potassium, may mediate the release of nitric oxide.

Introduction Garthwaite *et al.* (1988) showed that cell suspensions prepared from neonatal rat cerebellum released a substance with similar characteristics to endothelium derived relaxing factor (EDRF) when stimulated with glutamate or N-methyl-D-aspartate (NMDA). The response to NMDA was blocked by the specific antagonist D,L-2-amino-5-phosphonovalerate (AP-5). The chemical nature of EDRF in vascular tissue has been identified as nitric oxide or a nitric oxide-containing molecule which is formed from the amino acid precursor L-arginine (Palmer *et al.*, 1988). The identity of the EDRF-like substance released from cerebellar tissue has yet to be confirmed.

We have shown previously that endogenous glutamate is released in a calcium-dependent manner from cerebellar slices exposed to elevated potassium ion concentrations (Barnes *et al.*, 1988). The present study investigated the effects of 50 mM potassium chloride on nitric oxide and glutamate release from adult rat cerebellar slices; tissue guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels were also measured.

Methods Female Wistar rats (200–220 g) were killed by stunning and decapitation and parasagittal cerebellar slices (250 μm) prepared and immediately perfused with gassed (95%O_2/5%CO_2) artificial cerebrospinal fluid (ACSF) at 1.25 ml min^{-1} at 37°C as previously described (Barnes *et al.*, 1988). Five slices from a single rat were used for each experiment and provided one data point. The tissue was allowed to equilibrate for 30 min in normal ACSF followed by 30 min in nominally magnesium-free ACSF after slicing. All the experiments were carried out in magnesium-free ACSF. Four-minute aliquots of perfusate were collected and the nitric oxide levels assayed by a previously described technique (Garside, 1982). The method converts nitrite, a stable reaction product of nitric oxide with oxygen, to free nitric oxide which was measured with a commercially available nitric oxide analyser (Dasibi 2108, Quantitech, Milton Keynes, U.K.). Three ml of the perfusate was injected into the reaction flask. Nitric oxide levels were quantified in parts per billion in the perfusate (ppB, 10^9). A standard curve for sodium nitrite was linear over the concentration-range 125 pmol to 2 nmol (Figure 1a). Glutamate levels in the perfusate were assayed by high performance liquid chromatography (h.p.l.c.) with fluorescence detection following pre-column derivatization with *o*-phthaldialdehyde (Barnes *et al.*, 1988). Tissue cyclic GMP levels were measured by radio-immunoassay with a commercially available kit (New England Nuclear) and expressed as pmol mg^{-1} tissue protein.

Three 4 min samples of perfusate were collected to assess basal release of nitric oxide. During the collection of the fourth sample a 4 min pulse of 50 mM potassium chloride in ACSF was perfused. This was followed by the collection of a further three samples in normal ACSF. The results given in Figure 1b are plotted as a percentage of 'basal' release calculated from the mean of the first three unstimulated samples. Statistical analysis was carried out by two-way analysis of variance between tissues and sample. The error mean square from this analysis was used to perform *a priori* contrasts between the basal and stimulated release.

For the measurement of glutamate and cyclic GMP, slices were prepared as described above except that the experiment was terminated following the collection of sample 4. The slices were immediately frozen in liquid nitrogen and stored until assayed for cyclic GMP as described previously (Collins *et al.*, 1988). The perfusate from samples 1–4 was assayed for glutamate. Control tissues were perfused as described above but not stimulated with 50 mM potassium. Statistical analysis for cyclic GMP and glutamate were performed by use of Student's *t* test for unpaired data.

Results Basal release of nitric oxide showed a wide variation between tissues with a mean ± s.e.mean of 160 ± 53 ppB for the first three samples collected. The pulse of 50 mM potassium during sample 4 produced a significant increase in release in both samples 4 and 5 ($P < 0.05$, $P < 0.01$ respectively, Figure 1b). The mean stimulated release of nitric oxide in samples 4 and 5 was 262 ± 70 ppB which was equivalent to approximately 660 pmol nitrite. Nitric oxide levels in samples 6 and 7 were not different from basal levels. Tissue cyclic GMP levels increased following stimulation with 50 mM potassium from 91 ± 8 s.e.mean to 169 ± 27 pmol mg^{-1} protein ($P < 0.05$), while glutamate levels in the perfusate increased from 205 ± 51 pmol mg^{-1} to 2255 ± 421 pmol mg^{-1} ($P < 0.001$) following 50 mM potassium.

Discussion Knowles *et al.* (1989) showed that nitric oxide is formed from L-arginine in nervous tissue which points to a possible role in transduction mechanisms in the central nervous system. The report by Garthwaite *et al.* (1988) implicates glutamate in the release of EDRF (nitric oxide) from isolated cerebellar cells. This, together with the fact that 50 mM K^+ produced a ten fold increase in endogenous glutamate release over basal suggests that stimulation of postsynaptic glutamate receptors could account for the release of neuronal nitric oxide seen in the present study. It has been postulated that nitric oxide, following its release from the postsynaptic neurone may modulate neurotransmitter release by an action on the presynaptic terminal (Garthwaite *et al.*, 1988). This action might be mediated via activation of guanyl

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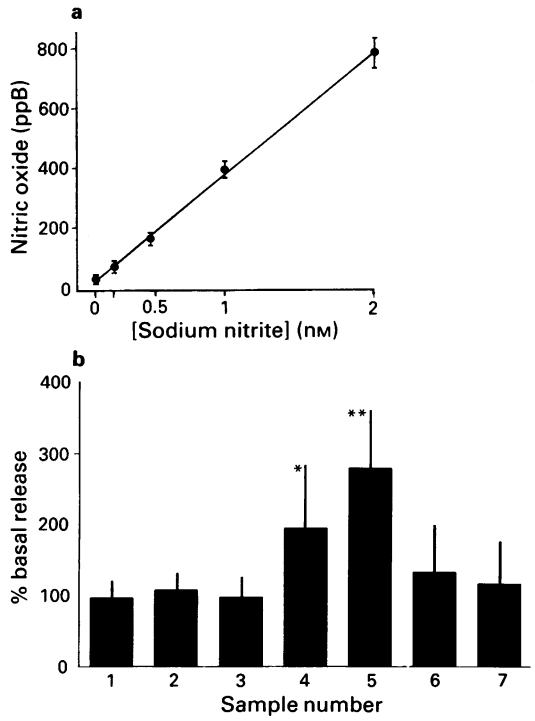

Figure 1 (a) Standard curve for sodium nitrite. Values are means with s.e.mean shown by vertical bars ($n = 8$). (b) The effect of 50 mM potassium, perfused during the collection of sample 4, on the release of nitric oxide (parts per billion) from cerebellar slices. Values are means ($n = 7$) with s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$.

cyclase and a consequent increase in cyclic GMP. It has been reported that glutamate will stimulate cyclic GMP production in the cerebellum (Garthwaite & Garthwaite, 1987) and in the present experiments the release of glutamate following a 50 mM pulse of potassium could account for the increase in cyclic GMP through a nitric oxide-dependent mechanism. It is unlikely that the increase in cyclic GMP was due to a direct effect of potassium as Collins *et al.* (1988) showed that 120 mM potassium did not elevate cyclic GMP in rat aorta.

The possibility exists that the nitric oxide detected in the perfusate may have originated from the vascular tissue present in the slice. However, increased potassium ion concentration does not elevate intracellular calcium ion concentration in endothelial cells (Colden-Stanfield *et al.*, 1986) which is considered to be a prerequisite for release of EDRF from vascular tissue. It is also unlikely that the glutamate released in the cerebellar slice initiated the release of nitric oxide from endothelium as Hardebo *et al.* (1989) found no evidence for excitatory amino acid effects on rat basilar arteries during perfusion with high concentrations of glutamate, while Beart *et al.* (1988) were unable to demonstrate the presence of NMDA receptors on cerebral microvessels. We have also investigated the effects of glutamate on the release of EDRF from rat aorta with negative results (data not given). Thus, it would appear that the release of nitric oxide in the present experiments is not due to a glutamate-mediated response on cerebral vessels.

In conclusion, all the available evidence supports the view that the stimulated release of nitric oxide described in this paper has a neuronal origin.

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SPECIAL REPORT

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Regional haemodynamic changes during oral ingestion of N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester in conscious Brattleboro rats

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Homozygous Brattleboro (i.e. vasopressin-deficient) rats were chronically instrumented with pulsed Doppler probes and intravascular catheters to permit continuous monitoring of regional haemodynamics. Over a 9 h period, rats drinking water showed no systematic changes in heart rate or mean arterial blood pressure although renal, mesenteric and hindquarters vascular conductances fell. These changes showed diurnal rhythyms, probably related to the nocturnal habits of rats. In separate groups of animals spontaneous oral ingestion of N^G-monomethyl-L-arginine (L-NMMA; 1 mg ml^{-1}) or N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mg ml^{-1}) caused marked hypertension but no significant bradycardia. Compared to control animals, rats drinking L-NMMA for 9 h showed significantly greater mesenteric and hindquarters vasoconstrictions, and rats drinking L-NAME showed greater vasoconstrictions in all 3 vascular beds.

Introduction Endothelial cell nitric oxide production is involved in the regulation of blood flow and pressure in mammals, including man (Vallance *et al.*, 1989; Gardiner *et al.*, 1990a; see Moncada *et al.*, 1989), and inhibition of nitric oxide synthesis by N^G-monomethyl-L-arginine (L-NMMA) leads to vasoconstriction and systemic hypertension (see Moncada *et al.*, 1989; Gardiner *et al.*, 1990a). Intravenous (i.v.) administration of bolus doses of L-NMMA in conscious, Long Evans rats causes marked decreases in regional vascular conductances that can be attenuated, to variable extents, by L- but not by D-arginine (Gardiner *et al.*, 1990a). These observations raise interesting questions about the long-term cardiovascular effects of prolonged inhibition of nitric oxide production.

Although techniques are available for chronic i.v. administration of drugs in rats, they are not without problems or expense. An attractive alternative would be a system in which prolonged inhibition of nitric oxide production could be achieved by spontaneous ingestion of an orally active compound, but normal rats drink relatively small volumes (20–30 ml 24 h^{-1}), with most drinking occurring during the dark period in association with eating; hence addition of substances to the drinking water might not achieve effective dosing. However, homozygous Brattleboro (i.e. vasopressin-deficient) rats have marked and continuous polydipsia (fluid intake 250–350 ml 24 h^{-1}). Therefore, these animals are a good test system, since their continuous drinking means that oral drug intake is relatively constant and allows low concentrations in the drinking water to be effective. In pilot experiments we determined that Brattleboro rats responded to i.v. L-NMMA in a similar way to Long Evans rats, and then investigated regional haemodynamic changes in Brattleboro rats drinking water that contained L-NMMA (1 mg ml^{-1}).

Recently it has been found that N^G-nitro-L-arginine is a more potent inhibitor of endothelium-dependent vasodilatation than is L-NMMA *in vitro* (Moore *et al.*, 1990). However, N^G-nitro-L-arginine is not readily soluble in water, whereas N^G-nitro-L-arginine methyl ester (L-NAME) is easily dissolved. Therefore, we extended the present study by also investigating the regional haemodynamic changes in Brattleboro rats drinking a solution of L-NAME at a concentration of 0.1 mg ml^{-1} since preliminary experiments showed that i.v. L-NAME had similar effects to i.v. L-NMMA, but was about 10 fold more potent. A preliminary account of some of this work has been given (Gardiner *et al.*, 1990b).

Methods Male, homozygous Brattleboro rats (350–450 g), bred in the Animal Unit at Nottingham, were anaesthetized (sodium methohexitone 60 mg kg^{-1}, i.p., supplemented as required) and had pulsed Doppler probes (Haywood *et al.*, 1981) sutured around left renal and superior mesenteric arteries and the distal abdominal aorta, as described previously (Gardiner *et al.*, 1990a). Animals were given an i.m. injection of 7 mg kg^{-1} ampicillin (Penbritin, Beechams Ltd) and returned to their home cages with free access to food and water. Between 7–14 days later animals were re-anaesthetized for the implantation of intravascular catheters; experiments were begun the following day when animals were fully conscious and unrestrained. Continuous recordings were made of mean blood pressure, instantaneous heart rate, and renal, mesenteric and hindquarters Doppler shifts signals (Crystal Biotech VF-1 system operating at a pulse repetition frequency of 125 kHz, and using HVPD-20 modules; Crystal Biotech, Holliston, MA, U.S.A.). Percentage changes in the mean Doppler shift signals are a good index of changes in volume flow (Haywood *et al.*, 1981) and percentage changes in conductances were calculated from mean Doppler shift and mean blood pressure signals (Gardiner *et al.*, 1990a).

Animals were separated into three groups: those drinking water ($n = 8$); those drinking L-NMMA (1 mg ml^{-1}, $n = 8$) and those drinking L-NAME (0.1 mg ml^{-1}, $n = 8$).

Baseline recordings were made between 06 h 30 min and 07 h 00 min and then, in groups 2 and 3, the drinking water was replaced with the solution of L-NMMA or L-NAME, respectively. Hourly intakes of water, L-NMMA or L-NAME and cardiovascular variables were recorded over the following 9 h. At each time point average values for the variables were taken over periods of about 5 min.

L-NAME methyl ester hydrochloride was obtained from Sigma; L-NMMA acetate was synthesized at Wellcome Research Laboratories (Beckenham).

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Within-group analysis of data was by Friedman's test comparing all values to the baseline state. Inter-group comparisons were made using the Kruskal-Wallis Test. A P value < 0.05 was taken as significant.

Results In Brattleboro rats drinking water the mean fluid intake was $9.0 \pm 0.4\,ml\,h^{-1}$ ($n = 8$) over 9 h. Heart rate and mean arterial blood pressure showed no systematic changes, although there were reductions in renal, mesenteric and hindquarters vascular conductances (Table 1). These changes in vascular conductances showed diurnal rhythms, probably related to the noctural eating habits of the rats.

The mean fluid intake of rats drinking L-NMMA was $8.8 \pm 0.5\,ml\,h^{-1}$ ($n = 8$). These animals showed marked increases in mean arterial blood pressure but no significant, sustained bradycardia (Table 1). By the end of the 9 h observation period there were decreases in mesenteric and hindquarters vascular conductances that were significantly greater than those seen in the control animals (Table 1).

Rats given L-NAME drank $8.5 \pm 0.8\,ml\,h^{-1}$ ($n = 8$) and showed marked hypertension but no significant bradycardia; there were renal, mesenteric and hindquarters vasoconstrictions (Table 1). In all 3 vascular beds the changes in conductances were greater than those seen in control rats and after 6 and 9 h, the hindquarters vasoconstriction was greater also than that seen in animals drinking L-NMMA (Table 1). Three of the animals drinking L-NAME died over-night; there were no deaths in the control or L-NMMA groups.

Discussion The present work in Brattleboro rats has shown that spontaneous oral ingestion of L-NMMA or L-NAME caused (within 1 h) hypertension which was sustained for the 9 h observation period. The hypertension was not accompanied by consistent bradycardia. Present evidence indicates that L-NMMA and L-NAME are potent inhibitors of endothelium-dependent nitric oxide synthesis and vasodilatation (Rees *et al.*, 1989; Moore *et al.*, 1990), but these compounds would be expected also to inhibit many other processes dependent upon nitric oxide production (Moncada *et al.*, 1989). Thus, although it is likely that the hypertension and increased vasoconstriction during L-NMMA or L-NAME ingestion were due, in part, to inhibition of vascular endothelial cell nitric oxide production we cannot exclude contributions from other mechanisms. Indeed, the absence of sustained bradycardia in the presence of hypertension indicates that cardiac baroreflex mechanisms, which in our experience are intact in Brattleboro rats (Gardiner & Bennett, 1988), were not operating normally in the presence of L-NMMA or L-NAME. Since there is evidence that nitric oxide might be involved in nociceptor afferent signalling (Duarte *et al.*, 1990), it is feasible that baroreceptor afferents might utilize a similar system and hence be influenced by L-NMMA or L-NAME. If baroreflex control of vasomotor efferent outflow was also blunted under these conditions this would have exacerbated the hypertension. These results contrast with those following acute i.v. injection of L-NMMA or L-NAME when the hypertension is accompanied by bradycardia in Long Evans rats (Gardiner *et al.*, 1989; 1990a,c) and in Brattleboro rats (unpublished observations).

By the end of the 9 h observation period the hypertension seen in rats drinking L-NMMA or L-NAME was associated with particularly marked hindquarters vasoconstriction; indeed, only in this vascular bed was flow reduced below baseline. Since, following acute i.v. administration of L-NMMA (Gardiner *et al.*, 1990a) or L-NAME (Gardiner *et al.*, 1990c) both renal and mesenteric vascular beds initially show substantial and greater vasoconstrictions than the hindquarters, the present results indicate the former two vascular beds are able to adjust for the inhibition of nitric oxide-mediated mechanisms in a way not possible in the hindquarters.

Although rats drinking L-NAME showed more marked hindquarters vasoconstriction than those drinking L-NMMA, both groups showed similar degrees of hypertension. Thus, it is probable that rats drinking L-NAME had a greater reduction in cardiac function than those drinking L-NMMA.

Table 1 Cardiovascular changes in Brattleboro rats drinking water (control), or solutions of N^G-monomethyl-L-arginine (L-NMMA; $1\,mg\,ml^{-1}$) or N^G-nitro-L-arginine methyl ester (L-NAME; $0.1\,mg\,ml^{-1}$)

Time (h)		1	3	6	9
Heart rate (beats min^{-1})	Control	-10 ± 6	4 ± 7	7 ± 12	5 ± 11
	L-NMMA	-11 ± 3*	-10 ± 7	-6 ± 6	-8 ± 7
	L-NAME	-25 ± 12	-20 ± 14	4 ± 8	1 ± 9
Mean blood pressure (mmHg)	Control	1 ± 1	4 ± 2	3 ± 3	-1 ± 1
	L-NMMA	13 ± 3*†	23 ± 6*†	23 ± 4*†	28 ± 7*†
	L-NAME	19 ± 6*†	33 ± 3*†	28 ± 2*†	32 ± 5*†
Renal flow (%)	Control	4 ± 5	1 ± 4	-3 ± 7	-13 ± 3*
	L-NMMA	15 ± 6°	6 ± 4	1 ± 7	1 ± 7
	L-NAME	-3 ± 4	-7 ± 7	-14 ± 7	-10 ± 7
Mesenteric flow (%)	Control	-15 ± 4*	-14 ± 3*	-18 ± 4*	-9 ± 6
	L-NMMA	-11 ± 3*	-19 ± 4*	-14 ± 5*	-13 ± 7
	L-NAME	-19 ± 5*	-26 ± 5*	-25 ± 6*	-12 ± 8
Hindquarters flow (%)	Control	-4 ± 5	-5 ± 6	-15 ± 4*	-18 ± 3*
	L-NMMA	-18 ± 3*	-31 ± 6*†	-33 ± 6*	-37 ± 6*
	L-NAME	-4 ± 5	-37 ± 4*†	-50 ± 2*†	-55 ± 4*†
Renal conductance (%)	Control	4 ± 5	-2 ± 4	-6 ± 6	-12 ± 3*
	L-NMMA	4 ± 4	-10 ± 5	-16 ± 6*	-18 ± 7*
	L-NAME	-17 ± 4*°	-29 ± 6*†	-32 ± 5*†	-31 ± 4*†
Mesenteric conductance (%)	Control	-16 ± 4*	-16 ± 4*	-19 ± 5*	-8 ± 6
	L-NMMA	-20 ± 3*	-32 ± 4*	-27 ± 5*	-28 ± 6*†
	L-NAME	-29 ± 3*†	-43 ± 4*†	-41 ± 5*†	-31 ± 7*†
Hindquarters conductance (%)	Control	-4 ± 6	-8 ± 7	-16 ± 6*	-18 ± 3*
	L-NMMA	-26 ± 2*†	-41 ± 6*†	-44 ± 6*	-48 ± 6*†
	L-NAME	-17 ± 7*	-51 ± 3*†	-60 ± 1*†°	-65 ± 4*†°

Values are mean ± s.e.mean, $n = 8$ for all the groups.
* $P < 0.05$ versus baseline (Friedman's test); † $P < 0.05$ versus control at same time (Kruskal-Wallis test); ° $P < 0.05$ L-NMMA versus L-NAME at same time.

It remains to be determined if direct or indirect cardiac effects, or both, of L-NAME were responsible for the deaths in this group.

We conclude that the experimental model used in the present work will be a useful tool in the investigation of the roles of nitric oxide in cardiovascular function in normal and pathophysiological states.

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SPECIAL REPORT

Inhibitory actions of prostaglandin E_1 on non-adrenergic non-cholinergic contraction in guinea-pig bronchi

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We have investigated the effect of prostaglandin E_1 (PGE_1) on non-adrenergic, non-cholinergic (NANC) contraction in guinea-pig bronchial strips. PGE_1 (10 nM to 10 μM) did not alter baseline tension but reduced NANC contractions induced by electrical field stimulation (EFS) in a concentration-dependent fashion ($-\log EC_{50}$ was 6.60 ± 0.10 M and maximum inhibition was $88.7 \pm 2.9\%$). PGE_1 (> 0.3 μM) also reduced the contraction induced by substance P (1 μM). Removal of epithelium did not alter the effects of PGE_1 on NANC contraction. These results suggest that PGE_1 exerts both pre- and post-junctional inhibitory actions on NANC contraction.

Introduction Airway tissue from a variety of species, including man, has been found to release prostaglandins of the E series (Steel *et al.*, 1979). Release of the E series prostaglandins from airway smooth muscle was accelerated by contractions induced by a variety of mediators (Orehek *et al.*, 1975). The PGE series relaxes tracheobronchial smooth muscle (Main, 1964) and inhibits cholinergic neurotransmission (Ito & Tajima, 1981).

Tachykinins are localized to C-fibre nerve endings in the airways of many species, including man (Lundberg *et al.*, 1984). Non-adrenergic, non-cholinergic (NANC) bronchoconstriction evoked by vagal stimulation may be due to the release of neuropeptides such as tachykinins from the sensory nerves (Lundberg *et al.*, 1983). These neuropeptides have potent inflammatory effects in airways (Barnes, 1986). We have therefore studied the effects of the PGE series on the NANC bronchoconstrictor response evoked by electrical field stimulation (EFS) to determine whether the PGE series has an inhibitory action on sensory neurones.

Methods Male Hartley guinea-pigs (300–500 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Lungs were removed rapidly and were immersed in Krebs-Henseleit solution equilibrated with 95% O_2 and 5% CO_2. The two main bronchi were dissected free of loose connective tissue, opened longitudinally along the anterior side, and cut into strips. These strips were mounted in organ baths containing 5 ml Krebs-Henseleit solution maintained at 37°C. Isometric tension was continuously measured with a force transducer (TB612-T, Nihon Kohden, Japan) connected to a pen recorder (HORIZ-8K, San-ei Co., Japan). Bronchial strips were placed between two rectangular platinum electrodes (6 × 40 mm) for EFS. The tissues were stretched initially to a tension of 1 g for 30 s and thereafter maintained for 60 min under a resting tension of 0.25 g, which was found to be optimal for measuring changes in tension. EFS (supramaximal voltage of 50 V, a frequency of 10 Hz and 1 ms duration) was applied for 20 s. The contractile responses induced under these conditions were completely abolished by tetrodotoxin (1 μM), confirming the neural nature of the response.

Experimental protocol After the baseline response to EFS was obtained, we added increasing concentrations of PGE_1 (from 10 nM to 10 μM) and repeated the stimulation 5 min after the administration of each concentration of PGE_1, when the effects of PGE_1 on the response to EFS reached their maximum. PGE_1 was administered after the preceding contraction had returned to baseline. We also tested the effects of PGE_1 on contraction induced by capsaicin (1 μM). After the baseline response to capsaicin (1 μM) was obtained, the bath was rinsed for 15 min. Five minutes after the administration of either PGE_1 (0.3 μM) or the vehicle for PGE_1, capsaicin (1 μM) was administered again. We compared the second response to the first one (control).

To determine whether removal of the epithelium altered the effects of PGE_1 on the response to EFS, we performed parallel studies on paired bronchi from the same animals after removing the epithelium from one randomly chosen bronchus by rubbing the luminal surface gently with gauze held with a pair of fine forceps. Removal of the epithelium was confirmed histologically.

The effects of PGE_1 (from 10 nM to 10 μM) on contractile responses to exogenous substance P (SP) (1 μM, which produced a contractile response equivalent to that produced by EFS) were also evaluated. All experiments were carried out in the presence of atropine sulphate (1 μM), propranolol (1 μM), indomethacin (1 μM) and phentolamine (10 μM).

Values are expressed as mean ± s.e.mean. Statistical analysis was performed by one way analysis of variance. Significance was accepted at $P < 0.05$.

Results EFS produced a long lasting NANC contraction that was reproducible within any given tissue (Figure 1a). PGE_1 had no effect on resting tension but decreased the amplitude of both EFS- (Figure 1b) and SP-induced contractions. The inhibition was concentration-dependent ($-\log EC_{50} = 6.60 \pm 0.10$ M for EFS and 5.86 ± 0.03 M for SP) (Figure 1c). PGE_1 at 0.3 μM inhibited EFS-induced contraction (by $47.8 \pm 8.1\%$; $P < 0.01$) but did not significantly alter the contractile response to exogenously administered SP (by $3.7 \pm 2.9\%$; $P > 0.20$). At 10 μM, PGE_1 inhibited EFS-induced contraction (by $86.2 \pm 3.4\%$; $P < 0.001$) and SP-induced contraction (by $38.8 \pm 2.5\%$; $P < 0.001$). Inhibitory actions of PGE_1 were significantly greater on EFS-induced contractions than on SP-induced contractions in terms of both $-\log EC_{50}$ ($P < 0.001$) and maximum inhibition ($P < 0.001$). Likewise, capsaicin (1 μM)-induced contraction was significantly inhibited by PGE_1 (0.3 μM) ($51.5 \pm 3.0\%$ of control with PGE_1 and $86.3 \pm 10.5\%$ of control in the presence of the vehicle for PGE_1; $P < 0.05$, $n = 5$). Removal of the epithelium increased EFS-induced contractions by $20.5 \pm 6.8\%$ ($P < 0.05$, $n = 5$). However, PGE_1 inhibited EFS-induced contraction by $50.6 \pm 1.2\%$ at 0.3 μM ($P < 0.01$, $n = 5$) and by $88.7 \pm 2.9\%$ at 10 μM ($P < 0.01$, $n = 5$) without

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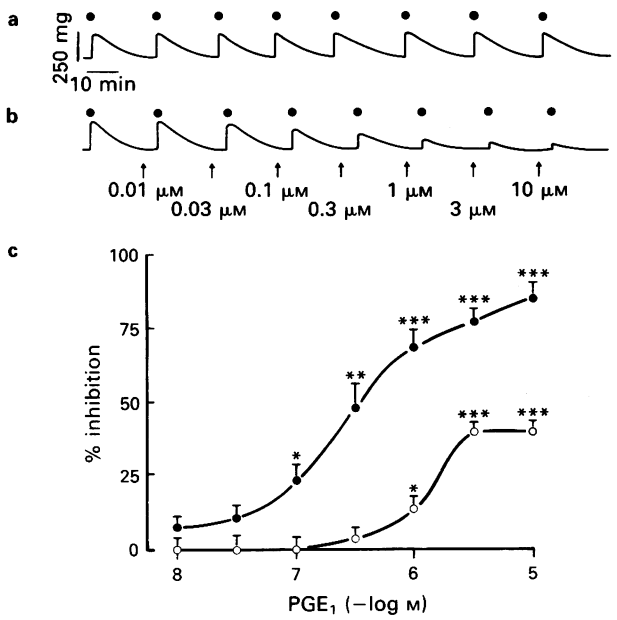

Figure 1 Typical tracing showing the contraction induced by electrical field stimulation (●) (EFS) in the presence of atropine (1 μM), propranolol (1 μM), phentolamine (10 μM) and indomethacin (1 μM) in guinea-pig bronchi *in vitro*. (a) Long-lasting contraction caused by EFS was reproducible several times, and (b) inhibited by prostaglandin E_1 (PGE_1). (c) PGE_1 inhibited EFS- (●) and substance P (1 μM)-(○) induced contraction concentration-dependently. Data are shown as mean of values from 6 guinea-pigs; s.e.mean indicated by vertical bars. Significant differences from control values are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

epithelium. Thus, the inhibitory effects that PGE_1 had on the responses to EFS did not differ significantly between bronchi with and without epithelium at both concentrations ($P > 0.30$).

In contrast to PGE_1, PGE_2 increased resting tension with a threshold concentration of 10 nM, and the subthreshold concentration of PGE_2 (3 nM) did not alter EFS-induced contraction significantly ($n = 5$, $P > 0.50$).

Discussion Our study shows that PGE_1 reduces the neurally-mediated broncho-constrictor response evoked by EFS in guinea-pig bronchi. This excitatory response resulting from nerve stimulation in the presence of atropine and propranolol is NANC in nature, and probably due to the release of neuropeptides such as SP and neurokinin A from sensory nerve endings (Lundberg *et al.*, 1983).

In order to establish whether the inhibitory effects of PGE_1 are due to its effect on neuronal terminals or on the tachykinin receptors of the airway smooth muscle, we compared the effect that PGE_1 had on the contractile response to EFS and capsaicin with the effect that it had on the contractile response to exogenous SP. PGE_1 at 0.3 μM inhibited approximately half of the NANC contraction without having an inhibitory effect on the contractile response to SP. This implies that PGE_1-induced inhibition, at concentrations of up to 0.3 μM, is not related to an alteration of smooth muscle function, but probably represents a prejunctional mechanism such as the inhibition of tachykinin release from sensory nerve endings. However, further increases in the concentrations of PGE_1 decreased the contractile response to SP, suggesting that higher concentrations of PGE_1 inhibit NANC contraction via both pre- and post-junctional mechanisms.

Removal of epithelium is reported to enhance the contractile response to tachykinins in guinea-pig trachea (Devillier *et al.*, 1988). Therefore, PGE_1 might reduce the NANC contraction via mechanisms related to epithelium. However, epithelial removal did not alter PGE_1-induced effects on the NANC contraction, suggesting that PGE_1 inhibits the NANC contraction independently of the epithelium.

Sensory nerves may play an important role in the neurogenic inflammation of the airways (Barnes, 1986). Regulation of the release of sensory neuropeptides from nerves may therefore reduce neurogenic inflammation. Our study suggests that PGE_1 has an inhibitory action on neuropeptide release from sensory nerve endings. Therefore, PGE_1 may be useful in the treatment of neurogenic inflammation in lung disease.

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Bay K 8644, modifier of calcium transport and energy metabolism in rat heart mitochondria: a new intracellular site of action

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1 The dihydropyridine Ca^{2+} channel agonist Bay K 8644 (10–200 μM) produced a concentration-dependent increase in State 4 respiration in the rat heart mitochondria with the highest concentration (200 μM) increasing the rate from 33.1 ± 0.7 to 187.0 ± 13.3 ng atoms O_2 consumed $min^{-1} mg^{-1}$ protein.

2 Bay K 8644 (200 μM) reduced State 3 respiration from 247.2 ± 11.7 to 174.4 ± 0.06 ng atoms $O_2 min^{-1} mg^{-1}$ protein, reduced the respiratory control index (RCI) from 5.3 ± 0.45 to 1.1 ± 0.03 and reduced the ADP:O ratio from 2.75 ± 0.03 to 1.3 ± 0.15.

3 A similar, but smaller, stimulation of State 4 respiration was seen with nitrendipine (25–200 μM), the rate increasing from 22.6 ± 1.0 to 33.1 ± 1.8 ng atoms O_2 consumed $min^{-1} mg^{-1}$ protein in the presence of 200 μM nitrendipine.

4 Bay K 8644 (10–60 μM) increased the total Ca^{2+} uptake into rat heart mitochondria, the total increasing from 248.8 ± 8.4 to 406.9 ± 17.6 ng Ca^{2+} mg^{-1} protein at 60 μM Bay K 8644 (EC_{50} = 18.9 ± 1.4 μM).

5 Bay K 8644 (10–60 μM) produced a concentration-dependent reduction in the Ca^{2+} influx rate (IC_{50} = 52.5 ± 2.8 μM). Similar effects were seen with (+)-Bay K 8644 and (−)-Bay K 8644.

6 Nitrendipine (40–120 μM) stimulated Ca^{2+} efflux from mitochondria preloaded with the ion; the efflux rate increasing from 2.9 ± 0.05 to 114.2 ± 6.2 nmol Ca^{2+} $min^{-1} mg^{-1}$ protein (EC_{50} = 57.3 ± 1.3 μM).

7 These data indicate dihydropyridine-induced changes in the activity of the mitochondrial Na^+/Ca^{2+}. antiporter pathway; nitrendipine causing stimulation and Bay K 8644 causing inhibition.

Introduction

Calcium ion (Ca^{2+}) accumulation *in vitro* is an important feature of mitochondria isolated from a variety of vertebrate tissues (Akerman & Nicholls, 1983). Under normal physiological conditions, mitochondrial Ca^{2+} transport occurs via specific unidirectional carrier systems which catalyze either the nett influx or nett efflux of the ion (Fiskum, 1984; Crompton, 1985).

In cardiac mitochondria, the key carriers for Ca^{2+} are the electrophoretic uniporter pathway for influx, and the Na^+/Ca^{2+} antiporter pathway associated with efflux. The uniporter system involves the diffusion of Ca^{2+} down an electrochemical gradient with the nett transfer of the two positive charges associated with the entry of each Ca^{2+} ion into the mitochondrion (Vercesi *et al.*, 1978). This process, although primarily dependent on respiration, may also be driven via the hydrolysis of adenosine 5'-triphosphate (ATP) (Lehninger *et al.*, 1978). In contrast, the antiporter system is supported by coupled respiration and, in the presence of Na^+, promotes the active extrusion of Ca^{2+} from the mitochondrial matrix against an electrochemical gradient (Nicholls, 1978a).

Under normal physiological conditions this process produces a sigmoidal relationship between the electrochemical gradient and the efflux rate, with the inner membrane potential remaining unchanged (Affolter & Carafoli, 1980). However, in order to establish the steady-state distribution of Ca^{2+}, Na^+ and H^+ across the inner mitochondrial membrane, the contribution of the Na^+/H^+ antiporter pathway must also be taken into consideration (Brierley, 1976; Crompton *et al.*, 1978).

The two major functions of the Ca^{2+} cycle appear to be: (a) modulation of free Ca^{2+} levels within the mitochondrial matrix and (b) buffering of cytosolic free Ca^{2+} at the 'set point' when the rate of influx of the ion into the mitochondrion equals the rate of efflux from the organelle (Nicholls, 1978b). Furthermore, changes in the level of free Ca^{2+} within the mitochondrial matrix contribute towards the control of Ca^{2+}-sensitive dehydrogenase enzymes associated with oxidative metabolism, thus providing a mechanism by which energy demand and supply may be balanced (Denton *et al.*, 1980; Hansford, 1985; McCormack & Denton, 1985; Denton & McCormack, 1986).

Providing that the fluctuations in free Ca^{2+} levels within the cell are transient, the buffering capacity of the mitochondrion may be of value in protecting the cell from cytosolic Ca^{2+} overload. However, changes in cytosolic Ca^{2+} levels are now thought to be associated with a number of pathophysiological conditions, such as that seen following reperfusion and reoxygenation occurring after an ischaemic attack or hypoxia and the 'Ca^{2+} paradox', all of which increase the susceptibility of the cell to Ca^{2+} overload (Zimmerman *et al.*, 1967; Shen & Jennings, 1972; Jennings & Ganote, 1976; Bourdillon & Poole-Wilson, 1981; Nakanishi *et al.*, 1982; Nayler *et al.*, 1985; Cheung *et al.*, 1986; Allen & Orchard, 1987; Murphy *et al.*, 1987).

Under these conditions excess Ca^{2+} is accumulated and retained within the mitochondrial matrix in the form of an inorganic phosphate precipitate (Denton & McCormack, 1985). Such matrix precipitation results in the loss of the steady-state distribution of Ca^{2+} across the mitochondrial membrane, mitochondrial swelling, loss of membrane integrity, deposition of amorphous densities in the matrix, decreased tissue high energy phosphate stores and loss of cellular homeostasis, eventually leading to cell necrosis (Schwartz *et al.*, 1973; Regitz *et al.*, 1984; Piper *et al.*, 1985; Lochner *et al.*, 1987).

Recent studies have shown that the Ca^{2+} overload associated with myocardial damage may be reduced, and oxidative phosphorylation maintained, by the prophylactic use of Ca^{2+} antagonists (Fleckenstein, 1983; Baydoun *et al.*, 1986a). More recently, studies on the Ca^{2+} antagonist nitrendipine and the endogenous acyl carnitine, palmitoyl carnitine (PC), have shown that these compounds have the ability to alter Ca^{2+} transport into the mitochondrion by promoting release of the ion from the organelle (Watts *et al.*, 1987; Baydoun *et al.*,

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1988). In the present study, we describe the ability of Bay K 8644 to modify the energy metabolism and Ca^{2+} handling associated with rat heart mitochondria.

Methods

Preparation of mitochondria

Tightly-coupled heart mitochondria were prepared from female Wistar rats (250–500 g) according to the method of Vercesi *et al.* (1978). The rats were stunned by a blow to the head, bled and the hearts rapidly removed into an ice-cold isolation medium containing 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl buffer (pH 7.4) and 0.1 mM EGTA. The hearts were minced, washed and incubated with Nagarse (1 mg g^{-1} wet weight) for 10 min at 4°C. After incubation, excess Nagarse was washed off, the tissue homogenised in the isolation medium with a glass UNI-FORM Type H homogeniser, and the resulting homogenate centrifuged at 500 *g* for 10 min in an MSE High-Speed centrifuge at 0–4°C. The resulting supernatant was recentrifuged at 10,000*g* for 7 min to obtain the mitochondrial pellet. The pellet was washed with ice-cold buffer containing 210 mM mannitol, 70 mM sucrose and 5 mM Tris-HCl (pH 7.4) to give a final concentration of 15 mg ml^{-1}. Protein was measured by the method of Gornall *et al.* (1949).

Measurement of respiration

Oxygen consumption was measured polarographically at 37°C with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) coupled to a BBC SE 120 pen recorder, according to the method of Sweetman & Weetman (1972). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl buffer (pH 7.4), 5 μM potassium dihydrogen orthophosphate and 2.0 mg mitochondrial protein in a final volume of 2.0 ml. Following a one minute incubation period, State 4 respiration (substrate in excess, adenosine 5'-diphosphate (ADP) absent) was initiated by the addition of 5 mM glutamate (monopotassium salt) plus 5 mM malate (sodium salt). One minute later, State 3 respiration (substrate in excess, ADP present) was initiated by the addition of 400 nmol ADP. When present, racemic Bay K 8644 (10–200 μM), racemic nitrendipine (25–200 μM), oxidised nitrendipine (25–200 μM) were added to the chamber one min before the initiation of State 3 respiration.

Measurement of Ca^{2+} ion movement

Ca^{2+} ion movements were followed at 37°C by a Corning Ca^{2+}-specific electrode coupled to a Petracourt PM 10 pH meter, and a BBC SE 120 pen recorder, according to the method of Crompton *et al.* (1983). The incubation medium contained 250 mM sucrose, 5 mM succinate (Tris salt), 2 mM potassium dihydrogen orthophosphate and 5 mM Tris-HCl buffer (pH 7.4) in a final volume of 10 ml. The amount of Ca^{2+} present as an impurity was determined before each individual study, and only those incubation mixtures found to contain Ca^{2+} levels below those shown to produce mitochondrial damage (sub-uncoupling levels) were used. Ca^{2+} uptake was initiated by the addition of 2.5 mg of mitochondrial protein. When present, ruthenium red (RR; 2–12 nM), racemic Bay K 8644 (10–100 μM), (−)-Bay K 8644 (5–100 μM), (+)-Bay K 8644 (5–100 μM), racemic nitrendipine (20–100 μM), oxidised nitrendipine (20–100 μM), (−)-nitrendipine (20–100 μM), (+)-nitrendipine (20–100 μM) were added one min before the addition of the mitochondrial suspension. The logarithmic responses of the Ca^{2+} electrode were linearised graphically according to the method of Fiskum *et al.* (1979).

The data are the means ± s.e.mean of at least four determinations in different preparations. Stimulative effects are presented as EC_{50} and inhibitory effects as IC_{50}; both parameters referring to the concentrations necessary to produce 50% of the maximal effect.

Materials

Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses (Poole, Dorset, U.K.). Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was donated by Bayer A.G., Pharmaceutical Centre, F.R.G.; Nagarse (crystallised lyophylized bacterial AL-Protease 120 × 10^4 PUN/g) was purchased from Hughes and Hughes Ltd. (Romford, U.K.). All other experimental compounds were kindly donated by Imperial Chemical Industries Plc, Pharmaceutical Division, Cheshire, U.K. All water soluble compounds were dissolved in distilled and deionised water at room temperature. Water insoluble compounds were dissolved in dimethylsulphoxide (DMSO), the final concentration of the solvent in the reaction medium not exceeding 1% (v/v). At these concentrations DMSO had no effect on either oxidative phosphorylation or Ca^{2+} transport.

Results

NAD^+-linked oxidation of glutamate plus malate

Racemic Bay K 8644 (10–200 μM) produced a concentration-dependent inhibition of State 3 respiration in rat heart mitochondria (Figure 1). In the presence of 200 μM racemic Bay K 8644 the State 3 rate of oxygen consumption was reduced from 247.2 ± 11.7 to 174.4 ± 0.06 ng atoms O_2 min^{-1} mg^{-1} protein ($n = 5$). At concentrations of racemic Bay K 8644 ⩽ 60 μM the inhibitory effect was not statistically significant ($P < 0.05$). Over the same concentration range (10–200 μM) racemic Bay K 8644 produced a concentration-dependent stimulation of State 4 respiration, the rate of oxygen consumption increasing from 33.1 ± 0.07 to 187.6 ± 13.3 ng atoms O_2 min^{-1} mg^{-1} protein ($n = 5$; Figure 1).

At concentrations of Bay K 8644 greater than 60 μM, these effects indicate a loss of respiratory control (i.e. stimulation of State 4 and inhibition of State 3 $P < 0.05$), a fact which was confirmed by the observation that, at concentrations > 60 μM, Bay K 8644 produced a significant reduction in both the res-

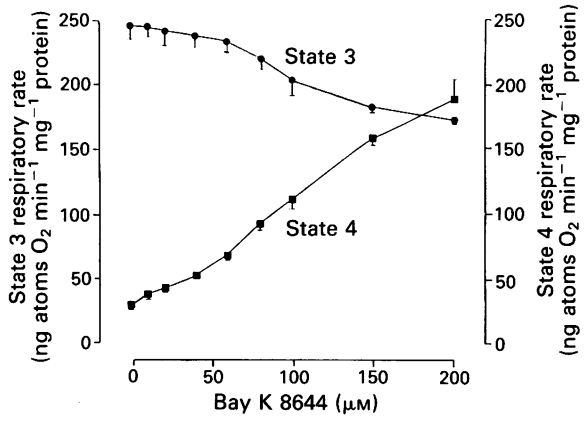

Figure 1 The effect of Bay K 8644 on the NAD^+-linked substrate oxidation of glutamate plus malate in rat heart mitochondria. Mitochondrial respiratory activity was recorded polarographically by a Clark-type oxygen electrode coupled to a BBC SE120 single pen recorder running at a chart speed of 3.0 cm min^{-1} and calibrated to give a full-scale deflection of 10 mV. The reaction medium contained 315 μmol mannitol, 105 μmol sucrose, 7.5 μmol potassium dihydrogen orthophosphate and 7.5 μmol Tris-HCl, pH 7.4. All experiments were carried out at 37°C, and each experiment was started by the addition of 2 mg mitochondrial protein to 1.5 ml of the above medium. The mitochondrial suspension was allowed to equilibrate for 1 min and State 4 respiration induced by the addition of 7.5 μmol glutamate plus 7.5 μmol malate. State 3 respiration was initiated 1 min later by the addition of 400 nmol ADP. When present, Bay K 8644 (15–300 nmol) was added before the addition of mitochondria. Results are the means of five determinations; vertical lines show s.e.mean.

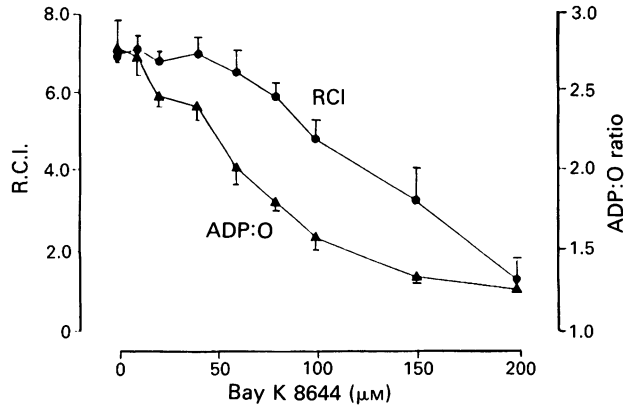

Figure 2 The effect of Bay K 8644 on the respiratory control index and ADP:O ratios in rat heart mitochondria. Experimental conditions were as described in the legend to Figure 1. When present, Bay K 8644 (15–300 nmol) was added before the addition of mitochondria. Results are the means of five different determinations and vertical lines show s.e.mean.

piratory control index (RCI) and the ADP:oxygen (ADP:O) ratio (Figure 2). Table 1 presents a summary of the effects of racemic Bay K 8644 (10–200 μM), nitrendipine (25–200 μM) and oxidised nitrendipine (25–200 μM) on the NAD^+-linked oxidation of glutamate plus malate. Nitrendipine (25–200 μM) produced a concentration-dependent stimulation of State 4 respiration and inhibited State 3 respiration, but did not alter the RCI or ADP:O ratio. Oxidised nitrendipine (25–200 μM) was without effect on mitochondrial oxidation of glutamate plus malate over the concentration range used.

Calcium ion movements

Figure 3a and b shows the effects of 10–100 μM racemic Bay K 8644, (+)- and (−)-Bay K 8644 on both the total Ca^{2+} uptake (a) and the rate of Ca^{2+} uptake (b) into rat heart mitochondria. Both the isomers and the racemic mixture produced concentration-dependent increases in total Ca^{2+} ion uptake (racemic Bay K 8644 $EC_{50} = 18.9 \pm 1.4\,\mu$M; (+)-Bay K 8644 $EC_{50} = 23.8 \pm 0.7\,\mu$M; (−)-Bay K 8644 $EC_{50} = 24.4 \pm 1.4\,\mu$M; $n = 4$), but reduced the rate of ion uptake into mitochondria (racemic Bay K 8644 $IC_{50} = 52.5 \pm 2.8\,\mu$M; (+)-Bay K 8644 $IC_{50} = 55.1 \pm 4.7\,\mu$M; (−) Bay K 8644 $IC_{50} = 51.1 \pm 3.0\,\mu$M; $n = 4$).

Ruthenium red (RR: 2–25 nM; a known inhibitor of the uniporter pathway; Reed & Bygrave, 1974) produced a concentration-dependent decrease in both the total Ca^{2+} ion uptake ($IC_{50} = 5.2 \pm 0.25$ nM; $n = 4$) and the rate of ion influx ($IC_{50} = 4.5 \pm 0.37$ nM; $n = 4$). However, Bay K 8644 (10–80 μM) reversed the reduction in Ca^{2+} ion uptake induced by RR (25 nM; Figure 4).

Replacement of either Bay K 8644 or RR by nitrendipine (20–10 μM) produced a concentration-dependent inhibition of both the Ca^{2+} influx rate from 272.1 ± 12.8 to 52.1 ± 5.3 nmol Ca^{2+} $min^{-1}mg^{-1}$ protein ($IC_{50} = 75.3 \pm 2.1\,\mu$M) and the total Ca^{2+} uptake from 231.1 ± 13.5 to 45.6 ± 4.2 nmol Ca^{2+} mg^{-1} protein ($IC_{50} = 92.0 \pm 0.9\,\mu$M); and reduced the Ca^{2+} retention time from 16.0 ± 0.6 to 3.0 ± 0.3 min. Direct studies on Ca^{2+} efflux revealed that racemic nitrendipine (40–120 μM) at the equilibrium point (influx rate equals efflux rate) after the mitochondria had accumulated a load of 242.1 nmol Ca^{2+} mg^{-1} protein, induced a concentration-dependent stimulation of Ca^{2+} efflux from 2.9 ± 0.05 to 114.2 ± 6.2 nmol Ca^{2+} $min^{-1}mg^{-1}$ protein ($EC_{50} = 57.3 \pm 1.3\,\mu$M).

Comparative studies on the rates of influx and efflux of the

Table 1 The effect of Bay K 8644 and selected Ca^{2+} antagonists on the NAD^+-linked substrate oxidation of glutamate plus malate by rat heart mitochondria

	Mitochondrial respiration (ng atoms O_2 consumed $min^{-1}mg^{-1}$ protein)		*Respiration ratios*	
Compound	*State 4*	*State 3*	*RCI*	*ADP:O*
(±)-Bay K 8644				
Control	33.1 ± 0.7	247.2 ± 11.7	5.3 ± 0.45	2.75 ± 0.03
12.5 μM	42.9 ± 1.94	222.1 ± 7.1	5.91 ± 0.38	3.0 ± 0.05
50 μM	45.6 ± 4.26	189.3 ± 8.6	4.24 ± 0.26	2.92 ± 0.05
100 μM	101.0 ± 6.60	179.6 ± 9.5	2.06 ± 0.12	2.29 ± 0.10
200 μM	187.0 ± 13.3	174.4 ± 0.06	1.1 ± 0.03	1.3 ± 0.15
Verapamil				
Control	31.9 ± 1.8	255.4 ± 17.4	4.6 ± 0.36	2.9 ± 0.06
100 μM	33.2 ± 1.7	242.7 ± 20.8	3.8 ± 0.24	2.9 ± 0.07
400 μM	42.0 ± 2.6	184.9 ± 12.4	2.5 ± 0.14	2.8 ± 0.08
800 μM	51.5 ± 4.0	119.0 ± 3.9	1.4 ± 0.04	1.7 ± 0.09
Diltiazem				
Control	28.4 ± 1.4	246.1 ± 14.3	5.0 ± 0.22	2.9 ± 0.07
100 μM	28.4 ± 1.4	233.3 ± 19.6	4.3 ± 0.24	2.7 ± 0.10
200 μM	32.7 ± 2.1	232.1 ± 16.9	4.3 ± 0.31	2.6 ± 0.10
800 μM	53.3 ± 2.7	219.2 ± 13.8	2.7 ± 0.15	2.4 ± 0.09
1600 μM	75.9 ± 5.1	149.7 ± 8.3	1.4 ± 0.11	1.8 ± 0.07
(±)-Nitrendipine				
Control	26.0 ± 1.0	222.2 ± 11.7	5.8 ± 0.20	3.0 ± 0.05
25 μM	26.9 ± 1.8	220.0 ± 10.1	5.8 ± 0.14	2.9 ± 0.06
100 μM	33.1 ± 1.8	211.4 ± 13.7	5.0 ± 0.30	2.86 ± 0.11
200 μM	35.8 ± 2.2	194.5 ± 9.0	5.1 ± 0.49	2.9 ± 0.06
Oxidised nitrendipine				
25 μM	27.5 ± 1.5	245.5 ± 9.5	5.91 ± 0.02	3.0 ± 0.06
200 μM	29.0 ± 1.0	241.5 ± 10.0	5.8 ± 0.03	2.9 ± 0.03

Experimental conditions were as described in the legend to Figure 1. When present, Bay K 8644 (18.75 nmol), verapamil (150–2000 nmol), diltiazem (150–2000 nmol), nitrendipine (37.5–300 nmol) and oxidised nitrendipine (37.5–300 nmol) were added individually before the addition of mitochondria. Results are the means ± s.e.mean of five different determinations. RCI = respiratory control index.

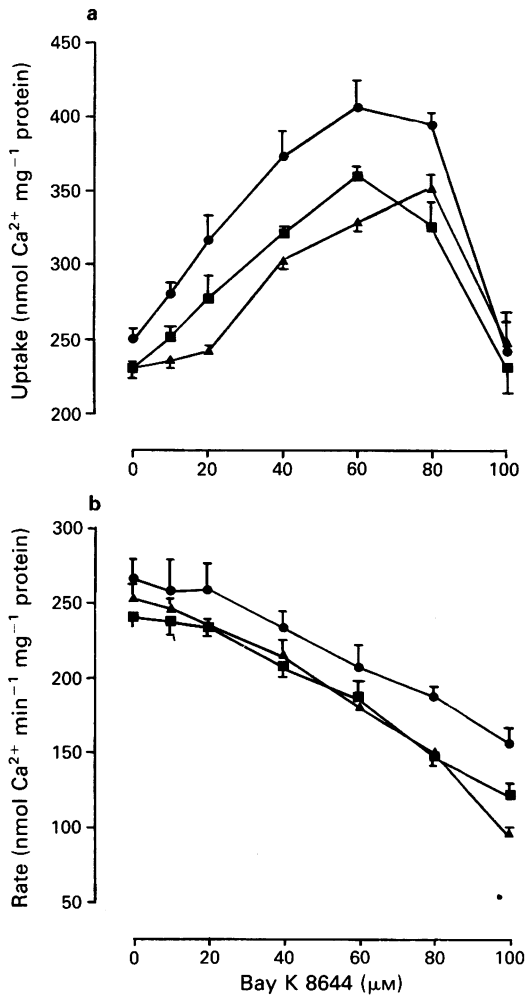

Figure 3 Effects of Bay K 8644 and its isomers on Ca^{2+} influx rate (b) and total Ca^{2+} uptake (a) in rat heart mitochondria. Mitochondrial Ca^{2+} movements were monitored by a Corning Ca^{2+}-specific electrode coupled to a Petracourt PM10 pH meter and a BBC SE 120 pen recorder. The reaction medium contained 2500 μmol sucrose, 50 μmol succinate (Tris salt), 20 μmol potassium dihydrogen orthophosphate and 50 μmol Tris-HCl, pH 7.4. (a) Ca^{2+} uptake was initiated by the addition of 2.5 mg mitochondrial protein to 10 ml of the reaction medium at 37°C. When present, 100–1000 nmol racemic Bay K 8644 (●), 100–1000 nmol (−)-Bay K 8644 (▲), or (+)-Bay K 8644 (■) were added to the reaction medium before the initiation of Ca^{2+} uptake. Results are the means of five separate determinations and vertical lines show s.e.mean.

ion show that, at a concentration of 68.8 ± 1.7 μM racemic nitrendipine, the rate of Ca^{2+} influx equalled the rate of Ca^{2+} efflux. At this concentration of racemic nitrendipine, the influx rate was stimulated by 68.4%, indicating that the predominant effect of nitrendipine is the stimulation of Ca^{2+} efflux. Replacement of racemic nitrendipine with either the (+)- or the (−)-isomer produced quantitatively similar effects on mitochondrial Ca^{2+} efflux (EC_{50}(+)-isomer = 56.6 ± 1.1 μM; EC_{50}(−)-isomer = 59.8 ± 2.5 μM). In contrast, the inactive oxidised nitrendipine (20–100 μM) produced no effect on either Ca^{2+} influx or efflux.

Discussion

A common feature of the Ca^{2+} antagonists is their ability to inhibit the slow transsarcolemmal Ca^{2+} inward current (Godfraind *et al*, 1986) as a result of the blockade of voltage-operated channels (VOCs) in the plasma membrane. Clinically, these compounds have been shown to protect against some of the deleterious effects, such as mitochondrial damage

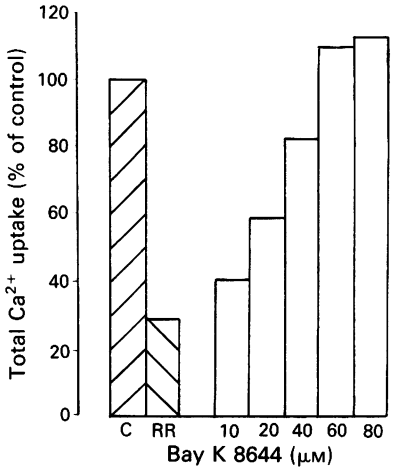

Figure 4 Effect of Bay K 8644 on ruthenium red induced Ca^{2+} efflux. Experimental conditions were as described in the legend to Figure 3. Ca^{2+} efflux was initiated by the addition of ruthenium red (250 pmol) to mitochondria preloaded with Ca^{2+}. When present, racemic Bay K 8644 (100–800 nmol) was introduced into the reaction chamber together with ruthenium red (250 pmol). C represents the total Ca^{2+} uptake under control conditions and RR in the presence of ruthenium red and no Bay K 8644.

and dysfunction, which result from cardiac ischaemia and following reperfusion (Jennings *et al*., 1985). In contrast, some analogues of these compounds, the so-called Ca^{2+} channel agonists, have the ability to increase transsarcolemmal Ca^{2+} current. Such a compound is the dihydropyridine Bay K 8644, an activator of VOCs (Schramm *et al*., 1983).

Direct studies on the changes in mitochondrial function by these agents are limited, although the following Ca^{2+} antagonist effects having been demonstrated: prevention of mitochondrial swelling (Matlib, 1985), inhibition of total Ca^{2+} uptake (Leblondel & Allain, 1984), inhibition of the Na^+/Ca^{2+} antiporter system (Wolkowicz *et al*., 1983) and stimulation of the Na^+/Ca^{2+} antiporter (Baydoun *et al*., 1989b). Autoradiographic and electron microscopic studies have confirmed that diltiazem, verapamil, nifedipine, bepredil and nitrendipine all accumulate within the mitochondrial matrix, suggesting a possible intramitochondrial site of action for these compounds (Sato *et al*., 1971; Nakajima *et al*., 1975; Lullman *et al*., 1979; Pang & Sperelakis, 1983; Tagami *et al*., 1985). The data presented here now identify a possible intracellular site of action for Bay K 8644.

Concentrations of racemic Bay K 8644 (≤ 60 μM) have been found to stimulate mitochondrial respiratory activity. However, the observations that phosphorylation of ADP was followed by a return to State 4 respiration, the RCI and ADP:O ratios were unchanged and that, at the concentrations used, State 3 respiration was unchanged confirm that this was not the result of an uncoupling effect of oxidative phosphorylation. Classically, the uncoupling of electron transport from ATP synthesis results in a decrease in the State 3 respiratory rate. Stimulation of the respiratory rate suggests that Bay K 8644 has the ability either to act as an additional respiratory substrate, or to increase intramitochondrial Ca^{2+} levels. Experiments where the respiratory substrate was replaced by Bay K 8644 confirmed that the dihydropyridine could not act as an alternative substrate. Thus, we would conclude that the stimulation of mitochondrial respiration seen with Bay K 8644 results from an increase in intramitochondrial Ca^{2+} levels in a manner similar to the phenomenon of Ca^{2+}-stimulated respiration (Chance, 1965), which in turn could result in the stimulation of Ca^{2+}-sensitive enzymes associated with energy metabolism (Hansford, 1985). It should be noted that exposure to ≥ 1 mM Ca^{2+} can result in 'massive loading' of mitochondria, causing uncoupling and irreversible damage to the organelle (Lehninger *et al*., 1967).

Previous studies with Ca^{2+} modifying agents have shown

that all compounds tested, with the exception of nitrendipine or its oxidised form, inhibit mitochondrial respiratory activity (Baydoun *et al.*, 1986a,b). These inhibitory effects were evident at concentrations of 100 μM, and result from non-specific membrane disruption consequent upon a 'detergent-like' effect on the mitochondrial membrane (Helenius & Simon, 1975) and the resultant collapse in the electrochemical gradient. Other workers have indicated that verapamil and diltiazem may reduce the capacity of mitochondria to produce ATP, either as a result of the accumulation of the catabolites of adenine nucleotides (Nigdikar *et al.*, 1986), or by a reduction in mitochondrial oxygen demand (De Jong *et al.*, 1984).

Studies on mitochondrial Ca^{2+} transport with the same concentration range used in the resipiratory studies indicate a number of effects specific to Bay K 8644 and the Ca^{2+} antagonist tested. Both racemic Bay K 8644 and the (+)- and (−)-isomers were able to increase the total Ca^{2+} uptake into tightly coupled cardiac mitochondria, in the same concentration range which has previously been shown to increase mitochondrial respiration. This increase in total Ca^{2+} uptake did not occur as a result of an increase in the uptake rate, but rather as a result of a gradual accumulation of the ion due to a change in balance between ion influx and efflux.

The proven ability of RR to stimulate Ca^{2+} efflux (Reed & Bygrave, 1974) was therefore used to confirm this change in balance by the addition of Bay K 8644 to mitochondria preloaded with Ca^{2+} causing the blockade of the dye-induced Ca^{2+} efflux. These findings, together with those on mitochondrial respiration, indicate that Bay K 8644-induced Ca^{2+} uptake is related to a stimulation in State 4 respiration and/or inhibition of the Ca^{2+} efflux pathway following blockade of the Na^+/Ca^{2+} antiporter. The former would, however, appear to be unlikely in view of its inability to act as an alternative respiratory substrate, or to stimulate key citric acid cycle enzymes (Fox *et al.*, 1989), thus indicating that stimulation is a direct result of Ca^{2+} uptake. This is in direct contrast to the stimulating effects seen with nitrendipine or palmitoyl carnitine (Baydoun *et al.*, 1988).

With the exception of oxidised nitrendipine (Baydoun *et al.*, 1986a,b), all other compounds tested caused inhibition of the rate of Ca^{2+} influx into cardiac mitochondria. In these studies, RR was confirmed to be a non-competitive inhibitor of the electrophoretic uniporter pathway associated with influx (Reed & Bygrave, 1974; Jurkowitz *et al.*, 1983), and nitrendipine to be a stimulator of efflux by a specific activation of the Na^+/Ca^{2+} antiporter (Baydoun *et al.*, 1986b; Lukacs & Fonyo, 1986). Unlike RR, verapamil- and diltiazem-induced inhibition of influx was not accompanied by efflux (Reed & Bygrave, 1974) indicating decreased uniporter activity, and not direct inhibition resulting from a possible decrease in membrane potential. The decrease in membrane potential results from incorporation of the antagonist into the lipid phase of the membrane producing a 'detergent-like' effect. The concentrations used in the transport studies show that the Ca^{2+} influx pathway is more sensitive to changes in oxidative phosphorylation, and that changes in influx are not secondary to the uncoupling of oxidation from phosphorylation (Baydoun *et al.*, 1986a,b).

Previous pharmacological studies with Bay K 8644 have shown it to be an activator of L-type Ca^{2+} channels associated with plasma membranes, to relax smooth muscle and to produce either negative or positive inotropy. The data presented here, therefore, indicate an intracellular site of action for Bay K 8644, different modes of action for the antagonists, and differences in stereospecificity for the action of dihydropyridine with respect to the plasma membrane (Vaghy *et al.*, 1988) and the inner mitochondrial membrane. In these studies, the different effects may also be divided into subgroups of similar action (verapamil/diltiazem; nitrendipine; Bay K 8644) which are similar to those subgroups previously identified for K^+-depolarised smooth muscle (Spedding & Berg, 1984; Spedding, 1988), and more recently for liver cells (Thurman *et al.*, 1988).

Further analysis of these data shows that the mode of action of Bay K 8644 on the Ca^{2+} efflux pathway is directly opposite to that of the endogenous Ca^{2+} channel agonist palmitoyl carnitine (Baydoun *et al.*, 1988), whilst plasma membrane studies suggest a similar mechanism of action for these compounds (Spedding & Mir, 1987).

Thus, we conclude that the effects of Bay K 8644 at the level of the mitochondrion are to increase mitochondrial Ca^{2+} by inhibition of the Na^+/Ca^{2+} antiporter without disruption of the mitochondrial membrane potential, resulting in an overall reduction in available cytosolic Ca^{2+}.

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Separate [^{3}H]-nitrendipine binding sites in mitochondria and plasma membranes of bovine adrenal medulla

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1 Two binding sites for the 1,4-dihydropyridine (DHP) derivative [^{3}H]-nitrendipine have been found in the bovine adrenal medulla. The high-affinity site (K_d = 0.48 nM and B_{max} = 128 fmol mg^{-1} protein) was specifically located in purified plasma membranes. The low-affinity site (K_d = 252 nM and B_{max} = 169 pmol mg^{-1} protein) was located only in mitochondria. Chromaffin granule membranes lacked specific binding sites for [^{3}H]-nitrendipine.

2 Kinetic analysis of the rates of association and dissociation of [^{3}H]-nitrendipine, saturation isotherms and displacement experiments with unlabelled nitrendipine and PN200-110 revealed single, homogeneous populations of high- and low-affinity sites in plasma and mitochondrial membranes, respectively.

3 The high affinity site was sensitive to Ca^{2+} deprivation and heating; it was practically unaffected by changes in ionic strength of the medium and its optimal pH was slightly alkaline. This site exhibited a strong DHP stereoselectivity; diltiazem increased and verapamil decreased the affinity of [^{3}H]-nitrendipine.

4 In contrast, binding of [^{3}H]-nitrendipine to the low affinity site was more heat resistant and less affected by Ca^{2+} removal. Its optimal pH was slightly acid and the increase in ionic strength enhanced the number of available sites. The site had no DHP stereoselectivity. Verapamil decreased the dissociation constant of [^{3}H]-nitrendipine acting in a non-competitive manner; diltiazem did not affect equilibrium binding parameters of [^{3}H]-nitrendipine.

5 These results suggest that both binding sites reflect different receptor entities. The high-affinity binding site corresponds to the dihydropyridine receptor associated with the L-type calcium channel. The function of the mitochondrial, low-affinity binding site is, at present, unknown.

Introduction

Blocker and activator 1,4-dihydropyridines (DHP) inhibit or stimulate, respectively, Ca^{2+} uptake and catecholamine release in cultured chromaffin cells or perfused adrenal glands (Ceña *et al.*, 1983; Garcia *et al.*, 1984), suggesting the presence of a DHP receptor coupled to specific voltage-dependent Ca channels.

Nitrendipine has been routinely used to identify DHP receptors and to determine their pharmacological and biochemical properties in heart (Belleman *et al.*, 1981), muscle (Bolger *et al.*, 1982) and brain (Murphy & Snyder, 1982). The high-affinity binding site is considered to be a part of, or closely related to plasmalemmal L-type voltage-sensitive Ca channels; this has been demonstrated through electrophysiological (Hess *et al.*, 1984), biochemical (Flockerzi *et al.*, 1986) or functional experiments (Artalejo *et al.*, 1988). A low-affinity site has been recently demonstrated in guinea-pig heart mitochondria (Brush *et al.*, 1987). Further experiments showed that the inner mitochondrial membrane was enriched in low-affinity sites (Zernig & Glossmann, 1988).

The present study was designed to elucidate the nature of DHP binding sites in adrenal medulla. We demonstrate here that the bovine adrenal medulla contains two separate binding sites for [^{3}H]-nitrendipine; the high-affinity site is located in the plasmalemma of chromaffin cells and the low-affinity site in the inner mitochondrial membrane. Marked differences in their kinetic constants and their biochemical and pharmacological properties suggest that they are unrelated receptor entities.

Methods

Purification of subcellular fractions

Procedures to prepare subcellular fractions were performed at 4°C. All buffers used contained 0.1 mM phenylmethylsulphonyl fluoride and 1 mM iodoacetamide as protease inhibitors.

For each preparation, 20 bovine adrenal glands were obtained from a local slaughterhouse within 20–30 min of death of the animals and placed on ice. The superficial fat was removed, the adrenal cortex dissected away and medullae finely minced, placed in 6 volumes (w/v) of ice-cold 0.32 M sucrose, 50 mM tris(hydroxymethyl)aminomethane/HCl (Tris) buffer pH 7.4.

Plasma membranes Medullae were homogenized in a Polytron at setting 7 for 15 s, 3 times. In order to purify plasma membranes, the following modifications of the method of Meyer & Burger (1979) were introduced: (1) the 1000 *g* supernatant was centrifuged at 100,000 *g*. (2) The 100,000 *g* pellet was layered on 1.6 M sucrose and centrifuged at 100,000 *g* for 1 h. (3) The 0.32/1.6 M sucrose interface was layered on a discontinuous sucrose gradient (1/1.2/1.9 M sucrose; 10/10/4 ml) and centrifuged for 4 h at 100,000 *g*. The final plasma membrane fraction was resuspended in 50 mM Tris/HCl buffer pH 7.4 and stored at −80°C until use.

Mitochondria and chromaffin granules Medullae homogenization was carried out in a Polytron, at setting 4 for 15 s twice. The crude homogenate was filtered and centrifuged at 1000 *g* for 15 min and the supernatant spun down at 9800 *g* for 15 min. The pellet was resuspended and layered on 1.6 M sucrose and centrifuged as described by Bartlett & Smith (1974). In order to obtain chromaffin granules, the 1.6 M sucrose pellet was resuspended in 50 mM Tris/HCl buffer pH 7.4 and washed three times at 9800 *g* for 15 min. The mitochondria fraction was obtained from the 0.32/1.6 M sucrose interface by use of the same washing procedure as for chromaffin granules. In both cases, membranes were stored at −80°C until use.

Submitochondrial fractions were obtained according to the procedure of Sottocasa *et al.* (1967).

[^3H]-nitrendipine binding

Membranes (50 to 200 μg; 0.5 to 1 ml, final volume) were incubated for 1 h at 25°C with increasing concentrations of [^{3}H]-nitrendipine (diluted 1 : 100 with unlabelled nitrendipine when concentrations were higher than 2 nM) in 50 mM Tris/HCl buffer pH 7.4 for binding to plasma membranes, or 50 mM 2-(N-morpholino)ethanesulphonic acid (MES) pH 6, containing 500 mM NaCl for binding to mitochondria, and 0.5 mM $CaCl_2$ in both cases. At the end of the incubation period, samples were cooled in an ice water bath for 5 min (except for plasma membranes), filtered under vacuum through Whatman GF/C glass fibre filters and washed 4 times with cold incubation buffer. In all cases, the washing volume was 5 fold the incubation volume. Radioactivity remaining in the filters was counted in a Beckman LS2800 liquid scintillation counter. Non-specific binding was determined in the presence of 10 μM unlabelled nitrendipine. Incubations were carried out in glass test tubes because it is known that [^{3}H]-DHPs stick to plastic. All experiments were performed under sodium vapour light to prevent DHP breakdown. All DHPs were dissolved in ethanol. The highest concentration of ethanol used in the assays was 1%, as in previous controls it was shown that this concentration did not affect [^{3}H]-nitrendipine binding.

Analysis of experimental equilibrium data were performed by LIGAND, a non-linear least squares computer programme (Munson & Rodbard, 1980). In all cases, saturation experiments fitted significantly to a one-binding site model according to F tests. The kinetic association experiments were linearized according to the following pseudo-first order equation (Weiland & Molinoff, 1981): $\ln(B_{eq}/B_{eq} - B) = k_{+1}t[L]_T B_{max}/B_{eq}$.

The kinetic dissociation data were linearized according to the first-order integrate rate equation (Weiland & Molinoff, 1981): $\ln(B/B_0) = -k_{-1}t$, where B_{eq} represents concentration of radioligand-receptor complex at equilibrium, $[L]_T$ total concentration of radioligand, B_0 the concentration of radioligand-receptor prior to the addition of excess unlabelled ligand, and B the concentration of radioligand-receptor at time t.

In all cases, values are given as means ± s.e.mean.

Other assays

Acetylcholinesterase (AChE) (EC 3.1.1.7) was determined according to the method of Ellman et al. (1961); dopamine β-hydroxylase (DBH) (EC 1.14.17.1) according to Nagatsu & Udenfriend (1972); succinate dehydrogenase (SDH) (EC 1.3.99.1), as described by King (1967); monoamine oxidase (MAO) (EC 1.4.3.4.), according to Jarrott & Langer (1971). In all cases, one unit of enzyme activity corresponded to one nmol production formed min^{-1}. Protein content was determined according to Lowry et al. (1951) with bovine serum albumin used as standard protein.

Materials

[^{3}H]-nitrendipine was purchased from New England Nuclear (West Germany). Nitrendipine was a gift from Professor F. Hoffmeister (Bayer AG; Wuppertal, West Germany). (+)- and (−)-PN200-110 (isradipine) were generously donated by Dr R.P. Hof (Sandoz, Basel, Switzerland). Verapamil was a gift of Knoll (West Germany). All other reagents were from Sigma (U.S.A.).

Results

Preliminary experiments carried out on microsomal fractions showed the presence of two binding sites for [^{3}H]-nitrendipine. To study the location of the two DHP binding sites, subcellular fractions were purified and [^{3}H]-nitrendipine binding measured in these subcellular organelles.

Separation and subcellular localization of high- and low-affinity [^3H]-nitrendipine binding sites

Table 1 shows the activity of various marker enzymes and [^{3}H]-nitrendipine binding to high- and low-affinity sites present in subcellular organelles obtained according to the procedure used to purify plasma membranes. The 1/1.2 M sucrose interface was considerably enriched in AchE activity, a specific marker for plasma membranes (Rosenheck, 1987). High affinity [^{3}H]-nitrendipine binding co-purified with AChE activity in this fraction. In contrast, it was clear that the mitochondrial enzyme SDH, the chromaffin granule marker DBH and the low affinity [^{3}H]-nitrendipine binding did not purify in this fraction. Displacement of [^{3}H]-nitrendipine binding by unlabelled nitrendipine in the 1/1.2 M sucrose interface revealed a K_i of 0.57 ± 0.08 nM (Table 3).

Table 2 presents data concerning the procedure for purification of mitochondria and chromaffin granules. The 0.32/1.6 M sucrose interface was enriched in SDH activity (inner mitochondrial marker) and low affinity [^{3}H]-nitrendipine binding. The K_i of unlabelled nitrendipine to block specific [^{3}H]-nitrendipine binding in this fraction was 273 ± 41 nM (Table 3). Further separation of submitochondrial fractions (Figure 1) revealed that [^{3}H]-nitrendipine binding was located in the inner mitochondrial membrane (heavy fraction), as demonstrated by SDH activity. DBH activity (marker of chromaffin granules) predominated in the 1.6 M sucrose pellet. Neither high- nor low-affinity [^{3}H]-nitrendipine binding purified in this fraction. Furthermore, increasing concentrations of unlabelled nitrendipine from 1 pM to 10 μM did not inhibit total binding of [^{3}H]-nitrendipine to the chromaffin granule fraction, indicating that specific [^{3}H]-nitrendipine binding sites are not present in this fraction.

Table 1 Plasma membrane isolation: distribution of marker enzymes and [^{3}H]-nitrendipine binding

Purification steps	*Marker enzymes*			*[^3H]-nitrendipine binding*	
	AChE	*SDH*	*DBH*	*high-affinity*	*low-affinity*
Supernatant 800 *g*	24	12	207	6.1	6.1
Pellet 100,000 *g*	92	17	522	18.7	24.3
Interface 0.32 M/1.6 M sucrose	175	52	977	45.1	37.0
Pellet 1.6 M sucrose	2	15	3350	6.2	10.4
Interface 0.32/1 M sucrose	253	50	1068	ND	ND
Interface 1/1.2 M sucrose	521	14	171	99.7	11.6
Interface 1.2/1.9 M sucrose	95	131	1886	8.3	56.0

Enzyme activity is expressed in u mg^{-1} protein. Binding of [^{3}H]-nitrendipine to high (0.8 nM)- and low (200 nM)-affinity sites was done in each case as described in Methods. Binding to high-affinity sites was defined as specific binding displaced by 2.5 μM (±)-Bay K 8644, and is expressed in fmol mg^{-1} protein. Specific binding non-displaceable by 2.5 μM (±)-Bay K 8644 was defined as binding to low affinity sites, and is expressed in pmol mg^{-1} protein. Data correspond to the mean of 2 experiments performed in duplicate. AChE, acetylcholinesterase. SDH, succinate dehydrogenase. DBH, dopamine β-hydroxylase. ND = Not detectable.

Table 2 Mitochondria purification: distribution of marker enzymes and [^{3}H]-nitrendipine binding

Purification steps	*Marker enzymes*			*[^{3}H]-nitrendipine binding*	
	AChE	*SDH*	*DBH*	*high-affinity*	*low-affinity*
Supernatant 800 *g*	25	11	126	5.9	6.5
Supernatant 10,000 *g*	33	28	347	4.5	14.3
Pellet 10,000 *g*	15	66	533	5.2	24.3
Interface 0.32 M/1.6 M sucrose	16	128	673	6.5	81.6
Pellet 1.6 M sucrose	14	11	2612	7.3	5.6

Determinations were performed as described in the legend of Table 1. Data correspond to the mean of 2 experiments performed in duplicate. For abbreviations used see Table 1.

Kinetic and equilibrium properties of [^{3}H]-nitrendipine binding

Plasma membranes At 25°C, association and dissociation kinetics of binding of [^{3}H]-nitrendipine to plasma membranes were monophasic (Figure 2a,b). From kinetic constants (Table 3), the calculated K_d for [^{3}H]-nitrendipine was 0.2 nM.

Table 3 Constants of [^{3}H]-nitrendipine binding to plasma membrane and mitochondria of bovine adrenal medulla

Conditions	*Plasma membrane*	*Mitochondria*
Saturations		
Control: K_d	0.48 ± 0.03	252 ± 45
B_{max}	128 ± 5	169 ± 25
	(4)	(4)
0 NaCl: K_d		255 ± 56
B_{max}		*19* ± 3
		(2)
+ 10 μM diltiazem: K_d	0.12 ± 0.01	215 ± 34
B_{max}	106 ± 4	156 ± 20
	(4)	(4)
+ 10 μM verapamil: K_d	1.16 ± 0.26	533 ± 80
B_{max}	130 ± 22	149 ± 18
	(4)	(4)
Preheating 60°C: K_d	ND	231 ± 37
B_{max}	ND	136 ± 18
	(2)	(2)
Kinetics		
Association: k_{+1}	7.46 ± 0.63 × 10^7	1.21 ± 0.04 × 10^5
	(3)	(3)
Dissociation		
+ 10 μM nitrendipine: k_{-1}	0.015 ± 0.001	0.028 ± 0.002
	(3)	(3)
+ 10 μM verapamil: k_{-1}		0.058 ± 0.005
		(3)
Displacements		
(±)-Nitrendipine: K_i	0.57 ± 0.08	273 ± 41
	(2)	(2)
(+)-PN200-110: K_i	0.030 ± 0.007	2340 ± 580
	(3)	(3)
(−)-PN200-110: K_i	2.6 ± 0.6	1680 ± 350
	(3)	(3)
EGTA: % displacement	44	17
	(3)	(3)

K_d and K_i values are expressed in nM. B_{max} values are expressed in fmol mg^{-1} protein (plasma membrane) and pmol mg^{-1} protein (mitochondrial membrane). k_{+1} and k_{-1} are expressed in M^{-1} min^{-1} and min^{-1}, respectively. ND = binding sites not detectable. Numbers in parentheses represent the number of duplicate experiments.

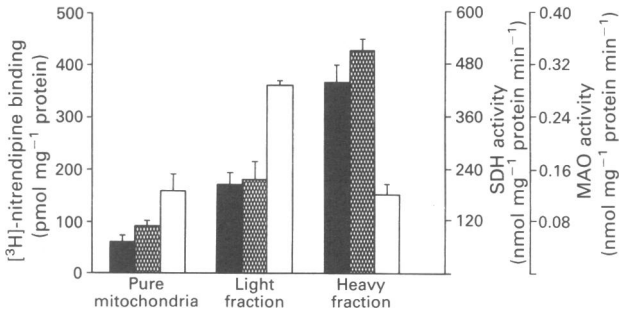

Figure 1 Isolation and characterization of submitochondrial fractions from bovine adrenal medulla. Light and heavy fractions were isolated from purified mitochondria. In each fraction, the specific binding of 200 nM [^{3}H]-nitrendipine and the activities of monoamine oxidase (MAO) and succinate dehydrogenase (SDH), were measured; they reflect the outer and inner mitochondrial membrane enzyme markers, respectively. As can be seen, the light fraction corresponds to the outer membrane with some contamination of the inner membrane. The heavy fraction contains the inner mitochondrial membrane. Columns show means and bars s.e.mean of 6 experiments performed with different batches of purified mitochondria. Solid columns, [^{3}H]-nitrendipine binding; hatched columns, SDH activity; open columns, MAO activity.

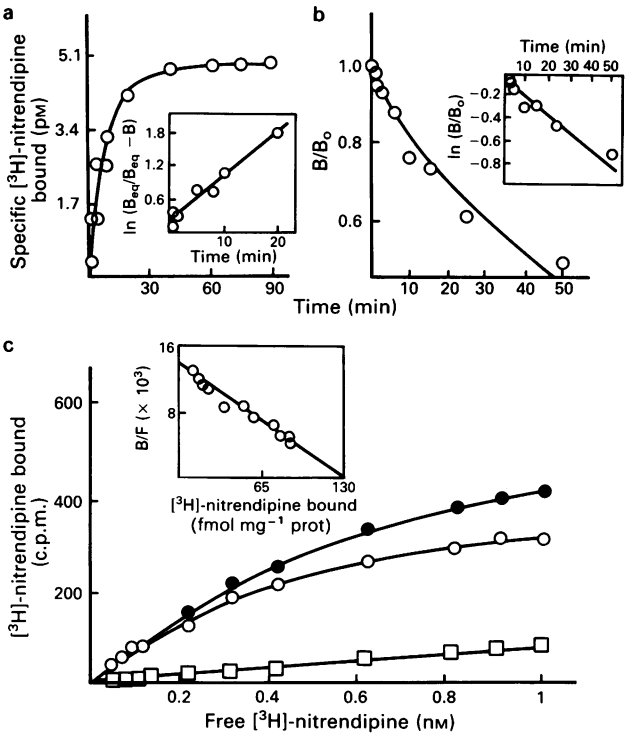

Figure 2 Binding of [^{3}H]-nitrendipine to partially purified chromaffin cell plasma membranes. (a) Plasma membranes (50 μg ml^{-1}) were incubated with 0.8 nM [^{3}H]-nitrendipine for increasing times, and then filtered as described in Methods. Data are means of 3 experiments performed in duplicate. Inset: experimental data were linearized, as described in Methods. (b) Dissociation kinetics were started after one hour incubation with 0.8 nM [^{3}H]-nitrendipine by adding 10 μM unlabelled nitrendipine. Samples were taken and filtered at different times as described before. Data correspond to 3 experiments performed in duplicate. Inset: experimental data were linearized as described in Methods. (c) Saturation isotherm of equilibrium binding of [^{3}H]-nitrendipine to purified plasma membranes (50 μg protein ml^{-1}). Total binding (●) was measured by adding [^{3}H]-nitrendipine in a range of 0.04 to 1 nM. Non-specific binding (□) was determined in the presence of 10 μM unlabelled nitrendipine. Specific [^{3}H]-nitrendipine binding (○) was calculated as the difference between total and non-specific binding. Data correspond to the means of 4 experiments performed in duplicate. Inset: Scatchard plot of the same data adjusted by the LIGAND programme. Equilibrium binding constants are given in Table 3.

Specific and total binding of [^{3}H]-nitrendipine tended to saturate, while non-specific binding was linear ($r^2 > 0.9$) (Figure 2c). Scatchard analysis of data showed the presence of a single binding site, with K_d of 0.48 ± 0.03 nM, and a density of receptors of 128 ± 5 fmol mg^{-1} protein (Figure 2, inset in c). The K_d was quite close to that obtained from association and dissociation kinetic constants.

Mitochondrial membranes At 25°C, association and dissociation kinetics for binding of [^{3}H]-nitrendipine to mitochondrial membranes were monoexponential (Figure 3a,b). From kinetic constants (Table 3), we calculated a K_d for [^{3}H]-nitrendipine of 231 nM.

In order to study equilibrium binding parameters, saturation experiments were performed. The non-specific binding, determined in the presence of 10 μM unlabelled nitrendipine, was linear ($r^2 = 0.96$), while the total and specific binding tended to saturate (Figure 3c). Scatchard analysis of experimental data gave a single binding site with a K_d of 252 ± 14 nM and a B_{max} of 169 ± 20 pmol mg^{-1} protein (Figure 3c, inset). The K_d obtained in these experiments was in good agreement with that calculated from kinetic constants.

Differences between mitochondrial and plasmalemmal [^{3}H]-nitrendipine binding sites

When [^{3}H]-nitrendipine binding was performed in the presence of 1 mM EGTA to test Ca^{2+}-dependence of binding to both sites, it was seen that in plasma membranes the sensitivity of binding sites to Ca^{2+} depletion was higher than in mitochondrial membranes (Table 3).

Thermosensitivity of DHP receptors was tested by heating mitochondria and plasma membranes for 10 min at 60°C; after which, saturation of [^{3}H]-nitrendipine was performed as in control conditions. Plasma membrane receptors were completely destroyed by this treatment, while mitochondrial receptors were more heat-resistant (Table 3).

Increasing concentrations of NaCl (from 50 to 700 mM) greatly enhanced [^{3}H]-nitrendipine (0.8 nM) binding to mitochondrial membranes; this reached a plateau at a NaCl concentration of 500 mM (Figure 4a). K_d values for [^{3}H]-nitrendipine in the presence or absence of 500 mM NaCl were similar. However, B_{max} in the presence of 500 mM NaCl was 10 fold higher than in the absence of NaCl (Table 3). In contrast, binding to plasma membranes was only slightly enhanced by increasing NaCl concentrations (Figure 4a).

The dependence of the [^{3}H]-nitrendipine binding on pH was clearly different in both subcellular fractions (Figure 4b). Mitochondrial binding sites showed a narrower pH-dependence range and a more acidic optimal pH than plasmalemmal binding sites. In fact, at pH 6 in which we observed maximal binding to mitochondria, binding to plasma membranes was minimum.

Another definite difference between the two organelles concerns the stereoselectivity of the DHP receptors (Figure 5). The enantiomer of the DHP Ca channel blocker (+)-PN200-110 efficiently displaced [^{3}H]-nitrendipine binding from plasma membranes, having a K_i 76 fold lower than the (−)-enantiomer (Table 3), indicating that the plasmalemmal DHP receptor exhibits a high degree of stereoselectivity. In contrast,

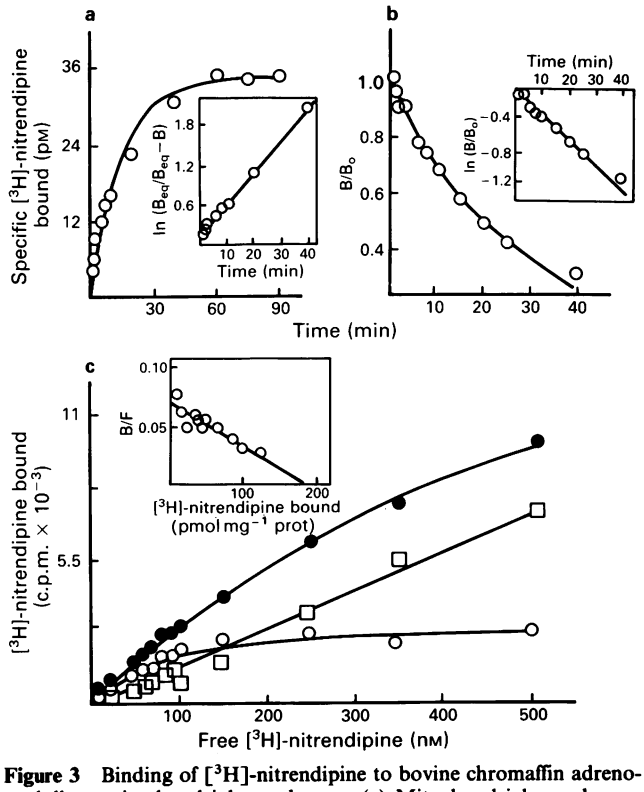

Figure 3 Binding of [^{3}H]-nitrendipine to bovine chromaffin adrenomedullary mitochondrial membranes. (a) Mitochondrial membranes (50 μg 0.5 ml^{-1}, final volume) were incubated with 0.8 nM [^{3}H]-nitrendipine for increasing time periods, and then filtered as described in Methods. Data are means of 3 experiments in duplicate. Inset: experimental data were linearized, as described in Methods. (b) Dissociation kinetics were started after one hour incubation with 0.8 nM [^{3}H]-nitrendipine by adding 10 μM unlabelled nitrendipine. Samples were taken and filtered at different times. Data are means of 3 experiments in duplicate. Inset: experimental data were linearized according to a first order equation. (c) Saturation isotherm of binding of [^{3}H]-nitrendipine to purified mitochondrial membranes. Specific [^{3}H]-nitrendipine binding (10 to 500 nM) to 50 μg protein 0.5 ml^{-1} (○), was calculated as the difference between total binding (●) and non-specific binding (□). Inset: Scatchard plot of the same data adjusted by the LIGAND programme. The equilibrium binding constants are given in Table 3.

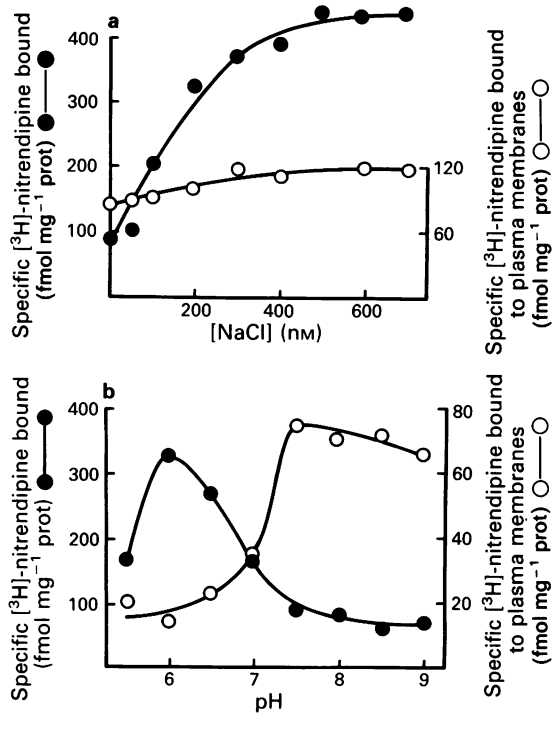

Figure 4 Influence of ionic strength (a) and pH (b) on specific [^{3}H]-nitrendipine binding to mitochondria and plasma membranes. In (a) binding was measured by adding 0.8 nM [^{3}H]-nitrendipine to mitochondrial (●) or plasma membranes (○) (50 μg protein in 0.5 ml in both cases) in 50 mM Tris/HCl buffer pH 7.4 with increasing concentrations of NaCl of 50 to 700 mM. Each point represents the mean of 2 experiments in duplicate. In (b), binding of 0.8 nM [^{3}H]-nitrendipine to 50 μg protein of mitochondrial (●) or plasma membranes (○) in 0.5 ml of 50 mM MES buffer (pH 5.5 to 7) or 50 mM Tris/HCl buffer (pH 7 to 9). Each point represents the mean of 2 experiments in duplicate.

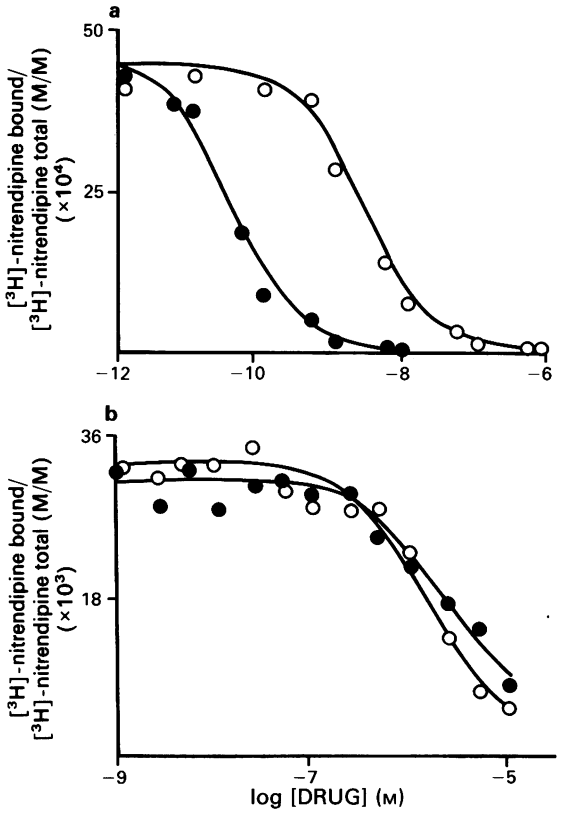

Figure 5 Inhibition of binding of [^{3}H]-nitrendipine to plasma membranes (a) and mitochondrial membranes (b) by (+)-PN 200-110 (●) and (−)-PN200-110 (○). Concentration of [^{3}H]-nitrendipine was 0.8 nM for plasma membranes and 250 nM for mitochondrial membranes. Data are means of 3 experiments in duplicate in both cases. K_i values are given in Table 3.

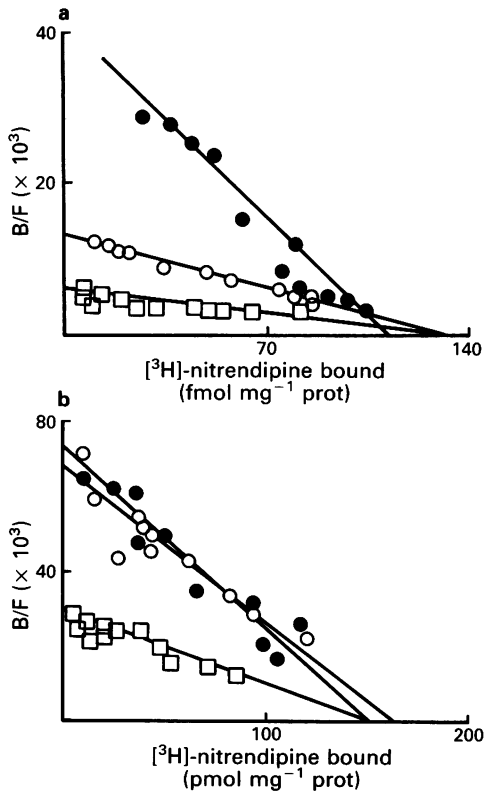

Figure 6 Scatchard analysis showing the effects of verapamil and diltiazem on [^{3}H]-nitrendipine binding to plasma membranes (a) and mitochondria (b). Verapamil (□) and diltiazem (●) were present at 10 μM in both cases. Saturations in absence of drugs (○) to plasma and mitochondrial membranes are also depicted. Assays were carried out as described in Methods, except for verapamil in (b), where incubation time was 150 min. Data are means of 4 duplicate experiments. Binding parameters are given in Table 3.

in mitochondrial membranes both enantiometers of PN200-110 displaced [^{3}H]-nitrendipine binding with similar K_i values (Table 3).

The effects of phenylalkylamines and benzothiazepine derivatives on DHP binding to high affinity receptors in different systems are well known (Janis & Triggle, 1984). In bovine adrenomedullary plasma membranes the presence of 10 μM verapamil increased the K_d of [^{3}H]-nitrendipine about 2.5 fold (Figure 6a), while the B_{max} was unchanged (Table 3). Similar effects were observed in mitochondrial membranes (Figure 6b); in the presence of verapamil (10 μM), the K_d for [^{3}H]-nitrendipine was doubled but the B_{max} was unaltered (Table 3). Verapamil modulated [^{3}H]-nitrendipine binding to mitochondrial membranes in a non-competitive manner; the k_{-1} of [^{3}H]-nitrendipine when dissociation kinetics were performed in the presence of verapamil plus nitrendipine was higher than in controls (nitrendipine alone) (Table 3).

Diltiazem revealed a new difference between plasmalemmal and mitochondrial membranes. Diltiazem (Figure 6a) enhanced the affinity of [^{3}H]-nitrendipine for the plasma membrane high-affinity binding site, while it had no effect on the B_{max} (Table 3). In contrast, this benzothiazepine did not affect the equilibrium parameters of [^{3}H]-nitrendipine in mitochondria (Figure 6b and Table 3).

Discussion

The present work demonstrates the presence in bovine adrenal medulla of two separate DHP receptors. The high-affinity site was located in the plasma membrane, and the low-affinity site in the inner mitochondrial membrane. Observed differences in properties of both DHP binding sites support the hypothesis that high- and low-affinity sites are associated with different molecular entities.

The dihydropyridine receptor of chromaffin cell plasma membranes

According to its properties, the plasmalemmal high-affinity site is probably associated with the L-type calcium channel, as it has already been established in other tissues; in fact, L-type Ca channels have been identified in bovine chromaffin cells (Fenwick *et al.*, 1982). The calculated K_d of [^{3}H]-nitrendipine for this binding site is in the range obtained in other tissues, excluding skeletal muscle (Janis *et al.*, 1987). Other properties of the adrenomedullary plasma membrane DHP receptors studied in the present work (divalent cation dependence, stereoselectivity, thermosensitivity, and allosteric regulation by verapamil and diltiazem) support this assumption.

The dihydropyridine receptor of chromaffin mitochondrial membranes

A low-affinity, DHP binding site associated with mitochondria has been previously shown in different tissues. However, the presence of this receptor in neuronal or adrenal medulla tissues has not been demonstrated previously. Our results suggest that there are no significant differences between these paraneuronal and non-neuronal mitochondrial DHP receptors. The low-affinity binding site has clear differences from the high affinity binding site, including different location, pH and ionic strength-dependence, heat-resistance, and lack of regulation by diltiazem. The effect of verapamil on DHP binding to these low-affinity receptors is controversial. Our

results demonstrate that verapamil regulates the binding of [^{3}H]-nitrendipine to mitochondrial receptors in an allosteric manner. In equilibrium studies, the effect of verapamil was to reduce the affinity of [^{3}H]-nitrendipine for this receptor, with no effect on B_{max}. Additional dissociation kinetics demonstrated that verapamil is acting in a non-competitive manner, as it enhanced the dissociation kinetics constant. These results are in contrast with those of Brush *et al.* (1987), who demonstrated no effect of verapamil on [^{3}H]-nitrendipine binding to guinea-pig cardiac mitochondrial membranes. In line with our results, is another study in which verapamil was shown to displace completely [^{3}H]-nitrendipine binding from guinea-pig cardiac mitochondrial membranes (Zernig & Glossmann, 1988). However, to date, the effect of verapamil on equilibrium or kinetic [^{3}H]-nitrendipine binding constants has not been studied.

A series of effects of DHPs on different molecular entities unrelated to calcium channels are known (see recent review by Zernig, 1990). However, the functional importance of each one is unclear. Mitochondrial DHP receptors could have a physiological role in Ca-sequestering or other metabolic functions of mitochondria and we are now testing these hypotheses.

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Evidence that an atypical β-adrenoceptor mediates the inhibition of spontaneous rhythmical contractions of rabbit isolated jejunum induced by ritodrine and salbutamol

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1 The nature of the adrenoceptors mediating the inhibitory action of noradrenaline, ritodrine and salbutamol on the spontaneous activity of longitudinal muscle of the rabbit jejunum *in vitro* was investigated by use of a range of adrenoceptor antagonists.

2 The actions of ritodrine and salbutamol were antagonized competitively by propranolol. The pA_2 values of 6.4 and 6.6 respectively were smaller than those found elsewhere for β_1- and β_2-adrenoceptors.

3 In contrast, the responses to ritodrine and salbutamol were antagonized only by high concentrations ($>2.7\,\mu M$) of phentolamine and were unaffected by yohimbine ($2.6\,\mu M$), mepyramine ($2.5\,\mu M$) or cimetidine ($4.0\,\mu M$).

4 Ritodrine which is less potent than salbutamol in tissues with typical β_2-adrenoceptors was found to be 8 times more potent than salbutamol in the rabbit jejunum.

5 It is suggested that in the rabbit jejunum ritodrine and salbutamol may act at an atypical β-adrenoceptor, at which propranolol is a competitive but not very potent antagonist.

Introduction

Catecholamines inhibit spontaneous, rhythmical contractions of longitudinal muscle of the rabbit small intestine (Finkleman, 1930), and this response is mediated by both α- and β-adrenoceptors (Furchgott, 1960; Bowman & Hall, 1970). These β-adrenoceptors were initially classified as β_1 (Lands *et al.*, 1967) but, when more selective β_1 and β_2 agonists and antagonists became available, evidence was found for the presence of β_2-adrenoceptors also (Wagner *et al.*, 1981). Nevertheless, certain paradoxes remained unresolved; for example Bowman & Hall (1970) found that noradrenaline exhibited a higher degree of β-selectivity than adrenaline in the rabbit small intestine, and Wagner *et al.* (1981) obtained several findings which are hard to reconcile with typical β_1- or β_2-adrenoceptor-mediated activity. In the present study the nature of the adrenoceptors mediating inhibitory effects of sympathomimetic drugs in the rabbit small intestine have been investigated further by use of noradrenaline, two selective β_2-adrenoceptor agonists (salbutamol and ritodrine) and various antagonists.

Methods

New Zealand White rabbits of either sex, weighing 0.8–0.95 kg, were killed by a blow on the neck and subsequent exsanguination. Segments of jejunum, 1 cm long, were suspended in isolated organ baths containing Krebs solution at 37°C, equilibrated with 95% O_2 and 5% CO_2. The aboral end of each segment was fixed near the bottom of the bath and the oral end was connected via a thread to an isotonic transducer, counterbalanced by a 1 g load, enabling longitudinal muscle activity to be recorded. Each segment was allowed to equilibrate for 45 min by which time regular spontaneous activity was seen and consistent inhibitory responses to submaximal concentrations of noradrenaline were obtained.

Concentration-response curves to noradrenaline, ritodrine or salbutamol were performed in the absence and then in the presence of 3 or more concentrations of either propranolol or phentolamine, each equilibrated for 20 min. Control tissues were treated similarly but were not exposed to an antagonist.

The responses to submaximal concentrations of ritodrine ($0.3\,\mu M$) and salbutamol ($3.3\,\mu M$) were also recorded in the presence of yohimbine ($2.6\,\mu M$), mepyramine ($2.5\,\mu M$) and cimetidine ($4.0\,\mu M$), to test whether their inhibitory action might be mediated through α_2-adrenoceptors or H_1- or H_2-receptors.

Drugs and solutions

The following drugs were used: cimetidine (Smith Kline & French), mepyramine (May & Baker), (−)-noradrenaline bitartrate (Sigma), phentolamine mesylate (Ciba), (±)-propranolol hydrochloride (Sigma), ritodrine hydrochloride (Duphar), salbutamol (Sigma) and yohimbine (Sigma).

All agonists were dissolved and diluted in acid saline (NaCl 161 mM, HCl $10\,\mu M$ and ascorbic acid 0.6 mM). All antagonists were dissolved in saline (NaCl 161 mM) and subsequently diluted in Krebs solution.

The Krebs solution had the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.6, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 23.8 and glucose 11.1.

Calculations and statistics

The inhibitory response to each agonist drug concentration was calculated as the % decrease in spontaneous contraction amplitude. From each concentration-response curve, an EC_{50} value was determined as the concentration of agonist causing 50% of the maximum response to that agonist, and these values (when obtained in the absence of antagonist) were used to calculate pD_2 values ($-\log EC_{50}$). When antagonism appeared competitive the ratios of the EC_{50} values in the presence and absence of antagonist were calculated for each concentration of antagonist used. For each agonist, the data from six experiments were pooled to construct a Schild plot (Arunlakshana & Schild, 1959). The slope and x-intercept of the regression line were calculated by linear least squares regression analysis as described by Colton (1974). The x-intercept was taken as the pA_2 value if the slope of the regression line was not statistically significantly different from 1.

The significance of any deviation from 1 of the slopes of the Schild plots was determined by Student's *t* test. The statistical significance of the difference between original and subsequent

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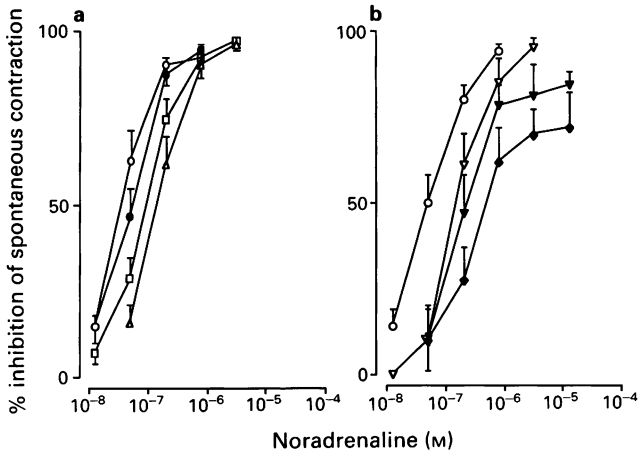

Figure 1 Inhibition of spontaneous activity in the rabbit jejunum. Concentration-response curves for noradrenaline in the absence (○) and presence of (a) propranolol (●, 34 nM; □, 340 nM; △, 3.4 μM), or (b) phentolamine (▽, 270 nM; ▼, 2.7 μM; ◆, 27 μM). Each point is the mean of 6 observations. Vertical lines show s.e.mean.

responses obtained in control tissues was tested by the Wilcoxon signed rank sum test. A difference was assumed to be significant when $P < 0.05$.

Results

Noradrenaline (13 nM–810 nM) inhibited spontaneous contractions of the rabbit jejunum in a concentration-dependent manner. The concentration-response curve for noradrenaline was shifted to the right by both propranolol (34 nM, 340 nM, 3.4 μM) and phentolamine (270 nM, 2.7 μM, 27 μM) (Figure 1). The highest concentration of phentolamine also suppressed the maximum response to the agonist.

Ritodrine (150 nM–4.9 μM) and salbutamol (840 nM–53 μM), like noradrenaline, inhibited spontaneous contraction of the rabbit jejunum in a concentration-dependent manner. The concentration-response curves for ritodrine and salbutamol were also shifted to the right by propranolol (340 nM, 1.1 μM, 3.4 μM, 10 μM) (Figure 2a and c). At a low concentration phentolamine (2.7 μM) had no significant ($P > 0.05$) effect (Figure 2b and d), but at a high concentration (27 μM) it suppressed the maximum responses to ritodrine and salbutamol.

The pD_2 values for noradrenaline, ritodrine and salbutamol are given in Table 1, together with Schild plot slopes and x-intercepts for propranolol antagonizing the action of these 3 agonists. The Schild plots are shown in Figure 3. In the cases of ritodrine and salbutamol the difference between the Schild plot slopes and 1 is not statistically significant, indicating competitive antagonism and thus allowing the x-intercepts for these agonists to be taken as the pA_2 values. However, with

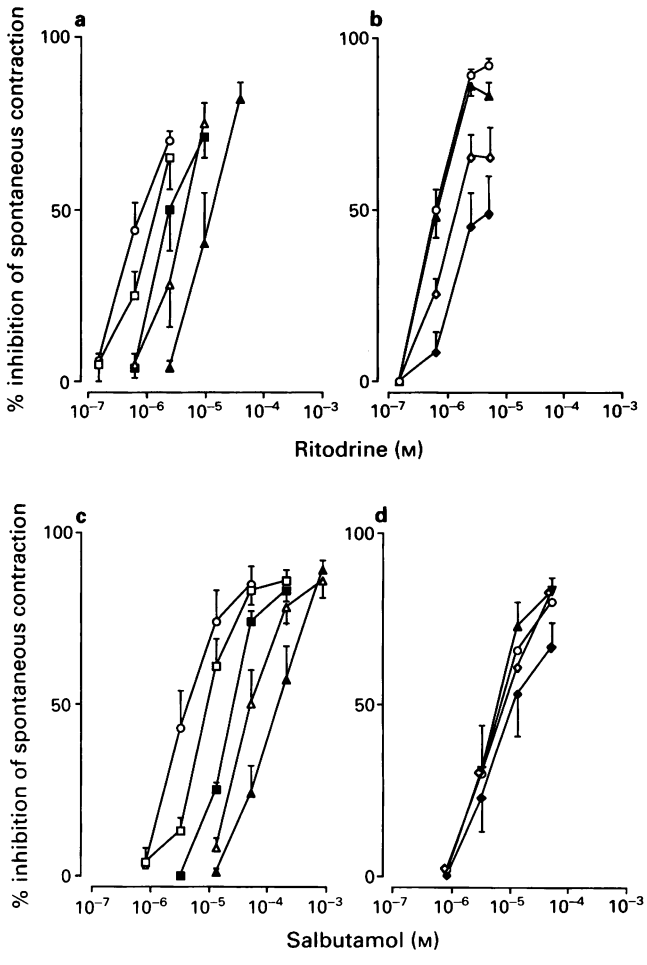

Figure 2 Inhibition of spontaneous activity in the rabbit jejunum. Concentration-response curves for ritodrine in the absence and presence of (a) propranolol and (b) phentolamine, and for salbutamol in the absence and presence of the same antagonists (c and d). (○) Indicates absence of antagonist. Propranolol concentrations used were: (□) 340 nM; (■) 1.1 μM; (△) 3.4 μM; (▲) 10 μM. Phentolamine concentrations used were: (▼) 2.7 μM; (◇) 8.8 μM; (◆) 27 μM. Each point is the mean of 6 observations. Vertical lines show s.e.mean. On two concentration-response curves in (d) s.e.mean values have been omitted for clarity.

noradrenaline the difference between the slope and 1 is highly significant ($P < 0.001$), so the antagonism is either non-competitive or not occurring at some of the receptors at which noradrenaline is acting.

In control tissues the difference between original and subsequent responses to agonist were not statistically significant ($n = 6$ for each agonist). In addition the effects of ritodrine

Table 1 Inhibition of spontaneous activity in the rabbit jejunum. pD_2 values, Schild plot slopes, x-intercepts and pA_2 values obtained with various agonists and propranolol

		Propranolol Schild plot	
Agonist	*pD_2 value*[a]	*Slope*[b]	*x-intercept or pA_2 value*
Noradrenaline	7.4 ± 0.09 (12)*	0.28 ± 0.13 (6,18)†	6.9‡
Ritodrine	6.2 ± 0.04 (12)	0.88 ± 0.11 (6,24)	6.4§
Salbutamol	5.3 ± 0.10 (12)	0.93 ± 0.16 (6,24)	6.6§

Values shown as mean ± s.e.mean[a] or ± s.e.[b].
* Number of experiments.
† Number of experiments, number of points on Schild plot.
‡ x-intercept (slope significantly different from 1, $P < 0.05$).
§ pA_2 value (slope not significantly different from 1, $P > 0.05$).

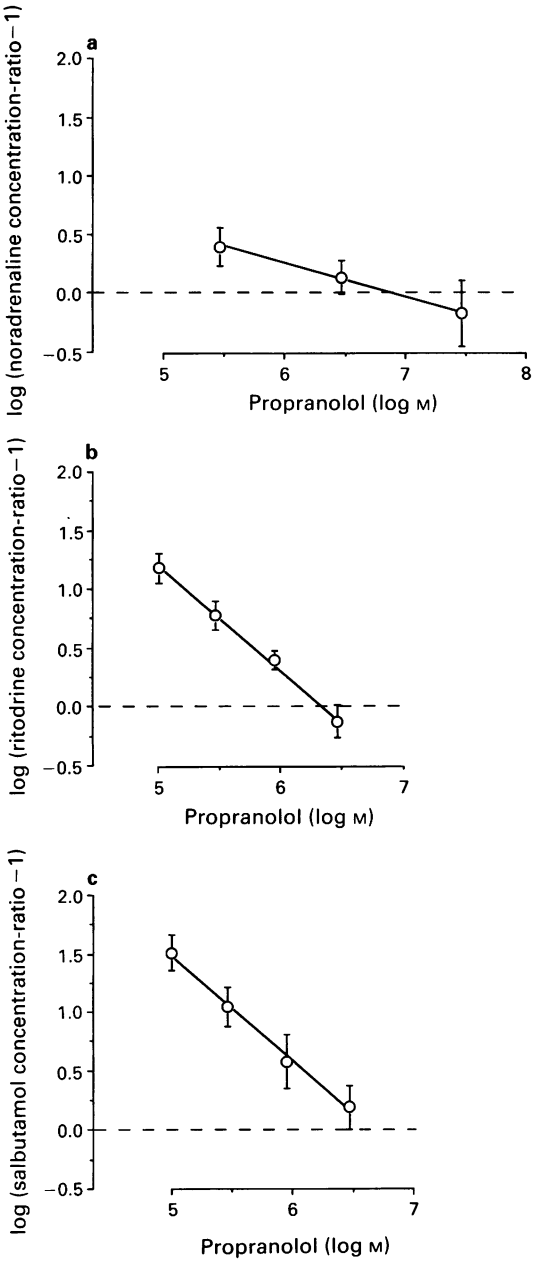

Figure 3 Schild plots (Arunlakshana & Schild, 1959) for propranolol antagonizing (a) noradrenaline, (b) ritodrine and (c) salbutamol in the rabbit jejunum. The regression lines shown were calculated by use of linear least squares regression analysis. Each point illustrated is the mean of 6 values. Vertical lines show s.e.mean.

(0.30 μM) or salbutamol (3.3 μM) were unaffected by the presence of yohimbine (2.6 μM), mepyramine (2.5 μM) or cimetidine (4.0 μM) ($n = 6$ for each agonist).

Discussion

The results are consistent with the existence in the rabbit jejunum of a β-adrenoceptor distinct from the well characterized β_1- or β_2-subtypes. Evidence for this can be drawn from both the pA_2 values obtained for propranolol and the relative potencies of salbutamol and ritodrine.

Established pA_2 values for propranolol acting at β_1- or β_2-adrenoceptors lie between 8.0 and 9.0 (Bristow *et al.*, 1970; Farmer & Coleman, 1970; Farmer & Levy, 1970; Lulich *et al.*, 1976), while the pA_2 value for propranolol acting against noradrenaline in the presence of phentolamine in the rabbit ileum has been determined as 9.8 (Wagner *et al.*, 1981). The considerable difference between these pA_2 values and those obtained in the present study (6.4 and 6.6 with ritodrine and salbutamol, respectively) indicates that in the rabbit jejunum propranolol antagonizes ritodrine and salbutamol at receptors different from conventional β_1- or β_2-adrenoceptors. As the slope of the Schild plots obtained with each agonist is close to 1 it is likely that both agonists are acting mainly at receptors where propranolol is a competitive antagonist. Furthermore, as the pA_2 values obtained with propranolol against ritodrine or salbutamol are similar both agonists are probably acting mainly at the same receptor.

These atypical adrenoceptors may co-exist with β_1-adrenoceptors identified in this tissue by Lands *et al.* (1967). It seems unlikely that the inhibition of spontaneous activity in the rabbit jejunum observed in our study is mediated by β_2-adrenoceptors because ritodrine was approximately 8 times as potent as salbutamol, as illustrated by a difference in pD_2 values for these agonists of just under 1 log unit, whereas ritodrine is less potent than salbutamol on conventional β_2-adrenoceptors in tissues such as the rat uterus or guinea-pig lung strip. For example, in uterus preparations from pregnant or ovariectomised rats, pD_2 values for ritodrine inhibiting the amplitude of longitudinal muscle contraction ranged from 7.24 (Ikeda & Tamaoki, 1984) to 7.3 (as calculated from the EC_{50} value, Izumi & Kishikawa, 1982), while corresponding values for salbutamol were in the range of 8.36 to 9.72 (Hollingsworth & Schnieden, 1973; Granger *et al.*, 1985). Likewise in the guinea-pig lung strip the pD_2 value for ritodrine-mediated relaxation was approximately 5.4 (as estimated from graphed data of Broadley *et al.*, 1986), while for salbutamol the pD_2 was approximately 7.0 (as estimated from graphed data of Schreurs *et al.*, 1980). These differences between relative potencies in tissues with conventional β_2-adrenoceptors compared with rabbit jejunum suggests that ritodrine and salbutamol are acting at receptors in the rabbit jejunum which are not typical β_2-adrenoceptors. However, the possibility that there is atypical coupling rather than an atypical receptor cannot be excluded.

To justify the designation of these receptors as a β-adrenoceptor subtype the possibility of these responses being mediated through a previously detected receptor not classified as a β-adrenoceptor needs to be excluded. α-Adrenoceptors have been shown to inhibit rabbit ileum contractile activity (Furchgott, 1960), but the present findings that only high concentrations of phentolamine (> 2.7 μM) antagonized the effects of ritodrine and salbutamol and that this antagonism was non-competitive are not consistent with blockade of conventional α_1-adrenoceptors. Moreover, although the responses to noradrenaline were antagonized competitively by low concentrations of phentolamine, at a high concentration the antagonism was of a non-competitive nature. The possibility that salbutamol and ritodrine act at α_2-adrenoceptors or H_1- or H_2-receptors can probably be excluded because yohimbine, mepyramine or cimetidine respectively at concentrations known to be effective at these receptors in other tissues (Arunlakshana & Schild, 1959; Brimblecombe *et al.*, 1975; Fuder *et al.*, 1983), did not alter the action of either adrenoceptor agonist in the rabbit jejunum.

There have been a number of reports of atypical β-adrenoceptors in various tissues. Directly relevant to the results given above is the atypical β-adrenoceptor which mediates lipolysis in both brown and white adipocytes (Harms *et al.*, 1974; Arch *et al.*, 1984; Wilson *et al.*, 1984; Grassby *et al.*, 1987). At this receptor the pA_2 values obtained for propranolol range from 6.2 to 6.84 (Harms *et al.*, 1974; Wilson *et al.*, 1984), while the pD_2 value for ritodrine is approximately 6.0 (as estimated from the graphed results of Grassby *et al.*, 1987) and the pD_2 values for salbutamol range from 4.9 to 5.6 (as calculated from EC_{50} values obtained by Wilson *et al.*, 1984 and Arch *et al.*, 1984). These values correspond fairly closely to those described here for the atypical β-adrenoceptor in the rabbit jejunum. Coleman *et al.* (1987) demonstrated, in the

guinea-pig gastric fundus, an atypical β-adrenoceptor which mediated relaxation and which appears to be similar in its sensitivity to propranolol and salbutamol to the rabbit jejunal β-adrenoceptor described here. Thus, at least two other tissues may possess atypical β-adrenoceptors which are similar in agonist and antagonist affinities to those atypical β-adrenoceptors described here. BRL 35135A is a potent and selective agonist at the atypical β-adrenoceptors in both these tissues (Wilson *et al.*, 1984; Colman *et al.*, 1987), but the action of this agent in the rabbit jejunum remains to be tested. An atypical adrenoceptor which inhibits longitudinal muscle tension development in the guinea-pig ileum has also been described (Bond & Clarke, 1988). However, this adrenoceptor, unlike that described here, is resistant to blockade by propranolol. There is also evidence for an atypical heart β-adrenoceptor in a number of different species (Kaumann, 1989).

In conclusion, the pA_2 values for propranolol acting against ritodrine and salbutamol, and the relative potencies of these agonists, differ in the rabbit jejunum from values for tissues containing typical β_1- or β_2-adrenoceptor subtypes. This constitutes provisional evidence for a β-adrenoceptor subtype not previously described in the rabbit jejunum but which appears to resemble the adrenoceptors of adipocytes and guinea-pig gastric fundus. However, further experimentation with a broader range of antagonists is required.

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PAF-induced bronchial hyperresponsiveness in the rabbit: contribution of platelets and airway smooth muscle

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1 Aerosol administration of platelet activating factor (PAF) to normal rabbits induced an enhanced airway responsiveness to inhaled histamine, 6 and 24 h after exposure. Following exposure to bovine serum albumin (BSA) as the carrier molecule for PAF, there was an increase in airway responsiveness to histamine 6 h after challenge, although by 24 h this was not significantly different from the responsiveness of airways to histamine before BSA.

2 PAF-induced bronchial hyperresponsiveness at 24 h was associated with a substantial increase in the number of neutrophils and mononuclear cells and a small, but significant increase in the number of eosinophils in the lungs as assessed by bronchoalveolar lavage. BSA exposure failed to alter the total number of cells in the lungs, although there was a significant increase in the number of neutrophils in the bronchoalveolar lavage fluid.

3 Selective platelet depletion with a guinea-pig anti-rabbit platelet serum inhibited PAF-induced bronchial hyperresponsiveness. In addition, there was an attenuation of PAF-induced airway inflammation in animals rendered thrombocytopenic.

4 The contractile potency to histamine, methacholine and carbachol was similar in intrapulmonary bronchi taken from rabbits exposed to an aerosol of BSA or PAF. Furthermore, the relaxant potency to the non-selective β-adrenoceptor agonist isoprenaline, was unaltered in PAF-treated rabbits. In contrast, there was a 2.58 fold reduction in the relaxant potency to theophylline in rabbits exposed to PAF compared with rabbits exposed to BSA.

5 These results suggest that in the rabbit, PAF-induced bronchial hyperresponsiveness at 24 h is associated with airways inflammation and is dependent upon platelet activation, but is not related to changes in airway smooth muscle function.

Introduction

Platelet activating factor (PAF) has been implicated in the pathogenesis of bronchial asthma as PAF has a number of biological properties of relevance to this disease (Page, 1988). PAF is of particular interest as it is able to induce a long-lasting and non-specific bronchial hyperresponsiveness both in experimental animals (Mazzoni *et al.*, 1985; Chung *et al.*, 1986; Barnes *et al.*, 1987; Robertson *et al.*, 1988) and man (Cuss *et al.*, 1986). However, the precise mechanisms underlying this heightened airway responsiveness are unknown. It has been suggested that bronchial hyperresponsiveness is secondary to airway inflammation as a number of stimuli capable of inducing bronchial hyperresponsiveness also induce an infiltration of inflammatory cells, principally neutrophils and eosinophils into the airways (Chung *et al.*, 1985; Murphy *et al.*, 1986). Moreover, depletion of circulating leucocytes has been demonstrated to inhibit the development of bronchial hyperresponsiveness in experimental animals (O'Byrne *et al.*, 1984; Marsh *et al.*, 1985), but this has not been observed by other investigators (Murlas & Roum, 1985; Thompson *et al.*, 1986; Cibulas *et al.*, 1988). Other investigators have also suggested that alterations in airway smooth muscle function (Antonnissen *et al.*, 1979), bronchial wall oedema (Persson, 1986) or geometric factors (James *et al.*, 1989) may contribute to bronchial hyperresponsiveness.

It is now recognized that some of the biological activities of PAF are secondary to platelet activation (Vargaftig *et al.*, 1980; Mazzoni *et al.*, 1985; Lellouch-Tubiana *et al.*, 1988) and that the platelet may act as an inflammatory cell in its own right (Page, 1989).

The present study has therefore attempted to investigate whether PAF-induced bronchial hyperresponsiveness is secondary to changes in airway smooth muscle function or platelet activation.

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Methods

In vivo *experiments*

New Zealand White rabbits (3–3.5 kg) were anaesthetized with diazepam (5 mg ml^{-1}, 5 mg kg^{-1} i.p.) and fentanyl citrate (0.315 mg ml^{-1}, 0.4 ml kg^{-1} i.m.) and placed in a supine position. Rabbits were intubated with a 3.0 mm endotracheal tube which was connected to a pneumotachograph (Bore 00) and the flow determined by a Validyne pressure transducer. Pleural pressure was estimated by placing an oesophageal balloon in the lower third of the oesphagus to obtain the maximum expiratory pressure. Thoracic pressure was measured by a sidehole catheter on the distal end of the tracheal tube. Transpulmonary pressure, the difference between thoracic and pleural pressure was measured with a differential pressure transducer (Validyne). The flow was integrated to obtain a continuous recording of tidal volume. Measurements of total lung resistance (R_L) and dynamic compliance (C_{dyn}) were calculated by a pulmonary mechanical analyser (Buxco, Model 6, Sharon, CT, U.S.A.) as described previously (Giles *et al.*, 1974). Anaesthesia was maintained throughout the course of the experiment by administration of 0.2–0.3 ml fentanyl citrate intramuscularly every 30–40 min, according to the protocol described by Flecknall (1987).

Assessment of airway responsiveness to histamine

Airway responsiveness to histamine was determined by exposing rabbits to increasing concentrations of histamine (1.25–80 mg ml^{-1}) administered to the lungs directly via an endotracheal tube. Aerosols were generated by a Devilbiss ultrasonic nebuliser which has previously been demonstrated to generate particles of which 80% are less than 0.5 μm in diameter.

After measurement of baseline lung function, animals were exposed to an aerosol of sterile saline for 2 min immediately

followed by lung function measurements. Increasing concentrations of histamine were administered, with each dose being given for 2 min, until there was at least a doubling in R_L or a 30% fall in C_{dyn}. The provocation dose (PD) of histamine which produced a 100% increase in R_L (PD_{100}) and 30% fall in C_{dyn} (PD_{30}) was determined for each animal and used as an index of airway responsiveness.

On day 2 animals were re-anaesthetized and a PAF challenge performed. After exposure to an aerosol of 0.25% bovine serum albumin (BSA) for 2 min, rabbits were exposed to PAF ($80\,\mu g\,ml^{-1}$) administered over a 1 h period. Lung function was then monitored for a 1 h period after exposure to PAF. Control animals received an aerosol of BSA followed by exposure to an aerosol of BSA containing the same concentration of ethanol as that received by the PAF-treated animals (approx. 1%).

On day 3, increasing concentrations of histamine were administered to the anaesthetized rabbits as on day 1 and the PD_{100} for histamine determined.

Bronchoalveolar lavage

On completion of the concentration-effect curve to histamine on day 1 and day 3, a bronchoalveolar lavage (BAL) was performed. Rabbits were lavaged by passing a cannula into the airways via the endotracheal tube until it was wedged. Three millilitres of 10% normal rabbit serum in 0.9% sterile saline was injected and immediately aspirated from the lungs. Total cell counts were determined under light microscopy by an improved Neubauer haemocytometer. Cytospins were prepared, fixed and stained with Lendrum's stain. A total of 200 cells were counted differentially and classified as neutrophils, eosinophils or mononuclear cells based on standard morphological criteria. The return volume of BAL was 1.97 ± 0.13 ml ($n = 15$) and had a coefficient of variation of 26%.

Preparation of anti-platelet serum

Guinea-pig anti-rabbit platelet serum (APS) was a kind gift of Dr K.D. Butler (Ciba-Geigy Pharmaceuticals, Horsham, U.K.), prepared according to the protocol described by Butler & Smith (1982).

Administration of anti-serum

Animals were treated with $0.5\,ml\,kg^{-1}$ of APS approximately 16 h before PAF or BSA challenge. Normal guinea-pig serum (NGPS) was administered in an identical manner to serve as an appropriate control. In 2 rabbits, administration of APS induced only a 70% depletion of platelets. In such circumstances, 0.2–0.4 ml of APS was administered slowly over a 1 h period via the marginal ear vein until the platelet count was reduced by at least 90%. In 5 rabbits, APS was administered 16 h before a second histamine dose-response curve to determine if thrombocytopenia *per se* modified airway responsiveness to histamine.

Enumeration of platelet and white blood cell counts

Before and 15 min after exposure to PAF, 3 ml of blood was taken from the central ear artery into 0.3 ml 3.8% sodium tricitrate. Total white blood cell counts were obtained under light microscopy following dilution with Turks reagent. Two millilitres of blood was then spun at $200\,g$ for 3 min and the platelet-rich plasma removed. The blood platelet count was enumerated under phase contrast light microscopy following dilution with brilliant cresyl blue and 3.8% sodium citrate. In addition, blood samples were taken from the ear vein before and 16 h after administration of either APS or NGPS and total blood counts performed as described above. Blood smears were also obtained, stained with Lendrum's stain and a total of 200 cells counted differentially.

In vitro *experiments*

New Zealand White rabbits (3–3.5 kg) treated with NGPS and exposed to an aerosol of either 0.25% BSA or PAF ($80\,\mu g\,ml^{-1}$) for 1 h and then challenged 24 h later with histamine, followed by BAL, were killed by exsanguination whilst under anaesthesia. The lungs were quickly removed and placed in cold, oxygenated Krebs-Henseleit solution. Rabbit intrapulmonary bronchi (1–3 mm i.d.) were dissected free of parenchymal tissue and visible blood vessels. Bronchial rings were suspended under 1 g tension in organ baths containing Krebs-Henseleit solution aerated with 95% O_2, 5% CO_2 and maintained at 37°C. Changes in isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on a 6 channel ectromed. All bronchial preparations were allowed to equilibrate for 45 min and decreases in resting tension which may have occurred during this period were compensated for by readjustment of tension to 1 g. The bathing solution was changed every 15 min before the addition of pharmacological agonists.

Following equilibration in Krebs-Henseleit solution, each bronchial preparation was exposed to a single concentration of carbachol (1 μM). When the contractile response had plateaued the preparations were once again washed and allowed to equilibrate for a further 45 min. Cumulative concentrations of either carbachol (0.1–100 μM), histamine (1–100 μM), methacholine (0.1–100 μM), isoprenaline (0.01–10 μM) or theophylline (0.01–5 mM) were added to bronchial preparations chosen randomly. In the case of isoprenaline and theophylline, bronchial preparations were contracted with a concentration of carbachol which produced 25% of the maximal response (EC_{25}) and cumulative concentrations of isoprenaline or theophylline superimposed on this contractile response. The pharmacological testing of all agonists in bronchi from 0.25% BSA and PAF-treated animals was performed blind. In a separate experiment, PAF (0.001–1 μM; dissolved in Krebs containing 0.25% BSA) was added to epithelium-intact bronchi from naïve rabbits, suspended in silicone-treated organ baths. Only one concentration of PAF was tested in each preparation to avoid the effects of tachyphylaxis.

Analysis of results

Results from *in vivo* studies are expressed as mean ± s.e.mean. *In vivo* histamine potency derived from measurements of resistance (PD_{100}) and dynamic compliance (PD_{30}) are expressed as geometric mean ± s.e.mean. Some of the data are expressed as the derived PD_{100} (antilog PD_{100}) together with upper and lower values for s.e.mean.

For the *in vitro* data, agonist potency ($pD_2 = \log_{10} EC_{50}$) and the maximal tension generated (E_{max}) were obtained. On each experimental day a mean pD_2 and E_{max} value respectively were obtained for each agonist. On completion of the study, the mean values were grouped according to the treatment and a grand mean ± s.e.mean was obtained for both pD_2 and E_{max}.

The probability (P) of differences between mean values was determined by Student's two tailed, paired or non-paired t test and was considered significant if $P < 0.05$. Analysis of variance (ANOVA) was used to analyse baseline R_L and C_{dyn} data. Analysis of covariance (ANCOVA) was used to analyse the BAL data and Kruskal-Wallis one-way analysis of variance was used to analyse the eosinophil data.

Drugs

Carbamylcholine hydrochloride, methacholine hydrochloride, histamine dihydrochloride, histamine diphosphate, (±)-isoprenaline hydrochloride, theophylline, bovine serum albumin Grade V (endotoxin free) and normal rabbit serum were all obtained from Sigma (Poole, Dorset): PAF was purchased from Novabiochem (Nottingham, U.K.); diazepam (Roche, U.K.); fentanyl citrate (Janssen Pharmaceutical Ltd, U.K.).

Anti-platelet serum was a kind gift of Dr K.D. Butler, Ciba-Geigy Pharmaceuticals (Horsham, U.K.). Composition of the Krebs-Henseleit solution (mM): NaCl 117.6, $NaHCO_3$ 25, D-glucose 11.1, KCl 5.4, $MgSO_4$ 0.57, KH_2PO_4 1.03 and $CaCl_2$ 2.5. Isoprenaline was dissolved in 0.9% NaCl solution containing 20 $\mu g\,ml^{-1}$ ascorbic acid.

Results

In vivo *studies*

PAF (80 $\mu g\,ml^{-1}$) induced acute bronchoconstriction in control animals, R_L increased by 146.2 ± 40.7% ($n = 6$) and C_{dyn} decreased by 30.6 ± 4.7% ($n = 6$) (Figure 1). PAF-induced changes in pulmonary function diminished over the next hour, although the values for R_L and C_{dyn} did not return completely to baseline (Figure 1). Exposure to 0.25% BSA induced a small but significant bronchoconstriction (R_L +10.7 ± 2.3%, C_{dyn} −7.2 ± 3.2%, $P < 0.05$). Platelet depletion reduced the magnitude of PAF-induced bronchoconstriction compared with rabbits treated with NGPS (R_L +29.3 ± 5.5%, C_{dyn} −11.8 ± 6.6%, $n = 5$, $P < 0.05$, Figure 2).

Effect of PAF on airway responsiveness to histamine

Resistance The concentration of histamine required to increase R_L by 100% (PD_{100}) was 47.1 mg ml^{-1} (42.0–52.8; $n = 10$). A significant increase in airway responsiveness to histamine was observed 6 h (12.0 mg ml^{-1} (9.5–15.1), $P < 0.05$; $n = 4$) and 24 h (19.0 mg ml^{-1} (14.4–25.1), $P < 0.05$; $n = 5$) but not 72 h (52.5 mg ml^{-1} (44.2–62.4), $P > 0.05$; $n = 4$) following exposure to an aerosol of PAF.

Airway responsiveness to histamine was not significantly different 6 h (33.1 mg ml^{-1} (25.9–42.4), $P > 0.05$; $n = 2$), 24 h (44.7 mg ml^{-1} (38.5–51.8), $P > 0.05$; $n = 5$) and 72 h (61.4 mg ml^{-1} (45.5–82.8), $P > 0.05$; $n = 4$) following exposure to an aerosol of BSA. The mean results shown in Figure 3a were obtained after logarithmic transformation of the data.

Compliance The concentration of histamine required to decrease C_{dyn} by 30% (PD_{30}) was 60.7 mg ml^{-1} (55.3–66.0; $n = 10$). A significant increase in airway responsiveness to histamine was observed 6 h (20.4 mg ml^{-1} (12.0–34.7), $P < 0.05$; $n = 5$) and 24 h (23.4 mg ml^{-1} (18.2–30.2), $P < 0.05$; $n = 5$) but not 72 h (74.1 mg ml^{-1} (61.6–100.0), $P > 0.05$; $n = 4$) following exposure to an aerosol of PAF.

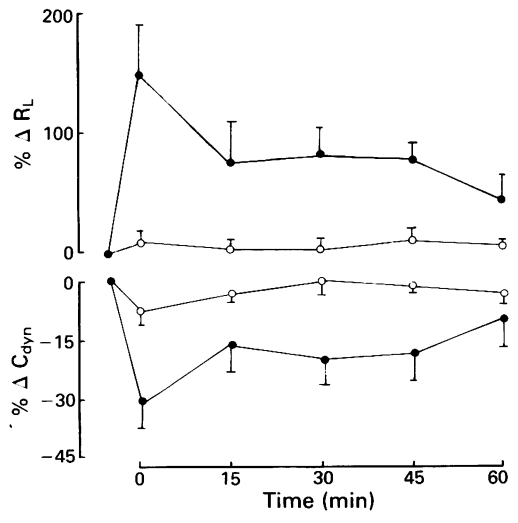

Figure 1 Changes in total lung resistance (R_L) and dynamic compliance (C_{dyn}) recorded over a 1 h period following a 1 h aerosol exposure to 0.25% bovine serum albumin (○) or PAF 80 $\mu g\,ml^{-1}$ (●) in rabbits. Each point is the mean from 6 animals and vertical lines show s.e.mean.

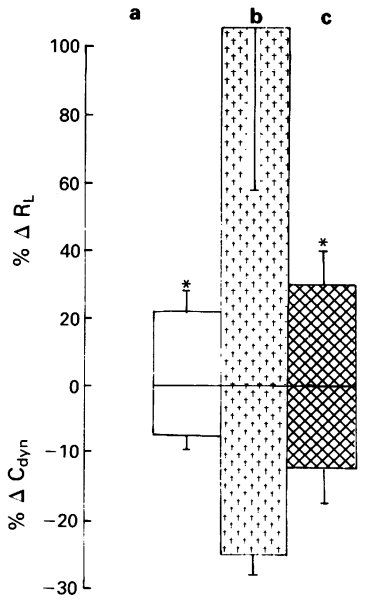

Figure 2 Changes in total lung resistance (R_L) and dynamic compliance (C_{dyn}) in rabbits treated with normal guinea-pig serum (NGPS) (columns a and b, $n = 5$) and in rabbits rendered thrombocytopenic (cross-hatched columns (c), $n = 5$) following a 1 h aerosol exposure to 0.25% bovine serum albumin (a, open columns) or 80 $\mu g\,ml^{-1}$ PAF (columns b and c). Results expressed as mean and bars show s.e.mean. * PAF-induced bronchoconstriction significantly reduced in platelet-depleted rabbits compared with NGPS-treated rabbits ($P < 0.05$, non-paired t test).

Airway responsiveness to histamine was significantly increased 6 h after exposure to BSA (30.9 mg ml^{-1} (25.6–37.2), $P < 0.05$; $n = 4$), but to a lesser extent than that observed to PAF. Airway responsiveness to histamine was not significantly different 24 h (60.2 mg ml^{-1} (54.0–67.3), $P > 0.05$;

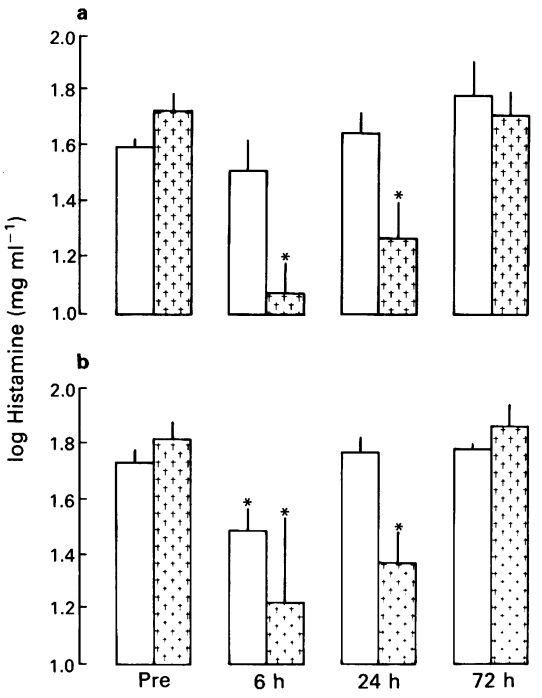

Figure 3 Airway responsiveness to histamine (a) log PD_{100} resistance and (b) log PD_{30} compliance, 6, 24 and 72 h after aerosol exposure to 0.25% bovine serum albumin (BSA) (open columns) or 80 $\mu g\,ml^{-1}$ PAF (shaded columns). Results are expressed as geometric mean from 4–5 observations and bars show s.e.mean. * $P < 0.05$ (paired t test) compared with airways responsiveness to histamine before exposure to BSA or PAF (pretreatment (Pre) value).

$n = 5$) and 72 h (61.6 mg ml^{-1} (57.8–65.8), $P > 0.05$; $n = 4$) following exposure to an aerosol of BSA. The mean results shown in Figure 3b were obtained after logarithmic transformation of the data.

Mean pretreatment baseline R_L and C_{dyn} values were 15.8 $\pm$ 1.5 cmH_2O l^{-1} s^{-1} ($n = 10$) and 3.2 $\pm$ 0.4 ml cmH_2O^{-1} ($n = 10$), respectively. Furthermore, there was no significant difference in baseline R_L and C_{dyn} at 6, 24 and 72 h ($P > 0.05$, ANOVA).

Effect of PAF on circulating platelets and leucocytes

Aerosol administration of PAF failed to alter the number of circulating platelets (pretreatment: 3.39 $\pm$ 0.35 $\times$ 10^5 cells μl^{-1}; post-treatment: 3.50 $\pm$ 0.53 $\times$ 10^5 cells μl^{-1}; $n = 3$) or leucocytes (pretreatment: 1.28 $\pm$ 0.25 $\times$ 10^4 cells ml^{-1}; post-treatment: 1.08 $\pm$ 0.50 $\times$ 10^4 cells ml^{-1}; $n = 3$). No differential cell count was perfomed on the latter cell type.

Effect of administration of anti-platelet or normal serum on circulating platelet and white blood cell count

Administration of either anti-platelet serum (APS) or normal guinea-pig serum (NGPS) failed to alter the total number of circulating leucocytes compared with untreated animals (Table 1). A significant increase in the number of neutrophils and a decrease in the number of mononuclear cells following administration of APS compared with control animals was observed. However, administration of the NGPS induced a similar neutrophilia and reduction in the number of mononuclear cells which was not significantly different from that induced by APS (Table 1).

Effect of platelet depletion on histamine-induced bronchoconstriction

The R_L PD_{100} following histamine administration was 50.1 mg ml^{-1} (42.7–58.9; $n = 5$). Administration of APS failed to modify histamine-induced bronchoconstriction (PD_{100} 44.7 mg ml^{-1} (33.9–58.9), $n = 5$; $P > 0.05$).

Effect of platelet depletion on PAF-induced bronchial hyperresponsiveness

In another series of experiments, an increase in airways responsiveness to histamine was observed 24 h following exposure to PAF in naïve (pretreatment PD_{100}: 51.3 mg ml^{-1} (43.2–60.8); 24 h post-treatment: PD_{100} 30.2 mg ml^{-1} (24.0–38.0), $n = 9$, $P < 0.05$, Figure 4a) and NGPS-treated rabbits (pretreatment: 58.9 mg ml^{-1} (47.0–73.8); (post-treatment: 28.8 mg ml^{-1} (21.9–38.0), $n = 6$, $P < 0.05$, Figure 4a). NGPS treatment alone failed to alter the PAF-induced increase in airway responsiveness to histamine ($P < 0.05$). PAF increased airway responsiveness to histamine by 2 fold or greater in 5 rabbits, by 1.4 fold in 7 rabbits and minimally in 3 rabbits (Figure 4a). In contrast, airway responsiveness to histamine was not increased 24 h following exposure to BSA in NGPS-treated rabbits (pretreatment: 45.3 mg ml^{-1} (32.2–63.7): post-treatment: 43.2 mg ml^{-1} (32.1–58.2), $n = 5$, $P > 0.05$, Figure 4b). In animals rendered thrombocytopenic, PAF failed to increase airway responsiveness to histamine (pretreatment: 56.0 mg ml^{-1} (43.6–72.4); post-treatment: 54.2 mg ml^{-1} (38.4–76.6), $n = 5$, $P > 0.05$, Figure 4c). The individual data for the 3 groups of rabbits are shown in Figure 4.

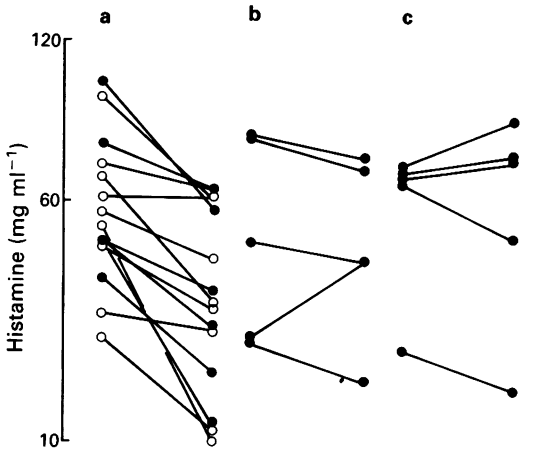

Figure 4 Individual histamine PD_{100} values before and 24 h after aerosol exposure to (a) PAF 80 μg ml^{-1} in naïve rabbits (○) or rabbits treated with normal guinea-pig serum (●), (b) 0.25% bovine serum albumin or (c) PAF 80 μg ml^{-1} in rabbits treated with guinea-pig anti-rabbit platelet serum.

Bronchoalveolar lavage

Before PAF exposure, mononuclear cells comprised greater than 95% of the resident cell population with a small (generally less than 5%) number of neutrophils. The mean pretreatment cell counts from the 3 groups were not significantly different ($P > 0.05$, ANOVA) and consequently the pretreatment cell counts from each group were pooled, as presented in Table 2. PAF induced a significant increase in the total number of leucocytes in the airways as assessed by BAL, 24 h after PAF exposure in animals treated with NGPS (ANCOVA $F(2,11) = 6.2$, $P < 0.01$; Table 2). Although there was an increase in the numbers of neutrophils and monocytes in BAL following PAF this was not significant ($P > 0.05$, ANCOVA). In contrast, a small but significant increase in the numbers of BAL eosinophils following PAF was observed ($P < 0.05$). Exposure to an aerosol of BSA in rabbits treated with NGPS induced a small but insignificant increase in the

Table 1 Effect of normal guinea-pig serum (NGPS) and anti-platelet serum (APS) on circulatory cell numbers in the rabbit

	Pretreatment	*Post NGPS*	*Post APS*
		(cells μl^{-1})	
Platelets	381545 $\pm$ 56056	436800 $\pm$ 79867	29286 $\pm$ 4082*
		($\times 10^3$ cells μl^{-1})	
White blood cells	13.2 $\pm$ 1.00	11.4 $\pm$ 1.30	12.2 $\pm$ 1.70
Neutrophils	4.80 $\pm$ 0.46	6.43 $\pm$ 1.23*	5.89 $\pm$ 0.65*†
Eosinophils	0.25 $\pm$ 0.03	0.30 $\pm$ 0.05	0.29 $\pm$ 0.04
Mononuclear cells	6.60 $\pm$ 1.00	3.64 $\pm$ 0.37*	4.91 $\pm$ 1.00*†
Basophils	1.00 $\pm$ 1.10	1.03 $\pm$ 0.22	1.07 $\pm$ 0.16

Results are expressed as mean $\pm$ s.e.mean. $n = 5$–6 rabbits.
* Significant difference compared with pretreatment value ($P < 0.05$, paired t test).
† No significant difference compared with post NGPS value ($P > 0.05$, non-paired t test).

Table 2 Effect of PAF on pulmonary cell accumulation in rabbits treated with normal guinea-pig serum (NGPS) or anti-platelet serum (APS)

	Total	*Neutrophils*	*Eosinophils*	*Monocytes*
		($\times 10^5$ cells ml^{-1})		
Pretreatment	3.38 ± 0.44	0.16 ± 0.05	0	2.79 ± 0.39
BSA	4.78 ± 0.83	1.78 ± 0.28	0.006 ± 0.006	2.96 ± 0.84
PAF/NGPS	15.88 ± 4.26*	5.57 ± 2.14	0.568 ± 0.207*	9.69 ± 5.0
PAF/APS	3.57 ± 1.18†	2.11 ± 0.59	0.014 ± 0.006†	2.04 ± 0.81

Results are expressed as mean ± s.e.mean. $n = 5$ animals in each group. The prechallenge lavage data are the mean of 15 rabbits.
* Significant increase in total cell numbers ($P < 0.01$, non-paired t test) and eosinophils ($P < 0.05$, Mann-Whitney test) compared with BSA group.
No significant difference in total cell numbers or eosinophils compared with BSA group. († $P > 0.05$).

total cell infiltrate (Table 2). However, there was a significant increase in the number of neutrophils in the airways after BSA ($P < 0.05$, paired t test). BSA treatment induced no significant changes in the number of eosinophils or mononuclear cells. Depletion of circulating platelets reduced the PAF-induced leucocyte infiltration ($P < 0.05$) and this was associated with a reduction in the number of eosinophils (Table 2).

In vitro *studies*

Contractile agonists Carbachol, methacholine and histamine produced concentration-dependent contractions in bronchial preparations from rabbits exposed to BSA or PAF. However, there was no significant difference in either the contractile potency (pD_2) or maximal tension generated (mg mg^{-1} wet weight) for carbachol, methacholine and histamine in bronchi from rabbits exposed to BSA or PAF ($P > 0.05$; Tables 3 and 4). PAF (0.001–1 μM) itself failed to contract isolated bronchi from naïve rabbits ($n = 3$ rabbits providing 18 bronchial preparations).

Table 3 Contractile and relaxant potency (pD_2) of various agonists in bronchial preparations from rabbits exposed 24 h previously to 0.25% BSA or PAF (80 μg ml^{-1})

	BSA	*PAF*
	pD_2	
Contractile agonists		
Carbachol	−6.356 ± 0.064 5(17)	−6.211 ± 0.047 5(17)
Methacholine	−5.768 ± 0.071 5(18)	−5.773 ± 0.150 5(17)
Histamine	−5.100 ± 0.156 5(23)	−4.974 ± 0.159 5(21)
Relaxant agonists		
Isoprenaline	−6.871 ± 0.226 5(11)	−6.690 ± 0.130 4(7)
Theophylline	−4.101 ± 0.105 5(12)	−3.691 ± 0.068* 5(14)

Results are expressed as mean ± s.e.mean. $pD_2 = \log_{10} EC_{50}$.
n (number below each result) = number of animals and n (number in parentheses) indicates the total number of bronchial preparations. * Relaxant potency for theophylline significantly reduced in rabbits exposed to PAF compared with BSA ($P < 0.01$, non-paired t test).

Table 4 Maximal tension generated (E_{max}) by various contractile agonists in rabbits treated 24 h previously with 0.25% BSA or PAF (80 μg ml^{-1})

	BSA	*PAF*
	E_{max} (mg mg^{-1} wet weight)	
Carbachol	200 ± 50	136 ± 27
Methacholine	202 ± 48	167 ± 41
Histamine	168 ± 46	131 ± 27

Results are expressed as mean ± s.e.mean. n = similar to those given in Table 2.

Relaxant agonists Isoprenaline and theophylline produced concentration-dependent relaxation of carbachol-induced tone in bronchial preparations from both 0.25% BSA-treated and PAF-treated rabbits. The maximal relaxant response was 70 ± 11% ($n = 5$) and 78 ± 22% ($n = 4$) in rabbits exposed to BSA and PAF, respectively. Similarly, for theophylline the maximal relaxant response was 154 ± 19% ($n = 5$) and 147 ± 12% ($n = 5$) in rabbits exposed to BSA and PAF, respectively. The relaxant potency of isoprenaline was not significantly different in bronchial preparations from rabbits exposed to BSA compared with PAF ($P > 0.05$, Table 3). In contrast the relaxant potency to theophylline was reduced by 2.58 fold in bronchial preparations from rabbits exposed to PAF compared with those from rabbits exposed to BSA (Table 3).

Discussion

It is becoming increasingly apparent that PAF may play a significant role in the pathogenesis of asthma, as it mimics many of the features which are characteristic of asthma (Barnes *et al.*, 1988; Page, 1988). We have therefore investigated the ability of PAF to induce airway hyperresponsiveness and inflammation in the rabbit and assessed the relative contribution of the platelet and airway smooth muscle function to these responses.

Exposure of rabbits to an aerosol of PAF induced acute bronchoconstriction. PAF has previously been shown to induce a platelet-dependent bronchoconstriction in this and other species following intravenous administration (Vargaftig *et al.*, 1980; Halonen *et al.*, 1985). Similarly in our experiments, aerosol administration of PAF induced a platelet-dependent bronchoconstriction. Furthermore, isolated bronchial preparations failed to contract to PAF under conditions where they readily contracted to other spasmogens. Our *in vitro* results are consistent with the inability of PAF to contract canine trachea (Popovich *et al.*, 1988), guinea-pig trachea (Cerrina *et al.*, 1983) and human bronchus (Schellenberg *et al.*, 1983) unless platelets are present (Schellenberg *et al.*, 1983; Popovich *et al.*, 1988). However, our results are in contrast to those of Lefort *et al.* (1984), who demonstrated that whilst bronchoconstriction induced by systemically administered PAF was platelet-dependent in the guinea-pig, an aerosol of PAF induced a platelet-independent bronchoconstriction.

No change in the number of circulating platelets or leucocytes was observed following aerosol exposure to PAF. This is

consistent with studies performed in the guinea-pig (Lefort *et al.*, 1984). However, a change in a minority cell population may still have occurred which we did not detect as we did not perform differential leucocyte counts on peripheral blood samples. In man, a transient neutropenia without thrombocytopenia was observed following inhalation of PAF (Roberts *et al.*, 1988). The lack of an effect on circulating platelets in man and rabbit suggests that platelet aggregation *per se* is not necessary for PAF-induced effects in the lung, consistent with observations made in other species (Robertson & Page, 1987).

Following exposure to PAF, airway responsiveness to histamine was increased at 6–24 h later. Such effects are consistent with those obtained following systemic administration or inhalation of PAF in the dog (Chung *et al.*, 1986), guinea-pig (Mazzoni *et al.*, 1985; Barnes *et al.*, 1987; Robertson & Page, 1987; Coyle *et al.*, 1988a; Robertson *et al.*, 1988), rabbit (Nieminen *et al.*, 1988) and in man (Cuss *et al.*, 1986). In our study the ability of PAF to induce airway hyperresponsiveness to histamine was relatively small (approx 1.5–4 fold) and did not occur in all animals. This observation is of interest for it has rescently been found that PAF will not induce bronchial hyperresponsiveness in all subjects (Jenkins *et al.*, 1989) and in the rabbit at least, is susceptible to changes in body temperature (Nieminen *et al.*, 1989).

The underlying mechanisms whereby PAF can induce an increase in airway responsiveness is unclear. However, platelet activation appears to be important, as platelet depletion with a selective anti-serum inhibited PAF-induced airway hyperresponsiveness. Our results confirm previous observations in the guinea-pig that PAF induces a platelet-dependent increase in airway responsiveness (Mazzoni *et al.*, 1985).

Studies performed in both experimental animals and man suggest airway inflammation is important in heightened airway responsiveness (Chung, 1987). In the present study, PAF induced a 4 fold increase in the number of leucocytes in the airways as assessed by BAL and our results are consistent with previous *in vivo* findings that PAF can elicit airway inflammation (Camussi *et al.*, 1983; Lellouch-Tubiana *et al.*, 1988; Coyle *et al.*, 1988a). The differential cell counts revealed a trend towards an increase in the number of neutrophils and mononuclear cells which, due to the variability in the magnitude of the change between animals, did not reach statistical significance. However, there was a significant increase in the number of eosinophils in BAL fluid obtained from rabbits 24 h after exposure to PAF, which was small when compared with the increase in the numbers of neutrophils and mononuclear cells. Exposure to BSA also produced an increase in the number of neutrophils, probably as a result of non-specific inflammation.

PAF has been demonstrated to be a potent activation stimulus for neutrophils (Kurihara *et al.*, 1989), mononuclear cells (Yasaka *et al.*, 1982) and eosinophils (Wardlaw *et al.*, 1986; Tamura *et al.*, 1987). Furthermore, specific binding sites for PAF have recently been indentified on the surface of these cell types (Kurihara *et al.*, 1989). PAF has also been demonstrated to induce neutrophil and mononuclear cell accumulation *in vivo* following intracutaneous adminstration in healthy individuals (Archer *et al.*, 1985), whereas local administration of PAF to the skin of allergic subjects induces predominantly an eosinophil-rich infiltrate (Henocq & Vargaftig, 1986). Aerosol administration of PAF in man induces a transient neutropenia which probably reflects sequestration of neutrophils in the pulmonary vasculature (Roberts *et al.*, 1988). PAF exposure also induced an increase in the number of mononuclear cells in the BAL 24 h after challenge and these observations are consistent with those observed following intratracheal administration of PAF in the rabbit (Camussi *et al.*, 1983).

In these experiments, the ability of PAF to induce leucocyte infiltration into the airways was dependent on platelet activation, since the total number of inflammatory cells found in lavage fluid after exposure to PAF was significantly reduced in thrombocytopenic animals. The differential cell counts only revealed significance for the effect of APS on PAF-induced eosinophil infiltration, although there was a tendency for the number of neutrophils and mononuclear cells to fall as well. The precise mechanisms underlying platelet-dependent cellular infiltration are unknown, but our results are consistent with a platelet-dependent eosinophilia observed in the rabbit following exposure to allergen (Coyle *et al.*, 1988b) and in guinea-pig following exposure to PAF or allergen (Lellouch-Tubiana *et al.*, 1988). However, recent studies in the guinea-pig have not found any significant effect of platelet depletion on eosinophil infiltration in the lung induced by an aerosol of PAF (Sanjar *et al.*, 1990). Nevertheless, it is recognized that platelets can release a variety of chemotactic factors for neutrophils (e.g. platelet factor 4 (PF4), platelet derived growth factor (PDGF) and certain lipoxygenase metabolites of arachidonic acid), eosinophils (PF4) and mononuclear cells (e.g. PF4, transforming growth factor-beta (TGF-β)) (Page, 1989). Our results are of interest because platelet activation has recently been suggested to occur chiefly in allergic asthmatics, and increased platelet turnover (Gresele *et al.*, 1987), reduced platelet survival time (Taytard *et al.*, 1986) and prolonged bleeding times (Szczeklik *et al.*, 1986) have all been found to be features of allergic asthma. However, such changes have not been observed consistently within the allergic population (Hemmendinger *et al.*, 1989). Nevertheless, platelet aggregates have recently been found in the lung vasculature (Beasley *et al.*, 1989), platelets have been observed on damaged airway epithelium in symptomatic asthmatics (Jeffery *et al.*, 1989) and in BAL fluid obtained from allergic asthmatics (Metzger *et al.*, 1986) following allergen challenge. Furthermore, the exercise-induced rise in plasma levels of PF4 and β-thromboglobulin were attenuated in asthmatics following treatment with the PAF-antagonist BN 52036 (Wilkens *et al.*, 1990).

We have also investigated whether altered smooth muscle function could account for the bronchial hyperresponsiveness observed *in vitro*. The contractile potency to histamine, carbachol and methacholine was not enhanced in rabbits exposed to PAF compared with rabbits exposed to BSA. Similarly, the maximum tension developed in response to these spasmogens was similar in bronchi taken from both sources. These results are consistent with those found in guinea-pigs exposed to PAF (Robertson *et al.*, 1988) and suggest that alterations in airway smooth muscle function *per se* are not responsible for PAF-induced bronchial hyperresponsiveness. Similarly, no alteration in airway smooth muscle function has been observed in bronchi taken from asthmatic lung (Cerrina *et al.*, 1986; Goldie *et al.*, 1986) except in isolated cases (Schellenberg & Foster, 1984; De Jongste *et al.*, 1987).

We also assessed whether PAF-induced bronchial hyperresponsiveness was associated with a loss in airway β-adrenoceptor function. There was no alteration in the relaxant potency to isoprenaline in bronchi from rabbits exposed to PAF compared with BSA-treated rabbits, consistent with previous findings in other species (Barnes *et al.*, 1987). However, other investigators have demonstrated that incubation of lung membranes with PAF did result in a reduction in β-adrenoceptor density (Agrawal & Townley, 1987), an effect presumably due to the high concentrations of PAF utilised *in vitro*. Furthermore, our observations in the rabbit are consistent with those obtained in mild asthma where no alteration in β-adrenoceptor function has been observed (Svedymyr *et al.*, 1977). However, in severe asthma, β-adrenoceptor dysfunction has been demonstrated (Cerrina *et al.*, 1986; Goldie *et al.*, 1986). In contrast, the potency of theophylline was reduced in bronchi taken from rabbits previously exposed to PAF. The reasons for this remain unclear but may involve a cyclic AMP-independent mechanism, since β-adrenoceptor function was unaltered.

PAF has recently been shown to be a potent substrate at inducing vascular permeability in the airways (Evans *et al.*, 1987) and airway oedema has been suggested to contribute to airway hyperresponsiveness (Persson, 1986). However, recent investigations in the guinea-pig have suggested no causal relationship between airway vascular permeability and bron-

chial hyperresponsiveness (Roberts & Barnes, 1989). Chronic administration of PAF has also been shown to induce changes in smooth muscle thickening (Touvay *et al.*, 1990) and airway connective tissue (Schellenberg, 1987) which may occur via a different mechanism from that described in the present study. The contribution of platelets and airways inflammation to those long-term anatomical changes remains unknown.

In summary, we have demonstrated that PAF induces both bronchial hyperresponsiveness and airway inflammation which are dependent on some aspect of platelet activation. However, this increase in airway responsiveness is not a consequence of changes in airway smooth muscle function *per se*.

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The effects of acute and chronic lithium treatment on pilocarpine-stimulated phosphoinositide hydrolysis in mouse brain *in vivo*

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1 Measurements were made of the *in vivo* formation of inositol phosphates in the brains of C57/B1/601a mice treated acutely or chronically with lithium chloride (LiCl).

2 A single injection of LiCl (10 mEquiv kg^{-1}, s.c.) 18 h before death increased the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-Ins P's) in the brains of mice injected i.c.v. with [^{3}H]-myo-inositol 24 h previously.

3 Pilocarpine (200 mg kg^{-1}, i.p.) injected 15 min before death further enhanced the formation of [^{3}H]-Ins P's in the brains of LiCl-treated, but not saline-treated, mice. The enhancement due to pilocarpine was abolished by injection of atropine sulphate (10 mg kg^{-1}, i.p.) 10 min earlier.

4 Chronic (14 days) LiCl feeding produced an accumulation of [^{3}H]-Ins P's significantly less than that due to a single injection of LiCl, but the response to pilocarpine was markedly greater in mice chronically fed with LiCl when compared with mice acutely injected with LiCl.

5 Mass measurements of endogenous inositol 1,4,5 triphosphate revealed increases due to pilocarpine and chronic LiCl feeding alone. A combination of the two treatments produced levels greater than either alone.

6 These results demonstrate that LiCl treatment enhances both basal and pilocarpine-stimulated inositol phospholipid hydrolysis *in vivo* and this might be relevant to its therapeutic effects.

Introduction

Although lithium salts have been widely employed for many years as an effective therapy for acute mania and as a prophylactic treatment for manic-depressive disorders, the neurochemical basis for their effectiveness is still unclear (Wood & Goodwin, 1987).

Recently, attention has focused on the post-receptor effects of lithium in central nervous system preparations. Lithium is known to inhibit the formation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) stimulated by activation of a number of receptors (Ebstein *et al.*, 1980) and has now been shown to interact with another second messenger generating system, i.e. the phosphoinositide cycle (Berridge, 1984).

It has been suggested by Berridge *et al.* (1982) that because lithium inhibits inositol 1 phosphate phosphatase and thereby prevents the re-synthesis of phosphatidylinositol, the beneficial effects of lithium might be related to a reduction in the functional activity of Ca^{2+}-mobilizing receptors. In support of this, various *ex vivo* studies have demonstrated a reduction in [^{3}H]-inositol phosphate production in brain slices from lithium-treated animals, following incorporation of [^{3}H]-myo-inositol and stimulation with calcium-mobilizing receptor agonists or sodium fluoride *in vitro* (Kendall & Nahorski, 1987; Godfrey *et al.*, 1989; Whitworth & Kendall, 1988b).

Other *in vitro* studies have shown that lithium has multiple effects on the generation of inositol polyphosphates; most notably an inhibition of inositol tetrakisphosphate production in both rat and mouse brain slices (Batty & Nahorski, 1987; Whitworth & Kendall, 1988a) indicating that the mechanism of action of lithium could be more complex than reducing the synthesis of inositol phospholipids.

In this study, we have examined the effects of lithium treatment on the *in vivo* stimulation of inositol phosphates in mouse brain by the muscarinic agonist, pilocarpine, and have demonstrated that the results of *in vivo* and *ex vivo* experiments are radically different.

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Methods

Drug administration

Male C57/B1/601a mice (20–25 g; Olac, Bicester) were housed in groups of ten on a 12 h light: 12 h dark cycle. They were maintained on a diet containing lithium chloride (LiCl) (0.4% w/w) for 14 days. Control animals were fed a similar diet without added lithium. Water and 0.9% NaCl solution (saline) were provided *ad libitum* for both groups of animals. Although LiCl-fed animals exhibited an approximate 14% weight loss, while control animals showed an 8% gain, there was no associated mortality and mice appeared to be generally healthy.

In experiments involving acute lithium treatment, animals were given a loading dose of 10 mEquiv kg^{-1} LiCl dissolved in saline and injected subcutaneously (s.c.), 18 h before they were killed.

Those animals treated with pilocarpine, received a single intraperitoneal injection of 200 mg kg^{-1}, 15 min before they were killed.

Atropine sulphate was administered as a single injection (10 mg kg^{-1}, i.p.) 10 min before pilocarpine, or, in the absence of pilocarpine, 25 min before the mice were killed.

For those experiments concerned with the measurement of [^{3}H]-inositol phosphates ([^{3}H]-Ins P's), the mice were given intracerebroventricular (i.c.v.) injections while under brief halothane anaesthesia (Heal, 1984) of 4 μCi [^{3}H]-myo-inositol in 4 μl saline for acute or chronic experiments, 24 h before being killed.

Lithium ion (Li^+) levels

Portions of brain from LiCl fed rats were homogenized in 10% perchloric acid (PCA) (1 ml 300 mg $^{-1}$ wet weight tissue),

centrifuged (1000 *g*, 20 min) and the supernatant assayed for Li^+ by flame photometry.

Total [^{3}H]-inositol phosphate accumulation

Mice were killed by focused microwave irradiation (2.25 kW at 2450 MHz for 2 s directed onto the head and neck region) and the brains were removed and dissected on ice. Portions containing the cerebral cortex and hippocampus were separated and sonicated in 1 ml ice-cold perchloric acid (PCA) (10% w/v) for 20–30 s to extract the [^{3}H]-Ins P's. The remaining portions of brain were frozen and stored for later analysis of the Li^+ levels by flame photometry.

Sonicated samples were neutralised with KOH (1.5 M) and left on ice for at least 20 min before centrifugation at 2000 *g* for 20 min. Then 1 ml of the supernatant was added to 2.25 ml Tris buffer (50 mM), mixed and taken for analysis of total [^{3}H]-Ins P's accumulation by anion exchange chromatography on Dowex chloride form columns. The columns were washed with 20 ml H_2O before elution of the [^{3}H]-Ins P's from the columns with 3 ml HCl (1 M).

Excess supernatant was discarded and the remaining tissue was added to 0.94 ml of chloroform : methanol : conc HCl (100 : 200 : 1) followed by further aliquots of chloroform (0.32 ml) and water (0.32 ml) to extract the [^{3}H]-inositol phospholipids. The 200 μl samples of the chloroform phase containing [^{3}H]-inositol phospholipids were placed in scintillation vials and left to evaporate overnight. Scintillation fluid was added to all the [^{3}H]-Ins P's or [^{3}H]-inositol phospholipid samples and the radioactivity determined by liquid scintillation counting.

Separation of [^{3}H]-Ins 1,4,5 trisphosphate ([^{3}H]-Ins 1,4,5 P_3)

Samples were prepared and total [^{3}H]-Ins P's extracted into 10% PCA as described above. Subsequently 1.5 ml of neutralised supernatant was added to a HEPES/EDTA buffer, (final concentration HEPES 25 mM; EDTA 5 mM) pH 7.0. [^{3}H]-Ins 1,3,4 P_3 contained within these samples was then subjected to selective hydrolysis by the addition of the enzyme preparation obtained from a rat whole brain homogenate (Kennedy *et al.*, 1989) (25% w/v 25 mM HEPES; 2 mM $MgCl_2$) centrifuged at 100 000 *g* for 90 min at 4°C. Supernatant from this preparation was added to the [^{3}H]-Ins P's sample to give a final concentration of 5% w/v. Samples were mixed and incubated at 37°C for 30 min. Incubations were terminated by addition of ice-cold trichloroacetic acid (TCA) to give a final concentration of 1 M and the supernatants were treated with 5 × 2 volumes of diethyl ether to remove TCA before being neutralised with $NaHCO_3$ (50 mM). The [^{3}H]-Ins P's were separated on Dowex AG 1-X8 anion exchange resin (200–400 mesh, formate form). Sequential elution of the individual [^{3}H]-Ins P's was based on the scheme of Batty & Nahorski (1985). Although this type of separation is unable to distinguish between the various Ins P's isomers collected in each fraction, parallel analysis by high performance liquid chromatography (h.p.l.c.) anion exchange chromatography (Whitworth & Kendall, 1988a) verified the fact that the [^{3}H]-inositol trisphosphate fraction contained only the [^{3}H]-Ins 1,4,5 P_3 isomer following the rat brain enzyme treatment.

Sonicated tissue samples were again assayed for [^{3}H]-inositol phospholipid content.

All samples were quantified by liquid scintillation counting.

Mass measurement of inositol 1,4,5 trisphosphate (Ins 1,4,5 P_3)

In a second series of experiments, mice were killed and the brains removed and dissected as described above for determination of endogenous Ins P's. Portions containing cortex and hippocampus were sonicated in 1 ml PCA (10%, w/v) and the Ins P's extracted with a 1 : 1 mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine (0.5 ml/0.3 ml PCA) containing 12.5% (v/v) EDTA (10 mM). Samples were vortexed and centrifuged before removing and retaining the supernatant. Samples other than those of the control were diluted both 100 and 200 fold before being assayed for Ins 1,4,5 P_3 accumulation by the commercial radioreceptor binding assay system supplied by Amersham. There was good agreement between the results obtained with these two dilutions.

Sonicated tissue samples were also assayed for protein concentration by the method of Bradford (1976).

Materials

[2-^{3}H]-myo-inositol was obtained from New England Nuclear and the [^{3}H]-Ins 1,4,5 P_3 assay system for the measurement of cold Ins 1,4,5 P_3 assay was purchased from Amersham. All other drugs and chemicals were purchased from Sigma Chemicals or Fisons PLC.

Equipment

The microwave applicator was an EF-R unit supplied by Elnode Services Ltd, Luton, Bedfordshire.

Results

Selection of the pilocarpine dose

Preliminary studies were undertaken to select an appropriate dose of pilocarpine. In agreement with the findings of Turski *et al.* (1984), some mice treated with i.p. doses of 200 mg kg^{-1} pilocarpine displayed tremor, sedation and increased salivation. Although these parameters were not quantified, prior treatment with LiCl (10 mEquiv kg^{-1}) appeared to increase the intensity of this syndrome. At a dose of 300 mg kg^{-1}, pilocarpine induced pronounced tremor and salivation and occasional myoclonic seizures. In animals treated 1 h beforehand with LiCl (10 mEquiv kg^{-1}), 300 mg kg^{-1} pilocarpine induced myoclonic and tonic seizures and was lethal in most cases.

A dose of 200 mg kg^{-1} of pilocarpine was therefore used in all succeeding experiments.

Acute LiCl treatment

The effects of a single injection of pilocarpine or LiCl on total [^{3}H]-Ins P's Mice were injected intracerebroventricularly with 4 μl [^{3}H]-myo-inositol. Twenty-four hours later, these animals were injected with saline (i.p.) 1 h before being killed by focussed microwave irradiation. Under these control conditions, [^{3}H]-myo-inositol treatment resulted in the appearance of approximately 5000 d.p.m. in the combined extract of cortex and hippocampus brain regions (Figure 1a).

When [^{3}H]-Ins P's were measured 15 min after an injection of pilocarpine (200 mg kg^{-1}, i.p.), this treatment did not significantly increase their accumulation in comparison with that of saline-treated controls (Figure 1a). Eighteen hours after injection of LiCl (10 mEquiv kg^{-1}, s.c.), the accumulation of [^{3}H]-Ins P's was increased with respect to saline-treated controls, but not to the pilocarpine-treated group (Figure 1a). Administration of pilocarpine (200 mg kg^{-1}, i.p.) to mice which had been previously injected with LiCl (10 mEquiv kg^{-1}, s.c.) produced a marked increase in [^{3}H]-Ins P's formation, which was not only greater than that observed in the saline-treated controls, but also greater than that found in mice injected with either pilocarpine or LiCl alone (Figure 1a).

In addition to measurements of the accumulation of [^{3}H]-Ins P's, the incorporation of [^{3}H]-myo-inositol into the phospholipid fraction was also determined. Although there was some variability between the four groups studied, the only sig-

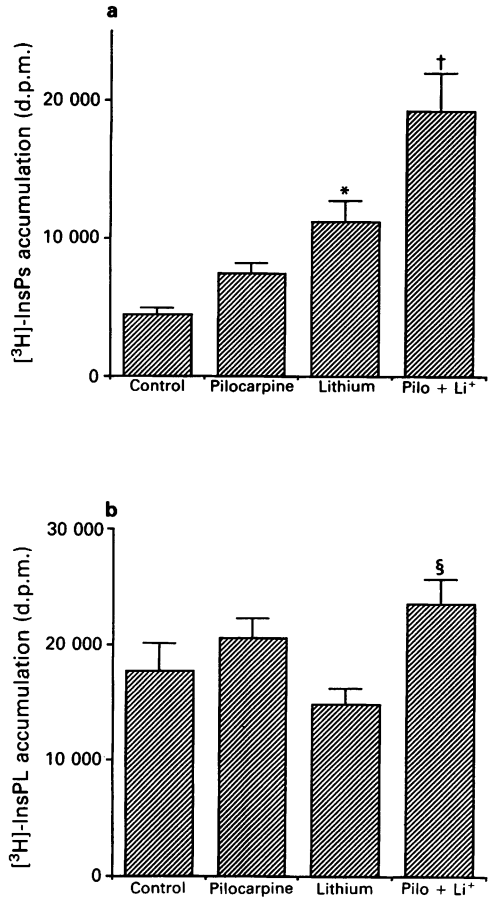

Figure 1 Effect of acute lithium treatment (10 mg kg^{-1}, s.c.) on the accumulation of total [^{3}H]-inositol phosphates ([^{3}H]-Ins P's) (a) and total [^{3}H]-inositol phospholipid ([^{3}H]-Ins PL) (b) due to *in vivo* stimulation by pilocarpine (200 mg kg^{-1}, i.p.). Mice received 4 μCi injections of [^{3}H]-myo-inositol i.c.v. and results are expressed as mean d.p.m. for ten mice in each group; s.e.mean shown by vertical bars. * Significantly different compared with control group; § significantly different compared with lithium group; † significantly different compared with all other groups. $P < 0.05$, 2 way analysis of variance.

nificant change was a greater incorporation of [^{3}H]-myo-inositol in the pilocarpine plus LiCl-treated group when compared with those mice receiving LiCl alone (Figure 1b).

In mice treated 10 min previously with atropine sulphate (10 mg kg^{-1}) there were no signs of tremor, sedation or abnormal salivation after treatment with pilocarpine, irrespective of whether they were LiCl-treated or not. Atropine had no effect on the increased accumulations of [^{3}H]-Ins P's in the brains of mice treated 18 h previously with LiCl (10 mEquiv kg^{-1}) but it abolished the enhancement due to pilocarpine treatment (Figure 2). Accumulations of [^{3}H]-Ins P's following atropine alone or atropine plus pilocarpine treatments were not different from control (data not shown).

The Li^+ concentration in the brains of mice injected with LiCl (10 mEquiv kg^{-1}, s.c.) was 0.96 ± 0.07 mmol kg^{-1} wet weight ($n = 10$).

Chronic LiCl treatment

The effects of a single pilocarpine injection on total [^{3}H]-Ins P's in mice maintained on a LiCl diet When the control group of mice, maintained on LiCl-free diet for 14 days, were injected with [^{3}H]-myo-inositol (4 μCi i.c.v.) and killed 24 h later by microwave irradiation, this resulted in 5000 d.p.m. appearing as [^{3}H]-Ins P's in the cortex/hippocampus extract (Figure 3a). This was almost identical to the accumulation observed in the acute LiCl studies. In this control group of

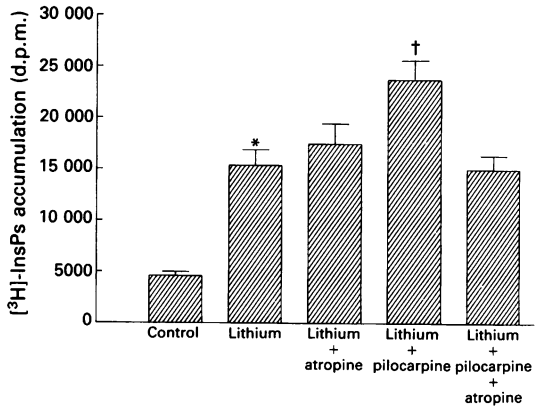

Figure 2 Effect of atropine sulphate (10 mg kg^{-1}, i.p.) on the *in vivo* accumulation of [^{3}H]-Ins P's due to acute LiCl and pilocarpine treatment (conditions as for Figure 1). Mice received 4 μCi injections of [^{3}H]-myo-inositol i.c.v. and results are expressed as mean d.p.m. for each group of 9 or 10 mice; s.e.mean shown by vertical bars. * Significantly different compared with control; † significantly different compared with lithium group. $P < 0.05$, 2 way analysis of variance.

mice, pilocarpine (200 mg kg^{-1}, i.p.) did not significantly increase the accumulation of [^{3}H]-Ins P's and this is again in agreement with results obtained in the acute LiCl experiments (Figure 3a). In contrast to the effects of an acute LiCl injection, when mice were fed a diet containing LiCl (0.4% w/w)

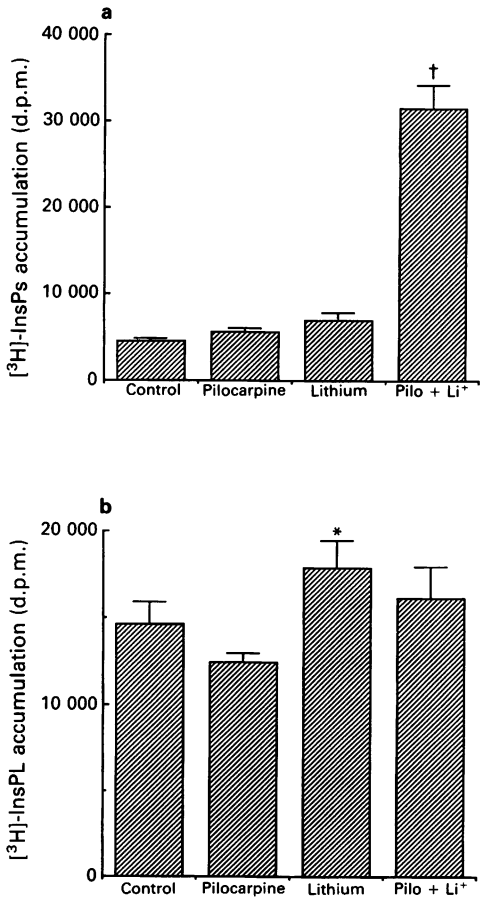

Figure 3 Effect of chronic lithium treatment (diet containing LiCl 0.4% w/w for 14 days) on the accumulation of (a) total [^{3}H]-inositol phosphates ([^{3}H]-Ins P's) and (b) total [^{3}H]-inositol phospholipids ([^{3}H]-Ins PL) due to *in vivo* stimulation by pilocarpine (200 mg kg^{-1}, i.p.). Results are expressed as d.p.m. and data are mean for ten mice in each group; s.e.mean shown by vertical bars. * Significantly different compared with pilocarpine group; † significantly different compared with all other groups. $P < 0.05$, 2 way analysis of variance.

for 14 days, there was no enhancement of total [^{3}H]-Ins P's accumulation compared with mice maintained on the LiCl-free diet (Figure 3a). However, treatment of LiCl-fed mice with pilocarpine (200 mg kg^{-1}, i.p.) produced a very marked increase in [^{3}H]-Ins P's formation which was significantly greater than that due to pilocarpine treatment in LiCl-free mice or that of the group fed the LiCl diet alone (Figure 3a). In addition, the accumulation of [^{3}H]-Ins P's induced by pilocarpine in excess of saline controls after chronic LiCl treatment was approximately two fold higher than that observed after a single LiCl injection (mean d.p.m. ± s.e.mean: chronic LiCl = 27,001 ± 2287, $n = 9$; acute LiCl 14,864 ± 1714, $n = 10$; significantly different, $P < 0.001$, Student's unpaired t test).

The incorporation of [^{3}H]-myo-inositol into the phospholipid fraction was again variable. In these chronic studies, the significant difference was a greater incorporation in the LiCl-fed mice compared with those LiCl-free animals given an injection of pilocarpine (200 mg kg^{-1}, i.p.) (Figure 3b).

The brain levels of Li^+ in the LiCl-fed mice was 0.78 ± 0.02 mmol kg^{-1} wet weight ($n = 10$).

The effects of a single pilocarpine injection on individual [^{3}H]-Ins P's in mice maintained on a LiCl diet In addition, to the assessment of total [^{3}H]-Ins P's accumulation, measurements were also made of the formation of higher phosphates. Pilocarpine (200 mg kg^{-1}) but not chronic LiCl feeding increased [^{3}H]-Ins 4,5-biphosphate ([^{3}H]-Ins 4,5 P_2) accumulation. Neither pilocarpine injected into LiCl-free mice, nor chronic LiCl feeding, had any effect on the accumulation of [^{3}H]-inositol 1,4,5-trisphosphate ([^{3}H]-Ins 1,4,5 P_3) or [^{3}H]-inositol tetrakisphosphate ([^{3}H]-Ins P_4) (Figure 4). Increases in all of the higher phosphates were observed when pilocarpine (200 mg kg^{-1} i.p.) was administered to mice maintained on the LiCl (0.4% w/w) diet for 14 days (Figure 4).

The h.p.l.c. profile shown in Figure 5 demonstrated that the [^{3}H]-inositol trisphosphate fraction consisted of a single peak which eluted in the same position as authentic [^{3}H]-Ins 1,4,5 P_3 isomer after treatment of the brain slice extract with a rat brain supernatant (as described in Methods).

The effects of a single pilocarpine injection on mass measurement of endogenous Ins 1,4,5 P_3 in mice maintained on a LiCl diet In contrast to its lack of effect on the accumulation of [^{3}H]-Ins 1,4,5 P_3 (Figure 4b), a single injection of pilocarpine (200 mg kg^{-1}, i.p.) to LiCl-free mice produced a significant enhancement of endogenous Ins 1,4,5 P_3 accumulation (Figure 6). Chronic LiCl treatment also produced a significant increase in the formation of this inositol phosphate (Figure 6). However, in agreement with the [^{3}H]-myo-inositol pre-

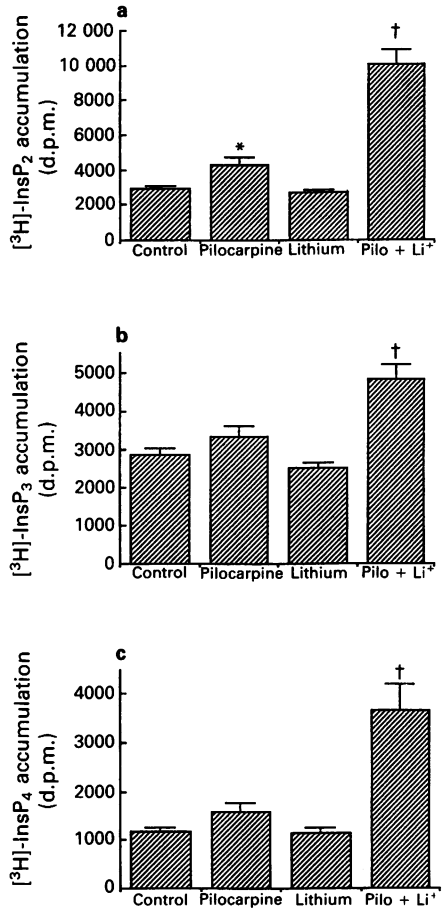

Figure 4 Effect of chronic lithium treatment on the accumulation of (a) [^{3}H]-inositol 4,5 bisphosphate ([^{3}H]-Ins 4,5 P_2), (b) [^{3}H]-inositol 1,4,5 trisphosphate ([^{3}H]-Ins 1,4,5 P_3) and (c) [^{3}H]-inositol tetrakisphosphate ([^{3}H]-Ins P_4) due to *in vivo* stimulation by pilocarpine (Pilo, 200 mg kg^{-1}, i.p.). Results are expressed in d.p.m. and data are mean for five mice in each group; s.e.mean shown by vertical bars. * Significantly different compared with lithium group; † significantly different compared with lithium groups. $P < 0.05$, 2 way analysis of variance.

labelling experiments, treatment of LiCl-fed mice with pilocarpine (200 mg kg^{-1}, i.p.) resulted in an accumulation of Ins 1,4,5 P_3 which was very much greater than that due to either pilocarpine or LiCl treatment alone (Figure 6).

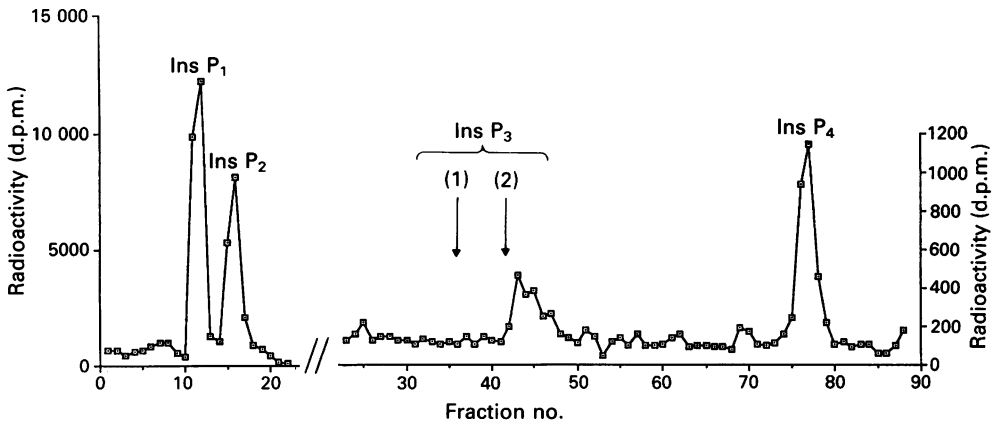

Figure 5 H.p.l.c. profile of individual [^{3}H]-inositol phosphates ([^{3}H]-Ins P's) accumulation due to chronic lithium treatment and *in vivo* pilocarpine (200 mg kg^{-1}, i.p.) administration. Samples were treated with rat brain supernatant (see Methods) and the profile demonstrates that the [^{3}H]-inositol trisphosphate fraction contains a single peak which eluted in the same position as authentic [^{3}H]-inositol 1,4,5 trisphosphate ([^{3}H]-Ins 1,4,5 P_3) (2). There was no peak corresponding to authentic [^{3}H]-inositol 1,3,4-trisphosphate ([^{3}H]-Ins 1,3,4 P_3) (1), as determined in separate h.p.l.c. runs.

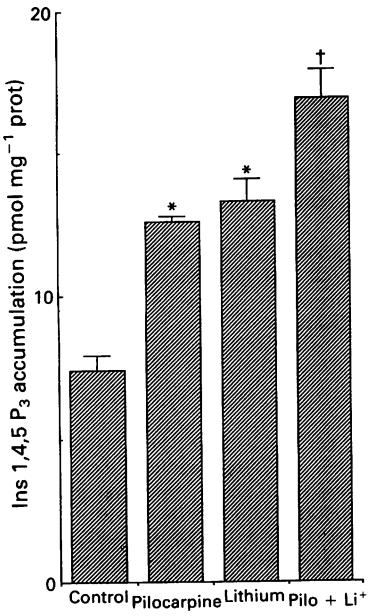

Figure 6 Effect of pilocarpine (200 mg kg^{-1}, i.p.), with and without prior chronic lithium treatment, on the amounts of endogenous inositol 1,4,5 trisphosphate (Ins 1,4,5 P_3) present in mouse cerebral cortex. Results are expressed in pmol mg^{-1} protein. Data are mean of five mice for each group; s.e.mean shown by vertical bars. * Significantly different compared with control group; † significantly different compared with all other groups. $P < 0.05$, 2 way analysis of variance.

Discussion

In previous studies, it has been reported that lithium treatment causes a reduction in muscarinic receptor-stimulated inositol phospholipid hydrolysis in rat brain slices (Kendall & Nahorski, 1987; Whitworth & Kendall, 1988b; Godfrey *et al.*, 1989). Since the treatment does not affect the number or affinity of muscarinic receptors (Maggi & Enna, 1980; Godfrey *et al.*, 1989), it would seem likely that the effect is mediated at a post-receptor level. It has been suggested by Berridge *et al.* (1982) that the reduction in brain myo-inositol level which results from inositol monophosphate phosphatase inhibition (Hallacher & Sherman, 1980) could lead to a depletion of inositol phospholipids, and therefore, to a general reduction in receptor-mediated inositol phosphate production. In fact, *in vitro* repletion of tissue myo-inositol actually enhances the reductions in cholinoceptor-stimulated [^{3}H]-Ins P's formation in rat brain slices (Kendall & Nahorski, 1987; Whitworth & Kendall, 1989) in the absence of any gross depletion of brain myo-inositol levels. This is probably because lithium induces changes in the specific activity of receptor-linked pools of [^{3}H]-myo-inositol-labelled phosphoinositides and this hypothesis is supported by the finding that lithium treatment causes an increase in [^{3}H]-myo-inositol incorporation into Ptd Ins 4,5 P_2 (Godfrey *et al.*, 1989).

Recent *in vitro* studies have shown that lithium reduces [^{3}H]-myo-inositol polyphosphate formation (Batty & Nahorski, 1987; Whitworth & Kendall, 1988), but does not alter phospholipase C activity (Kendall & Nahorski, 1987). Taking into account the finding that lithium treatment also reduces [^{3}H]-Ins P's formation due to a receptor-independent stimulus (i.e. sodium fluoride; Godfrey *et al.*, 1989), a reasonable working hypothesis is that lithium reduces inositol phospholipid hydrolysis by altering receptor/effector coupling and Avissar *et al.* (1988) have recently provided data which implicates GTP-binding proteins as possible targets for lithium's actions. However, the studies outlined above have been of the *ex vivo* design; consisting of *in vivo* lithium treatment of animals followed by *in vitro* pre-labelling of brain slice phospholipids with [^{3}H]-myo-inositol and agonist stimulation. The interpretation of the results of such experiments is necessarily complicated by the possibility of effects due to post-mortem changes in tissue function and possibly also brain lithium concentrations. In the present study, we have therefore carried out *in vivo* investigations, employing mice because of their ease of i.c.v. injection as compared with larger rodents (Heal, 1984).

A number of important differences between the results of these experiments and *ex vivo* studies are readily evident. Following chronic feeding with lithium, the accumulation of total [^{3}H]-Ins P's was greatly reduced compared with the accumulation after a single acute lithium treatment (Figures 1a, 3a).

The accumulation of [^{3}H]-Ins P's in the presence of lithium alone presumably reflects the effect of the ion on normal neuronal activity and one interpretation of the data is that chronic lithium treatment reduces this activity. This unstimulated neuronal activity is apparently unrelated to muscarinic receptors because it was not inhibited by atropine (Figure 2). An alternative possibility is that the reduction in total [^{3}H]-Ins P's (which is predominantly [^{3}H]-inositol monophosphate ([^{3}H]-Ins P_1) (Figure 5)) is a consequence of lithium inhibiting the formation of the inositol polyphosphates. However, consideration of Figure 4b and c makes it plain that the formation of [^{3}H]-Ins 1,4,5 P_3 and [^{3}H]-Ins P_4 were not reduced by lithium treatment.

With regard to the responses to the muscarinic agonist pilocarpine, both acute (Figure 1a and Figure 2) and chronic (Figure 3a) lithium treatments enhanced the accumulations of total [^{3}H]-Ins P's and there was a significant enhancement in the response to pilocarpine following chronic lithium feeding compared with that following acute treatment. The effects of pilocarpine appear to be mediated by muscarinic receptors because they were abolished by pretreatment with atropine (Figure 2). It is possible that these results reflect poorly the actual changes in endogenous inositol phosphate levels as a result of lithium-induced changes in [^{3}H]-phospholipid specific activity, since in previous *ex vivo* studies, reductions in the accumulation of [^{3}H]-Ins P's have only been apparent after treatment of rat brain slices with exogenous myo-inositol (Whitworth & Kendall, 1989). However, mass measurements of Ins 1,4,5 P_3 produced a similar picture, in that chronic lithium feeding again enhanced the effects of pilocarpine (Figure 6). However, these data differ from those generated in the [^{3}H]-myo-inositol prelabelling experiments in that lithium treatment alone produced a significant Ins 1,4,5 P_3 response of the same magnitude as that due to pilocarpine. The reasons for these differences are not clear at present, but might represent specific activity changes in the prelabelling assay, as has been proposed to explain similar discrepancies seen *in vitro* by Challis *et al.* (1988).

A likely explanation for the large responses to lithium and pilocarpine is that they act synergistically in the intact animal as reported by Honchar *et al.* (1983) causing, at the top of the dose range, seizures and irreversible brain damage. This is thought to be due to massive releases of acetylcholine (Jope *et al.*, 1987). Since pilocarpine is a weak partial agonist with regard to Ins P's production in brain (Fisher & Bartus, 1985), release of the more efficacious acetylcholine could account for the increased Ins 1,4,5 P_3 response *in vivo* in the presence of lithium and pilocarpine. This is consistent with the blockade of the response by atropine. Under the conditions used here, the combination of pilocarpine and lithium treatments had a muscarinic receptor-mediated proconvulsive effect and it is pertinent to ask whether the changes in phosphoinositide were the cause or effect of this action. Kindling has been reported to enhance excitatory amino acid-stimulated phosphoinositide hydrolysis (Iadorola *et al.*, 1986), so activation of this transduction system does seem to be involved in the development of seizures and the anticonvulsant carbamazepine (McDermott & Logan, 1989) reduces basal and muscarinic receptor-stimulated [^{3}H]-Ins P's accumulation, again suggesting that products of phosphoinositide hydrolysis have a causal

role in seizure production, rather than being a response to the condition.

Further work is necessary in order to reconcile *in vivo* and *in vitro* models, but given that there are very large post-mortem increases and decreases in brain concentrations of inositol phosphates and polyphosphoinositides, respectively (Soukup *et al.*, 1978; Meek, 1986) which could alter the activity of phospholipase C-linked receptor systems, and that these changes are avoided by microwave irradiation, the *in vivo* model may be preferable in this regard. The increase in Ins 1,4,5 P_3 formation due to chronic lithium treatment alone (Figure 6) is harder to explain, but it could be related to the enhanced release of some endogenous transmitter substance that stimulates inositol phospholipid hydrolysis, as has been reported for 5-hydroxytryptamine (Friedman & Wang, 1988).

In future experiments, it will therefore be necessary to examine the effects of a variety of receptor antagonists on the endogenous Ins 1,4,5 P_3 response to lithium and to assess the responses to other agonists which do not result in the complication of acetylcholine release associated with pilocarpine.

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The effects of (−)-daurisoline on Ca^{2+} influx in presynaptic nerve terminals

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1 The effects of (−)-daurisoline on $^{45}Ca^{2+}$ uptake and [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release from synaptosomes of rat cerebral cortex and on contractile activity of rat aorta were examined.

2 Application of (−)-daurisoline (1–100 μM) produced concentration-related inhibition of high K^+-stimulated $^{45}Ca^{2+}$ uptake and [^{3}H]-GABA release (IC_{50} = 7.7 ± 0.9 μM and 10.0 ± 1.5 μM, respectively) in synaptosomes but verapamil was only weakly active.

3 Neither (−)-daurisoline (100 μM) nor verapamil (100 μM) modified $^{45}Ca^{2+}$ uptake in control medium (5 mM K^+, resting uptake) and [^{3}H]-GABA release in Ca-free medium (45 mM K^+ basal release).

4 High K^+ and noradrenaline-evoked contractions of rat aorta were inhibited by both (−)-daurisoline and verapamil.

5 In conclusion, (−)-daurisoline, which differed from verapamil in its mode of blocking Ca^{2+} influx may be a potent Ca^{2+} antagonist of Ca^{2+} channels in neurones.

Introduction

(−)-Daurisoline (Figure 1) is a tetrahydroisoquinoline (TIQ) isolated from the roots of *Menispermum dauricum*. TIQs, which possess a diversity of pharmacological activities, have been shown to have Ca^{2+} channel blocking properties. Recently, some TIQs, such as tetrandrine (Han *et al.*, 1987) and dauricine (Dongke *et al.*, 1987) were used clinically for the treatment of hypertension, supraventricular tachycardia and congestive heart failure. Like other Ca^{2+} channel antagonists, these TIQs block L-type Ca^{2+} channels in cardiovascular muscle (King *et al.*, 1980). We considered that (−)-daurisoline might have a similar effect on Ca^{2+} influx to tetrandrine and dauricine due to their chemical similarity and we therefore examined its Ca^{2+} antagonistic effects.

Pinched off nerve terminal endings (synaptosomes) retain the functional capacity to take up $^{45}Ca^{2+}$ during depolarization (Nachshen & Blaustein, 1980) and release neurotransmitter in response to the influx of Ca^{2+} (Drapeau & Blaustein, 1983; Suszckiw *et al.*, 1985). Although the time course of radioflux measurements (seconds) does not compare well with that of synaptic transmission as it is measured electrophysiologically (milliseconds) several features correlated well between the two systems, such as their sensitivity to block by divalent inorganic cations (Manalis *et al.*, 1984; Suszkiw *et al.*, 1985) and their similar lack of sensitivity to organic Ca channel blockers (Gotgiff & Magazanile, 1977; Suszkiw *et al.*, 1986; Atcheson & O'Leary, 1987). Because of these similarities, and because the $^{45}Ca^{2+}$ uptake and transmitter release process by synaptosomes have been well studied, we have taken advantage of this system as a model to test the effects of (−)-daurisoline on depolarization-induced Ca^{2+} influx and [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release.

The stimulated 'fast' phase of uptake, which occurs during the first 5 s of depolarization (Nachshen, 1985; Suszkiw *et al.*, 1986), is presumed to be mediated by a voltage-dependent Ca^{2+} channel and has been associated with release of a number of transmitters from synaptosomes (Floor, 1983; Leslie *et al.*, 1985). The goal of the present study was to investigate the effects of (−)-daurisoline on 'fast' phase $^{45}Ca^{2+}$ influx and transmitter release in synaptosomes. In addition, the effects of (−)-daurisoline on contractile activity of rat aorta stimulated by noradrenaline (NA) and high K^+ were tested to ascertain any selectivity for Ca^{2+} channel blockade between neurones and smooth muscle.

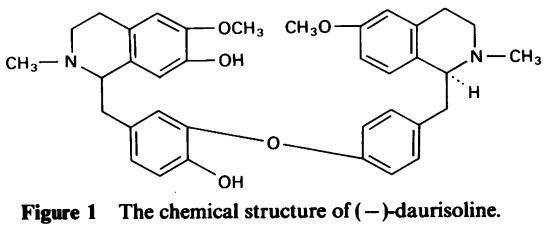

Figure 1 The chemical structure of (−)-daurisoline.

Methods

Preparation of synaptosomes

Synaptosomes were prepared from rat cerebral cortex which was removed, pooled, and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a Teflon coated pestle followed by centrifugation at 4°C for 10 min at 1000 *g*. Crude synaptosomes were obtained by centrifugation at 17000 *g* for 60 min at 4°C and resuspension of the pellet in 10 volumes 0.32 M sucrose. Synaptosomes were separated by sucrose gradient centrifugation at 55000 *g* for 45 min at 4°C. Synaptosomes in the 0.8 M sucrose fraction from the sucrose gradient were equilibrated with physiological saline by the addition of 2.5 volumes of ice-cold standard HEPES buffer (composition, mM: NaCl 145, KCl 5, $MgCl_2$ 1.2, NaH_2PO_4 2.4, and HEPES 20, adjusted to pH 7.4 at 25°C with Tris). The diluted synaptosomes were centrifuged at 10000 *g* for 10 min at 4°C and the pellet was resuspended in cold HEPES buffer.

Measurement of $^{45}Ca^{2+}$ influx

The synaptosome suspensions (300 μl; containing approximately 0.25 mg protein) were preincubated for 10 min at 30°C (with or without drug) before initiating test uptake of $^{45}Ca^{2+}$. Aliquots of the warmed synaptosome suspensions were added to 100 μl incubation solutions containing 1 μCi of $^{45}Ca^{2+}$. Uptake into synaptosomes of $^{45}Ca^{2+}$ was measured in either standard HEPES buffer or stimulating buffer, containing 75 mM K^+ (final concentration), substituted for equimolar concentration of sodium. After a test incubation lasting 20 s, $^{45}Ca^{2+}$ uptake was stopped by addition of 4.5 ml of ice-cold HEPES buffer. The diluted suspensions were immediately filtered by suction through Whatman glass fibre filters (GF/C 2.4 cm diameter). The filters were rapidly washed twice with 4.5 ml ice-cold HEPES. The filters were then suspended in vials containing 5 ml of scintillation liquid (toluene containing

Table 1 Effects of drugs on K-stimulated ^{45}Ca uptake in synaptosomes

Drugs (μM)	*Ca uptake* (nmol mg^{-1} protein) *Standard buffer*	*Stimulating buffer*	ΔK	% *inhibition*
Control	0.83 ± 0.09	1.78 ± 0.85	0.95 ± 0.07	
Daurisoline (5)	0.80 ± 0.07*	1.37 ± 0.63**	0.57 ± 0.04	40.0
Verapamil (50)	0.81 ± 0.10*	1.58 ± 0.75**	0.77 ± 0.08	18.9

Synaptosomes were preincubated with or without drug in standard buffer and then incubated with or without drug with ^{45}Ca, in standard buffer or stimulating buffer. Stimulated ^{45}Ca uptake ΔK, was then calculated as the difference between ^{45}Ca uptake from stimulating buffer and standard buffer.
* $P > 0.1$ (NS). ** $P < 0.001$, two-tailed t-test.

0.5% PPO, 0.01% POPOP) and the $^{45}Ca^{2+}$ content of the material trapped by the filters was determined by liquid scintillation spectrometry. The protein concentration was determined by the method of Lowry *et al.* (1951). The extra uptake of $^{45}Ca^{2+}$ (K^+-stimulated uptake = ΔK) was calculated as the difference between the uptake from standard HEPES buffer and the uptake from the stimulating buffer. In each experiment $^{45}Ca^{2+}$ uptake determinations were made on 6 replicates, as indicated in the table and figure legends.

Measurement of [^{3}H]-GABA release

Rat hippocampus synaptosome suspensions (1 ml containing approximately 0.8 mg protein) were incubated with 4 nM [^{3}H]-GABA for 15 min at room temperature (25–30°C). Uptake was stopped by the addition of 1 ml ice-cold HEPES buffer. Synaptosomes were separated by centrifugation at 35000 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was used for measurement of [^{3}H]-GABA release. Stimulated release was studied, based on the technique of Haycock *et al.* (1977). The synaptosomes preloaded with [^{3}H]-GABA, were washed by addition of six consecutive 1 ml ice-cold Ca^{2+}-free wash solutions (composition, mM: NaCl 100, KCl 45, $MgCl_2$ 1.2, NaH_2PO_4 2.4, EGTA 20 and HEPES 20, pH 7.4). [^{3}H]-GABA release was stabilized by the fifth wash (Cotman *et al.*, 1976). The washed synaptosomes were separated by centrifugation at 35000 *g* for 5 min at 4°C. The supernatant was discarded, and the pellet was suspended in HEPES buffer for the measurement of [^{3}H]-GABA release. Suspensions were preincubated for 10 min at 30°C (with or without drug) before initiating test [^{3}H]-GABA release. [^{3}H]-GABA release was measured in either 1.2 mM $CaCl_2$ wash (stimulated release) or Ca^{2+}-free wash buffer (basal release). The reaction was quenched rapidly with 1 ml ice-cold Ca^{2+}-free wash buffer 15 min after addition of 1.2 mM $CaCl_2$ to the test medium (Ca^{2+}-free wash buffer). Net Ca^{2+}-dependent release of [^{3}H]-GABA was calculated by subtracting the % basal release values from % stimulated release values. All values represent the mean ± s.e.mean of six determinations.

Effects of (−)-daurisoline on noradrenaline- and K^+-induced contractions

In these experiments, inhibition of the contractions induced by noradrenaline (NA) and high K^+-depolarization was measured as a function of the efficacy of (−)-daurisoline or verapamil. Rats were killed, exsanguinated and the aortae removed and cut into aortic rings (3 mm long). These were mounted in all-glass 20 ml muscle chambers at 37°C. The bathing fluid was Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, at 37°C pH 7.4. It was continuously gassed with 95% O_2:5% CO_2. Responses were recorded isometrically. First, a control maximal contraction produced with either 1 μM NA or 75 mM K^+. All data are expressed as a percentage of this control contraction. This was followed by a contraction induced by a concentration of the agonist used for a specific (−)-daurisoline or verapamil concentration to be tested. The aorta was then challenged in the presence of (−)-daurisoline or verapamil with the agonist. Subsequently, the agonist was washed out in the presence of the next higher concentration of (−)-daurisoline or verapamil and after 30 min, again challenged by the same agonist concentration. The process was repeated until the entire (−)-daurisoline or verapamil concentration-response curve at this agonist concentration was obtained. Only one concentration-response curve was obtained from each muscle ring. The magnitude of the control contraction did not change significantly during the time course of the experiments.

Materials

$^{45}Ca^{2+}$ (specific activity, 25 mCi g^{-1}) and [^{3}H]-GABA (specific activity, 35 Ci mmol^{-1}) were obtained from China Atomic Energy Institute. Verapamil and noradrenaline were obtained from Tianjing Research Institute of Pharmaceutical Industry. (−)-Daurisoline was obtained from the Department of Pharmachemistry, China Pharmaceutical University. HEPES was obtained from U.S. Biochemical Co.

Results

Effects of (−)-daurisoline on $^{45}Ca^{2+}$ uptake in synaptosomes

Triggered $^{45}Ca^{2+}$ uptake in high K^+ buffer during the first 20 s of depolarization was approximately 2–3 fold of the basal $^{45}Ca^{2+}$ uptake in control buffer (Table 1). (−)-Daurisoline (100 μM) had little or no effect on basal uptake. The most prominent effect produced by (−)-daurisoline was concentration-dependent inhibition of stimulated $^{45}Ca^{2+}$ uptake (IC_{50} = 7.7 ± 0.9 μM, n = 6). Meanwhile, for 40% inhibition of maximal $^{45}Ca^{2+}$ uptake, a verapamil concentration of 100 μM was required. These results are shown in Figure 2.

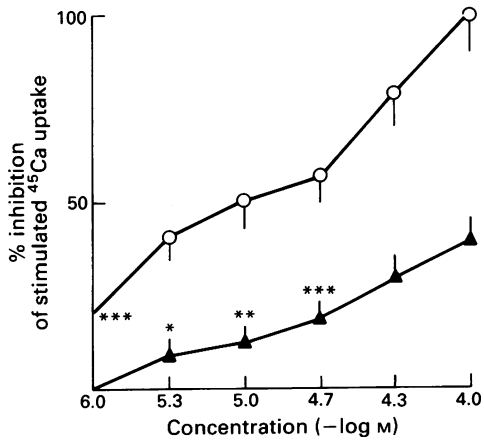

Figure 2 Effects of (−)-daurisoline on $^{45}Ca^{2+}$ uptake in synaptosomes: (▲) inhibitory effect of verapamil; (○) blocking action of (−)-daurisoline on the effects of incubating synaptosomes with high external K^+ (75 mM). Points in the curves represent the mean of 6 experiments; s.e.mean shown by vertical lines. Test incubation with $^{45}Ca^{2+}$ lasted 20 s. All buffers contained 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$. $^{45}Ca^{2+}$ uptake from control buffer averaged 0.81 ± 0.09 nmol mg^{-1} protein. * $P < 0.02$ (values significantly less than control); ** $P < 0.01$; *** $P < 0.001$, one-tailed t test.

Effect of (−)-daurisoline on [^{3}H]-GABA release

Addition of $CaCl_2$ (0.2–1.2 mM) produced a concentration-related increase in [^{3}H]-GABA release (Figure 3). The % maximal release was 2.98 ± 0.33. The effect of (−)-daurisoline (1–100 μM) on K^+-stimulated [^{3}H]-GABA release is seen in Figure 4. When (−)-daurisoline concentration was increased from 1 μM to 100 μM, inhibition produced by (−)-daurisoline was 15.3 ± 1.8% and 70.6 ± 5.4% respectively (IC_{50} = 10.0 ± 1.3, n = 7). At a concentration of 10 μM, verapamil only slightly inhibited [^{3}H]-GABA release (% inhibition = 10.9 ± 1.8, n = 7). Neither (−)-daurisoline (100 μM) nor verapamil (100 μM) decreased the basal release in Ca-free buffer significantly.

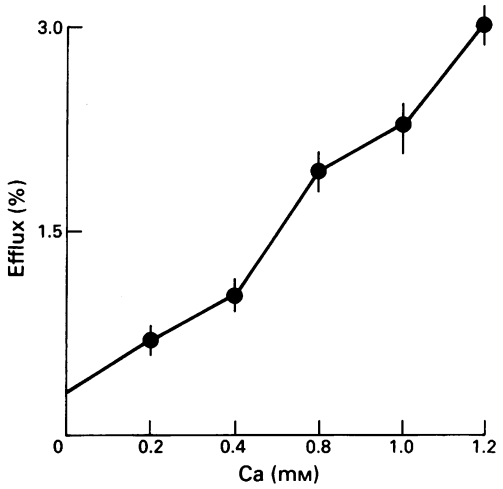

Figure 3 The influence of external Ca^{2+} on [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release. Points indicate [^{3}H]-GABA release in various Ca^{2+} concentrations. Each point represents mean for 7 determinations; s.e.mean shown by vertical lines. Test incubation with indicated Ca^{2+} concentration lasted 15 min at room temperature (25–30°C). All buffers contained 1.2 mM $MgCl_2$ and 45 mM K^+. Radioefflux in a given supernatant is represented as the total radioactivity present in the pellets immediately after the centrifugation.

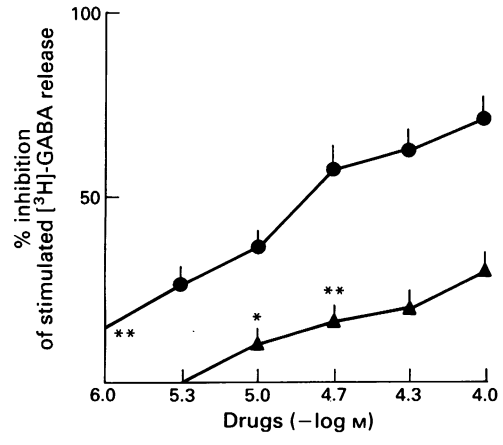

Figure 4 The effects of drugs on [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release in synaptosomes. Synaptosomes loaded with [^{3}H]-GABA were preincubated for 10 min at 30°C with drugs at the indicated concentrations before initiating [^{3}H]-GABA release: (▲)(−)-daurisoline; (●) verapamil [^{3}H]-GABA release from synaptosomes was measured in 1.2 mM $CaCl_2$ wash buffer for 15 min. Values represent the mean for 7 determinations; s.e.mean shown by vertical lines. Percentage inhibition is expressed as Net release without drug − Net release with drug/Net release without drug (%). * $P < 0.02$ (values significantly less than control); ** $P < 0.01$, one-tailed t test.

Effects of (−)-daurisoline on contractile activity of aorta

As shown in Figures 5 and 6, (−)-daurisoline produced a concentration-dependent inhibition of contraction induced by NA (1 μM) and high K^+ (75 mM) with an IC_{50} of 39.8 ± 4.1 μM and 3.9 ± 0.2 μM (n = 5). The IC_{50} of verapamil was 31.6 ± 2.9 μM and 0.21 ± 0.04 μM (n = 5), respectively.

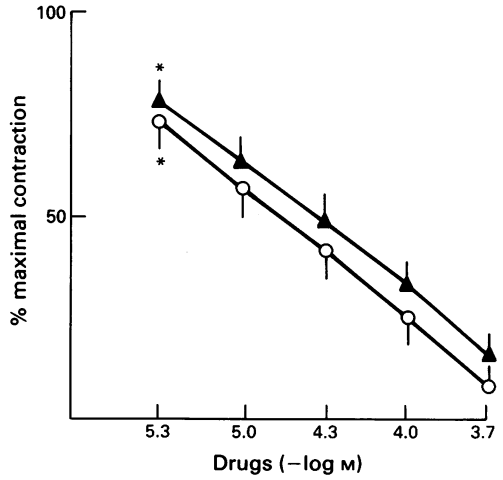

Figure 5 Concentration-response curves for the inhibition by (−)-daurisoline (▲) and verapamil (○) of noradrenaline (1 μM)-induced contractions of rat aortic rings. For details, see Methods. Each point represents a mean of five determinations; s.e.mean was less than 10% of the mean. * $P < 0.05$ (values significantly less than control), two-tailed t test.

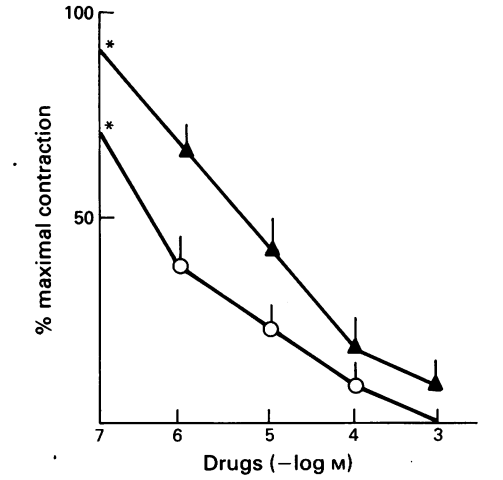

Figure 6 Concentration-response curves for the inhibitory efficacy of (−)-daurisoline (▲) and verapamil (○) against contractions produced by K^+ (75 mM). Each point represents mean of five determinations; s.e.mean was less than 10%. * $P < 0.05$, two-tailed t test.

Discussion

$^{45}Ca^{2+}$ uptake stimulated by high K^+, was maximal within 2 min at 30°C and consisted of a 'fast' phase which occurs during the first 5 s of depolarization and a 'slow' phase which appears 20 s after prolonged depolarization (Figure 7). This result is in agreement with previous reports (Nachshen & Blaustein, 1979). The 'fast' phase of uptake is presumed to be mediated by voltage-dependent Ca^{2+} channel and is associated with release of the transmitter from synaptosomes (Floor, 1983; Leslie *et al.*, 1985). The exact nature of the 'slow' phase is as yet unclear, but is probably due in part to exchange processes. To our knowledge, it is likely that only depolarization-induced transmembrane Ca^{2+} movements are involved in synaptic effects of (−)-daurisoline in this study, since effects of

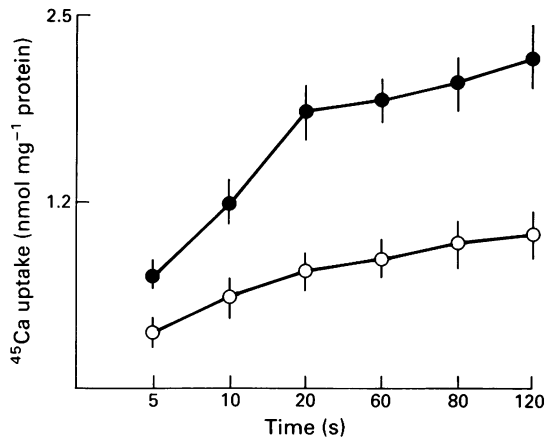

Figure 7 Synaptosomes were preincubated in standard HEPES buffer and then incubated with $^{45}Ca^{2+}$ standard HEPES buffer or stimulating buffer for the indicated test-incubation period: $^{45}Ca^{2+}$ uptake in the test incubation period with stimulating buffer (●) or standard HEPES buffer (○). The values represent the mean for 7 determinations; s.e.mean shown by vertical lines. * $P < 0.01$ (values significantly less than resting uptake), one-tailed t test.

(−)-daurisoline on $^{45}Ca^{2+}$ uptake were observed only at 'fast' phase and since the data presented in this investigation show that the resting $^{45}Ca^{2+}$ uptake and basal [^{3}H]-GABA release were not altered by (−)-daurisoline. (−)-Daurisoline did not chelate Ca^{2+} directly (data not shown).

In the [^{3}H]-GABA release experiments, only 45 mM K^+ was used to minimize the possibility of influencing intraterminal mitochondral Ca^{2+} metabolism (Haycock *et al.*, 1977).

In a separate set of experiments, we also investigated the effect of (−)-daurisoline on contractile force of rat aorta evoked by NA and high K^+. In vascular smooth muscle, L-type Ca^{2+} channels operated by depolarization have been reported (Nilius & Tsien, 1985). L-type Ca^{2+} channels with highest K^+ depolarization threshold are sensitive to organic Ca^{2+} antagonists such as verapamil. Marked inhibition of Ca^{2+} conductance in vascular smooth muscle is observed with concentrations of verapamil of the order of 10 nM to 10 μM (Fleckenstein, 1977). Our results revealed that verapamil sharply inhibited the contractile force of rat aorta evoked by NA and high K^+ with an IC_{50} of 31.6 ± 2.9 μM and 0.21 ± 0.04 μM, respectively. But the effects of verapamil on stimulated $^{45}Ca^{2+}$ uptake and [^{3}H]-GABA release by synaptosomes showed only 1/20 of the efficacy of (−)-daurisoline. In contrast to verapamil, daurisoline was less sensitive in inhibiting the contractile force of the rat aorta stimulated by NA and high K^+. These results indicated that (−)-daurisoline is relatively selective as an antagonist of Ca^{2+} channels in neurones.

The mode of blocking Ca^{2+} influx of (−)-daurisoline and its specificity for a particular subpopulation of Ca channels were not explored. The electrophysiological correlate to the fast phase of tracer influx into synaptosomes is unclear, as is the actual type of Ca channel involved in neurotransmitter release (Nowycky *et al.*, 1985). Recently, Miller (1987) and Triggle (1989) have presented evidence that the N-type Ca channel is responsible for $^{45}Ca^{2+}$ uptake and transmitter release. The most prominent agent active at the N-channel is ω-conotoxin GVIA, a 27 residue peptide isolated from the toxin of molluscs of the *Conus* genus (Olivera & Cruz, 1987). ω-Conotoxin exerts potent inhibitory effects in many, but not all neuronal systems suggesting that neuronal communications may depend upon a variety of channel types differing in their pharmacological behaviour and possibly also in their cellular location. Radioligand binding studies of [^{3}H]-conotoxin may be useful in discerning which types of Ca^{2+} channels in neurones are affected by daurisoline. Further investigations are required to show that (−)-daurisoline is a specific antagonist of N-type Ca channels.

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Investigation into atropine-induced antinociception

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1 The effect of atropine on the nociceptive system was examined in mice and rats by use of the hot-plate, writhing and tail-flick tests.

2 Atropine dose-dependently produced analgesia, no effect and hyperalgesia. Analgesia was observed in both species with doses ranging from 1 to 100 $\mu g\,kg^{-1}$ while hyperalgesia was obtained with 5 $mg\,kg^{-1}$.

3 Atropine antinociception was prevented by pirenzepine (0.1 μg per mouse, i.c.v.), dicyclomine (10 $mg\,kg^{-1}$, i.p.), atropine-methylbromide (0.5 μg per mouse, i.c.v.) and hemicholinium-3 (1 μg per mouse, i.c.v.). Naloxone (1 $mg\,kg^{-1}$, i.p.), α-methyl-*p*-tyrosine (100 $mg\,kg^{-1}$, s.c.) and reserpine (2 $mg\,kg^{-1}$, i.p.) were ineffective.

4 The site of atropine analgesia is in the CNS since it exerts its antinociceptive effect also when injected i.c.v. (1–10 ng per mouse). Moreover drugs which do not cross the blood-brain barrier, such as hemicholinium-3, pirenzepine and atropine methylbromide, were unable to antagonize atropine analgesia if administered i.p.

5 Atropine also *in vitro*, showed a biphasic action on electrically-evoked guinea-pig ileum contractions. Concentrations between 10^{-14} and 10^{-12} M increased electrically and nicotine-evoked contractions but did not affect acetylcholine- and oxotremorine-evoked contractions. Concentrations above 10^{-9} M inhibited both electrically- and drug (acetylcholine, nicotine and oxotremorine)-evoked contractions while they were ineffective on unstimulated ileum.

6 On the basis of the above findings, amplification of cholinergic transmission by very low doses of atropine is postulated, through a selective blockade of presynaptic muscarinic autoreceptors, as the likely mechanism of action.

7 Atropine antinociception, unlike oxotremorine antinociception, was obtained without any impairment of mouse rota-rod performance.

8 The antagonism by pirenzepine and dicyclomine of oxotremorine and atropine antinociception suggests that M_1 muscarinic receptor subtypes are responsible for cholinergic analgesia.

Introduction

Atropine-like preparations were used by ancient Romans to relieve pain; Pliny the elder, in his *Historia Naturalis*, reported that the juice of *Mandragora officinarum* or *Hyosciamus niger* was administered to patients before surgery to produce analgesia. Similarly, Galen employed either *Mandragora* roots or *Hyosciamus* seeds to relieve pain such as toothache. Much more recently Hotovy (1956) has reported that atropine sulphate at a very low dose (30 $\mu g\,kg^{-1}$) is able to induce analgesia in rabbits. On the other hand it is well known that atropine administered at a much higher dosage (0.5–5.0 $mg\,kg^{-1}$) antagonizes antinociception induced by cholinomimetics such as physostigmine (Ireson, 1970) and oxotremorine (Paalzow & Paalzow, 1975; Bartolini *et al.*, 1987). Bartolini *et al.* (1980) reported that in rodents not only is atropine (1 $mg\,kg^{-1}$, s.c.) able to decrease the pain threshold but also clearly to potentiate naloxone hyperalgesia. Recently a selective block of the muscarinic presynaptic autoreceptors induced by both local anaesthetics (Bartolini *et al.*, 1987) and antiarrhythmic drugs (Ghelardini *et al.*, 1988) has been postulated to explain the antinociception caused by these drugs which is blocked by atropine (5 $mg\,kg^{-1}$, i.p.) and hemicholinium (1 μg per mouse, i.c.v.) but not by atropine methylbromide (5.5 $mg\,kg^{-1}$, i.p.). A similar mechanism of action might be responsible for the effect of atropine on the pain threshold since low doses of atropine might selectively antagonize presynaptic receptors while higher doses could additionally block postsynaptic receptors. In order to verify this hypothesis we investigated the antinociceptive effect of low doses of atropine and its underlying mechanism of action.

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Methods

Hot-plate test

Male albino Swiss mice (25–30 g) from the Morini breeding farm were used. The mice were placed inside a stainless steel container thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshops, Siena, Italy. The reaction time (s) for control animals was measured with a stop-watch before, and 15, 30 and 45 min after treatment. The endpoint used was the licking of the fore or hind paws. Those mice which scored below 12 and over 18 s in the pretreatment test were rejected. An arbitrary cut-off time of 45 s was adopted.

Writhing test

Male albino Swiss mice (25–30 g) were injected i.p. with a 0.5% aqueous solution of acetic acid (10 $ml\,kg^{-1}$). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Tail-flick test

Male albino Wistar and Fischer-344 rats (250–300 g), purchased respectively from Morini and Nossan were used. An analgesimeter from the Galileo Workshops, Florence, Italy was used to perform the tail-flick test described by D'Amour & Smith (1941), with minor modifications. The light from a project bulb situated beneath the platform where the animal

was placed, was focused through a small hole on the ventral part of the tail at a point about 4 cm from the tip. Withdrawal of the tail exposed a photocell to the light, which turned off thermal stimulus and automatically stopped the clock, measuring the latency period to the nearest 0.1 s. The intensity was regulated so that the reaction time varied between 3 and 5 s. A cut-off time of 10 s was used to prevent blistering. The analgesia was tested before, and 15, 30 and 45 min after treatment with atropine. Each value was derived from the mean of three consecutive readings in which the light was focused on three adjacent points of the tail.

Rota-rod test

Male Swiss albino mice (25–30 g) were tested on a rota-rod treadmill (Ugo Basile, Varese, Italy). The apparatus consisted of a base platform and a rotating rod of 3 cm in diameter with a non-slippery surface. This rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, using a rotating speed of 16 r.p.m. The integrity of motor coordination and resistance to fatigue were assessed on the basis of the endurance time of the animals on the rotating rod, which was automatically registered for each mouse. Three days before the test, the animals were trained 2–3 times a day. At the end of the three days, only the mice able to stay balanced on the rotating rod at least 120 s (cut-off time) were selected. On the fourth day, the performance time was measured before, and 15, 30 and 45 min after, treatment.

Isolated longitudinal muscle strip of guinea-pig ileum

The myenteric plexus longitudinal muscle was prepared according to Paton & Vizi (1969). The strip was suspended in a 12.5 ml thermoregulated (36–37°C) bath and stimulated by either an electrical field (0.1 Hz, 1 ms pulse duration and submaximal voltage) or by chemical stimulants such as nicotine, acetylcholine (ACh) and oxotremorine. The Krebs-Henseleit solution, bubbled with 95% O_2 and 5% CO_2, had the following composition (mM): NaCl 118.0, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_3 1.2, $NaHCO_3$ 25.0 and glucose 11.0.

Drugs and reagents

The following drugs were used: atropine sulphate, atropine methylbromide, hemicholinium-3 bromide (HC-3), DL-α-methyl-*p*-tyrosine methylester hydrochloride, propranolol hydrochloride, naloxone hydrochloride (Sigma), reserpine, L-(+)-ascorbic acid, acetylcholine chloride (Merck), oxotremorine (Fluka), baclofen and phentolamine (Ciba-Geigy), dicyclomine hydrochloride (Lepetit), pirenzepine hydrochloride (Istituto Sieroterapico Milanese), morphine hydrochloride (USL 10/D Florence), nicotine hydrogentartrate (BDH). Other chemicals were of the highest quality commercially available. All the drugs were dissolved in physiological saline solution except reserpine which was dissolved in a 20% aqueous solution of ascorbic acid. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by both s.c. and i.p. injection. The doses given in the text are expressed as salts. I.c.v. administration was performed during short ether anaesthesia by injecting the necessary dose dissolved in 5 μl according to the method described by Haley & McCormick (1957).

Statistical analysis

Results are given as the mean ± s.e. Dunnett's two-tailed test was used to verify the significance of differences among the means, and these were considered significant when P values were less than 0.05.

Results

Atropine antinociceptive effect by peripheral route

Subcutaneously-administered atropine induced a significant biphasic effect on the pain threshold of mice tested on the hot-plate (Figure 1a), inducing a clear antinociception in the range of 1–100 μg kg^{-1} s.c. and hyperalgesia at a higher dosage (5 mg kg^{-1}, s.c.).

Antinociception reached the maximum after 15 min (Table 1), persisting almost unchanged up to 30 min and then disappearing 45 min after administration. Antinociception with low doses of atropine (1 μg kg^{-1}, s.c.) was similarly observed in another analgesic test (writhing induced by acetic acid) where the number of writhes was halved 15 min after treatment.

Atropine was analgesic also in rats in the tail-flick test, where it induced the maximum effect 15 min after i.p. injection of 10–100 μg kg^{-1} (Figure 1b). Antinociception was obtained in both mice and rats without any visible change in the normal behaviour of animals, to the extent that the researchers, who were unaware of the treatment received by the animals, were unable to distinguish between various groups. This was confirmed in rota-rod experiments where atropine (1 μg kg^{-1}, s.c.) did not significantly change the time that mice remained on the rota-rod up to 45 min post-treatment. This was in contrast to oxotremorine (40 μg kg^{-1}, s.c.) which halved the 'on-rod' time to about 60 s, at 15 and 30 min post-treatment.

Atropine antinociceptive effect by intracerebroventricular route

Antinociception was also obtained in mice by low doses of atropine given i.c.v. Thus in the hot-plate test, significant enhancement (latency up to 20–25 s) was observed after atropine (1–10 ng, i.c.v.). Higher doses (50 and 100 ng, i.c.v.) were ineffective.

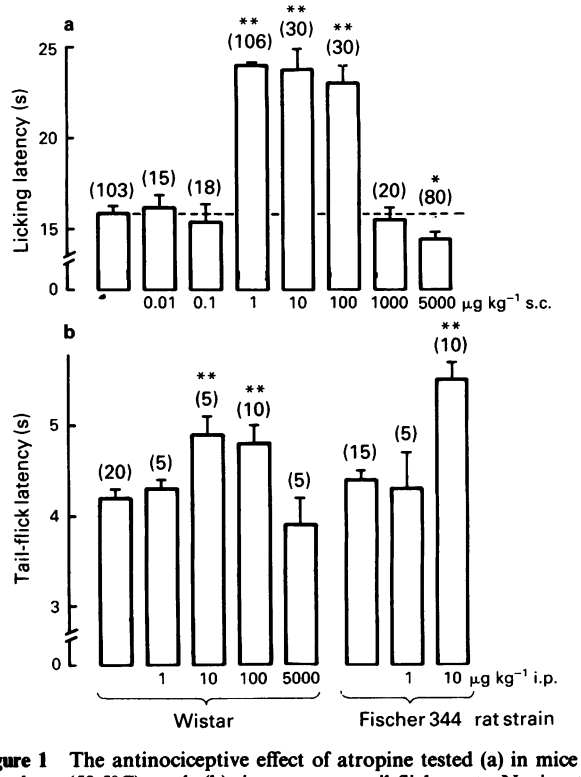

Figure 1 The antinociceptive effect of atropine tested (a) in mice on hot-plate (52.5°C) and (b) in rats on tail-flick test. Nociceptive responses were recorded 15 min after drug administration. Number of animals in parentheses. Vertical lines show s.e.mean. ** $P < 0.01$; * $P = 0.05$ in comparison with saline controls.

Table 1 Antagonistic effect by cholinolytic drugs but not by naloxone on atropine antinociception in mice

Pretreatment	Treatment (s.c.)	Hot-plate test Licking latency in mice (s) Before treatment	After treatment 15 min
Saline i.p. 10 ml kg^{-1}	Saline 10 ml kg^{-1}	14.6 ± 0.3 (103)	15.8 ± 0.4 (103)
	Atropine 1 μg kg^{-1}	14.9 ± 0.6 (106)	24.9 ± 0.2* (106)
HC-3 i.c.v. 1 μg per mouse	Saline 10 ml kg^{-1}	15.1 ± 1.0 (20)	16.5 ± 1.0 (20)
	Atropine 1 μg kg^{-1}	14.6 ± 1.1 (20)	15.8 ± 1.2 (20)
Atropine methylbromide i.c.v. 0.12 μg per mouse	Saline 10 ml kg^{-1}	14.3 ± 1.2 (10)	16.1 ± 1.3 (10)
	Atropine 1 μg kg^{-1}	15.1 ± 1.1 (10)	16.9 ± 1.1 (10)
Pirenzepine i.c.v. 0.1 μg per mouse	Saline 10 ml kg^{-1}	15.4 ± 0.9 (10)	16.1 ± 1.6 (10)
	Atropine 1 μg kg^{-1}	14.8 ± 1.3 (10)	16.4 ± 1.3 (10)
Dicyclomine i.p. 10 mg kg^{-1}	Saline 10 ml kg^{-1}	14.7 ± 0.7 (10)	14.9 ± 1.7 (10)
	Atropine 1 μg kg^{-1}	15.2 ± 0.9 (10)	15.7 ± 1.3 (10)
Pirenzepine i.p. 10 mg kg^{-1}	Saline 10 ml kg^{-1}	15.7 ± 0.9 (10)	16.3 ± 1.5 (10)
	Atropine 1 μg kg^{-1}	15.5 ± 1.2 (10)	26.1 ± 1.2* (10)
Naloxone i.p. 1 mg kg^{-1}	Saline 10 ml kg^{-1}	14.7 ± 0.7 (10)	14.9 ± 1.7 (10)
	Atropine 1 μg kg^{-1}	15.5 ± 1.2 (10)	24.1 ± 0.6* (10)
Atropine methylbromide i.p. 5.5 mg kg^{-1}	Saline 10 ml kg^{-1}	15.1 ± 0.6 (17)	16.0 ± 1.4 (17)
	Atropine 1 μg kg^{-1}	15.0 ± 0.7 (13)	23.7 ± 0.7* (13)

Pretreatments were performed 15 min before the treatments except hemicholinium-3 (HC-3) which was injected 5 h before treatment and pirenzepine which was administered simultaneously with treatment. The number of mice is shown in parentheses.
* $P < 0.01$ in comparison with saline controls.

Antagonism of the antinociceptive effect

The antinociceptive effect of atropine was not antagonized by naloxone while i.c.v. atropine methylbromide was able completely to reverse it (Table 1). Pirenzepine (0.1 μg per mouse i.c.v.) and dicyclomine (10 mg kg^{-1}, i.p.) antagonized atropine-induced antinociception in both the hot-plate and writhing models. The same results were also obtained by i.c.v. injection of HC-3 administered 5 h before the analgesic test. Also the fact that no analgesia was obtained with atropine administered at doses higher than 1–100 μg kg^{-1}, could be interpreted as an antagonistic effect of the higher versus the lower doses of atropine (Figure 1a).

Baclofen (2 mg kg^{-1}, s.c.) and morphine (4 mg kg^{-1}, s.c.) and oxotremorine (40 μg kg^{-1}, s.c.) all possessed antinociceptive activity equivalent to that of atropine (1 μg kg^{-1}, s.c.) in the hot-plate test. The activity of the former compounds, unlike that of atropine and oxotremorine, was not modified by dicyclomine (10 mg kg^{-1}, i.p.) or pirenzepine (0.1 μg per mouse i.c.v.). Against oxotremorine, dicyclomine was effective for 45 min whilst pirenzepine was only effective for 15 min (Table 2). HC-3 (1 μg per mouse i.c.v.) was ineffective against all three

Table 2 Effect of dicyclomine and pirenzepine on antinociception induced in mice by oxotremorine

Pretreatment	Treatment (s.c.)	Hot-plate test Licking latency in mice (s) Before treatment	After treatment 15 min	30 min	45 min
Saline 10 ml kg^{-1} i.p. or 5 μl i.c.v.	Saline 10 ml kg^{-1}	15.5 ± 1.2 (10)	16.7 ± 0.9 (10)	15.8 ± 1.6 (10)	16.8 ± 0.8 (10)
	Oxotremorine 40 μg kg^{-1}	15.6 ± 1.0 (10)	22.5 ± 1.2* (10)	25.8 ± 1.3* (10)	24.6 ± 1.6* (10)
Dicyclomine 10 mg kg^{-1} i.p. −15 min	Saline 10 ml kg^{-1}	15.7 ± 0.6 (25)	16.3 ± 0.8 (25)	17.1 ± 1.2 (25)	15.1 ± 0.9 (25)
	Oxotremorine 40 μg kg^{-1}	16.0 ± 0.7 (25)	17.5 ± 0.9 (25)	16.9 ± 0.9 (25)	15.6 ± 0.9 (25)
Pirenzepine 0.1 μg i.c.v.	Saline 10 ml kg^{-1}	15.0 ± 0.9 (15)	15.6 ± 0.5 (15)	15.1 ± 1.1 (15)	16.0 ± 1.0 (15)
	Oxotremorine 40 μg kg^{-1}	16.2 ± 1.1 (10)	15.4 ± 2.8 (10)	23.0 ± 2.2* (10)	17.4 ± 1.0 (10)

The number of mice is shown in parentheses.
* $P < 0.01$ in comparison with saline controls.

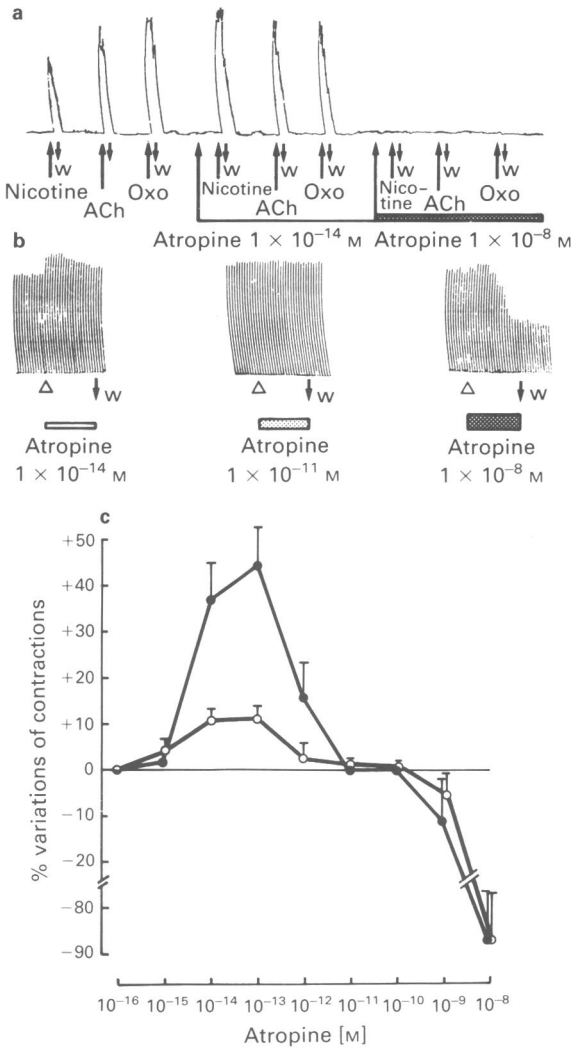

Figure 2 Effect of atropine on chemically- and electrically-evoked contractions of guinea-pig ileum myenteric plexus longitudinal muscle strip. (a) Tracing from a typical experiment showing the effect of atropine either at 10^{-14} M or at 10^{-8} M on contractions induced by nicotine 4×10^{-6} M, acetylcholine (ACh) 1×10^{-8} M and oxotremorine (Oxo) 5×10^{-8} M. (b) Tracing of a typical experiment showing the effect of atropine (10^{-14} M, 10^{-11} M and 10^{-8} M) on electrically evoked contractions (0.1 Hz, 1 ms duration, twice threshold voltage). (c) Percentage variation of electrically- (○) and nicotinic (●)-evoked contractions induced by graduated doses of atropine. Each point represents the mean of 4 experiments and vertical lines give s.e.mean.

agents. Pirenzepine administered i.p. at an equimolecular dose to dicyclomine was unable, unlike i.c.v. pirenzepine, to prevent atropine analgesia (Table 1), which was also unaffected by i.p. atropine methylbromide, a drug which like pirenzepine (Hammer & Koss, 1979) does not cross the blood-brain barrier (Table 1).

Atropine effect on longitudinal muscle strip of guinea-pig ileum

The effect of atropine proved to be biphasic. At low concentrations (below 10^{-11} M), atropine increased both nicotine- and electrically-induced twitches of the longitudinal muscle strip of guinea-pig ileum (Figure 2) and did not affect the contractions produced by exogenous ACh and oxotremorine (Figure 2a). Moreover, the same doses did not exert any effect on unstimulated ileum (Figure 2a). At higher concentrations (above 10^{-10} M) atropine drastically reduced all responses evoked by electrical, nicotinic and muscarinic stimulation (Figure 2). The potentiation of evoked contractions by low doses of atropine is about four times higher with nicotine than with electrical stimulation (Figure 2c). However, following treatments to block adrenoceptors or the adrenergic system (Table 3), the amplification of electrical stimulation induced by atropine equalled that induced by nicotine. As a matter of fact pretreatment of the guinea-pig with reserpine and α-methyl-*p*-tyrosine as well as treatment of guinea-pig isolated ileum with both phentolamine and propranolol (administered together) increased the potentiating effect of atropine on electrically-evoked contractions about four fold (Table 3).

Discussion

The present results indicate that atropine induced antinociception at low doses, did not modify the pain threshold at intermediate doses and provoked hyperalgesia at the highest ones. The antinociceptive effect induced by low doses of atropine was obtained consistently in both mice and rats in tests appropriate to each species. Moreover, analgesia was produced in animals without altering their normal performance on the rota-rod test.

The atropine analgesic site of action is likely to be located in the CNS for the following reasons: (1) It was possible to reach the same intensity of analgesia by injecting directly into the cerebral ventricles a dose of atropine (5 ng/mouse) which was considerably lower than that needed parenterally (1 $\mu g\,kg^{-1}$). (2) HC-3, atropine methylbromide and pirenzepine, all drugs that cross the blood-brain barrier with difficulty, antagonized atropine antinociception only when injected i.c.v. Since antinociception induced by low doses of atropine was blocked by i.p. dicyclomine and by i.c.v. pirenzepine and atropine methylbromide, it is likely that such analgesia is of the cholinergic type. In fact, pirenzepine, dicyclomine and atropine-methylbromide were all able to antagonize oxotremorine-induced analgesia. Moreover, drugs with no anticholinoceptor activity such as naloxone (Table 1), α-methyl-*p*-tyrosine and reserpine (data not shown) were unable to modify this analgesia by interfering with opioid, catecholaminergic and 5-hydroxytryptaminergic systems. A presynap-

Table 3 Percentage potentiation of electrically-evoked contractions of guinea-pig longitudinal muscle strip induced by atropine itself and in the presence of some sympatholytic treatments

	Atropine concentration				
Pretreatment	10^{-15}	10^{-14}	10^{-13}	10^{-12}	10^{-11}
None	0.9 ± 1.1	11.02 ± 2.0	11.3 ± 1.5	2.6 ± 1.1	1.4 ± 1.4
Reserpine	0	43.1 ± 11.2	43.3 ± 15.9	2.5 ± 2.5	0
α-Methyl-*p*-tyrosine	5.2 ± 2.3	36.9 ± 9.2	31.9 ± 3.9	10.5 ± 3.4	0
Phentolamine	4.0 ± 2.1	22.3 ± 5.9	25.7 ± 5.0	2.0 ± 2.0	0
Propranolol	3.2 ± 3.2	15.7 ± 6.7	12.6 ± 8.1	1.7 ± 1.7	0
Phentolamine + Propranolol	9.7 ± 4.9	37.4 ± 11.8	38.1 ± 4.6	4.0 ± 4.0	0

Reserpine (2 mg kg^{-1}, i.p.) and α-methyl-*p*-tyrosine (100 mg kg^{-1}, i.p.) were injected into guinea-pigs 48 h and 24 h respectively before the beginning of the experiment.
Both phentolamine (500 ng ml^{-1}) and propranolol (500 ng ml^{-1}) were injected into the bath 3 min before administration of atropine.

tic mechanism, facilitating cholinergic transmission, is likely to be involved in atropine analgesia because atropine antinociception was prevented by cerebral ACh depletion induced by i.c.v. HC-3. In agreement with this interpretation, antinociception induced by oxotremorine, a direct muscarinic agonist, was not decreased by i.c.v. HC-3.

The *in vitro* experiments have shown that concentrations of atropine ranging from 10^{-14} to 10^{-12} M were able to increase presynaptically induced (electrically- and nicotine-evoked) contractions of longitudinal muscle of guinea-pig ileum whereas postsynaptically evoked (ACh- and oxotremorine) contractions were unchanged. These results suggest the hypothesis that low doses of atropine act presynaptically to facilitate cholinergic transmission.

Nicotine-evoked ileum contractions were increased four times more than those electrically-evoked. This is probably due to the simultaneous activation, during electrically-evoked contractions, of both intramural cholinergic and sympathetic fibres; whereas during nicotine-evoked contractions only cholinergic neurones are likely to be activated. Noradrenaline released during electrical stimulation could be responsible for limiting the effect of the ACh released by low doses of atropine. This hypothesis is suggested by results obtained after sympathetic block (reserpine and α-methyl-*p*-tyrosine pretreatment, phentolamine and propranolol treatment) since in these conditions low concentrations of atropine were able to increase electrically-evoked contractions to the same extent as nicotine. Analgesia induced by the activation of the central cholinergic system may be mediated via M_1 muscarinic receptors, since pirenzepine and dicyclomine were able to antagonize both oxotremorine and atropine antinociception. Pirenzepine and dicyclomine are known to have an affinity for M_1 receptors at least 16 times higher than for other muscarinic receptors (Mitchelson, 1988). In our experiments, pirenzepine and dicyclomine were able to antagonize oxotremorine and atropine antinociception in the same molar range of i.c.v. atropine methylbromide and i.p. atropine (Bartolini *et al.*, 1987). It is quite possible therefore that postsynaptic receptors responsible for cholinergic antinociception are of the M_1 subtype. Moreover Potter *et al.* (1984), Raiteri *et al.* (1984), Mash *et al.* (1985), Mash & Potter (1986), Spencer *et al.* (1986) and Pohorecki *et al.* (1988) have all reported that in the CNS cholinergic inhibitory autoreceptors are of the M_2 subtype. If low doses of atropine induced analgesia by increasing ACh release through a selective block of muscarinic autoreceptors, it appears reasonable to argue that CNS autoreceptors are at least 1000 times more sensitive to atropine block than post synaptic ones (perhaps inhibitory heteroreceptors located on ascending nociceptive fibres). Other examples concerning the higher sensitivity of autoreceptors to blocking agents have already been described: for instance, Levine *et al.* (1979), Woolf (1980) and Kaiser & Guilband (1981) reported that very low doses of naloxone can induce analgesia and Ueda *et al.* (1986) showed that such analgesia is due to increased enkephalin release produced by blocking autoinhibition. Moreover Starke (1977) has reported that yohimbine enhances neural release of noradrenaline at lower concentrations than those required to block postsynaptic receptors. In conclusion, our results have shown that very low doses of atropine are able to induce analgesia by increasing cholinergic function. The amplification of cholinergic responses induced by atropine appears to be much more useful than direct stimulation with muscarinic agonists as shown by results obtained with the rota-rod test. Direct stimulation with muscarinic agonists exerts a strong and continuous activation of postsynaptic receptors, responsible for a large series of untoward effects at the same time as analgesia. Low doses of atropine, on the other hand, induce analgesia without any adverse symptomatology, as least as far as is shown by rota-rod results. Considering that by using an aqueous extraction the ancient Romans could obtain only very small quantities of *Hyosciamus niger* and *Mandragora officinarum* alkaloids, the present results provide one explanation of why they were able to relieve pain with this remedy.

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Pharmacological estimation of agonist affinity: detection of errors that may be caused by the operation of receptor isomerisation or ternary complex mechanisms

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1 Recent theoretical studies have questioned the pharmacological estimation of agonist affinity. They showed that when receptor isomerisation or ternary complex mechanisms operate, the receptor inactivation method can substantially overestimate affinity, whereas methods for partial agonist analysis are more accurate. We previously suggested that the operation of such mechanisms and therefore the presence of errors could be detected by analysing the same partial agonist by the receptor inactivation and comparative methods. This paper describes the practical application of this test.

2 The ternary complex mechanism was simulated for a partial agonist under various conditions relating receptor (R) and transducer (T) concentrations, one of which also corresponds to the receptor isomerisation mechanism. The theoretical data so generated were then analysed by the inactivation and comparative methods to quantify the magnitude of error of affinity estimation that could occur.

3 This analysis showed that for a partial agonist with approximately 85% of the activity of a full agonist, the inactivation method could produce an affinity (pK_A) estimate up to 0.7 $\log_{10}$ units higher than that produced by the comparative method. This difference would occur when the total receptor concentration ($[R_0]$) is less than or equal to the total transducer concentration ($[T_0]$). It also showed that the overestimation of affinity by the inactivation method was accompanied by drastic overestimation of E_m, the maximal effect parameter.

4 The test was then exemplified using the muscarinic receptor system in the guinea-pig isolated left atrial preparation, where there is evidence that a ternary complex mechanism operates. The test agonist was pilocarpine, which produced on average 83% of the activity of the full agonist, carbachol. Pilocarpine was analysed in comparison with carbachol and by receptor inactivation in the same tissue resulting in small and statistically insignificant differences in E_m (96.7% and 97.3% respectively) and pK_A (5.03 and 4.95 respectively).

5 In conclusion, in this experimental system, there was no evidence for the errors in agonist affinity estimation predicted by theory. Although this conclusion only applies to this system and application of the test to others is necessary to establish the generality of the present results, further examination of the theoretical basis for the predicted errors is required.

Introduction

In a number of recent articles (Colquhoun, 1987; Mackay, 1988; Kenakin, 1989; Leff & Harper, 1989) attention has been drawn to the theoretical unreliability of agonist affinity estimates obtained by pharmacological methods due to the operation of receptor isomerisation (del Castillo & Katz, 1957) and ternary complex (De Lean *et al.*, 1978) mechanisms.

In the case of the receptor isomerisation mechanism it has been shown that it is theoretically impossible, by use of traditional pharmacological methods (Stephenson, 1956; Furchgott, 1966; Barlow *et al.*, 1967), to estimate agonist affinity independently of efficacy, with the consequence that affinity itself is overestimated (Colquhoun, 1987). The same predictions are made in the case of the ternary complex mechanism when the concentration of agonist-receptor complexes is similar to or less than the concentration of transducer units with which they interact (Mackay, 1988; Leff & Harper, 1989). However, it is predicted for both mechanisms that the magnitude of overestimation of affinity will be larger in the case of full agonist analysis by the receptor inactivation method of Furchgott (1966) than in the case of partial agonist analysis by either the interaction method (Stephenson, 1956) or the comparative method (Barlow *et al.*, 1967). It follows, if these theoretical predictions are correct, that when the same agonist (which would necessarily be a partial agonist) is analysed by the inactivation method and either of the two latter methods, different estimates of affinity should be obtained. As suggested previously (Leff & Harper, 1989) this provides an experimental test for the operation of conditions under which erroneous affinity estimates may be made. This test was originally proposed to detect the operation of the ternary complex mechanism but it applies equally well to systems obeying the receptor isomerisation mechanism.

The present paper explains the practicalities of this test and illustrates its potential utility by its application to the analysis of a partial muscarinic agonist.

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Theory

The estimation of agonist affinity by pharmacological methods (Stephenson, 1956; Furchgott, 1966; Barlow *et al.*, 1967) implicitly assumes the validity of traditional receptor theory. With increasing knowledge of receptor mechanisms, it has become necessary to question the utility of traditional receptor theory as a basis for analysing agonist action (Colquhoun, 1987). The central issue is that the traditional theory assumes that affinity can be estimated independently of intrinsic efficacy. Analysis of two apparently plausible mechanisms for the activation of receptors by agonists indicates that this assumption is not valid. Below are given the main predictions of these analyses. For full theoretical details the reader is referred to the original papers.

Receptor isomerisation mechanism

This mechanism was originally proposed to explain how agonists exert their effects in ion-channel-linked receptor

systems (del Castillo & Katz, 1957):

$$A + R \overset{K_A}{\longleftrightarrow} AR \overset{E}{\longrightarrow} AR^*$$

R represents unoccupied receptors, AR represents occupied but unactivated receptors and AR* represents activated receptors. Thus, the generation of a pharmacological effect by the agonist depends on its ability to bind to the receptor and then to elicit its isomerisation into the active state. In this scheme, agonist affinity is determined by the dissociation constant, K_A, for the AR complex, and intrinsic efficacy is defined by the isomerisation constant, E (see Colquhoun, 1987).

Colquhoun (1987) gave the theoretical results of applying pharmacological methods for the estimation of agonist affinity in such systems. In the case of the irreversible receptor inactivation method (Furchgott, 1966), it was predicted that affinity would be overestimated, the estimated dissociation constant being:

$$K_{Aest} = K_A/(1 + E) \quad (1)$$

showing that the extent of overestimation would be proportional to the intrinsic efficacy of the agonist.

In contrast, in the case of the comparative (Barlow *et al.*, 1967) or interaction (Stephenson, 1956) methods for the analysis of partial agonists it was predicted that the estimated affinity would be close to the true value so long as the intrinsic efficacy of the test partial agonist was much less than that of the reference full agonist.

Ternary complex mechanism

This mechanism was originally proposed to explain how agonists exert their effects in receptor systems coupled to G-proteins (De Lean *et al.*, 1978). In principle, it may apply to any system in which the receptor interacts with a transducer unit in order to initiate a response:

$$A + R \overset{K_A}{\longleftrightarrow} AR + T \overset{K_{AR}}{\longleftrightarrow} ART$$

As with the isomerisation mechanism, R represents unoccupied receptors, AR represents occupied but unactivated receptors, and ART represents receptors in their active state due to coupling with transducers. K_A determines affinity in this mechanism as in the case of the isomerisation mechanism, whereas efficacy is now determined by the dissociation constant, K_{AR}, for the ternary complex, ART.

In this case, predictions relating to the application of pharmacological methods for affinity estimation depend on the relative concentrations of R and T (Leff & Harper, 1989). Denoting the total concentrations of these as $[R_0]$ and $[T_0]$ respectively, when $[R_0] \gg [T_0]$, the receptor inactivation method as well as the comparative method provides a correct K_A estimate. When $[R_0] \ll [T_0]$, the comparative method provides a correct estimate but the receptor inactivation method overestimates affinity, the estimated dissociation constant being:

$$K_{Aest} = K_A/(1 + [T_0]/K_{AR}) \quad (2)$$

This result, which was also given by Mackay (1988), is analogous to the prediction made for the isomerisation mechanism (equation 1), the extent of overestimation of affinity being dependent on intrinsic efficacy, in this case $1/K_{AR}$. When $[R_0] = [T_0]$, both the inactivation and comparative methods overestimate affinity but the size of the error is larger for the inactivation method.

A test for errors in affinity estimation

It follows, if these mechanisms operate, that the estimate of affinity for a partial agonist obtained using the receptor inactivation method will, in general, be higher than that obtained by the comparative method. Only in the case of the ternary complex mechanism, with $[R_0] \gg [T_0]$, are the estimates predicted to be the same, as would be assumed by traditional theory. This provides a test which may help to detect the operation of mechanisms which do not accord with traditional receptor theory and in doing so assist in avoiding erroneous affinity estimation. Alternatively, the test may serve to indicate that the traditional theory holds and that the associated affinity estimates are valid.

In the first part of this study, theoretical data are generated corresponding to the two mechanisms, and analysed in order to evaluate the magnitude of difference in affinity estimates that is likely to occur in practice. Having established the detection limits of the test in this way, it is then applied to experimental data obtained in a muscarinic receptor system.

Methods

Computer simulations

Agonist-concentration effect, E/[A], curves were generated with the formulation of the ternary complex model given by Leff & Harper (1989) and the reader is referred to that paper for full theoretical derivations; only brief details will be given here. According to theory, the equilibrium concentration of active ART complexes is given by a quadratic function of agonist concentration:

$$[ART]^2 + b[ART] + c = 0 \quad (3)$$

in which

$$b = -K_{AR}(1 + K_A/[A]) + [T_0] + [R_0]$$

$$c = [R_0][T_0]$$

[ART] has values defined by the roots of this equation. The pharmacologically meaningful one is:

$$[ART] = (-b - \sqrt{b^2 - 4c})/2 \quad (4)$$

E/[A] curves were simulated assuming that E equated with [ART], choosing parameter values to represent the conditions $[R_0] \gg$, = or $\ll [T_0]$.

Under the condition $[R_0] \ll [T_0]$, the [ART]/[A] relation can be written clearly (Leff & Harper, 1989):

$$[ART] = \frac{[R_0]\tau_T[A]}{K_A + (1 + \tau_T)[A]} \quad (5)$$

with τ_T defined as $[T_0]/K_{AR}$.

In the case of the isomerisation mechanism, the relation between [AR*] and [A] can be written (rearranging equation (3) in Colquhoun (1987)):

$$[AR^*] = \frac{[R_0]E[A]}{K_A + (1 + E)[A]} \quad (6)$$

which is clearly analogous to equation (5), the only difference being the mechanistic definition of efficacy.

This means that results obtained simulating equation (4) under the condition $[R_0] \ll [T_0]$ can be taken to represent either the ternary complex or isomerisation mechanisms.

All simulations were carried out on a Compaq 386/25 using the commercially available spreadsheet package, Symphony, produced by Lotus.

Guinea-pig isolated left atria

Male Dunkin-Hartley guinea-pigs (300–400 g) were killed by a blow to the back of the head. The left atria were quickly removed and suspended in 20 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.41, KCl 4.69, $MgSO_4$ 1.18, KH_2PO_4 1.18, glucose 11.10, $NaHCO_3$ 24.99 and $CaCl_2$ 2.52. This was maintained at 37°C and continually gassed with 5% CO_2 in O_2. The atria were

attached at the base to tissue-hook electrodes by cotton thread and connected at the top to isometric force transducers (Ormed Beam) with steel clips. The diastolic (resting) tension was set at an initial value of 1 g wt. The tissues were electrically stimulated with pulses of 5 ms duration, at a frequency of 2 Hz and a voltage which was 1.5 times that required for a threshold response (twitch). Generated force was recorded on a Devices M19 or an Advance Bryans flat bed recorder.

Experimental protocols

General All tissues were allowed a 60 min period of equilibration before the addition of any drugs. Agonist concentration-effect, E/[A], curves were constructed by cumulative additions of either carbachol or pilocarpine at 0.5 $\log_{10}$ unit increments. Up to 3 E/[A] curves were performed in each tissue. Responses were recorded as percentage inhibitions of the stimulated twitch.

Carbachol efficacy estimation by the receptor inactivation method In each tissue a carbachol E/[A] curve was constructed. After washing, tissues were incubated with the irreversible antagonist phenoxybenzamine (Pbz) (3 μM or 10 μM) or vehicle (40% polyethylene glycol) for 30 min. Following removal of the irreversible antagonist by several changes of the organ bath Krebs solution over a 40 min period, a second carbachol E/[A] curve was performed.

Pilocarpine affinity estimation by the comparative and irreversible receptor inactivation methods In each tissue a carbachol E/[A] curve was constructed, followed, after washing, by a pilocarpine E/[A] curve. Tissues were subsequently washed and then incubated with Pbz (0.3 μM, 0.5 μM or 1 μM) or vehicle for 30 min. Pbz was then removed by several washes over a 40 min period and a further pilocarpine E/[A] curve constructed.

Drugs

The following drugs were used: carbachol chloride (Sigma Chemical Company), pilocarpine hydrochloride (Evans Medical Ltd), phenoxybenzamine hydrochloride (Smith, Kline and French Laboratories), and atropine sulphate (Sigma Chemical Company).

Carbachol, pilocarpine and atropine were dissolved in distilled water. Phenoxybenzamine was dissolved in 40% polyethylene glycol.

Data analysis

Operational model fitting Computer-generated and experimental data were fitted using the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (7)$$

in which E and [A] are the pharmacological effect and the concentration of the agonist, respectively; E_m is the maximum possible effect; K_A is the agonist dissociation constant (this was estimated as the negative logarithm, that is, pK_A); τ is the efficacy of the agonist (estimated as a logarithm); n determines the steepness of the occupancy-effect relation.

The fitting procedures employed are described in detail elsewhere (Leff *et al.*, 1989); only brief details will be given here. As a multiple curve design was used, each tissue provided an estimate of K_A by each of the comparative and inactivation methods. In the case of the comparative method the partial agonist curve data were fitted to the operational model (equation 7) simultaneously to fitting the full agonist data to a logistic function of the form:

$$E = \frac{E_m [A]^n}{[A_{50}]^n + [A]^n} \quad (8)$$

where E_m and n are as defined above and $[A_{50}]$ is the location parameter for the full agonist curve. (Location parameters were estimated as negative logarithms ($p[A_{50}]$)). This allows K_A as well as τ for the partial agonist to be estimated as well as E_m and n from each pair of curves.

In the case of the inactivation method, the E/[A] curve data for the agonist (full or partial) before and after receptor inactivation were fitted to equation (7) providing a common estimate of E_m, n and K_A, and a value of τ for each curve in the pair. Average values of the parameter estimates are quoted with standard errors corresponding to between tissue variation. Comparisons of parameter estimates obtained by the comparative and inactivation methods were made by paired *t* test. Standard errors derived from the fitting procedure corresponding to internal fitting error (a measure of goodness-of-fit) are also quoted.

Logistic curve fitting In control experiments designed to determine any effect of drug vehicle on the response to carbachol and pilocarpine, individual E/[A] curves were fitted to a logistic function of the form:

$$E = \frac{\alpha [A]^m}{[A_{50}]^m + [A]^m} \quad (9)$$

where α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively (any vehicle effect would be shown by significant changes in one or more of these parameters).

All data fitting procedures were carried out with the statistical package, BMDP, and a Vax 11/780 mainframe computer.

Results

Analysis of theoretical data for a partial agonist

Figure 1 shows theoretical E/[A] curves generated using equation (4). Parameter values were chosen to represent the conditions $[R_0] \gg [T_0]$ (panel (a)), $[R_0] = [T_0]$ (panel (b)) and $[R_0] \ll [T_0]$ (panel (c)) in the ternary complex mechanism, the last condition also representing the isomerisation mechanism. Under each condition, an intrinsic efficacy ($1/K_{AR}$) value was chosen to simulate a partial agonist which can generate approximately 85% of the maximum effect. The same agonist was simulated using a lower value of $[R_0]$ representing the effects of receptor inactivation, and a full agonist curve was also generated in order to analyse the partial agonist by the comparative method.

'Data' points covering a realistic experimental range were sampled from the curves at 0.5 $\log_{10}$ unit intervals. These data are shown as symbols in Figure 1. These 'data' points refer to concentrations of [ART]. For curve fitting, [ART] was assumed to be directly proportional to effect, and the concentrations of [ART] were scaled up to cover a range of 0 to 100. E_m for the system is equivalent to the maximum theoretical concentration of [ART] that can be achieved. For each condition, the partial agonist curve was analysed by the comparative method and by the inactivation method. The resulting estimates of affinity and of the other operational parameters are collated in Table 1. This analysis showed that, under the condition $[R_0] \gg [T_0]$, accurate estimates of pK_A were obtained by both methods. Also, it is to be noted that E_m was accurately estimated by both methods and the standard errors associated with the estimated model parameters were small and similar. However, under the conditions $[R_0] = [T_0]$ and $[R_0] \ll [T_0]$, the inactivation method overestimated pK_A by some 0.7 $\log_{10}$ units, whilst the comparative method was

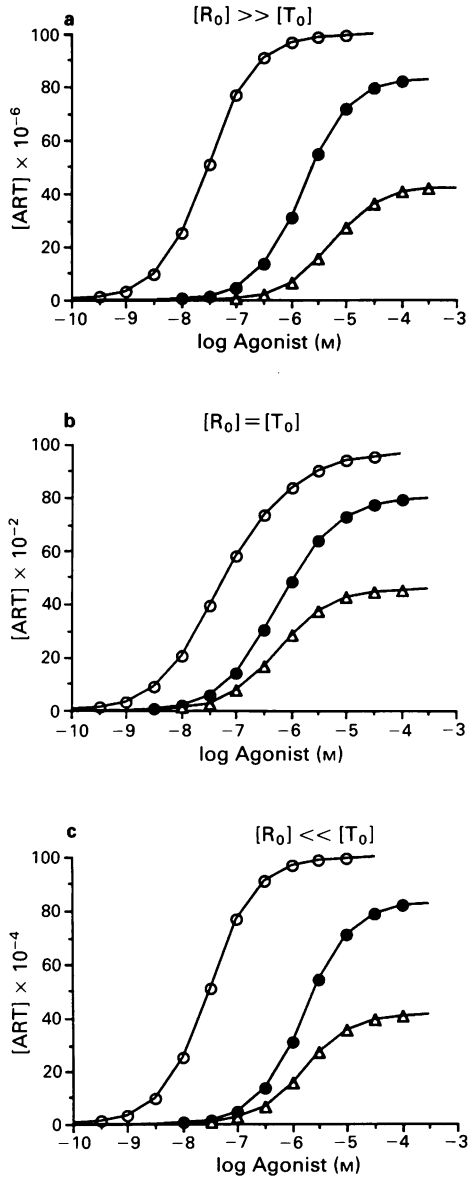

Figure 1 Theoretical E/[A] curves under various conditions relating receptor ($[R_0]$) and transducer ($[T_0]$) concentrations. Panels (a), (b) and (c) represent the conditions $[R_0] \gg [T_0]$, $[R_0] = [T_0]$ and $[R_0] \ll [T_0]$ respectively. The latter condition is equivalent to the isomerisation mechanism. Under each condition, theoretical curves were generated corresponding to a full agonist (○), a partial agonist (●) with approximately 85% of the activity of the full agonist and the same partial agonist after receptor inactivation (△).

The simulation parameters used were as follows:

	Affinity		*Intrinsic efficacy*				
	pK_A full	pK_A partial	pK_{AR} full	pK_{AR} partial	$[T_0]$	$[R_0]$ initial	$[R_0]$ inactivated
(a)	4.5	5.0	3.0	0.7	0.0001	1.0	0.15
(b)	4.5	5.0	3.0	1.3	1.0	1.0	0.5
(c)	4.5	5.0	3.0	0.7	1.0	0.01	0.005

The symbols represent the 'data' points that were sampled from computer generated curves and analysed by operational model-fitting (see Table 1).

accurate to within 0.1 $\log_{10}$ units. Also, E_m was overestimated by up to 500% by the inactivation method but accurately estimated to within 5% by the comparative method under both conditions. Furthermore, the standard errors associated with model fitting were considerably larger for the inactivation method than for the comparative method (see Table 1). For example under the condition $[R_0] = [T_0]$ the standard error on E_m was about 50%, whereas under the condition $[R_0] \gg [T_0]$ it was less than 1%. This is an indication of a poor quality of fit, which is anticipated since the theoretical data should not accord with the operational model under the conditions $[R_0] \leqslant [T_0]$. Indeed, when $[R_0] \ll [T_0]$ standard errors could not be estimated due to poorness of fit.

Table 1 Analysis of theoretical data for a partial agonist by comparative and inactivation methods

	Comparative Method		*Inactivation Method*	
	Estimate	*Standard error*	*Estimate*	*Standard error*
$[R_0] \gg [T_0]$				
E_m	98.9	0.4	98.6	0.8
n	1.02	0.01	1.03	0.02
log τ	0.69	0.01	0.70	0.02
pK_A	5.02	0.02	5.01	0.02
$[R_0] = [T_0]$				
E_m	95.0	0.8	342.6	175.7
n	0.75	0.02	0.56	0.08
log τ	0.96	0.04	−0.94	0.97
pK_A	5.10	0.05	5.72	0.26
$[R_0] \ll [T_0]$ (also represents the isomerisation mechanism)				
E_m	100.1	0.2	555.7	—
n	0.99	0.01	0.98	—
log τ	0.71	0.01	−0.77	—
pK_A	4.98	0.02	5.69	—

In the last fit, standard errors could not be computed due to degeneracies encountered during the fitting procedure. The 'correct' estimates of pK_A and E_m were 5.00 and 100.0 respectively.

Analysis of experimental data for the full agonist, carbachol

In order to apply the comparative method to the analysis of the partial agonist, pilocarpine (see below), it was necessary first to ensure that the reference agonist, carbachol, acts as a full agonist in the guinea-pig left atrial preparation. E/[A] curves for carbachol were obtained before and after Pbz-treatment (3 μM or 10 μM, 30 min). Control experiments showed that consecutive carbachol E/[A] curves obtained in the absence of Pbz treatment were effectively superimposed, confirming the validity of the paired curve analysis. The mean logistic parameters (± s.e.; $n = 5$) of these curves were as follows: 1st curve: $\alpha = 86.9$ (± 1.7); $m = 1.35$ (± 0.07); $p[A_{50}] = 6.68$ (± 0.04). 2nd curve: $\alpha = 85.8$ (± 1.5); $m = 1.40$ (± 0.06); $p[A_{50}] = 6.62$ (± 0.04).

Figure 2 illustrates typical results obtained in a single Pbz-treated tissue. The lines drawn through the data are the results of operational model-fitting. The substantial rightward shift and depression of the carbachol E/[A] curve produced by Pbz treatment indicated that the agonist has a high efficacy in this tissue. Analysis of 7 experiments gave an average estimate of efficacy (τ) of 72.4 (log $\tau = 1.86$ (± 0.11)) and an estimate of affinity (pK_A) of 4.73 (± 0.10). The high efficacy confirmed that carbachol was indeed a full agonist in this system. This is in agreement with the findings of other workers (Furchgott, 1966). The reliability of this analysis and that with pilocarpine requires that Pbz acts purely to decrease $[R_0]$ in this system. This was confirmed by experiments in which coincubation with the reversible competitive antagonist atropine was shown to protect the tissues from the effects of Pbz (data not shown, $n = 3$). Thus, E/[A] curves for carbachol obtained following incubation of tissues with atropine (0.1 μM) and Pbz (10 μM) then washout were indistinguishable from curves obtained following atropine treatment alone.

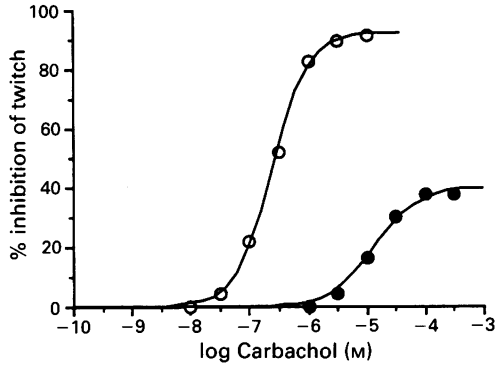

Figure 2 Effect of phenoxybenzamine (Pbz) treatment on carbachol E/[A] curves. Carbachol E/[A] curves were obtained before (○) and following 30 min exposure to 10 μM (●) Pbz. The symbols represent the data from a single tissue. The lines drawn through the data are the results of operational model fitting using the receptor inactivation method. For this particular tissue the estimated model parameters were as follows (standard errors in parentheses): E_m = 92.6 (± 1.3); n = 1.43 (± 0.08); τ_1 (control) = 58.9 (log τ_1 = 1.77 (± 0.07)); τ_2 (10 μM Pbz) = 0.8 (log τ_2 = −0.08 (± 0.04)); pK_A = 4.84 (± 0.07).

Analysis of experimental data for the partial agonist, pilocarpine

Pilocarpine E/[A] curves were obtained before and after Pbz treatment, following construction of an E/[A] curve for carbachol. As above, control experiments showed no differences between consecutive pilocarpine E/[A] curves obtained in the absence of Pbz treatment. The mean logistic parameters (± s.e.; n = 6) were as follows: 1st curve: α = 67.9 (± 4.8); m = 1.21 (± 0.10); $p[A_{50}]$ = 5.47 (± 0.07). 2nd curve: α = 68.6 (± 2.9); m = 1.40 (± 0.13); $p[A_{50}]$ = 5.42 (± 0.05).

Figure 3 illustrates a typical set of data obtained in a single Pbz-treated left atrium. Eight such experiments were conducted. In each instance, pilocarpine was analysed in comparison with carbachol and by the inactivation method.

The lines drawn through the data are the results of operational model-fitting. Table 2 shows the resulting parameter estimates and statistical analysis of the pK_A and E_m values obtained. Evidently, for each of these parameters the difference between the estimates provided by the two methods was very small and clearly not significant. Also, fitting errors associated with the pK_A and E_m estimates were small, on average 3% and 8% for the inactivation method and 2% and 2% for the comparative method (individual errors not shown).

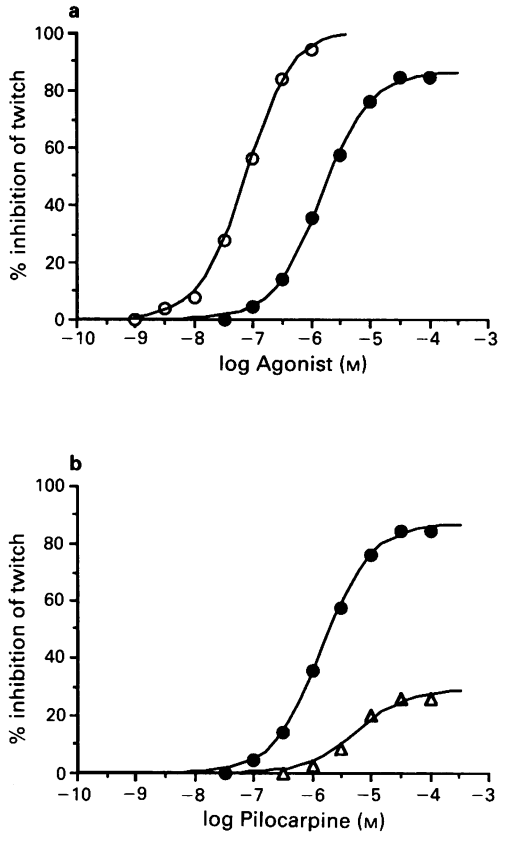

Figure 3 Analysis of pilocarpine E/[A] curve data by the comparative and inactivation methods. The data shown are from a single tissue for carbachol (○), pilocarpine (●) and pilocarpine after 30 min exposure to 1 μM phenoxybenzamine (Pbz, △). The lines drawn through the data are the results of operational model-fitting. Panel (a) illustrates the comparative analysis of pilocarpine using carbachol as the reference full agonist. The model parameter estimates were as follows (standard errors in parentheses): E_m = 101.0 (± 2.1); n = 1.08 (± 0.05); τ = 5.1 (log τ = 0.71 (± 0.05)); pK_A = 5.06 (± 0.06). Panel (b) illustrates the analysis of pilocarpine by receptor inactivation. The model parameter estimates were as follows (standard errors in parentheses): E_m = 105.6 (± 7.0); n = 1.06 (± 0.08); τ_1 (control) = 4.3 (log τ_1 = 0.63 (± 0.14)); τ_2 (1 μM Pbz) = 0.4 (log τ_2 = −0.40 (± 0.07)); pK_A = 5.12 (± 0.13).
For complete results see Table 2.

Discussion

The possibility that errors may occur in the estimation of agonist affinity by pharmacological methods makes it important that tests are available for their detection. This paper illustrates one such test.

Theoretical studies (Colquhoun, 1987; Mackay, 1988; Leff & Harper, 1989) of two plausible receptor models, the isomerisation (del Castillo & Katz, 1957) and the ternary complex (de Lean *et al.*, 1978) mechanisms, had already shown that the receptor inactivation method (Furchgott, 1966) is likely to provide higher estimates of agonist affinity than the interaction (Stephenson, 1956) or comparative (Barlow *et al.*, 1967) methods. It had also been suggested (Leff & Harper, 1989) that this difference could serve as the basis for a test to detect the operation of such receptor mechanisms and, therefore, to assess the validity of agonist affinity estimates obtained by pharmacological methods.

In practice, such a test can only be applied to partial agonists since only these can be analysed by both the inactivation and comparative methods. The first objective of the present study was to establish the magnitude of the difference in affinity estimates that the two tests would be likely to provide. This was done using theoretical data for a partial agonist operating by the ternary complex and isomerisation mechanisms and producing approximately 85% of the maximum possible effect. This effect level was chosen to be similar to that produced by the experimental example, pilocarpine. This

Table 2 Mean parameter estimates and statistical analysis of E_m values and pilocarpine pK_A values obtained from experimental data

	Comparative method		*Inactivation methods*		*Mean difference*	
	E_m	pK_A	E_m	pK_A	E_m	pK_A
Mean (n = 8)	96.7	5.03	97.3	4.95	0.6	0.08
s.e.	3.3	0.08	5.4	0.10	3.6	0.15
paired t test					$P > 0.5$	$P > 0.5$

theoretical analysis showed that, other than under the condition $[R_0] \gg [T_0]$ in the ternary complex model, the inactivation method was likely to produce an affinity estimate some five fold or 0.7 $\log_{10}$ units higher than the true value, whereas the comparative method was accurate to within 0.1 $\log_{10}$ units under all conditions. In addition, the overestimation of affinity by the inactivation method was accompanied by drastic overestimation of E_m. Therefore, in the context of the two mechanisms studied, it can be concluded that the operation of conditions unfavourable to affinity estimation can be detected by differences both in the values of K_A and E_m obtained by the two pharmacological methods.

In model-fitting terms, the overestimation of K_A and E_m are connected. The result of overestimating affinity is that the location of the control curve, obtained in the absence of receptor inactivation, is nearer to the estimated K_A than to the true K_A. Therefore, the partial agonist is treated by the fitting process as if it has lower efficacy than it actually does. In other words, the maximum effect of the partial agonist is treated as if it is lower in relation to the maximum possible effect, E_m, than it really is. Therefore, conversely, E_m is overestimated. This also accounts for the rather large standard error associated with E_m under the conditions $[R_0] = [T_0]$ and the inability to fit under the condition $[R_0] \ll [T_0]$. The lower the maximum effect of the partial agonist, the more the fitting process will have difficulty in projecting E_m from the curve depression caused by receptor inactivation.

The second objective of this study was to exemplify the test in a practical example. The muscarinic receptor system in the guinea-pig left atrium was chosen since there is evidence that it is a G-protein-linked system (Eglen *et al.*, 1987) and, therefore, could be anticipated to operate by the ternary complex mechanism. In this system, as demonstrated here, carbachol is a full agonist (see Figure 2 and analysis), permitting its use as the reference agonist in the comparative method. Pilocarpine demonstrates partial agonism in this tissue, achieving approximately 85% of the maximum effect of carbachol. Analysis of pilocarpine by receptor inactivation and by comparison with carbachol produced very similar, and statistically indistinguishable, estimates of pK_A (4.95 and 5.03 respectively) and E_m (97.3 and 96.7 respectively).

Also the standard errors associated with the parameters, in particular E_m, were small, giving no indication of the problems of goodness-of-fit associated with analysis of theoretical data.

Therefore, in this practical example there was no indication of the errors which the operation of the ternary complex mechanism can, in theory, produce. Waud (1969) performed a similar study on pilocarpine using the guinea-pig ileum preparation. He obtained a pK_A of 5.03 by the inactivation method and one of 5.36 by the comparative method. Although that study was not conducted with the above issues in mind, it confirms our findings.

The absence of the predicted theoretical errors in the present study does not rule out that the mechanism applies since, under one condition ($[R_0] \gg [T_0]$), similar estimates of pK_A and E_m are predicted by theory. If this is the correct explanation, the results would imply that although Pbz must have altered the ratio between $[R_0]$ and $[T_0]$ the reduction could not have been sufficient for $[R_0]$ to then approximate to $[T_0]$. That is, the condition $[R_0] \gg [T_0]$ would have applied before and after receptor inactivation. Of course, this is not to say that the same initial ratio between $[R_0]$ and $[T_0]$ would apply in other receptor systems and in such systems where $[R_0] \leqslant [T_0]$ the theoretically predicted errors may be demonstrable.

The results of the present study prompt the question as to whether the theoretical objections raised about agonist affinity estimation by pharmacological methods are justified since one possible interpretation of these results is that the theories and, therefore, their predictions are incomplete or wrong, as discussed elsewhere (Leff *et al.*, 1990). Clearly, further application of experimental tests to detect errors in agonist quantification and increased understanding of receptor-effector mechanisms will do much to clarify the issue.

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Anaesthetic suppression of transmitter actions in neocortex

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1 The effects of general anaesthetics were investigated on neuronal sensitivities to transmitter substances, which were determined by iontophoretic applications of acetylcholine, glutamate, N-methyl-D-aspartate (NMDA) and γ-aminobutyrate (GABA) during intracellular recording in *in vitro* slice preparations of neocortex (guinea-pig).

2 In most of the 65 neurones studied, perfusion of isoflurane (0.5–2.5 minimum alveolar concentration (MAC)) or Althesin (25–200 μM) and, in some cases, halothane (0.5–2 MAC), markedly reduced the depolarizing responses and associated membrane conductance changes evoked by dendritic applications of acetylcholine, glutamate, NMDA and GABA.

3 The order of depression was acetylcholine > glutamate or NMDA ≫ GABA. This selectivity could also be assessed from the EC_{50} for the isoflurane-induced depression of the just-maximal responses to acetylcholine, which was 0.9 MAC compared with an EC_{50} = 1.9 MAC for the suppression of glutamate responses. The selectivity was less pronounced in the case of the actions of Althesin, where the EC_{50}s were 75 μM for the depression of acetylcholine responses and 90 μM for the depression of glutamate responses.

4 The hyperpolarizing responses observed when GABA was applied near the perikaryon in 7 neurones, were slightly reduced (~15%) in 4, and unchanged in 3 neurones during anaesthetic application.

5 The pronounced depression of the responsiveness to the putative arousal transmitters and an observed blockade of acetylcholine-induced potentiation of glutamate actions suggest that anaesthetics produce unconsciousness, at least in part, by interfering with subsynaptic mechanisms of neocortical activation.

Introduction

The unconcious state produced by anaesthetic agents can be attributed to depressant actions at excitatory synapses and to a potentiation of inhibitory postsynaptic potentials (i.p.s.ps), particularly those mediated by γ-aminobutyrate (GABA; Richards, 1983). A depression of excitatory postsynaptic potentials (e.p.s.ps), has been observed in neocortical neurones on administration of either isoflurane or Althesin (El-Beheiry & Puil, 1989b). The depression of e.p.s.ps may be a consequence of reductions in presynaptic synthesis (Cheng & Brunner, 1978) and release of transmitter (Zorychta & Čapek, 1978; Quastel & Saint, 1986) or, may be attributed to alterations in postsynaptic transmitter actions (Catchlove *et al.*, 1972; Richards & Smaje, 1976; Barker & Ransom, 1978; Lambert & Flatman, 1981; Sawada & Yamamoto, 1985), as in the case of the enhanced i.p.s.ps (Scholfield, 1980; Gage & Robertson, 1985; Barker *et al.*, 1987).

The effects of anaesthetics on the electrical excitability of the neuronal membrane are often small (Nicoll & Madison, 1982; Berg-Johnsen & Langmoen, 1987; Puil & Gimbarzevsky, 1987; El-Beheiry & Puil, 1989a) or are not always observed when the e.p.s.ps are diminished (Takahashi & Takenoshita, 1987; Miu & Puil, 1989). These lines of evidence suggest that isoflurane or Althesin may interfere with the responsiveness of the subsynaptic membrane to transmitters likely to be involved in behavioural arousal (Krnjević, 1974; Krnjević & Puil, 1975; El-Beheiry & Puil, 1989b). This possibility was assessed in the present investigations from the effects of anaesthetics on neocortical neurones which are likely sites in the production of the unconscious state (Krnjević, 1974; Savaki *et al.*, 1983). Possible alterations in the actions of the transmitters – acetylcholine, glutamate and GABA — were investigated because of their long suspected roles in neocortical activation (Jasper *et al.*, 1965; Celesia & Jasper, 1966; Shute & Lewis, 1967; Krnjević, 1974; Lysakowski *et al.*, 1989). Some of these results have been published in a preliminary form (El-Beheiry & Puil, 1989c).

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Methods

Slice preparations and recording

Sensorimotor and anterior cingulate cortices were identified in the halothane-anaesthetized guinea-pig, excised, and cut into 500 μm thick slices according to previously described procedures (El-Beheiry & Puil, 1989a). The slices were submerged in artificial cerebrospinal fluid (CSF) oxygenated with a 95% O_2/5% CO_2 mixture and kept at room temperature until required for recording (bath temperature, 32–34°C). The constituents of the artificial CSF were (in mM): NaCl 124, KCl 3.75, KH_2PO_4 1.25, $MgSO_4 . 7H_2O$ 2, $CaCl_2 . 2H_2O$ 2, dextrose 10 and $NaHCO_3$ 26. The techniques for conventional intracellular recording using 3 M KCl or 0.6 M K_2SO_4 microelectrodes and measurement of input resistance in neurones of neocortical slices in a submersion type of chamber have also been described (El-Beheiry & Puil, 1989a).

Experimental procedures

The effects of anaesthetics were assessed by the following procedures: (1) after stable recording conditions were achieved, the iontophoretic current was adjusted in the range 30–350 nA (typically 80 nA), such that a submaximal (~ED_{50}) or a just-maximal response could be elicited with a transmitter substance; (2) the responses of relatively constant amplitude (usually 10–20 mV) were evoked at appropriate intervals (10–30 s) for a period of at least 5–8 min; (3) equi-amplitude responses to 2 or more transmitter substances were evoked in the same neurone; (4) perfusion of the anaesthetic was commenced during the continuing, intermittent transmitter applications; and (5) 3 or more responses to each transmitter substance obtained under each condition were averaged for the quantitative comparisons.

A slightly different procedure was used for investigation of NMDA responses because of the rapid development of desensitization or tachyphylaxis in the large pyramidal (layer IV–V) neurones. If a neurone was responsive to a low dose of NMDA the just-maximal effect was determined and 3 or 4 equi-amplitude responses were obtained at 3 min intervals before anaesthetic application was commenced.

Drugs and their applications

Isoflurane (Anaquest, Pointe Claire, Quebec) and halothane (Ayerst Laboratories, Montreal, Quebec) were vaporized at a $1\,l\,min^{-1}$ flow rate with 95% O_2/5% CO_2 gas mixture by 5 Fluotec Mark 3 vaporizers (Cyprane Limited, Keighley, Yorkshire) and bubbled into 5 reservoirs containing artificial CSF for at least 25 min. These agents were then perfused at the designated minimum alveolar concentration (MAC) values (cf. Miu & Puil, 1989). Althesin (Glaxo Canada Limited), a twin steroid preparation, was administered by perfusion. Althesin contained alphaxalone and alphadalone (one-half the anaesthetic potency of alphaxalone) dissolved in cremophor EL in a 3:1 ratio.

Acetylcholine (0.5 M, pH 4.5), glutamate (0.5 M, pH 8.5), N-methyl-D-aspartate (NMDA; 100 mM, pH 9) and GABA (100 mM, pH 4.5) (all obtained from Sigma Chemical Co., U.S.A.), or NaCl for control applications, were administered by iontophoresis from a 3- or 5-barrelled micropipette which was either glued in parallel to the recording electrode at an intertip distance of $\sim 30\,\mu m$ for somatic applications or, inserted separately into the dendritic region at a distance of $\sim 50\,\mu m$ from the presumed location of neuronal impalement. Except for NMDA, these substances were applied at recurring intervals (10–60 s) before, during and after concomitant perfusion of an anaesthetic. The duration of application was usually 4 or 5 s for NMDA or GABA, 6 s for glutamate and 7 to 20 s for acetylcholine. In ~75% of the neurones, tetrodotoxin (TTX; 1–1.5 μM; Sigma Chemical Co.) was applied to block spikes and hence indirect actions of the transmitter substances on neighbouring neurones.

Statistical analysis

Results are presented as mean values ± s.e.mean. Statistical analysis was performed by Student's t test. Overall significance was determined by ANOVA.

Results

The effects of isoflurane or Althesin were investigated in 65 neurones that exhibited stable resting potentials for periods of 0.5–5 h. The interactions of halothane were examined in 10 of the neurones. The mean resting potential (V_m) and input resistance (R_i) were -69 ± 2.3 mV and 51 ± 2.5 MΩ, respectively ($n = 65$). All neurones fired short duration spikes (> 75 mV amplitude) in response to depolarizing current pulse injections. Spontaneous discharge was observed in 23% of the 65 neurones.

As previously demonstrated (El-Beheiry & Puil, 1989a; Miu & Puil, 1989), the anaesthetics produced no significant alterations in V_m, R_i or membrane time constant except when applied at the highest doses. Application of isoflurane (2.5 MAC), halothane (2.5 MAC), or Althesin (200 μM) but not the vehicle, cremophor EL (cf. Pennefather *et al.*, 1980), hyperpolarized neurones by 3–5 mV and decreased R_i by 15–20%. In such cases, d.c.-injection was employed to maintain a constant V_m for observations of interactions with transmitters.

Acetylcholine responses

Acetylcholine applied to the apical dendritic region evoked a slowly developing depolarization (12.3 ± 1.5 mV) in 36 out of 50 neurones (Figure 1). The responses were associated with increased R_i (e.g., ~30% in Figures 1 and 3a) and were maximal in voltage for 10–30 s after termination of the application. In 3 neurones, the responses were blocked by the application of scopolamine (20 μM), suggesting actions of acetylcholine at muscarinic receptors (cf. Krnjević, 1987). An additional feature was the appearance of postsynaptic potentials (mostly e.p.s.ps) on the falling phase of the depolarizations, even during concomitant application of TTX. This suggested an involvement of Ca-spikes or presynaptic actions at nicotinic receptors on nerve terminals (Rovira *et al.*, 1983). In 14 neurones, the acetylcholine-evoked depolarization was preceded by a transient hyperpolarization (Figure 1a) that could be blocked by application of TTX.

Anaesthetic administration reversibly depressed the depolarizing and conductance responses to acetylcholine in almost all neurones (Table 1). These effects were observed even after low doses (0.5–1 MAC) of isoflurane. Recovery was usually observed 8–10 min after such applications (Figures 1 and 3a). At least partial recovery was evident after application of a high dose of isoflurane (2–2.5 MAC) and Althesin (150–200 μM).

Glutamate and NMDA responses

All 65 neurones were depolarized by 20 ± 1.4 mV on application of glutamate. The responses were reduced by administration of isoflurane (0.5–2.5 MAC), halothane (1.5 MAC) or Althesin (25–200 μM) and typical effects of isoflurane and Althesin are illustrated in Figure 2, and summarized in Table 1. Recovery from an application of isoflurane was observed after 6–18 min and was usually more rapid than after Althesin

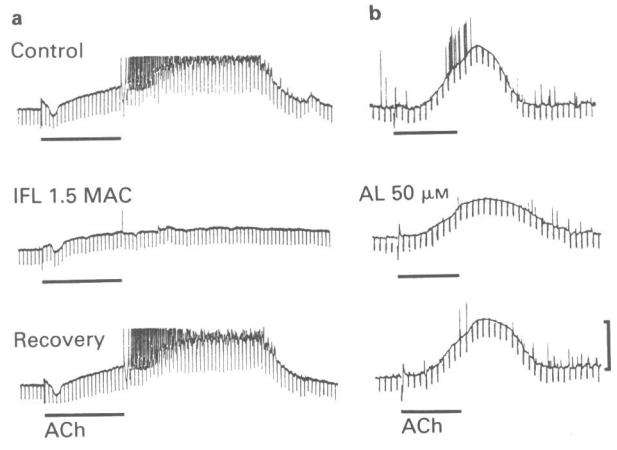

Figure 1 Depression by isoflurane (IFL) and Althesin (AL) of responses to acetylcholine (ACh; 100 nA for 20 s in (a), 125 nA for 9 s in (b) in 2 neurones (a), $V_m = -68$ mV; (b), $V_m = -72$ mV. Middle records were obtained at 9 min in (a) and 6 min in (b) of anaesthetic application. Negative voltage deflections represents tests for changes in input resistance. MAC = minimum alveolar concentration.

Table 1 Anaesthetic-induced depression of neuronal responses to transmitter substances

	Depolarization evoked by			*GABA evoked*	
Anaesthetic applied	*ACh* ($n = 36$)	*Glu* ($n = 65$)	*NMDA* ($n = 15$)	*hyperpolarization* ($n = 7$)	*depolarization* ($n = 29$)
Isoflurane	18/18	30/40	8/10	4/7	10/17
Althesin	15/16	25/32	7/9		11/15
Halothane	2/2	5/7			2/4

Depression was defined as >15% attenuation of control (just-maximal) response by the anaesthetic. The total number of neurones investigated was 65, i.e., all neurones were exposed to glutamate (Glu) and to at least one other transmitter substance. The anaesthetic doses were 0.5–2.5 minimum alveolar concentration (MAC) for isoflurane, 25–200 μM for Althesin and 0.5–2 MAC for halothane. ACh = acetylcholine and NMDA = N-methyl-D-aspartate.

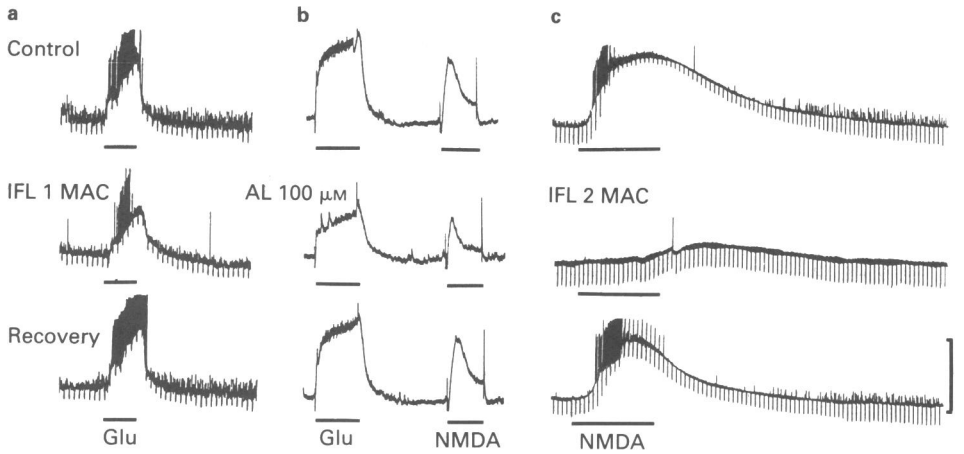

Figure 2 Anaesthetic depression of responses to glutamate (Glu; 100 nA for 5 s in (a) and 35 nA in (b)) and N-methyl-D-aspartate (NMDA; 55 nA for 5 s in (b), 110 nA for 15 s in (c)) in 2 neurones ($V_m = -70$ mV in (a), -75 mV in (b), -69 mV in (c)). Middle traces were obtained at ~10 min of anaesthetic application. Resistance test pulses were applied in (a) and (c). Tetrodotoxin (1.5 μM) was applied in (b). Voltage calibration: 25 mV in (a), 20 mV in (b), 30 mV in (c). For key to abbreviations used see legend of Figure 1.

application. Recovery from the depression due to the application of Althesin was observed after 15 min.

Only 15 of 37 neurones were depolarized by the application of NMDA (Figure 2b,c). In 4 of these, the responses to NMDA did not fade, even during relatively long applications (cf. Figure 2c). All NMDA responses were reduced by application of isoflurane (1–2 MAC) or Althesin (75–200 μM). These effects (Table 1) had magnitudes and recovery times similar to those for the depression of glutamate responses.

GABA responses

GABA application near the perikaryon evoked hyperpolarizing responses of 5–10 mV in 7 out of 7 neurones. Isoflurane application at 1 MAC had no significant effects on such responses, although at 2 MAC a slight reduction (~15%) was observed in 4 neurones.

When GABA was applied at a dendritic location just-maximal depolarizing responses (22.5 ± 2.0 mV) were observed in 29 out of 29 neurones (Figure 3a). The peak depolarizations which were accompanied by a decreased R_i (~50%) either waned, or maintained a constant amplitude during the application. Relatively low dose applications of isoflurane (0.5–1 MAC), or Althesin (25–100 μM) did not greatly alter these responses (Figure 3a). The maximal depression was ~40% with 2.5 MAC isoflurane, 1.5 MAC halothane or 200 μM Althesin (cf. Table 1).

Selectivity in depression

Anaesthetic interactions with transmitters When acetylcholine, glutamate and/or GABA were applied alternately to the same neurone, application of isoflurane, halothane or Althesin markedly attenuated the responses to acetylcholine after ~8 min, slightly reduced those to glutamate and produced only small changes in the depolarizing effects of GABA (cf. Figure 3 for isoflurane and Althesin). The selectivity was much more pronounced with isoflurane than Althesin application and no such effects were observed with applications of cremophor EL (Pennefather *et al.*, 1980; Cullen & Martin, 1982). In 7 neurones, low dose administration of isoflurane or Althesin produced no depression of the glutamate depolarization and sometimes prolonged its duration (cf. Figure 3b). In 5 neurones, the reductions in the acetylcholine responses were evident at 30–90 s i.e., before attenuation of glutamate responses. Full recovery from the reduction in glutamate depolarization was usually observed several minutes earlier than a complete return of the acetylcholine responses to control amplitude.

Dose-response relationships The dose-response relationships for the actions of isoflurane and Althesin on the just-maximal responses evoked by the transmitter substances are shown in Figure 4. The EC_{50} for the isoflurane depression of the acetylcholine responses was 0.9 MAC compared with 1.9 MAC for

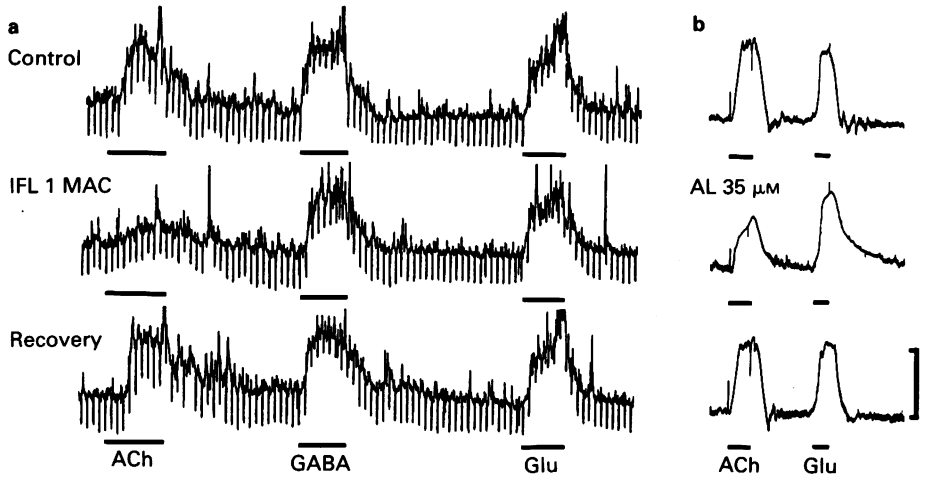

Figure 3 Effects of anaesthetic application on equi-amplitude responses to γ-aminobutyric acid (GABA; 75 nA for 6 s in (a)), glutamate (Glu; 85 nA in (a), 70 nA in (b)) and acetylcholine (ACh, 90 nA for 10 s in (a), 110 nA for 7 s in (b)) in 2 neurones ($V_m = -75$ mV in (a), -70 mV in (b)). Middle traces in (a) and (b) were obtained at 9 min of anaesthetic application. Resistance test pulses were applied in (a). Tetrodotoxin (1 μM) was applied in (b). Voltage calibration: 20 mV in (a), 10 mV in (b). For key to abbreviations used see legend of Figure 1.

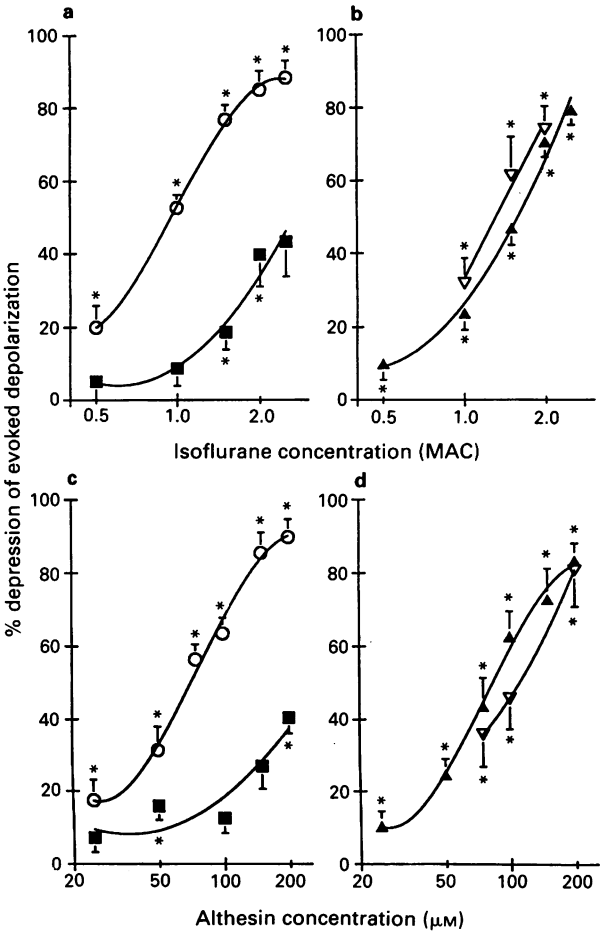

Figure 4 Pooled data from 42 neurones show dose-response relationships for (a,b) isoflurane- and (c,d) Althesin-induced depressions of depolarizations evoked by transmitter substances. Each point on the curve is the mean response from a neurone to at least 4 applications of isoflurane ($n = 22$) or Althesin ($n = 20$). * Indicates significant difference from control at $P < 0.05$. Overall significance was determined by ANOVA. Vertical lines show s.e.mean. In (a) and (c): (○) acetylcholine, (■) γ-aminobutyric acid. In (b) and (d): (▽) N-methyl-D-aspartate, (▲) glutamate.

the suppression of glutamate responses. Such differences in selectivity were also apparent in comparison with NMDA responses, although the data are more limited. The selectivity was less pronounced in the case of Althesin where the EC_{50} values were 75 μM for depression of the acetylcholine responses and 90 μM for depression of glutamate responses (Figure 4). The maximal reductions of the depolarizing responses induced by application of GABA were only about 40% at 2.5 MAC isoflurane or 200 μM Althesin.

Possible anaesthetic depression of the potentiation of glutamate-responses by acetylcholine This was examined in 7 neurones by briefly applying glutamate pulses ($\sim ED_{50}$) during the application of acetylcholine ($\sim ED_{50}$), and then concomitantly administering the anaesthetic for 6–9 min. As can be seen from Figure 5, the application of acetylcholine gradually depolarized the neurones and then enhanced and prolonged the effects of glutamate; when 1.5 MAC isoflurane ($n = 4$) or 75 μM Althesin ($n = 3$) was additionally administered, the effects of acetylcholine, including the enhancement were no longer evident. Note that such applications of anaesthetic also reduced the glutamate responses which recovered with the effects of acetylcholine.

Discussion

The unusual muscarinic actions of acetylcholine and its interactions with glutamate and anaesthetics led Krnjević (1974; 1987) to propose a depression of cholinergic activation in neocortex by the reticular activating system and intracortical transmission, as a basis for the anaesthetic state. Although the initial extracellular observations on the effects of systemic administration of various anaesthetics on cortical neurones in *in vivo* feline preparations were suggestive of this hypothesis (Catchlove *et al.*, 1972; Krnjević & Puil, 1975), there has not previously been sufficient evidence *a priori* for this mechanism. Similar *in vitro* studies on olfactory cortex showed that halothane, administered in the gas stream over the upper surface of the slice, or alphaxalone (the main active steroid in Althesin), applied in the saline perfusion as a liposome suspension, reduced glutamate-, and not acetylcholine-evoked neuronal firing (Richards & Smaje, 1976; Smaje, 1976). The reasons for this disparity probably relate to the differences in the

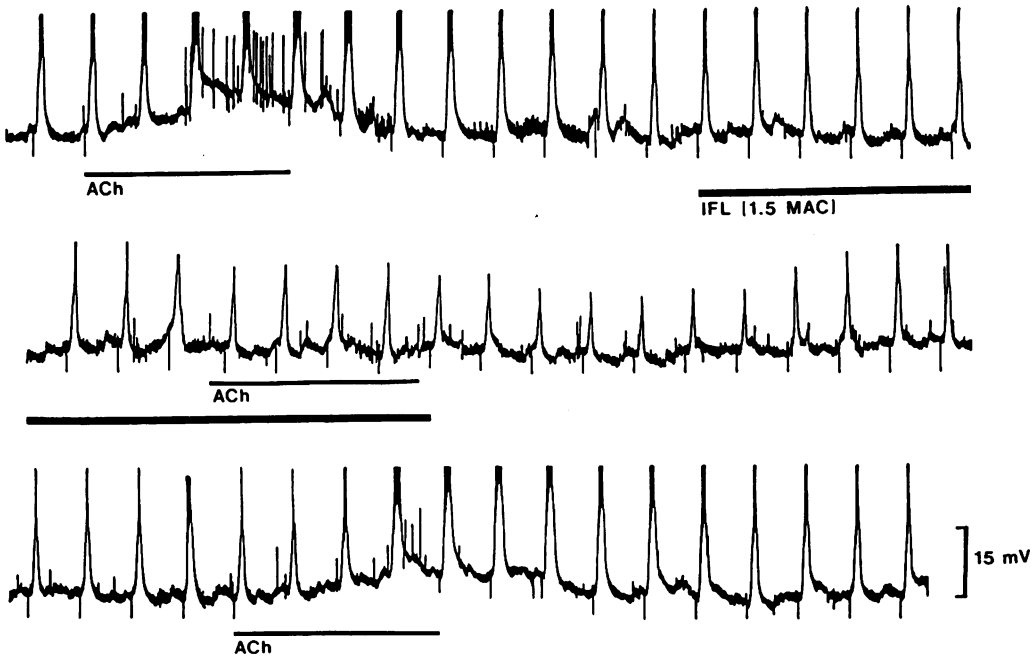

Figure 5 Continuous record of isoflurane (IFL)-induced depression of acetylcholine (ACh)-potentiation of glutamate (Glu)-induced actions on a sensorimotor neurone. Depolarizations evoked by Glu (cf. negative artefacts at onset of iontophoretic 70 nA current) and ACh (50 nA) are ~50% of their respective maximal responses. IFL was applied for 6 min (thick bar).

modes of anaesthetic application. Some recent investigations of neurones of the lateral geniculate nucleus have demonstrated that the enhancement of spontaneous spike discharge produced by electrical stimulation of the mesencephalic reticular formation, or acetylcholine application, is much more sensitive than that produced by glutamate- or NMDA-application, to the depressant effects of anaesthesia with N_2O and pentobarbitone or with N_2O and halothane (Francesconi *et al.*, 1988).

The present results in neocortical slices *in vitro* also show a degree of selectivity in the depression of arousal transmitter actions after administration of isoflurane, halothane and Althesin, i.e., the order of depression was acetylcholine > glutamate (or NMDA) ≫ GABA. Because changes in passive membrane properties of the neurones were not usually induced by anaesthetic application, the suppression may result from postsynaptic actions on the ionic channel-receptor complexes or on internal modulation of a secondary messenger system, subsequent to agonist-receptor interactions.

Acetylcholine responses

There are several ways in which anaesthetics can selectively interfere with the muscarinic system in cortical neurones. Anaesthetic application decreases the affinity of the receptor G-protein complexes for guanine nucleotides (Dennison *et al.*, 1987) and modifies channel proteins, thereby decreasing the number of activatable channels (Arimura & Ikemoto, 1986; Ikemoto *et al.*, 1988). Another explanation is that anaesthetics may bind allosterically with the receptor for acetylcholine and inhibit its specific binding (Young & Sigman, 1981).

Glutamate responses

The depression of the glutamate- or NMDA-evoked depolarizations by anaesthetics (Barker & Ransom, 1978; Lambert & Flatman, 1981; Sawada & Yamamoto, 1985; Thomson *et al.*, 1985; MacDonald *et al.*, 1987) may also be attributed to blocked (MacDonald *et al.*, 1987) or desensitized states of receptor-channel complexes (see Ikemoto *et al.*, 1988). These depolarizations result, at least in part, from a transmembrane Ca^{2+}-influx (Murphy & Miller, 1988; Puil & Benjamin, 1988). Anaesthetics may reduce the depolarization either directly by preventing receptor-activated Ca^{2+}-entry or, indirectly, by uncoupling Ca^{2+}-entry from its dependency on membrane voltage (Krnjević & Puil, 1988; Puil & Baimbridge, 1989).

GABA responses

In the neocortex, GABA application to perikarya elicited hyperpolarizing responses that were unaffected by isoflurane, whereas GABA application to dendrites resulted in depolarizing responses that were depressed by high concentrations of isoflurane or Althesin. GABA-evoked depolarizations have been demonstrated in pyramidal and granule neurones of hippocampal slice preparations; the ionic mechanism for the depolarization is unknown, but may involve an increased Cl^- conductance as in the generation of the hyperpolarization (Alger & Nicoll, 1982; Blaxter & Carlen, 1988). Isoflurane or Althesin administration to neocortical neurones suppressed the depolarizations evoked by GABA, as observed in other neurones during application of alphaxalone as well as high doses of other anaesthetics (Cullen & Martin, 1982; Brooks *et al.*, 1986).

A potentiation of Cl^--dependent responses to GABA has been observed in olfactory cortical (Scholfield, 1980) and spinal (Barker *et al.*, 1987) neurones on application of alphaxalone in low doses (1–10 μM); these doses correspond approximately to plasma concentrations in anaesthetized patients (Sear & Prys-Roberts, 1979). Preliminary investigations have revealed a brain concentration of 60 μM alphaxalone after systemic administration of Althesin to experimental animals (Smith *et al.*, 1974). In the absence of confirmatory data, the relevance of the concentrations of alphaxalone used in the above and the present studies to the clinical state remains unknown. However, it may be significant that the cremophor EL vehicle which did not have noticeable effects on neocortical neurones in our experiments, has been shown to reduce greatly the synaptosomal membrane:buffer partition coefficient of highly lipid soluble drugs (Roth & Williams, 1979); such observations may account for the higher EC_{50}s of alphaxalone found in the present (cf. Figure 4) and previous investigations (Pennefather *et al.*, 1980).

Significance

Given the special involvement of muscarinic pathways in determining conscious processes neocortical activation would be particularly susceptible to the depressant actions of anaesthetics (Shute & Lewis 1967; Krnjević, 1974; 1987). A sharp depression of cholinergic activity in the cortex could produce a 'surgical level' of anaesthesia, whereas deeper levels could reflect a more generalized impairment in cholinergic modulation of glutamatergic transmission. The anaesthetic depression in subsynaptic responsiveness to these putative arousal transmitters would arrest neocortical expression of the conscious state.

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The role of Na^+ in muscarinic receptor-mediated catecholamine secretion in the absence of extracellular Ca^{2+} in cat perfused adrenal glands

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1 The role of Na^+ in muscarinic receptor-mediated catecholamine secretion, which is independent of extracellular Ca^{2+}, was investigated by observing the effect of veratridine and ouabain in perfused adrenal glands of the cat.

2 Veratridine (10^{-4} M) markedly enhanced catecholamine secretion evoked by acetylcholine (ACh, 10^{-4} M) during perfusion with Ca^{2+}-free Locke solution containing hexamethonium (10^{-3} M). The enhancement tended to be larger for noradrenaline secretion than for adrenaline secretion. Qualitatively the same result was obtained in the response to pilocarpine (5×10^{-4} M).

3 Ouabain (10^{-4} M) also enhanced ACh- and pilocarpine-induced catecholamine secretions, especially noradrenaline secretion in the absence of extracellular Ca^{2+}.

4 Tetrodotoxin (10^{-6} M) blocked the enhancing effect of veratridine on ACh-induced catecholamine secretion, but not that of ouabain in the absence of extracellular Ca^{2+}.

5 When NaCl was replaced with sucrose, there was no secretory response to ACh regardless of the presence or absence of veratridine or ouabain. However, when ouabain, but not veratridine, was infused with Na^+ before the replacement of NaCl, the response to ACh was substantially augmented.

6 These results indicate that Na^+ is essential in the initiation of muscarinic receptor-mediated catecholamine secretion and its enhancement by veratridine and ouabain in the absence of extracellular Ca^{2+}. Both drugs seem to increase the intracellular concentration of Na^+ through different mechanisms and result in increases in the efficiency of Ca^{2+} mobilization from intracellular Ca^{2+} pools linked to muscarinic receptors.

Introduction

Catecholamine secretion induced by acetylcholine (ACh) from perfused bovine adrenal glands was inhibited, when the glands were exposed to Na^+-free medium for a long period of time (Banks *et al.*, 1969; Lastowecka & Trifaro, 1974), whereas it was somewhat augmented after Na^+ deprivation in cat perfused adrenal glands (Douglas & Rubin, 1963). Although the mechanisms thought to be involved in these phenomena are different and the reason for the difference between the two species remains unknown, these reports certainly indicate the importance of Na^+ for ACh-induced release of catecholamines from adrenal chromaffin cells.

Previously, ouabain was found to enhance catecholamine secretion evoked by activation of muscarinic, but not nicotinic, receptors during perfusion with Ca^{2+}-free Locke solution in cat adrenal glands (Yamada *et al.*, 1989a). We assumed that ouabain increased the intracellular concentration of Na^+ by inhibiting the Na^+-K^+ pump and resulted in an increase in Ca^{2+} storage in intracellular Ca^{2+} pools linked to muscarinic receptors (Nakazato *et al.*, 1988; Yamada *et al.*, 1989a). On the other hand, the operation of Na^+-induced Ca^{2+} release from intracellular storage sites has been suggested in many tissues (Baker & Crawford, 1975; Lowe *et al.*, 1976; Donatsch *et al.*, 1977; Silbergeld, 1977; Åkerman & Nicholls, 1981; Török, 1989). Therefore, intracellular Na^+ may have the ability to modulate the functions of the intracellular Ca^{2+} pool. It is possible that the increase in the intracellular concentration of Na^+ *per se* is sufficient for ouabain to enhance the catecholamine secretion evoked by muscarinic agonists, in addition to enhancement of the accumulation of Ca^{2+} in the storage sites.

Veratridine is known to increase Na^+ permeability by decreasing the rate of inactivation of Na^+ channels (Ohta *et al.*, 1973) and has no inhibitory action on Na^+-K^+ ATPase activity on nerve terminal membranes (Adam-Vizi & Ligeti, 1984). Like scorpion venom and grayanotoxin, veratridine increases catecholamine secretion from the perfused guinea-pig adrenal glands and this effect is completely blocked by either tetrodotoxin or removal of Ca^{2+}, indicating the presence of Na^+ channels on chromaffin cells similar to those on nerves (Ito *et al.*, 1978; 1979; 1980). It was reported that veratridine caused continuous secretion of catecholamines and influxes of $^{45}Ca^{2+}$ and $^{22}Na^+$, all of which were blocked by tetrodotoxin in bovine cultured adrenal chromaffin cells (Wada *et al.*, 1985). Thus, veratridine is a useful pharmacological tool to investigate the role of Na^+ in adrenal catecholamine secretion.

In the present experiments, the importance of Na^+ in catecholamine secretion induced by cholinoceptor agonists, especially in secretion evoked by muscarinic-receptor activation in the absence of extracellular Ca^{2+}, was evaluated by studying the effect of veratridine and ouabain during perfusion with Ca^{2+}-free and/or Na^+-deficient solutions in cat adrenal glands.

Methods

Cats of either sex, weighing 1.5 to 4.5 kg, were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Douglas & Rubin, 1961; Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.6 to 0.8 ml min^{-1} and maintained at room temperature (approximately 25°C). The effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) 3 and glucose 10. For Na^+-deficient solution, NaCl was replaced by an osmotically equivalent concentration of sucrose. In the Ca^{2+}-free solutions, $CaCl_2$ was omitted and glycoletherdiaminetetraacetic acid (EGTA) (10^{-5} M) was added. Experiments were started 40 to 60 min after isolation of the adrenal gland. Secretagogues were administered for 1 min beginning 1 min before the following 5 min collection periods because of the 1 min dead time of the arterial cannula. Samples collected were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

Adrenaline and noradrenaline were separated by high performance liquid chromatography (h.p.l.c., Jasco) and detected by an electrochemical detector (LC-4B, BAS). The detection limit was 10^{-8} M (0.01 nmol ml^{-1}) for either adrenaline or noradrenaline. The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982).

The following drugs were used: acetylcholine chloride (Ovisort; Daiichi), atropine sulphate (K & K), glycoletherdiaminetetraacetic acid (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), ouabain (Tokyo Kasei), physostigmine sulphate (Wako Pure Chem.), pilocarpine hydrochloride (Tokyo Kasei), tetrodotoxin (Sankyo) and veratridine (Sigma).

Results

Effect of veratridine on the secretory responses to acetylcholine and pilocarpine in the absence of extracellular Ca^{2+}

Nicotinic receptors were blocked by hexamethonium (10^{-3} M) in these experiments. A representative experiment is shown in Figure 1 and the results obtained from the same series of experiments are summarized in Table 1. First, ACh (10^{-4} M) was infused for 1 min during perfusion with standard Locke solution, which was then changed to Ca^{2+}-free Locke solution containing EGTA (10^{-5} M). In the presence of Ca^{2+}, ACh caused an increase in both adrenaline and noradrenaline secretions. The ratio of adrenaline to noradrenaline released was about 1:1 (Table 1). Fifteen minutes after the removal of Ca^{2+}, the adrenal glands were again stimulated with ACh. Under these conditions, ACh still caused a small increase in catecholamine secretion. As reported previously (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1989a), this residual response was repeatedly obtained as long as the adrenal gland was loaded with Ca^{2+} prior to stimulation with ACh. Veratridine (10^{-4} M) was added to the perfusion solution 5 min after the end of Ca^{2+} loading for 25 min and the gland was stimulated with ACh (10^{-4} M) for 1 min, 10 min after the start of veratridine infusion. Veratridine markedly enhanced ACh-induced catecholamine secretion and the magnitude of the restored

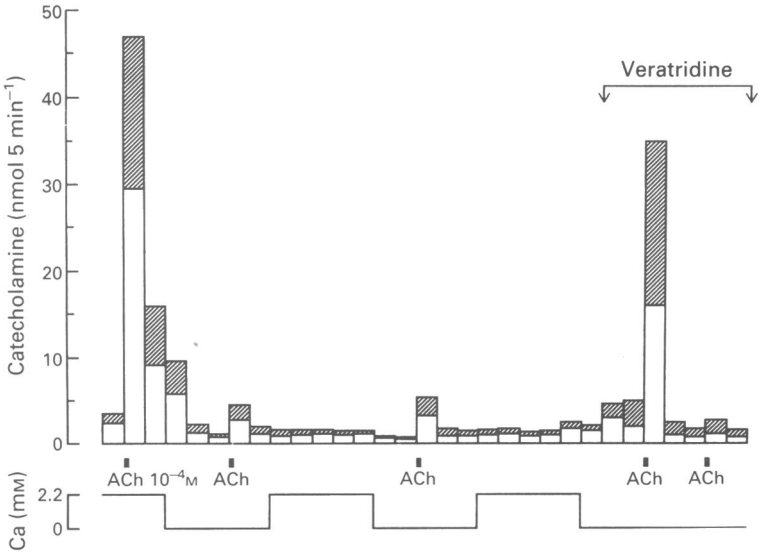

Figure 1 Effect of veratridine on acetylcholine-evoked catecholamine secretion in the absence of extracellular Ca^{2+}. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. Veratridine (10^{-4} M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

Table 1 Catecholamine secretions induced by acetylcholine (ACh) in the presence (A) and absence (B) of extracellular Ca^{2+} before and during treatment with veratridine (10^{-4} M) in cat perfused adrenal glands

	(A) *Locke*		(B) *Ca^{2+}-free Locke*			
			Control		*Veratridine* 10^{-4} M	
	Ad	NA	Ad	NA	Ad	NA
Resting release	6.3 ± 2.7	4.2 ± 1.4 (40%)	0.5 ± 0.1	0.5 ± 0.1 (50%)	1.3 ± 0.4	2.2 ± 0.4 (63%)
ACh 10^{-4} M	13.3 ± 2.0	12.5 ± 2.2 (48%)	1.3 ± 0.2	2.2 ± 0.4 (63%)	12.0 ± 3.4	14.1 ± 1.6 (54%)
No. of cats	7	7	9	9	3	3

Values indicate the mean (± s.e.) of adrenaline (Ad) and noradrenaline (NA) released in nmol 5 min^{-1} and the percentage of noradrenaline is given in parentheses under the various conditions indicated. The values of ACh-induced catecholamine secretions were obtained by subtracting the values of resting release from the respective evoked release of adrenaline and noradrenaline. In Ca^{2+}-free Locke solution, $CaCl_2$ was omitted and 10^{-5} M EGTA was added. All solutions contained hexamethonium (10^{-3} M).

responses attained almost the same level as those obtained in the presence of extracellular Ca^{2+} (Table 1). The percentage of noradrenaline released was increased a little. ACh applied 15 min after the first challenge failed to induce a response, even if veratridine was still present. As shown in Table 1, veratridine also caused a small increase in resting catecholamine secretion in the absence of extracellular Ca^{2+}. When 10^{-5} M veratridine was used, the response to ACh was also enhanced, but to a lesser extent.

In a similar fashion, the effect of veratridine on catecholamine secretion induced by pilocarpine was investigated without adding hexamethonium. In a manner similar to ACh-induced catecholamine secretion, veratridine also enhanced the secretory response to pilocarpine, especially noradrenaline secretion (Figure 2).

Effect of ouabain on the secretory responses to acetylcholine and pilocarpine in the absence of extracellular Ca^{2+}

The effect of ouabain (10^{-4} M) on catecholamine secretion evoked by muscarinic receptor activation was studied with a protocol similar to that for veratridine. As indicated in Figure 3a, catecholamine secretion induced by ACh (10^{-4} M) was enhanced by ouabain infused in the absence of extracellular Ca^{2+}. Unlike results with veratridine, the enhancement was greater in the response to the second challenge with ACh than to the first one. The response to pilocarpine (5×10^{-4} M) was also enhanced by ouabain (Figure 3b). The enhancement of noradrenaline secretion was much larger than that of adrenaline secretion in response to either ACh or pilocarpine.

The enhancement of ACh- and pilocarpine-induced secretory responses by veratridine and ouabain in the absence of extracellular Ca^{2+} was completely blocked by atropine (10^{-6} M) regardless of the presence or absence of hexamethonium (10^{-3} M).

Effects of tetrodotoxin on the enhancement of ACh-induced catecholamine secretion by veratridine

To confirm the involvement of voltage-dependent Na^+ channels in the enhancement by veratridine, the effect of tetrodotoxin on catecholamine secretion evoked by muscarinic receptor activation was studied. A representative result is illustrated in Figure 4. First, control responses to ACh (10^{-4} M) were successively obtained in the presence and absence of extracellular Ca^{2+}, then the adrenal gland was loaded with Ca^{2+} for 25 min. Fifteen minutes after the end of Ca^{2+} loading, ACh was again administered in the presence of veratridine (10^{-4} M) plus tetrodotoxin (10^{-6} M). Tetrodotoxin completely inhibited the enhancing effect of veratridine on ACh-induced catecholamine secretion that was unmasked after the removal of the toxin (Figure 4). On the other hand, tetrodotoxin failed to inhibit the enhancement of ACh-induced catecholamine secretion by ouabain (10^{-4} M) (data not shown).

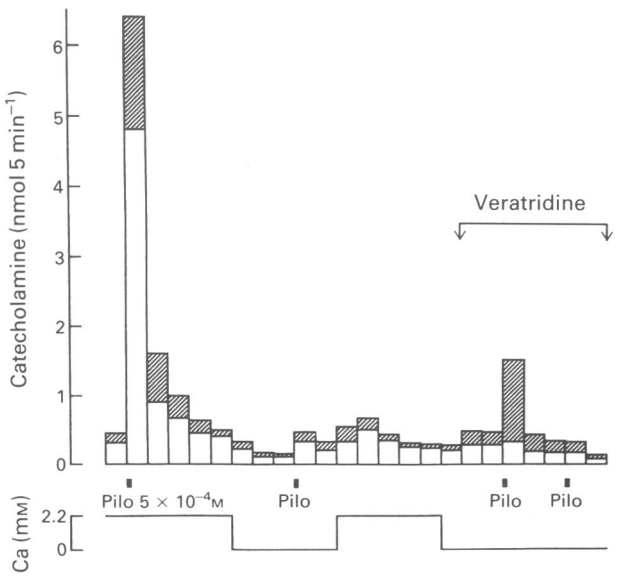

Figure 2 Effect of veratridine on pilocarpine-evoked catecholamine secretion in the absence of extracellular Ca^{2+}. Pilocarpine (Pilo, 5×10^{-4} M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. Veratridine (10^{-4} M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

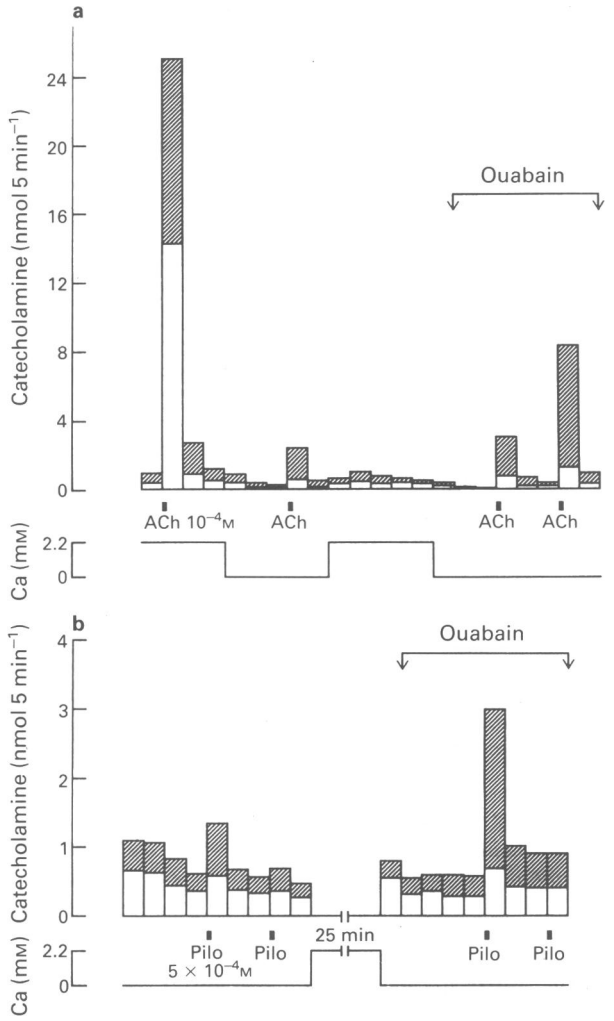

Figure 3 Effect of ouabain on catecholamine secretions evoked by acetylcholine and pilocarpine. Acetylcholine (ACh, 10^{-4} M) (a) and pilocarpine (Pilo, 5×10^{-4} M) (b) were sequentially applied for 1 min during perfusion with standard Locke solution and/or Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. Ouabain (10^{-5} M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Hexamethonium (10^{-3} M) was present throughout the experiment in (a). Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

Effect of Na^+ deprivation on muscarinic receptor-mediated catecholamine secretion

Experiments were carried out to determine whether the catecholamine secretion evoked by muscarinic receptor activation in the absence of extracellular Ca^{2+} could be observed in the absence of Na^+. Since Na^+ deprivation causes a marked increase in catecholamine secretion (Lastowecka & Trifaro,

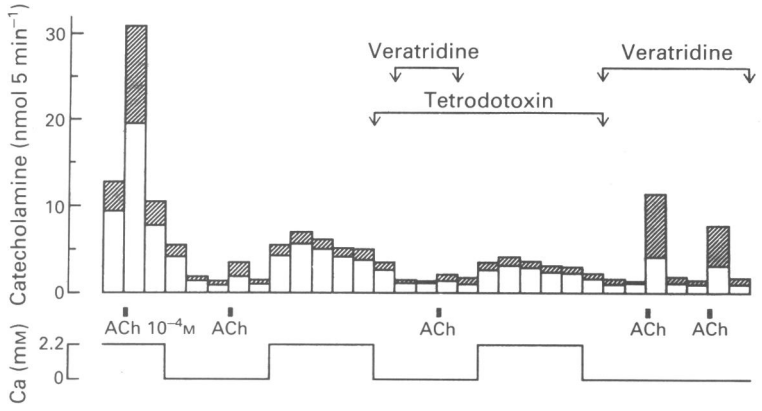

Figure 4 Effect of tetrodotoxin on the enhancement of acetylcholine-induced catecholamine secretion by veratridine. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. Veratridine (10^{-4} M) and tetrodotoxin (TTX, 10^{-6} M) were infused during the periods indicated by the horizontal bars delimited by arrows above the histograms. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

1974; Ito *et al.*, 1979), the glands were challenged with ACh (10^{-4} M) after the termination of the Na^+-deficient response. As shown in Figure 5, ACh failed to increase catecholamine secretion during perfusion with Na^+-deficient solution, but the response to ACh was completely restored to the control value after readmission of Na^+.

Requirement of Na^+ for the enhancement of muscarinic receptor-mediated catecholamine secretion by veratridine and ouabain

To study the requirement of Na^+ for the enhancement of ACh-induced catecholamine secretion by veratridine and ouabain, ACh (10^{-4} M) was applied during perfusion with Ca^{2+}-free, Na^+-deficient solution containing veratridine (10^{-4} M) or ouabain (10^{-4} M). Under these conditions, both drugs failed to enhance ACh-induced catecholamine secretion (data not shown). Then veratridine or ouabain was infused for 25 min with both Ca^{2+} and Na^+, and ACh (10^{-4} M) was applied 25 min after removal of the drugs and the cations. A representative experiment is illustrated in Figure 6, where veratridine (10^{-4} M) and ouabain (10^{-4} M) were sequentially infused at intervals of 95 min into the same adrenal preparation. Veratridine and ouabain caused a remarkable increase in catecholamine secretion (not illustrated in the figure) which quickly declined after the removal of the drugs and cations until it attained a new steady level. As shown in Figure 6, the response to ACh in the Ca^{2+}-free, Na^+-deficient environment was only slightly enhanced after treatment with veratridine, but was markedly increased after ouabain treatment. Finally, either veratridine or ouabain was infused for 25 min in the presence of Na^+ immediately after the end of Ca^{2+} loading, and ACh was administered 25 min after the removal of the drug and NaCl. As in the preceding experiments, ACh-induced catecholamine secretion was enhanced by ouabain, but not by veratridine. The effect of ouabain is illustrated in Figure 7.

Discussion

The present experiments show that Na^+ is essential for ACh to release catecholamine from cat adrenal glands through activation of muscarinic receptors in the absence of extracellular Ca^{2+}. Na^+ seems to play an important role in increasing the efficiency of ACh-induced Ca^{2+} mobilization from intracellular Ca^{2+} pools, which are postulated to link to muscarinic

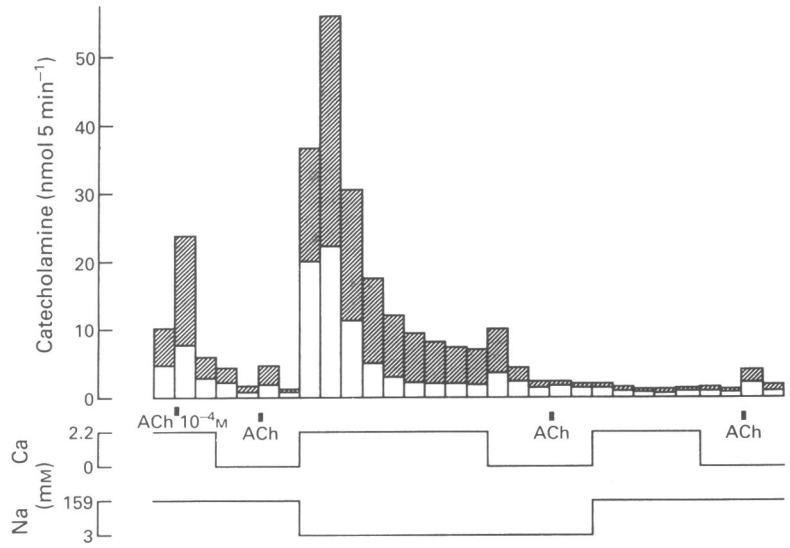

Figure 5 Catecholamine secretion evoked by acetylcholine during perfusion with Ca^{2+}-free, Na^+-deficient solution. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min in the presence or absence of Ca^{2+} and/or Na^+. Hexamethonium (10^{-3} M) was present throughout the experiment. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 10^{-5} M EGTA was added. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

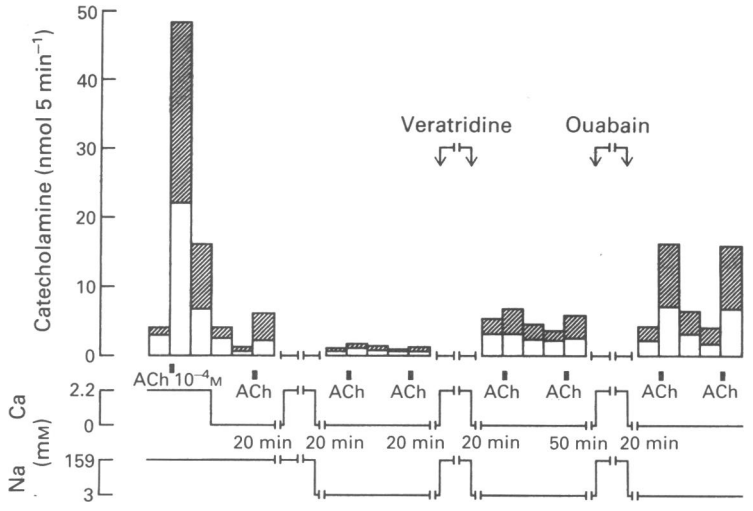

Figure 6 Catecholamine secretions evoked by acetylcholine during perfusion with Ca^{2+}-free, Na^+-deficient solution after exposure to veratridine and ouabain with Ca^{2+} and Na^+. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min in the presence and absence of Ca^{2+} and/or Na^+. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 10^{-5} M EGTA was added. Veratridine (10^{-4} M) and ouabain (10^{-4} M) were sequentially infused for 25 min at intervals of 95 min in the presence of both Ca^{2+} and Na^+. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

receptors. The effects of veratridine, ouabain and Na^+ deprivation provided some evidence supporting this view. First, catecholamine secretion induced by ACh was reversibly inhibited by Na^+ deprivation during perfusion with Ca^{2+}-free Locke solution. Second, veratridine and ouabain enhanced catecholamine secretions evoked by ACh and pilocarpine during perfusion with Ca^{2+}-free Locke solution. These potentiating effects were inhibited by removal of Na^+. Third, pretreatment with ouabain plus Na^+ with or without Ca^{2+} enhanced the secretory response to ACh obtained subsequently during perfusion with Ca^{2+}-free, Na^+-deficient solution.

It has been suggested that the increase in the intracellular concentration of Na^+ releases Ca^{2+} from internal stores in several systems (Baker & Crawford, 1975; Lowe *et al.*, 1976; Donatsch *et al.*, 1977; Silbergeld, 1977; Åkerman & Nicholls, 1981). The Na^+-induced Ca^{2+} release mechanism was also reported to operate in adrenal chromaffin cells (Gutman *et al.*, 1979). In the present study, spontaneous catecholamine secretion was only slightly enhanced by veratridine in the absence of extracellular Ca^{2+}, in contrast to the marked enhancement of muscarinic receptor-mediated secretory response. Therefore, the Na^+-induced Ca^{2+} release mechanism does not seem to play a major role in releasing spontaneous catecholamine secretion from adrenal chromaffin cells, if it does exist.

Previously, we found that ouabain enhanced catecholamine secretions evoked by splanchnic nerve stimulation, ACh and high K^+ in perfused guinea-pig adrenal glands (Nakazato *et al.*, 1986). In this previous work, we already noticed that the high K^+-induced secretory response enhanced by ouabain was completely inhibited by removal of extracellular Ca^{2+} and addition of Mg^{2+}, Co^{2+} or Ni^{2+}, but the secretory response to ACh was relatively resistant to these treatments. Later, it was found that the activation of muscarinic, but not

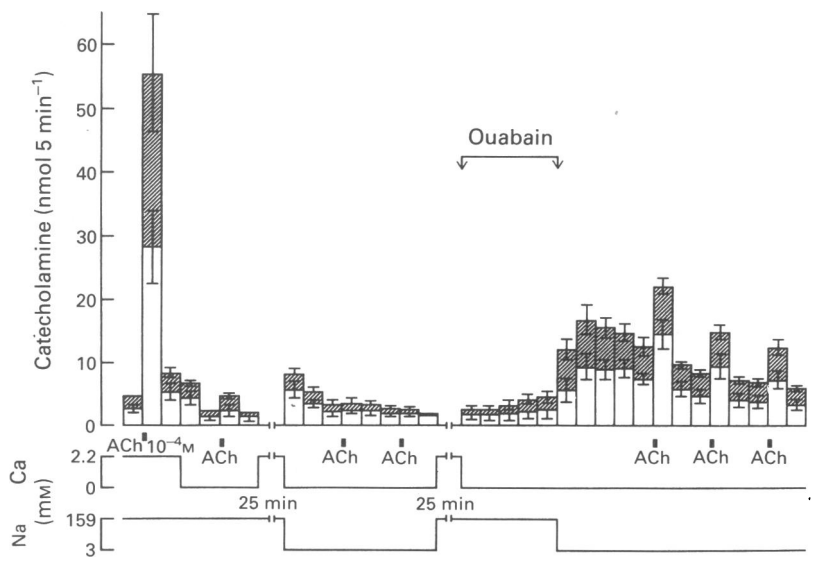

Figure 7 Catecholamine secretion evoked by acetylcholine during perfusion with Ca^{2+}-free, Na^+-deficient solution after exposure to ouabain with Na^+. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min in the presence or absence of Ca^{2+} and/or Na^+ before and after exposure to ouabain (10^{-4} M) in Ca^{2+}-free Locke solution. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 10^{-5} M EGTA was added. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the means of adrenaline (open) and noradrenaline (hatched) released in 5 experiments; vertical bars show s.e.mean.

nicotinic, receptors was partially effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} (Nakazato *et al.*, 1984; 1988). Furthermore, we reported that ouabain enhanced the secretory responses to stimulation of nicotinic and muscarinic receptors in the presence of extracellular Ca^{2+}, though only those induced by muscarinic receptor activation in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1989a). Our interpretation is that ouabain increases the intracellular concentration of Na^+ by inhibiting the Na^+-K^+ pump and results in an increase in the entry of Ca^{2+} in response to nicotinic stimulation or high K^+, and that at the same time ouabain causes an increase in storage of Ca^{2+} in intracellular Ca^{2+} pools linked to muscarinic receptors. Veratridine differs from ouabain in that it increases the entry of Na^+ through the activation of tetrodotoxin-sensitive Na^+ channels. Despite this fact, the effect of veratridine obtained in the present study suggests that the increase in the intracellular concentration of Na^+ augments the efficiency of the release of Ca^{2+} from internal stores and causes the potentiation of muscarinic receptor-mediated catecholamine secretion. This Na^+-dependent mechanism seems to be more marked in noradrenaline cells than in adrenaline cells, because both veratridine and ouabain increased noradrenaline secretion more than adrenaline secretion in response to ACh in the absence of extracellular Ca^{2+}.

Although a role for inositol 1,4,5-triphosphate has been suggested in bovine adrenal chromaffin cells (Stoehr *et al.*, 1986), there is little available information on the properties of intracellular Ca^{2+} pools responsible for agonist-induced catecholamine secretion. In previous works, we found that caffeine, which is known to enhance Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum of muscles (Endo, 1977), was effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} in perfused adrenal glands of the cat (Yamada *et al.*, 1988; 1989b). The catecholamine secretion induced by both caffeine and ACh was reversibly blocked by the intracellular Ca^{2+} antagonist TMB-8 (Yamada *et al.*, 1988) and was enhanced by ouabain (Yamada *et al.*, 1989a,b). However, pretreatment with caffeine did not entirely inhibit the subsequent secretory response to ACh (Yamada *et al.*, 1989b). Therefore, as in smooth muscle where Ca^{2+} stores consist of two classes (Iino *et al.*, 1988), adrenal chromaffin cells may possess heterogeneous Ca^{2+} pools in which the capacity to store Ca^{2+} and/or the ability to release Ca^{2+} is under the control of intracellular Na^+.

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Evidence for prejunctional M_2 muscarinic receptors in pulmonary cholinergic nerves in the rat

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1 The effects of muscarinic antagonists considered to be selective for M_1 receptors (pirenzepine) and for M_2 receptors (gallamine and methoctramine) were used to investigate the existence of prejunctional muscarinic receptors on cholinergic nerves in the rat lung. The tracheal tube preparation was used *in vitro*, and contraction of the trachealis muscle was induced by electrical field stimulation (EFS) and by application of an exogenous muscarinic agonist (pilocarpine), and measured as an increase in intraluminal pressure in the tube.

2 The muscarinic antagonists, gallamine and methoctramine, enhanced the contractions induced by nerve stimulation, while contractions elicited by exogenous application of pilocarpine were inhibited by the antagonists.

3 In contrast, pirenzepine blocked contractions induced by both EFS and pilocarpine in a dose-dependent manner (EC_{50} 0.1 μM) due to blockade of the postjunctional muscarinic receptors on airway smooth muscle. Potentiation of the response to EFS was never seen with this antagonist.

4 The muscarinic agonist, pilocarpine, caused a slow maintained increase in tone of the tracheal tube and at the same time reduced the contractions induced by EFS. This inhibitory effect was blocked by gallamine and methoctramine.

5 The results suggest that prejunctional inhibitory muscarinic receptors may be localised on the parasympathetic cholinergic nerve terminals innervating tracheal smooth muscle in the rat. This confirms previous findings obtained by measuring transmitter release in this species. The present results suggest that these receptors are of the M_2 subtype. Blockade of these autoreceptors with gallamine or methoctramine would increase the output of acetylcholine (ACh) and thereby enhance the nerve-induced contraction of tracheal smooth muscle.

Introduction

Prejunctional muscarinic receptors have previously been shown to inhibit neurotransmission in pulmonary cholinergic nerves in anaesthetized guinea-pigs (Fryer & Maclagan, 1984) and cats (Blaber *et al.*, 1985). In these species, gallamine was an antagonist for the autoreceptors and caused marked potentiation of vagally-induced bronchoconstriction, whereas the agonist, pilocarpine, inhibited vagally-mediated bronchoconstriction (Faulkner *et al.*, 1986). Using a different experimental approach *in vitro*, with radiolabelling techniques to measure transmitter release induced by potassium *in vitro*, Aas & Fonnum (1986) obtained evidence for muscarinic autoreceptors in the pulmonary cholinergic nerves in the albino rat. However, such receptors could not be demonstrated in anaesthetized rats *in vivo* with gallamine as an antagonist (Fryer & Maclagan, 1984).

In view of this apparent controversy, the experiments described in this paper were carried out to investigate whether muscarinic receptors could be demonstrated in cholinergic nerves in the isolated transmurally stimulated tracheal tube preparation, by the use of selective agonists and antagonists. Antagonists selective for M_1 receptors (pirenzepine) and M_2 receptors (methoctramine and gallamine) were used, together with the muscarinic agonist pilocarpine, which has been shown to activate pulmonary prejunctional muscarinic receptors in other species (Fryer & Maclagan, 1984; Faulkner *et al.*, 1986).

Methods

Albino rats (200–255 g) were anaesthetized with urethane (Sigma Ltd, 1.50 g kg^{-1} i.p.). The trachea was cannulated and removed, filled with Krebs-Henseleit buffer, and secured onto an electrode. The cannula was connected to a Statham Transducer (P23AC) forming a sealed system: the tube preparation was then suspended in an organ bath containing a physiological salt solution (composition in mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.16, $MgSO_4$ $7H_2O$ 1.19, $CaCl_2$ 2.0 and glucose 11.1), gassed with 95% O_2/5% CO_2 (pH 7.4) and maintained at 37°C (Farmer & Coleman, 1970).

One electrode was placed inside the tracheal tube and another outside for transmural stimulation. Stimulation (30 V, 0.2 ms, 30 Hz, maximal voltage at 40 s intervals) caused a rapid contraction of the trachealis muscle which was recorded as an increase in intraluminal pressure (ILP). Hexamethonium (50 μM) was present in the reservoir of buffer solution throughout the experiment to ensure that the postganglionic nerves were stimulated, and indomethacin (1 μM) was present to inhibit cyclo-oxygenase enzymes and so prevent prostaglandin formation (Walters *et al.*, 1984).

There was some variation between preparations in the size of the contraction elicited by transmural stimulation. The results from each tissue have therefore been expressed as the ratio (B_2/B_1) of the response after the addition of drugs (B_2) to the response before drugs (B_1). All drugs were added to the bath in a cumulative manner.

Chemicals

The drugs used were: pilocarpine nitrate and atropine sulphate (BDH); gallamine triethiodide (May & Baker); hexamethonium bromide (Koch-Light); indomethacin (Sigma); pirenzepine hydrochloride (Boehringer Ingelheim); methoctramine (gift from Dr C. Melchiorre, Department of Chemical Sciences, University of Camerino, Italy). All other chemicals were of analytical reagent grade. The drugs were dissolved and diluted in 0.9% saline except for indomethacin, which was dissolved by use of sonication in a buffer, containing KH_2PO_4 19.76 mM and Na_2HPO_4 118.34 mM, immersed for 60 min in hot water.

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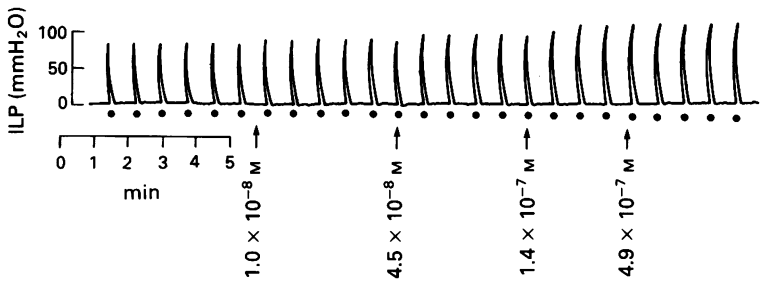

Figure 1 Increase in intraluminal pressure (ILP) of rat isolated tracheal tube preparation following transmural nerve stimulation (30 Hz, 0.2 ms, 5 s and 150 pulses, at dots). Methoctramine (final concentration in organ bath shown by arrows) enhanced contractions induced by transmural nerve stimulation but did not affect basal tone.

Statistics

Means and s.e.mean were calculated for all data. Student's *t* test was applied to the results to determine significant differences between the data of each group.

Results

In the isolated tracheal tube preparation from the albino rat, transmural stimulation induced a rapid, reproducible increase in intraluminal pressure, reflecting contraction of the trachealis muscle. The contractions were (1) dependent on stimulation frequency and on the length of the stimulus train, (2) atropine-sensitive and (3) unaffected by hexamethonium (50 μM). This indicates that contraction was due to stimulation of postganglionic cholinergic nerves. A relaxant response to transmural stimulation was not seen in these experiments.

Effects of muscarinic antagonists

The increase in intraluminal pressure induced by transmural stimulation was enhanced in the presence of methoctramine (Figures 1 and 3a) and gallamine (Figure 2a). The maximum effect obtained was an increase of 30% in the case of gallamine (Figure 2a) and an increase of 20% with methoctramine (Figure 3a). In parallel experiments, the antagonists reduced the contractions induced by exogenously applied pilocarpine as shown in Figures 2b and 3b, indicating that the muscarinic agonists had blocked the postjunctional muscarinic receptors on the trachealis muscle. The figures show that methoctramine was approximately 100 times more potent than gallamine for the postjunctional receptors on airway smooth muscle.

Different results were obtained with the muscarinic antagonist pirenzepine, which inhibited contractions induced by transmural nerve stimulation in a reversible manner at all doses used, with an EC_{50} of approximately 0.1 μM (Figure 4). Enhancement of nerve-induced muscle contraction was not seen with pirenzepine at any dose, in contrast to the effects seen with gallamine and methoctramine.

Effect of a muscarinic agonist

Addition of the muscarinic agonist, pilocarpine, to the bath caused a slow increase in intraluminal pressure due to con-

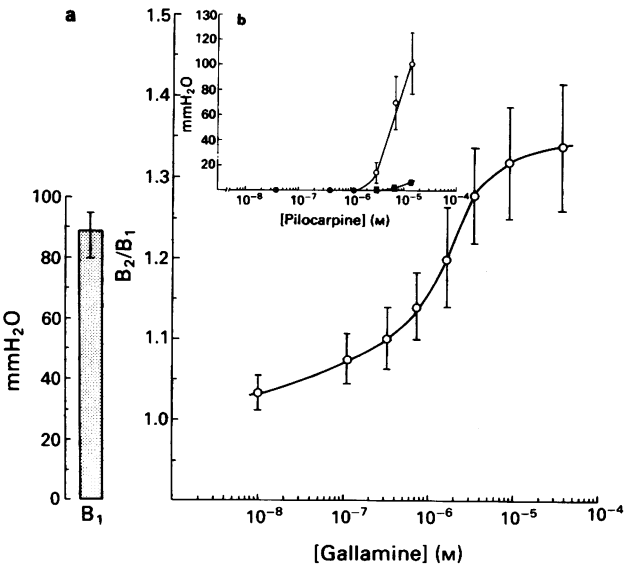

Figure 2 (a) The effect of gallamine on responses of the rat isolated tracheal tube preparation to transmural stimulation. The results are expressed as the ratio B_2/B_1 i.e. the responses after gallamine (B_2) divided by the response before the antagonist (B_1). Values of B_1, in absolute units, are represented by the column. Mean values are shown and vertical lines indicate s.e.mean (n = 5). (b) Dose-response curve to pilocarpine alone (increase in intraluminal pressure in mmH_2O, ○) and in the presence of gallamine (3.7×10^{-5} M, ●). A concentration of gallamine which was maximally effective in (a) (3.7×10^{-5} M) inhibited the postjunctional action of pilocarpine. Mean values are shown and vertical lines indicate s.e.mean (n = 5).

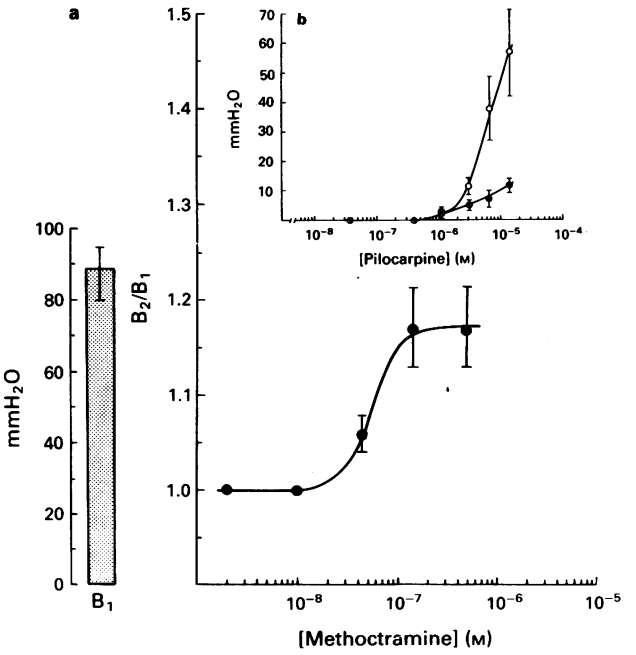

Figure 3 The effect of methoctramine on responses elicited by transmural stimulation (a) and on responses elicited by pilocarpine (b). The concentration of methoctramine which caused maximal facilitation of the response to nerve stimulation (4.9×10^{-7} M) in (a) inhibited the postjunctional effect of pilocarpine in (b) (●). Results are expressed as the mean B_2/B_1 ratio (n = 5) as in Figure 2. Vertical lines indicate s.e.mean.

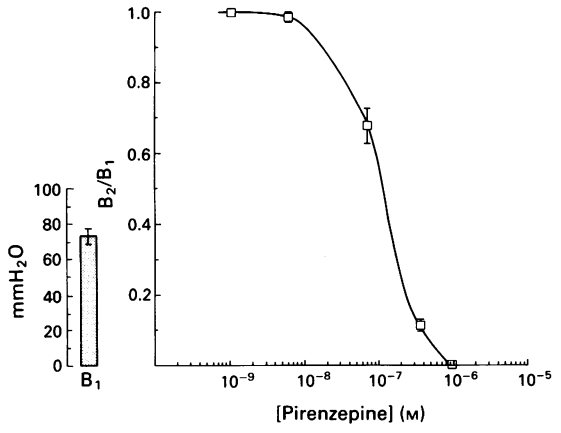

Figure 4 The effect of cumulative doses of pirenzepine on the transmurally-induced contraction of the rat isolated tracheal tube preparation. The inhibitory effect of pirenzepine is shown as a decrease in the ratio B_2/B_1 where B_2 is the response after exposure to pirenzepine. Control responses (B_1, mmH_2O) to electrical stimulation are shown on the left of the figure in absolute units. Results are expressed as the mean B_2/B_1 ratio ($n = 5$) as in previous figures. Vertical lines indicate s.e.mean.

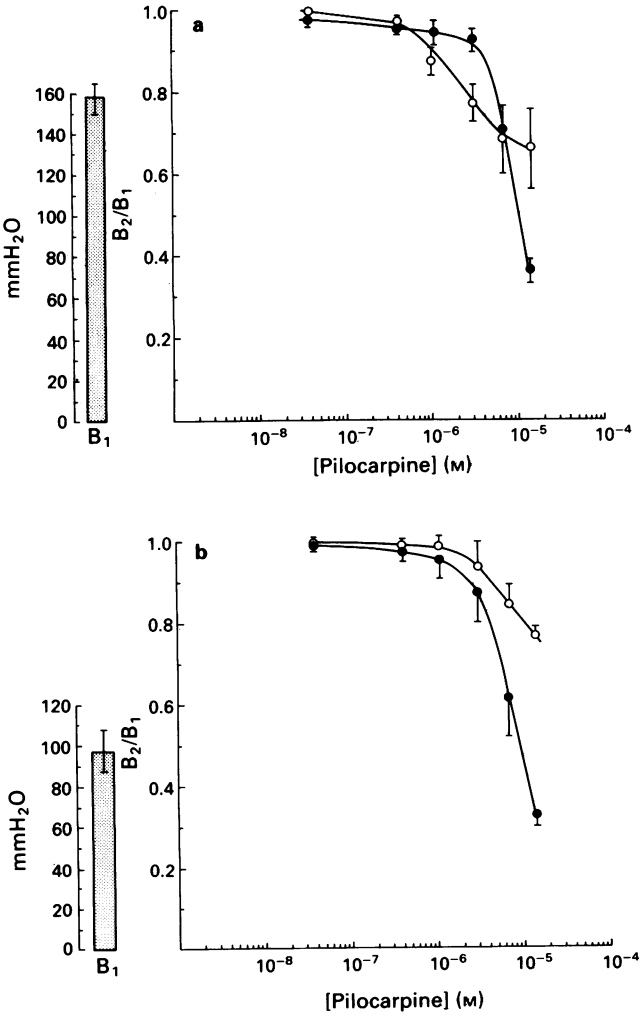

Figure 5 The effect of pilocarpine on transmurally-induced contractions of the rat tracheal tube preparation. The effect of pilocarpine is shown as a decrease in the ratio B_2/B_1 where B_2 is the response after exposure to the agonist. Cumulative doses of pilocarpine inhibited the contractions (●), and the effect of pilocarpine was reduced by gallamine (3.7×10^{-5} M, a) (○) and methoctramine (4.9×10^{-7} M, b) (○). Control responses (B_1, mmH_2O) elicited by transmural stimulation are shown to the left of the figure. Results are expressed as the mean B_2/B_1 ratio ($n = 5$) as in previous figures. Vertical lines show s.e.mean.

traction of the trachealis muscle. At the same time, contractions induced by nerve stimulation were reduced in a concentration-dependent manner (Figure 5). The inhibitory effect of pilocarpine on the nerve-induced response was significantly reduced by prior application of gallamine 3.7×10^{-5} M (Figure 5a) or methoctramine 4.9×10^{-7} M (Figure 5b).

Discussion

The aim of the present study was to investigate whether prejunctional muscarinic cholinoceptors exist in pulmonary cholinergic nerves in the rat, as they do in other species. The effect of one muscarinic agonist and three antagonists was studied on contractile responses of the isolated trachea elicited by post-ganglionic stimulation of the cholinergic nerves. In experiments of this type, if muscarinic receptors are present both on the nerve terminals and also on the effector cell, the effect obtained with muscarinic agonists and antagonists depends on the balance of potency of the drug for the neuronal and postjunctional receptors. If the antagonist has any effect on the postjunctional receptors, its potency for the prejunctional receptors will be underestimated.

In the lung, the postjunctional muscarinic receptors are apparently of the M_3 subtype in all species so far investigated (Barnes *et al.*, 1988), whereas the prejunctional receptors belong to the M_2 subtype in guinea-pig (Fryer & Maclagan, 1987), cat (Blaber *et al.*, 1985) and man (Minette & Barnes, 1988), and there are additional M_1 and M_3 receptors on dog pulmonary nerves (Ito & Yoshitomi, 1988). There is, at present, no antagonist sufficiently selective to block the neuronal receptors without an action on the postjunctional M_3 receptors. However, gallamine and pilocarpine have been shown to act as an antagonist and an agonist respectively, for the prejunctional receptors on pulmonary cholinergic nerves, at doses which are several fold lower than those needed to affect postjunctional muscarinic receptors on airway smooth muscle (Gardier *et al.*, 1974; 1978; Fryer & Maclagan, 1984; Faulkner *et al.*, 1986).

In the present experiments it was shown that stimulation of postganglionic cholinergic nerves in the rat trachea was potentiated by the selective muscarinic antagonists, gallamine and methoctramine. These results were not due to postjunctional mechanisms and are consistent with the existence of M_2 muscarinic inhibitory autoreceptors on the final terminals of the pulmonary cholinergic nerves in this species.

These results are similar to previous experiments *in vivo* in guinea-pig (Fryer & Maclagan, 1984) and cat (Blaber *et al.*, 1985), where gallamine potentiated the effect of parasympathetic nerve stimulation in the lungs. Methoctramine has recently been shown to be a potent antagonist of M_2-receptors in the myocardium in functional studies (Melchiorre *et al.*, 1987a,b) as well as in binding studies (Michel & Whiting, 1988). A prejunctional effect of methoctramine on neuronal muscarinic receptors has previously been demonstrated in guinea-pig lung *in vivo* by Watson *et al.* (1989), and similar results were obtained in the present experiments in the rat. This provides evidence that the prejunctional muscarinic-receptors in the rat pulmonary cholinergic nerves are also of the M_2 sub-type.

In earlier experiments in rat lung (Aas & Fonnum, 1986) the cholinoceptor agonist, oxotremorine, was shown to reduce the release of acetylcholine (ACh) from the pulmonary cholinergic nerves. In the present work, a similar conclusion was reached using pilocarpine as the agonist. Pilocarpine reduced the transmurally induced airway contraction, probably causing a reduction in the release of ACh due to activation of the prejunctional muscarinic receptors. This inhibitory effect of pilocarpine was reduced by prior incubation with either muscarinic antagonist, providing further evidence that these are M_2 receptors.

Concurrent with this reduction in transmurally-induced contraction, a postjunctional effect of the agonist was

observed, which caused an increase in the tone of the trachealis muscle. This pilocarpine-induced rise in ILP was reduced substantially by gallamine and methoctramine. Thus, both antagonists caused significant blockade of postjunctional M_3-receptors on the trachealis muscle, but methoctramine was about 100 times more potent than gallamine in this effect.

The results of the experiments with pirenzepine demonstrated clearly that the prejunctional receptors were not of the M_1-subtype, since there was no enhancement of the transmurally-induced contraction with low doses of this antagonist. However, pirenzepine showed a marked postjunctional effect and inhibited the transmurally induced airway contraction in a concentration-dependent manner. Thus at the doses used, pirenzepine was an antagonist for M_3 receptors on the trachealis muscle. Such a non-selective effect of pirenzepine has been demonstrated in other tissues (see review by Eglen & Whiting, 1986).

The results presented in this paper, with gallamine and methoctramine, demonstrate the existence of prejunctional muscarinic receptors in pulmonary cholinergic nerves in the rat trachea, which are of the M_2 subtype. This contrasts with previous work in the rat lung *in vivo*, where the use of gallamine failed to demonstrate these receptors. One possible explanation for this difference could be that the dose of gallamine used in the *in vivo* experiments, although an effective dose in other species, was insufficient to block muscarinic receptors in the rat lung. It appears that gallamine may have low potency as a cholinoceptor antagonist in the rat *in vivo*, for it has been found that the dose of gallamine needed to paralyse skeletal muscle in the anaesthetized rat is ten fold greater than that needed in other mammalian species (Bowman, 1964).

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The effect of peptidase inhibitors on bradykinin-induced bronchoconstriction in guinea-pigs *in vivo*

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1 Bradykinin (BK) instilled directly into the airway lumen caused bronchoconstriction in anaesthetized, mechanically ventilated guinea-pigs in the presence of propranolol (1 mg kg^{-1} i.v.) The geometric mean dose of BK required to produce 100% increase in airway opening pressure (PD_{100}) was 22.9 nmol (95% c.i. 11.7–44.6 nmol).

2 The dose-response curve for the effect of instilled BK was significantly shifted to the left by the angiotensin converting enzyme (ACE) inhibitor, captopril (5 and 50 nmol instillation, PD_{100} = 3.0, 95% c.i. 0.98–8.9, and 2.0 nmol, 95% c.i. 0.65–6.2 nmol, respectively).

3 The neutral endopeptidase (NEP) inhibitor, phosphoramidon (5 and 50 nmol instillation) also shifted the dose-response curve for the effect of instilled BK; the PD_{100} values = 2.2 (95% c.i. 0.40–11.7) and 1.8 nmol (95% c.i. 0.87–3.5 nmol), respectively.

4 After pretreatment with captopril (50 nmol) and phosphoramidon (50 nmol) in combination, the dose-response curve for the effect of instilled BK (PD_{100} = 1.1 nmol, 95% c.i. 0.37–3.2 nmol) was similar to that obtained in the presence of each inhibitor used alone.

5 The kininase I inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (50 nmol instillation) failed to alter the dose-response curve to instilled BK (PD_{100} = 14.6 nmol, 95% c.i. 6.7–32.0 nmol).

6 These data suggest that both ACE and NEP degrade BK in the airway lumen, but that kininase I is not involved.

Introduction

Bradykinin (BK) has been implicated in asthma (Barnes *et al.*, 1988), since BK is a potent bronchoconstrictor in asthmatic subjects, being significantly more potent than methacholine and histamine (Fuller *et al.*, 1987), but has no effect on airway function in normal subjects (Simonsson *et al.*, 1973; Newball *et al.*, 1975). Furthermore, asthmatic patients have an elevated kinin concentration in plasma (Abe *et al.*, 1967) and in bronchoalveolar lavage fluid after antigen challenge (Christiansen *et al.*, 1987).

BK may be formed from high-molecular weight kininogens in plasma which exude into the asthmatic airway by the action of enzymes such as tryptase released from airway mast cells (Proud *et al.*, 1988). The enzymes responsible for degradation of BK in airway, particularly when found in the airway lumen, have not yet been fully elucidated. A recent *in vitro* study showed that both neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) inhibitors potentiate BK-induced tracheal contraction in ferret (Dusser *et al.*, 1988; Frossard *et al.*, 1990). It seems likely that the mode of action of BK in the airways may be different *in vitro* and *in vivo*, because BK administered directly into the airway lumen causes bronchoconstriction mainly via a cholinergic reflex and release of tachykinins from sensory nerves rather than through a direct action on airway smooth muscle (Ichinose *et al.*, 1990). The purpose of this study was to investigate the role of enzymes such as ACE, NEP, and kininase I which may modulate BK-induced bronchoconstriction *in vivo*, in the degradation of BK. We examined the bronchoconstrictor effect of instilled BK in the presence of captopril, phosphoramidon, and DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTPA), which inhibit ACE, NEP and kininase I, respectively, in anaesthetized guinea-pigs. In this study, we investigated the effects of these agents administered directly into the airways by instillation, rather than intravenously, as this may more closely reflect the action of BK in asthmatic airways.

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Methods

Animal preparation

Male Dunkin-Hartley outbred guinea-pigs (Charles River U.K. Ltd., Kent, U.K.), weighing 350–400 g, were anaesthetized by an intraperitoneal injection of urethane (2 g kg^{-1}) and placed on a heated blanket (Homeothermic system, Harvard Apparatus Ltd., Edenbridge, Kent, U.K.) which maintained body temperature at about 37°C. The trachea was cannulated and the lungs were artificially ventilated with a small animal constant volume respirator (Harvard Apparatus Ltd.) at a frequency of 60 strokes min^{-1} and a tidal volume of 1 ml 100 g^{-1}. Airway opening pressure (P_{ao}) was recorded by a differential pressure transducer (Farnell Electronic Components Ltd., Leeds, U.K.) as an index of changes in tracheobronchial resistance to airflow. The carotid artery and jugular vein were cannulated to monitor systemic blood pressure and to inject drugs, respectively. We pretreated all animals with propranolol (1 mg kg^{-1} i.v.) 30 min before experimentation to block the bronchodilator effects of catecholamines which may be released after BK administration (Lewis & Reit, 1965; Piper *et al.*, 1967).

Studies with instilled BK

We constructed dose-response curves for the effects of BK instilled into airways in 7 groups of animals. Group 1 was pretreated with saline (vehicle for phosphoramidon, captopril, and MGTPA, n = 7), group 2 with captopril (5 nmol, n = 5), group 3 with captopril (50 nmol, n = 5), group 4 with phosphoramidon (5 nmol, n = 5), group 5 with phosphoramidon (50 nmol, n = 7), group 6 with phosphoramidon (50 nmol) and captopril (50 nmol) (n = 5), and group 7 with MGTPA

(50 nmol). Phosphoramidon, captopril, and MGTPA were administered by injecting 50 μl volumes together with 4 ml of air, directly into the airway via a side hole of the tracheal cannula 15 min before BK challenge. BK (0.5–50 nmol, 50 μl volumes) was then instilled directly into the trachea at 30 min intervals.

Drugs

Bradykinin (BK) and DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MGTPA) were kindly donated by Prof. H.P. Rang, Sandoz Institute for Medical Research (London). Phosphoramidon and urethane were obtained from Sigma Chemical Co. (Poole, Dorset). Propranolol hydrochloride was purchased from Imperial Chemical Industries plc. (Macclesfield, Cheshire), captopril from E.R. Squibb & Sons (Hounslow, Middlesex), and saline (0.9% w/v NaCl solution) from Travenol Laboratories (Thetford, Suffolk).

Statistical analyses

Data are expressed as means ± s.e. Differences between mean % increase in P_{ao} values were analysed by Student's unpaired t test (two-tailed). The provocative dose of BK required to produce 100% increase in P_{ao} (PD_{100}) was determined for each dose-response curve to BK. We used the geometric mean from each PD_{100} value and a Mann-Whitney U test (two-tailed). Probability values, $P < 0.05$ were considered significant.

Results

Baseline P_{ao}

The baseline P_{ao} was 10.2 ± 0.6 (mean ± s.e.) cmH_2O ($n = 39$). In some animals, saline, captopril, phosphoramidon, and MGTPA pretreatment caused a transient increase in P_{ao} (16/39 animals had an increase of 29.5 ± 4.2%), and the remaining animals (23/39) did not. However, 15 min later the P_{ao} returned to baseline values in all animals. In the BK challenge study, saline (vehicle for BK) instillation also caused a small increase in P_{ao} in some animals (20/39 animals had an increase of 22.4 ± 4.0%), we therefore took the P_{ao} after saline instillation as the baseline value.

Effect of captopril on BK-induced bronchoconstriction

BK (0.5–50 nmol, 50 μl) instillation caused a dose-related increase in P_{ao} after saline pretreatment (Figure 1) with a PD_{100} of 22.9 nmol (geometric mean) (Figure 2). Captopril pretreatment significantly enhanced the effect of BK in a dose-dependent manner, with a significant decrease in PD_{100} value to 3.0 and 2.0 nmol after 5 and 50 nmol captopril pretreatment, respectively ($P < 0.05$ and 0.01, Figure 2).

Effect of phosphoramidon on BK-induced bronchoconstriction

Phosphoramidon pretreatment also enhanced the BK-induced bronchoconstrictor response (Figure 3). The mean PD_{100} value decreased from 22.9 nmol (95% c.i. 11.7–44.6 nmol) after saline to 2.2 (95% c.i. 0.40–11.7) and 1.8 nmol (95% c.i. 0.87–3.5 nmol) after 5 and 50 nmol phosphoramidon, respectively (Figure 2).

Effect of combined captopril and phosphoramidon on BK-induced bronchoconstriction

We attempted to examine whether captopril and phosphoramidon had additive effects on BK-induced bronchoconstriction. Pretreatment with captopril (50 nmol) and phosphoramidon (50 nmol) in combination did not cause any further enhancement compared with captopril (50 nmol) or phosphoramidon (50 nmol) alone. The PD_{100} values were significantly altered after pretreatment with captopril, phosphoramidon, and the combination of captopril and phosphoramidon, by 1.1, 1.1 and 1.3 log units, respectively, from the PD_{100} after saline pretreatment (Figure 2). There was no significant difference between the PD_{100} values after the three drug treatments.

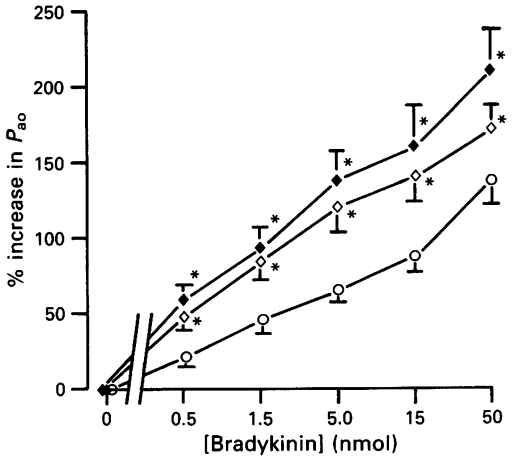

Figure 1 Dose-response curves for the bronchoconstrictor effect of instilled bradykinin (0.5–50 nmol), after pretreatment with saline ($n = 7$, ○), captopril 5 nmol ($n = 5$, ◇) and 50 nmol ($n = 5$, ◆), in guinea-pigs. The effect of saline (vehicle for bradykinin) instillation is shown at 0. Propranolol (1 mg kg^{-1} i.v.) was present throughout the experiments. All points are mean and vertical lines show s.e. P_{ao} = airway opening pressure. *$P < 0.05$ compared with the values of saline-pretreated animals (Student's unpaired t test).

Effect of DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid on BK-induced bronchoconstriction

MGTPA (50 nmol) pretreatment did not modify the airway response to BK. The PD_{100} obtained after MGTPA pretreatment was 14.6 nmol (95% c.i. 6.7–32.0 nmol), which was not significantly different from the value of saline-pretreated animals (Figure 2).

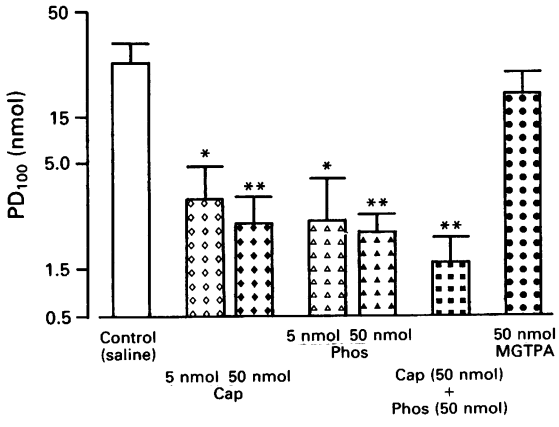

Figure 2 The dose of instilled bradykinin required to produce 100% increase in airway opening pressure (PD_{100}) in the guinea-pig after different pretreatments is shown. All columns indicate geometric mean and bars show s.e. of 5 to 7 animals. Abbreviations: Cap, captopril; Phos, phosphoramidon; MGTPA, DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid. Significant differences from control (saline pretreatment) values are indicated by *$P < 0.05$, **$P < 0.01$ (Mann-Whitney U test).

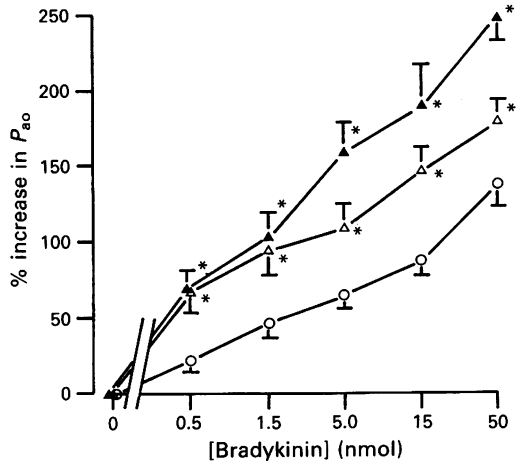

Figure 3 Dose-response curves for the bronchoconstrictor effect of instilled bradykinin (0.5–50 nmol) after pretreatment with saline ($n = 7$, ○), phosphoramidon (5 nmol, $n = 5$, △), and phosphoramidon (50 nmol, $n = 7$, ▲). The effect of saline (vehicle for bradykinin) instillation is shown at 0. Propranolol (1 mg kg^{-1} i.v.) was present throughout the experiments. All points are mean and vertical lines show s.e. P_{ao} = airway opening pressure. *$P < 0.05$ compared with the values of saline pretreated animals (Student's t test).

Discussion

The present data show that captopril, an inhibitor of ACE, and phosphoramidon, an inhibitor of NEP, significantly potentiate the bronchoconstriction induced by BK administered directly into the airway lumen in anaesthetized, mechanically ventilated guinea-pigs. The potency of these two peptidase inhibitors was almost equal, and there was no additive interaction between the two inhibitors. On the other hand MGTPA, a kininase I inhibitor, failed to modify the effect of BK in the airways.

ACE, NEP, and kininase I have different sites of action and different locations. These peptidases cleave BK at the dipeptide Phe^8-Arg^9, Pro^7-Phe^8, and C terminal Arg, respectively (Yang & Erdos, 1967; Regoli & Barabe, 1980; Dusser *et al.*, 1988; Proud & Kaplan, 1988), resulting in the formation of inactive products. ACE and NEP are membrane-bound peptidases (Johnson *et al.*, 1985) that exist in many tissues. In the lung, ACE is predominantly located on the surface of the lung endothelial cells (Regoli & Barabe, 1980), but may also be present in airways (Dusser *et al.*, 1988). By contrast NEP is localized to epithelium, submucosa and smooth muscle of airways (Johnson *et al.*, 1985; Sekizawa *et al.*, 1987a,b), whereas kininase I exists predominantly in blood (Regoli & Barabe, 1980; Proud & Kaplan, 1988). The effect of BK administered directly into the airway was potentiated by both captopril and phosphoramidon but not by MGTPA.

There are two possible mechanisms by which captopril and phosphoramidon may potentiate BK-induced bronchoconstriction. The most likely possibility is that these inhibitors reduce the rate of BK degradation. The other possibility is that degradation of tachykinins such as substance P (SP) and neurokinin A released by BK stimulation of afferent nerve endings in the airway is reduced. Both captopril and phosphoramidon potentiate BK-induced tracheal contraction *in vitro* in ferret but a SP-antagonist does not modify the bronchoconstrictor effect of BK (Dusser *et al.*, 1988), supporting the view that these inhibitors are potentiating BK itself. However, *in vivo*, BK stimulates vagal afferent C-fibres in the lung (Kaufman *et al.*, 1980), which may lead to bronchoconstriction via both a cholinergic reflex and release of peptides from sensory nerves. Indeed, in a previous study we demonstrated that BK administered directly into the airway lumen caused bronchoconstriction, which appeared to be largely mediated via a cholinergic mechanism and by the release of sensory neuropeptides; both atropine and neuropeptide depletion by capsaicin reduced the BK-induced bronchoconstriction, and the combination of these two pretreatments virtually abolished the effect of BK (Ichinose *et al.*, 1990). Furthermore, BK is capable of releasing tachykinins from sensory nerve endings in guinea-pig perfused lung (Saria *et al.*, 1988). Both ACE and NEP inhibitors potentiate SP-induced bronchoconstriction *in vitro* (Sekizawa *et al.*, 1987a,b) and *in vivo* (Shore *et al.*, 1988), presumably by preventing the degradation of SP. However, when SP is given by inhalation to guinea-pigs, while its effect is potentiated by an inhaled NEP inhibitor, inhaled captopril has no effect (Lotvall *et al.*, 1990) suggesting that NEP but not ACE is important in degrading SP delivered from the airway. For BK, captopril and phosphoramidon are equally effective which argues that inhibition of degradation of tachykinins released from sensory nerves by instilled BK cannot be the sole explanation for their effect. It is therefore possible that the potentiation of BK-induced bronchoconstriction by captopril and phosphoramidon is, in part, due to prevention of the degradation of neuropeptides which may be released from sensory nerves by BK stimulation and partly due to a direct effect of BK (presumably on C-fibre endings in the airway).

In conclusion, the bronchoconstrictor response to instilled BK is potentiated by both captopril, an inhibitor of ACE, and phosphoramidon, an inhibitor of NEP, but not by MGTPA, an inhibitor of kininase I, suggesting that both ACE and NEP contribute to the metabolism of BK in guinea-pig airways *in vivo*. These observations could be of relevance to the cough induced by ACE inhibitors (Fuller & Choudry, 1987) since, if ACE is involved in the degradation of BK in the respiratory tract, then any inhibition of this enzyme may potentiate the action of BK on sensory nerves in the respiratory tract, and thus lead to a lowered cough threshold.

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Endothelium-dependent vascular activities of endothelin-like peptides in the isolated superior mesenteric arterial bed of the rat

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1 The vasoconstrictor activities of endothelin-2, endothelin-3, sarafotoxin S6b, human proendothelin$_{1-38}$ and mouse vasoactive intestinal contractor (VIC) were studied in the isolated Krebs-Henseleit perfused mesenteric arterial bed of the rat in the presence and absence of the endothelium. The vasoconstrictor properties of endothelin-1 were studied in control preparations and in preparations treated with methylene blue or N_ω-nitro-L-arginine methyl ester (NAME). Finally, the direct vasodilator properties of endothelin-2, endothelin-3 and sarafotoxin S6b were studied in preparations preconstricted with methoxamine.

2 In the presence of an intact endothelium, all of the peptides caused dose-dependent increases in perfusion pressure and sarafotoxin S6b was a full agonist relative to the other peptides studied (maximum increase in perfusion pressure, $R_{max} = 106 \pm 11$ mmHg). Endothelin-1, endothelin-2 and VIC were more potent vasoconstrictors (ED_{50} 93.0 ± 40.0, 90.8 ± 20.5 and 106 ± 63 pmol, respectively) than endothelin-3 and sarafotoxin S6b, which were found to be equipotent (ED_{50} values 411 ± 195 and 345 ± 86 pmol, respectively). A full dose-response relationship could not be constructed for proendothelin, but the highest dose used (4 nmol) increased the perfusion pressure by 15.4 ± 1.6 mmHg.

3 Destruction of the endothelium with the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) significantly enhanced the pressor activity of all 5 peptides. The R_{max} for sarafotoxin S6b was not significantly altered by removal of the endothelium but its potency was significantly increased ($ED_{50} = 115 \pm 15$ pmol). Although their R_{max} values were significantly increased, endothelin-2 and VIC were still partial agonists relative to sarafotoxin S6b in CHAPS-pretreated preparations; their potencies were unchanged (ED_{50} values 118 ± 53 and 416 ± 196 pmol, respectively). Removal of the endothelium significantly reduced the potency of endothelin-3 (ED_{50}, 6.3 ± 2.2 nmol) but this peptide then exhibited full agonist activity ($R_{max} = 106 \pm 14$ mmHg). After endothelial cell destruction, the pressor responses to proendothelin were increased; 4 nmol gave a response of 38.8 ± 5.5 mmHg.

4 Exposure of preparations to either 100 μM NAME ($R_{max} = 42.6 \pm 2.4$ mmHg and $ED_{50} = 57.5 \pm 13.7$ pmol) or 10 μM methylene blue ($R_{max} = 36.0 \pm 5.1$ mmHg and $ED_{50} = 81.5 \pm 26.1$ pmol) significantly enhanced the maximum pressor responses to endothelin-1 (control: $R_{max} = 22.5 \pm 2.6$ mmHg; $ED_{50} = 93.0 \pm 40.0$ pmol). The values in the presence of NAME or methylene blue were not significantly different from those found previously for endothelin-1 after removal of the endothelium with CHAPS.

5 Endothelin-2, endothelin-3 and sarafotoxin S6b all caused vasorelaxation in preparations which had been precontracted with 100 μM methoxamine. This action was endothelium-dependent as it was abolished by perfusing the mesentery with CHAPS. Endothelin-3 and sarafotoxin S6b caused relaxation at much lower doses than were needed with endothelin-1 and endothelin-2.

6 The endothelium significantly modulates the vasoconstrictor activity of all the endothelin-like peptides studied, including the precursor peptide proendothelin (which was the least potent of the peptides). This modulation is likely to be due to the release of endothelium-derived relaxing factor, since similar results to destruction of the endothelium were obtained when endothelin-1 was investigated in the presence of either methylene blue or NAME (an inhibitor of nitric oxide formation) in the perfusion fluid. The vasodilator effects of the peptides were also endothelium-dependent. There was a different order of potency for vasoconstriction and vasodilatation supporting the suggestion that there are sub-types of receptor for the endothelin-like peptides in the vasculature; one type on the vascular smooth muscle and a second type on the endothelium.

Introduction

Since the initial characterization of porcine endothelin (Yanagisawa *et al.*, 1988b), the human genome has been found to contain coding for not only the same peptide sequence (Itoh *et al.*, 1988), now designated endothelin-1, but also for two other endogenous isopeptides (Inoue *et al.*, 1989), namely endothelin-2 and endothelin-3 (the latter sequence being identical to one originally found in the rat genome; Yanagisawa *et al.*, 1988a). It is thought that human endothelin-1 is formed from a 38 amino acid precursor, proendothelin$_{1-38}$ (Itoh *et al.*, 1988). All the endothelins contain 21 amino acids and 4 cysteine residues which are linked by disulphide bridges between residues 1 and 15 and between residues 3 and 11. Endothelin-2 shows the greatest homology with endothelin-1 and differs at only two positions in the smaller intramolecular loop (that between positions 3 and 11), whereas endothelin-3 has 6 substitutions relative to endothelin-1, 4 within the smaller intramolecular loop and 2 substitutions adjacent to bridge forming cysteinyl residues. Comparative studies on the vascular actions of endothelin-2 and endothelin-3 have shown that they are both less potent than endothelin-1 at contracting porcine

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coronary strips denuded of endothelium (Inoue *et al.*, 1989). However, endothelin-2 produced maximum responses which were 50% greater than those of endothelin-1 which, in turn, gave maximum responses twice as large as those of endothelin-3 (Inoue *et al.*, 1989).

In the rat isolated perfused mesenteric bed, endothelin-3 is a partial agonist relative to endothelin-1 but is more effective at causing endothelium-dependent relaxations (Warner *et al.*, 1989). All of the forms encoded in the human genome increase arterial pressure in the anaesthetized, chemically-denervated rat; endothelin-2 produced responses which are of the longest duration and endothelin-3 causes the largest initial depressor responses (Inoue *et al.*, 1989). A further endothelin-like peptide sequence of mammalian origin, with 3 substitutions relative to endothelin-1, was found in the mouse genome by Saida *et al.* (1989) and named vasoactive intestinal contractor (VIC).

A similar structure to the endothelins is possessed by the cardiotoxin sarafotoxin S6b, a constituent of the venom of the Palestinian burrowing asp *Atractaspis engaddensis* (Kloog *et al.*, 1988). Sarafotoxin S6b, like endothelin-1, is a 21 amino acid peptide which contains a hydrophobic carboxy terminus with a terminal tryptophan residue and has 2 disulphide bridges in the same positions as those of endothelin-1; of the 7 substitutions relative to endothelin-1, 4 are contained within the smaller intramolecular loop and 2 are in the 6 C-terminal residues. Functional homology between this toxin and endothelin-1 is suggested by the ability of sarafotoxin S6b to displace [^{125}I]-endothelin-1 from rat isolated cardiac membrane fragments (Gu *et al.*, 1989) and by the observations that sarafotoxins and endothelins also have common mechanisms of action on rat atria and brain (Ambar *et al.*, 1989).

We have previously shown that some alanyl-substituted analogues of endothelin-1 are active vasoconstrictors in the superior mesenteric arterial bed of the rat, even though each of these analogues is devoid of either one or both of the disulphide bridges possessed by the parent peptides (Randall *et al.*, 1989). Now we describe the role of the endothelium in controlling the constrictor effects of endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6b, human proendothelin$_{1-38}$ and vasoactive intestinal contractor (VIC) in the isolated, Krebs-Henseleit perfused mesenteric bed. This was assessed by using the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) to destroy the endothelium, or, in the case of endothelin-1, either methylene blue, to inhibit the stimulation of the soluble guanylate cyclase activated by endothelium-derived relaxing factor (EDRF), or N_ω-nitro-L-arginine methyl ester, (NAME), a putative inhibitor of EDRF synthesis (Moore *et al.*, 1990). Additionally, we studied the direct vasorelaxant properties of endothelin-2, endothelin-3 and sarafotoxin S6b in this preparation.

Methods

The isolated Krebs-Henseleit perfused superior mesenteric arterial bed of the rat

Adult male Wistar rats (250–400 g; Bantin & Kingman, Hull) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.: Sagatal; Rhône Poulenc, Dagenham) and heparinized (1000 u kg^{-1}, i.p.; Paines & Byrne, Greenford, Middlesex) with a single, combined intraperitoneal injection. The perfusion of the rat mesenteric arterial bed was carried out essentially according to the protocol of McGregor (1965). Briefly, a laparotomy was performed by a mid-line incision and the superior mesenteric artery identified and cleared of any adherent tissue. It was ligated, proximal to the abdominal aorta, cannulated with polythene PP50 tubing (Portex, Hythe, Kent) and the mesenteric arterial bed perfused, at 37°C and 2 ml min^{-1}, with oxygenated (95% O_2:5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and D-glucose 5.5. The Krebs-Henseleit solution contained 10 μM indomethacin; this concentration inhibits the production of eicosanoids in the rabbit thoracic aorta (Förstermann & Neufang, 1984) and has been used previously in the McGregor preparation, and a variety of other vascular tissues, for the same purpose (Warner *et al.*, 1989).

The perfused mesentery was carefully dissected away from the gastrointestinal tract, placed on a warmed Perspex block maintained at 37 ± 1°C and covered with Nescofilm (Nippon Shoji Kaishi Ltd, Osaka, Japan) to avoid the surface of the tissue being damaged by evaporation.

The perfusion pressure in the mesentery was recorded, from a T-piece inserted into the perfusion circuit close to the point at which the outflow cannula was inserted into the artery, using a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass model 7D polygraph. Zero pressure was calibrated to zero flow. Since the flow rate was kept constant, changes in perfusion pressure are directly proportional to changes in vascular resistance.

After a 30 min equilibration period, peptides were administered into the perfusion circuit in volumes of 100 μl or less. After the first dose, the subsequent administration of peptides took place 5 min after the tissue perfusion pressure had returned to the resting basal value following the previous dose of peptide.

When required, the endothelium was removed by perfusion of the vascular bed with a 0.3% (w/v) solution of the detergent CHAPS in distilled water for 120–150 s (Hiley *et al.*, 1987). Following this procedure, each tissue was allowed 45 min to re-equilibrate. In all preparations, before the construction of a dose-response curve for a peptide, the presence or absence of a functional endothelium was assessed by determining whether or not acetylcholine opposed noradrenaline-induced vasoconstriction (Randall *et al.*, 1989).

In addition to these experiments, the role of the endothelium in modulating the pressor actions of endothelin-1 was assessed by use of the guanylate cyclase inhibitor methylene blue (10 μM final concentration in the perfusate) and the putative inhibitor of nitric oxide synthesis N_ω-nitro-L-arginine methyl ester (100 μM final concentration).

The vasorelaxant properties of the endothelin/sarafotoxin peptides were assessed by recording reductions in the tone established by the infusion of 0.1 mM methoxamine. In order to avoid rapid loss of the methoxamine-induced tone it was found necessary to include bovine serum albumin in the perfusate at a concentration of 5 g l^{-1} (Byfield *et al.*, 1986). The flow rate had also to be increased to 4 ml min^{-1} in order to observe an appreciable increase in mesenteric tone in response to the infusion of methoxamine. In these experiments each peptide was administered in bolus doses of 30 μl or less.

Statistical and data analysis

All values are given as the mean ± s.e.mean. The values of n show the number of preparations in which an individual dose was examined but, since some doses of any given peptide were used on all the preparations, the greatest value of n shows the total number of preparations employed. Comparison between means was carried out by Student's unpaired t test.

Dose-response curves were analysed by fitting the logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the increase in perfusion pressure, A the dose of peptide, R_{max} the maximum pressor response, n_H the slope function and ED_{50} the dose of peptide giving the half-maximal response. A modified Marquardt procedure, as implemented in the Harwell routine VB01A on the Cambridge University IBM 3084Q mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

Drugs and peptides

All solutions, except those of the peptides, were made up on the day of the experiment. (−)-Noradrenaline bitartate (Koch-Light, Haverhill, Suffolk) was dissolved in 1 mg ml^{-1} ascorbic acid with saline as the diluent. Methoxamine hydrochloride, N_{ω}-nitro-L-arginine methyl ester and acetylcholine bromide (Sigma Chemical Company, Poole, Dorset) were dissolved in 0.9% NaCl (w/v). Indomethacin (Sigma) was dissolved to give a 1 mg ml^{-1} solution in 5% (w/v) sodium bicarbonate and then diluted to the required concentration in the Krebs-Henseleit solution. Fraction V bovine serum albumin and CHAPS were also obtained from Sigma and methylene blue was from Fisons, Loughborough.

Endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6b, human proendothelin$_{1-38}$ and mouse vasoactive intestinal contractor (VIC) were from the Peptide Institute, Osaka, Japan and were obtained through Scientific Marketing, London. Endothelin-1, endothelin-3 and sarafotoxin S6b were dissolved in distilled water to give 100 μM solutions. Endothelin-2, human proendothelin$_{1-38}$ and VIC were dissolved in distilled water containing 0.1% (v/v) acetic acid also to give a 100 μM solution. The 100 μM stock solutions were frozen in 100 μl aliquots at −20°C until the day of the experiment.

Results

Effect of NAME and methylene blue on the pressor activity of endothelin-1

In control preparations endothelin-1 (1 pmol–2 nmol) produced a dose-response curve (Figure 1) which was described by the following parameters obtained from the fitting procedure: R_{max} = 22.5 ± 2.6 mmHg, ED_{50} = 93.0 ± 40.0 pmol and n_H = 0.85 ± 0.12 (n = 4–20). Under similar conditions, the maximum pressor response that could be obtained to noradrenaline (30 nmol) was 42.8 ± 3.8 mmHg (n = 15). However, it may be seen from Figure 1 that in preparations which had been exposed to either 10 μM methylene blue or 100 μM NAME the R_{max} values determined for endothelin-1 were significantly enhanced to 36.0 ± 5.1 mmHg ($P < 0.01$; n = 4–17) and 42.6 ± 2.4 mmHg ($P < 0.001$; n = 3–7), respectively. Neither treatment produced significant changes in either n_H (1.63 ± 0.27, methylene blue; 1.58 ± 0.33, NAME) or ED_{50} values (81.5 ± 26.1 pmol, methylene blue; 57.5 ± 13.7 pmol, NAME) as compared to the control values. It is interesting to note that the R_{max} and ED_{50} values obtained for endothelin-1 following exposure of the preparations to either methylene blue or NAME were not only not significantly different from each other, but they were not different from those previously obtained for the responses to endothelin-1 after destruction of the endothelium with CHAPS (R_{max} = 50.3 ± 7.4 mmHg, ED_{50} = 133 ± 55 pmol and n_H = 0.84 ± 0.08; n = 6–13; Randall *et al.*, 1989).

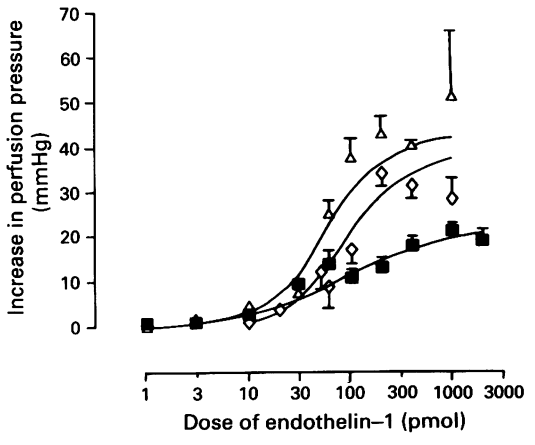

Figure 1 Pressor effects of endothelin-1 in control preparations (■; n = 4–20) of the rat isolated mesenteric arterial bed and in preparations perfused with either 100 μM N_{ω}-nitro-L-arginine methyl ester (△; n = 3–7) or with 10 μM methylene blue (◇; n = 4–17). Values are the mean, with the bars showing 1 s.e.mean, and the curves drawn are those obtained from the curve fitting procedure.

Pressor activities of other endothelin/sarafotoxin peptides in the presence of endothelium

Endothelin-2 (1 pmol–1 nmol), like endothelin-1, caused a dose-dependent increase in the perfusion pressure recorded in the isolated, Krebs-Henseleit perfused mesenteric arterial bed of the rat (Figure 2a). Its dose-response relationship had R_{max} = 11.4 ± 0.7 mmHg, ED_{50} = 90.8 ± 20.5 pmol and n_H = 2.02 ± 0.40 (n = 3–6). Thus, endothelin-2 was equipotent with endothelin-1 but its R_{max} was significantly ($P < 0.05$) less than that for the parent peptide. The increases in perfusion pressure observed following the administration of the peptide were similar in duration to those observed with endothelin-1 being slow to develop, reaching a maximum after around 2–3 min, and characteristically long lasting, taking on average in excess of 30 min to return to baseline.

Endothelin-3 (5 pmol–7 nmol) also caused dose-dependent increases in perfusion pressure (Figure 2b; Figure 3); the R_{max} was determined as being 7.7 ± 1.4 mmHg, the ED_{50} as 411 ± 195 pmol and n_H as 1.13 ± 0.24 (n = 4–5). Therefore, endothelin-3 was significantly less potent than either endothelin-1 ($P < 0.01$) or endothelin-2 ($P < 0.05$) and the maximum vasoconstriction induced by endothelin-3 was significantly smaller than that induced by either endothelin-1 or endothelin-2 ($P < 0.01$ and $P < 0.05$, respectively). A difference was also observed in the time course of the pressor responses to endothelin-3 (Figure 3) as compared to the other two endothelins in that they were shorter, typically lasting for around 10–15 min before they returned to the resting basal perfusion pressure (Figure 3a).

Sarofotoxin S6b (1 pmol–5 nmol) was also a pressor agent in the isolated mesentery (Figure 2c). Again the pressor responses were dose-dependent (Figure 4) and the fitted log dose-response curve had an R_{max} of 106 ± 11 mmHg, an ED_{50} of 345 ± 86 pmol and an n_H of 0.96 ± 0.05 (n = 3–5). Thus, compared to sarafotoxin S6b, endothelin-1, endothelin-2 and endothelin-3 are all partial agonists in this preparation (the significance of differences between the R_{max} for sarafotoxin S6b and those for endothelins 1–3 were $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). In addition, sarafotoxin S6b was equipotent with endothelin-3 but was significantly less potent than either endothelin-1 or endothelin-2 ($P < 0.01$ and $P < 0.01$, respectively). Also, as was observed with endothelin-3, the pressor responses obtained with sarafotoxin S6b were of a much shorter duration than was observed when either endothelin-1 or endothelin-2 were administered to the perfusate, characteristically lasting for only around 10–15 min (Figure 4a).

VIC, the last of the 21 amino acid peptides to be investigated, produced dose-dependent increases in perfusion pressure over the dose range 1–400 pmol, as shown in Figure 2d. The dose-response curve obtained had R_{max} = 12.5 ± 3.9 mmHg, ED_{50} = 106 ± 63 pmol and n_H = 1.35 ± 0.52 (n = 4–6). Hence VIC was equipotent with endothelin-1 and endothelin-2 but significantly more potent than sarafotoxin S6b ($P < 0.05$). Although the ED_{50} determined for VIC was much lower than that obtained with endothelin-3, the difference was not found to be significant. The R_{max} determined for VIC was very similar to that obtained for endothelin-2 but significantly less than that obtained with either sarafotoxin S6b ($P < 0.001$) or endothelin-1 ($P < 0.05$) and not significantly different from that for endothelin-3. The duration of the pressor responses obtained with this peptide was similar to those obtained with endothelin-2.

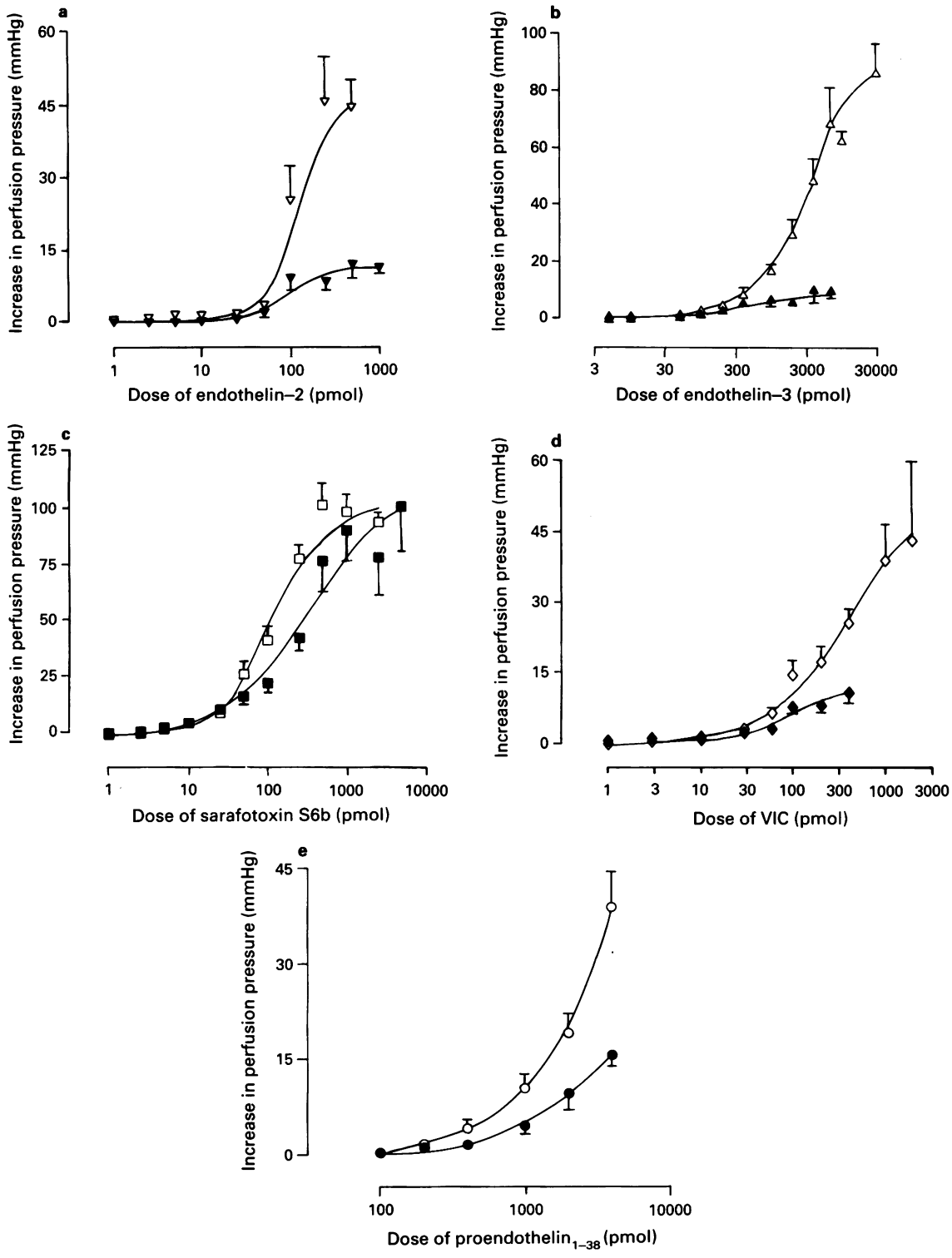

Figure 2 Pressor effects of endothelin-like peptides in control preparations of rat isolated mesenteric bed (solid symbols) and in preparations denuded of endothelium by perfusion with 0.3% CHAPS (open symbols). Data are shown as the mean with the vertical bars showing 1 s.e.mean and the curves drawn are those obtained from the curve-fitting procedure. Symbols and values of *n* are as follows: intact; (a) (▼) endothelin-2, 3–6; (b) (▲) endothelin-3, 4–5; (c) (■) sarafotoxin S6b, 3–5; (d) (◆) vasoactive intestinal contractor (VIC), 4–6; (e) (●) proendothelin, 5: endothelium-denuded preparations; (a) (▽) endothelin-2, 5–8; (b) (△) endothelin-3, 2–10; (c) (□) sarafotoxin S6b, 2–4; (d) (◇) VIC, 2–6; (e) (○) proendothelin, 5.

The final peptide studied, human proendothelin$_{1-38}$ (1 pmol–4 nmol) caused increases in mesenteric perfusion pressure in doses in excess of 400 pmol (Figure 2e). Unfortunately, the full dose-response curve to this peptide could not be determined due to its limited solubility. However, 4 nmol human proendothelin$_{1-38}$ caused a prolonged pressor response of 15.4 ± 1.6 mmHg ($n = 5$) which did not return to the basal perfusion pressure for approximately 30 min. Despite the inability to construct a full dose-response curve, it was clear that human proendothelin$_{1-38}$ was the least potent of the peptides examined with an ED_{50} in excess of 1 nmol.

Pressor activities of endothelin/sarafotoxin peptides following endothelial destruction with 0.3% CHAPS

With the exception of endothelin-1 (which has previously been investigated; Randall *et al.*, 1989), the peptides had their pressor activity determined in the isolated mesenteric bed fol-

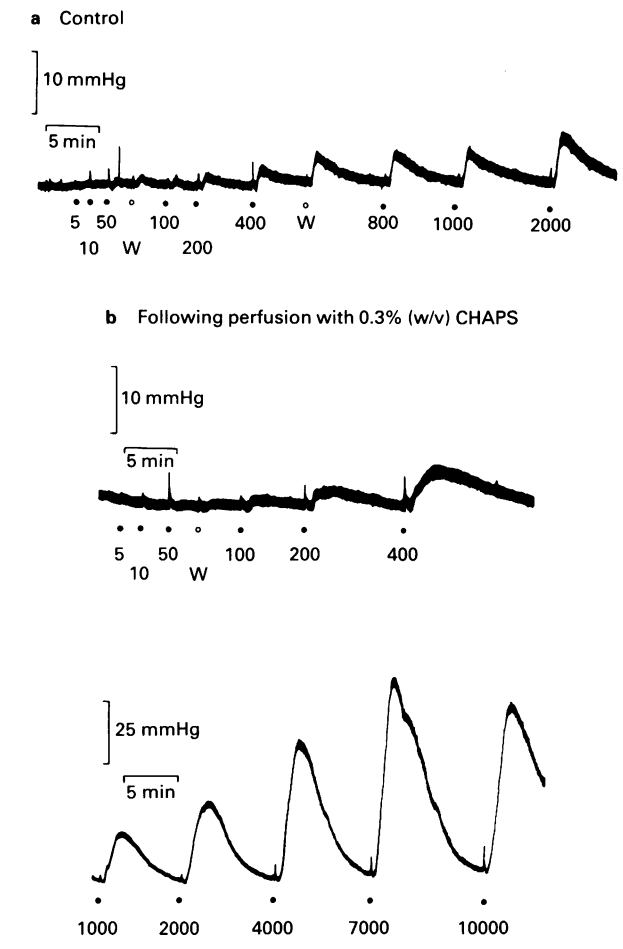

Figure 3 Experimental traces showing the effects on the pressor responses to endothelin-3 of removing the endothelium of the isolated perfused mesenteric bed of the rat with 0.3% CHAPS. Doses are given in pmol and W indicates washes of the peptide injection port.

lowing endothelial cell destruction by 0.3% CHAPS. The maximum pressor activity of endothelin-2 was enhanced (to 37.9 ± 6.9 mmHg; $P < 0.01$; $n = 5$–8), relative to that found in the presence of the endothelium, following CHAPS perfusion as shown in Figure 2a. The ED_{50} (118 ± 53 pmol) and n_H (2.31 ± 0.96) were not significantly different from those obtained in control preparations.

Similarly, Figure 3 shows that perfusion of the mesentery with CHAPS increased the responses to any given dose of endothelin-3. The log dose-response curve in Figure 2b shows that the R_{max} (106 ± 15 mmHg) obtained with endothelin-3 was significantly augmented ($P < 0.001$) but the n_H value of 0.89 ± 0.05 was unchanged. However, removal of the endothelium significantly ($P < 0.05$) reduced the potency of this peptide ($ED_{50} = 6.3 \pm 2.2$ nmol; $n = 2$–10). The maximum pressor response in the absence of endothelium was not significantly different from those for sarafotoxin S6b in the presence or absence of endothelium; thus, endothelin-3 appears to be a full agonist in the absence of the vascular endothelium.

Figure 2c shows that, for sarafotoxin S6b, CHAPS perfusion significantly changed neither R_{max} nor n_H relative to control (97.8 ± 3.8 mmHg and 1.50 ± 0.14 respectively; $n = 2$–4). However, in contrast to the findings obtained with endothelin-2 and endothelin-3, endothelial cell destruction significantly ($P < 0.01$) enhanced the potency of this peptide ($ED_{50} = 111 \pm 15$ pmol). Figure 4 shows the similarity in magnitude of responses to sarafotoxin S6b in the presence and absence of the endothelium and this contrasts with the responses for endothelin-3 which are illustrated in Figure 3.

As for endothelin-2 and endothelin-3, but in contrast to sarafotoxin S6b, the maximum pressor response to VIC was significantly enhanced (53.6 ± 12.7 mmHg; $n = 2$–6; $P < 0.01$) following perfusion of the mesentery with CHAPS (Figure 2d). However, the ED_{50} and n_H values were not altered following endothelial cell destruction (416 ± 196 pmol and 1.01 ± 0.10, respectively).

Even in preparations which had been perfused with CHAPS, a full dose-response curve for human proendothelin$_{1-38}$ could not be obtained (Figure 2e). However,

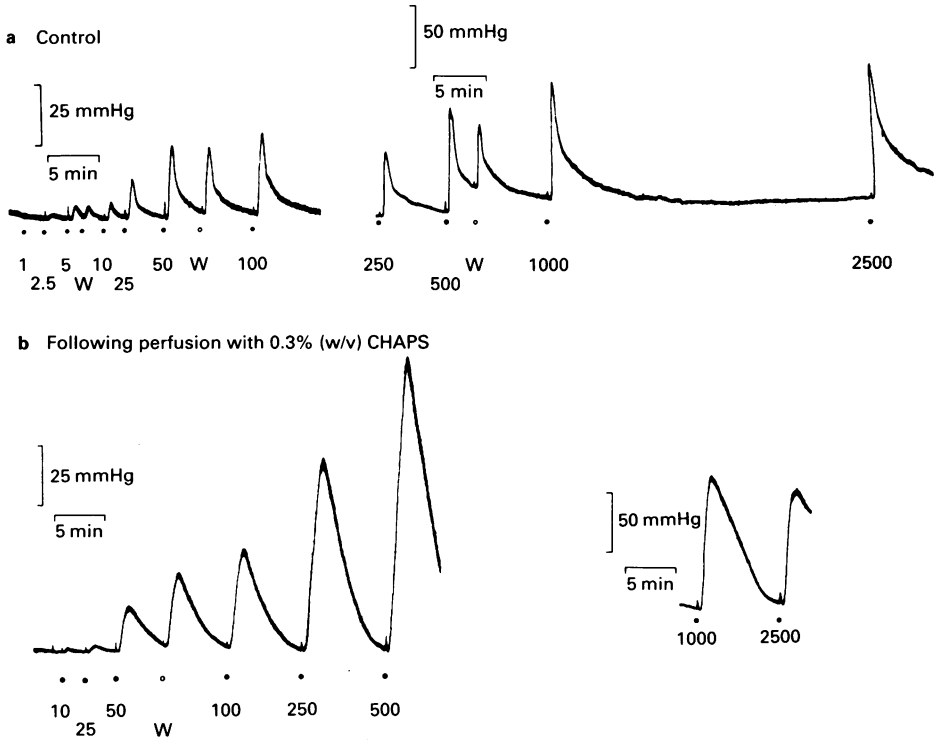

Figure 4 Experimental traces showing the effects of endothelial destruction with 0.3% CHAPS on the pressor responses to sarafotoxin S6b in the rat isolated mesenteric arterial bed. Doses are given in pmol and W indicates washes of the peptide delivery port.

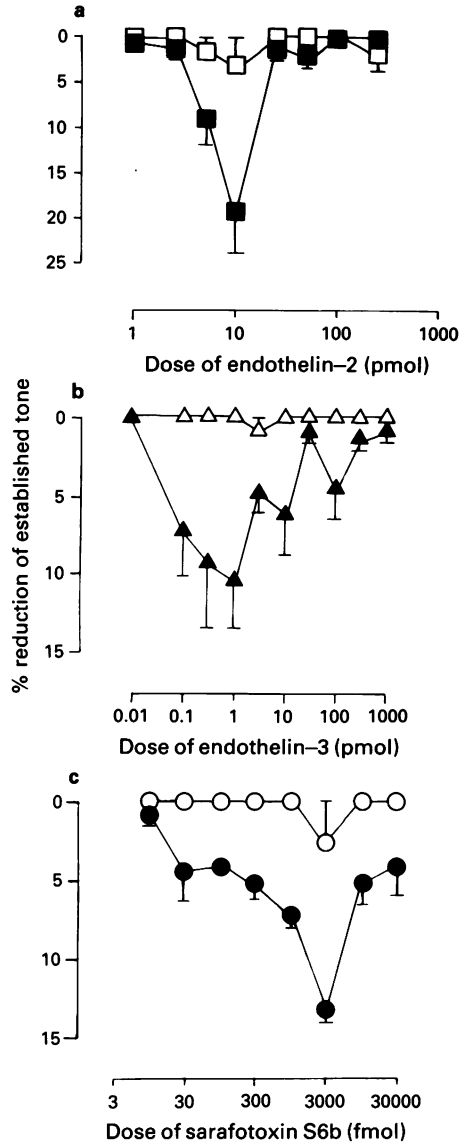

Figure 5 Dose-response relationships for the reduction by endothelin-like peptides of the tone established in the rat isolated perfused mesenteric bed by 100 μM methoxamine. The closed symbols show the responses (expressed as percentage reductions in the established tone and shown as the mean with the bars indicating 1 s.e.mean) in the presence of endothelium and the open symbols show the responses in preparations denuded of endothelium by perfusion with 0.3% CHAPS. Symbols and values of n are as follows: intact; (a) (■) endothelin-2, 5; (b) (▲) endothelin-3, 4–6; (c) (●) sarafotoxin S6b, 5: preparations without endothelium; (a) (□) endothelin-2, 4; (b) (△) endothelin-3, 4; (c) (○) sarafotoxin S6b, 3.

endothelial cell destruction clearly enhanced the pressor activity of this peptide and the responses to 400 pmol–4 nmol were significantly greater ($P < 0.05$) than those obtained with the same doses in preparations which possessed an intact endothelium.

Vasorelaxant properties of endothelin-like peptides

The 3 peptides studied, endothelin-2, endothelin-3 and sarafotoxin S6b, all caused transient decreases in the tone established in the isolated mesentery following the perfusion of the tissue with 100 μM methoxamine. These responses typically lasted for around 20–30 s. Endothelin-2, in doses between 2.5–50 pmol, caused significant reductions in the tone (which was 70.2 ± 6.8 mmHg; $n = 5$) established in the mesentery following the infusion of methoxamine into the perfusate (Figure 5a). In doses in excess of 50 pmol no relaxation could be observed. Doses of 10 pmol endothelin-2 or greater produced pressor responses superimposed on the methoxamine-induced tone. Table 1 and Figure 4a show that the maximum relaxation observed with endothelin-2 was obtained with 10 pmol.

Similarly, endothelin-3, between the doses of 100 fmol–10 nmol, transiently reduced the tone (54.3 ± 5.2 mmHg; $n = 6$) established in the mesentery by methoxamine (Figure 5b). The maximum relaxation observed occurred with 1 pmol endothelin-3 (Table 1). In doses in excess of 10 pmol, no relaxation occurred and only pressor responses could be obtained.

Figure 5c shows that sarafotoxin S6b, in doses from 30 fmol–10 pmol, also decreased transiently the tone established by methoxamine (45.4 ± 3.8 mmHg; $n = 5$). The maximum relaxant response was obtained with 3 pmol (Table 1). In doses of 1 pmol or greater, any relaxant response was rapidly followed by a more sustained pressor response. Both endothelin-3 and sarafotoxin S6b caused significant reductions in the tone established by methoxamine over much wider dose ranges than that for endothelin-2.

Effect of endothelial destruction on the vasorelaxant properties of endothelin-2, endothelin-3 and sarafotoxin S6b

Figure 5 shows that, following the perfusion of the mesentery with 0.3% CHAPS, neither endothelin-2 ($n = 4$), endothelin-3 ($n = 4$) nor sarafotoxin S6b ($n = 3$) were capable of opposing the increase in perfusion pressure (48.1 ± 4.5; $n = 11$) established by methoxamine.

Discussion

All the members of the endothelin/sarafotoxin family of peptides studied here in the isolated mesenteric bed of the rat have been shown to have pressor activity, including the putative precursor human proendothelin$_{1-38}$. This latter peptide was at least 30 times less potent than endothelin-1 when studied in intact tissues and at least 15 times so in tissues devoid of endothelium. These minimum estimates (the result of not being able to detect the top of the log dose-response curve for the precursor) are somewhat less than the potency ratio of approximately 100 fold found for human proendothelin$_{1-38}$ as a vasoconstrictor agent relative to endothelin-1 in rat isolated aorta (Kashiwabara *et al.*, 1989).

In comparing the maximum responses in the presence of the endothelium, it is noteworthy that the maximum response for endothelin-3 was the lowest whereas that for sarafotoxin S6b

Table 1 Relaxations to endothelin/sarafotoxin family peptides in rat isolated mesenteric bed precontracted with 100 μM methoxamine

Peptide	*Maximum relaxation* (% of preconstriction)	*Dose at which attained* (pmol)	n
Endothelin-1†	22.2 ± 3.4	50	7
Endothelin-2	19.4 ± 4.5	10	5
Endothelin-3	10.4 ± 3.0	1	6
Sarafotoxin S6b	13.2 ± 0.8	3	5

All values are given as the mean ± s.e.mean and n indicates the number of animals. The results were obtained in the presence of 10 μM indomethacin and a functional endothelium. The rate of perfusion was 4 ml min^{-1} and 0.5% (w/v) bovine serum albumin was present in the perfusate. † Data taken from Randall *et al.* (1989).

was not only the greatest but it was approximately twice that found for noradrenaline in identical conditions. As found previously for endothelin-1 (Randall *et al.*, 1989), removal of the endothelium with CHAPS affected the responses to all the peptides. The maximum responses were enhanced for endothelin-2, endothelin-3, proendothelin and VIC whereas that for sarafotoxin S6b was not significantly affected. It is interesting to note that, after endothelial destruction, the maximum response for endothelin-3 was not significantly different from that for sarafotoxin S6b. The ED_{50} values for the peptides were not affected by the CHAPS treatment, with the exceptions of sarafotoxin S6b and endothelin-3. For endothelin-3 the ED_{50} was significantly increased by approximately 15 fold. It is not clear what might be responsible for this change, but it might be related to the very low maximum response obtained in the presence of endothelium and the consequent difficulties in accurately defining the log dose-response curve. However, it is clear that the pressor responses to this peptide are virtually eliminated by the presence of the endothelium.

In the case of sarafotoxin S6b, the ED_{50} was reduced by 3 fold which shows that the vasoconstriction it causes is also subject to endothelial modulation. Since the maximum response obtained with this peptide was unaltered, it suggests that this is the greatest degree of constriction that can be obtained with a full agonist of the endothelin/sarafotoxin family. Since endothelin-3 was the only other peptide of the ones investigated that could cause this magnitude of pressor response, it appears that it and sarafotoxin S6b are the only full agonists amongst the natural peptides so far studied in this tissue. However, in our previous study of alanyl-substituted analogues of endothelin-1 (Randall *et al.*, 1989), we found that [$Ala^{3,11}$]endothelin-1 gave maximum responses that were not significantly different in the presence or absence of the endothelium, being 128 ± 10 and 110 ± 13 mmHg respectively; thus, despite its lacking one of the 2 disulphide bridges characteristic of the endothelin/sarafotoxin family, it too is a full agonist. The other alanyl-substituted derivative studied in the previous paper, [$Ala^{1,15}$]endothelin-1, also gave the same maximum responses as sarafotoxin S6b in the present study. Interestingly, destruction of the endothelium did not modify the pressor responses to either of these alanyl-substituted endothelins (Randall *et al.*, 1989).

The nature of the modulation of the pressor responses for these peptides was investigated with endothelin-1. This was done by examining the actions of methylene blue, an inhibitor of the stimulation of soluble guanylate cyclase by EDRF (Martin *et al.*, 1985), and N_{ω}-nitro-L-arginine methyl ester, an inhibitor of the biological production of nitric oxide (Collier & Vallance, 1989; Moore *et al.*, 1990) which accounts for the vasorelaxant action of EDRF. Both of these substances caused significant increases in the maximum pressor responses observed with endothelin-1 in the presence of endothelium. Further these maximum responses were neither significantly different from each other nor from that previously found by Randall *et al.* (1989) for endothelin-1 after the destruction of the endothelium. Thus, in the presence of indomethacin, the modulation of the pressor responses to the endothelin/sarafotoxin peptides by the endothelium in the rat mesenteric bed may be ascribed entirely to the actions of EDRF released either tonically or as a result of the actions of the peptides; the enhanced responses are clearly not the effect of removing a diffusional barrier. This latter possibility arose from the work of Pohl & Busse (1989) in which it was found that, in rabbit femoral artery and vein, endothelin-1 gave a pressor response when administered adventitially but not when given luminally; this difference was abolished by destruction of the endothelium.

Since the modulation of the pressor responses in the mesenteric bed was found to be due to the release of EDRF, it was decided to investigate the vasorelaxation responses which they should produce. Three of the peptides, endothelin-2, endothelin-3 and sarafotoxin S6b, were studied and all produced vasodepressor responses in the mesenteric arterial bed when it had been preconstricted with methoxamine. Since it was not possible to block selectively the pressor responses to the peptides, full dose-response relationships could not be determined for the vasorelaxant actions. Nevertheless, a striking feature of the results is that the vasorelaxation to endothelin-3 occurs at a much lower dose range (10 fmol–100 pmol) than the constrictor responses (30 pmol–10 nmol); these results therefore confirm those of Warner *et al.* (1989). Table 1 compares the maximum depressor responses and the doses at which they were obtained for the 3 peptides with those previously obtained for endothelin-1. It can be seen that the order of potency at producing the peak responses is endothelin-3 > sarafotoxin S6b > endothelin-2 > endothelin-1. This is in contrast to the order of potency of these same peptides as vasoconstrictors in the absence of endothelium (when, presumably, the only action of the peptides is that of vasoconstriction) which is sarafotoxin S6b $\geq$ endothelin-2 $\geq$ endothelin-1 $\gg$ endothelin-3. Coupled with the observations that neither of the dialanyl-substituted analogues previously studied showed endothelium-dependent modulation of their pressor responses, this suggests strongly that there are two different receptor types involved in activating the vascular smooth muscle to contract and the endothelium to release EDRF.

Maggi *et al.* (1989a,b) have also found evidence, from the use of agonists, that there are multiple endothelin receptors; the C-terminal hexapeptide, $endothelin_{16-21}$, can contract some smooth muscles, including the guinea-pig bronchus, rat vas deferens and rabbit pulmonary artery, but not others such as rat aorta, guinea-pig ileum and the human renal artery.

Further evidence for more than one type of endothelin receptor has been obtained by Watanabe *et al.* (1989) who found a single band on gel electrophoresis, of apparent molecular mass 53 kD, for [^{125}I]-endothelin-1 cross-linked with disuccidinyl tartrate to chick cardiac membranes whereas there were 3 bands for [^{125}I]-endothelin-3; two major bands of 34 and 46 kD and a minor one of 53 kD. They also determined the potency of each of endothelin-1, endothelin-2 and endothelin-3 at displacing the radioiodinated forms of the three peptides; endothelin-1 and endothelin-2 were generally equieffective in displacing the radioligands but endothelin-3 was more potent than either at displacing [^{125}I]-endothelin-3 and less potent than the other two at displacing [^{125}I]-endothelin-1 or [^{125}I]-endothelin-2. Although this difference in relative potency of endothelin-3 is similar to the potency sequences found in the present study; with all three radioligands sarafotoxin S6b was less potent than any of the other 3 peptides.

Kloog *et al.* (1989), in binding experiments with [^{125}I]-sarafotoxin S6b, found different orders of potency for a number of endothelin/sarafotoxin family members in displacing the radioligand in rat aorta, atria, cerebellum and caudate nucleus which suggested three receptor subtypes: one typical of the muscles and the others occurring in one or other of the two brain regions. However, in none of these tissues was endothelin-3 more potent than either sarafotoxin S6b or endothelin-1. It would therefore seem that none of the studies are in general agreement as to a possible receptor classification, though it is possible that one of the functional receptors occurring in the mesenteric vasculature might correspond with one or other of those found by Maggi *et al.* (1989a,b).

One further point that can be made about the possible receptor subtypes found in the rat mesenteric arterial bed is that endothelium-dependent modulation of their pressor responses was not observed for the two dialanyl substituted analogues of endothelin-1, [$Ala^{1,15}$]endothelin-1 and [$Ala^{3,11}$]endothelin-1, studied previously (Randall *et al.*, 1989). Thus, whilst both the disulphide bridges are not necessary for contractile action it might be concluded that the extra structural rigidity that the presence of both bonds confers is necessary for the activation of EDRF production.

The principal conclusions of the present investigation, taken

with our previous study of alanyl-substituted analogues of endothelin-1, are therefore three fold. Firstly, that there are possibly two distinct receptor subtypes for the endothelin/sarafotoxin family in the mesenteric arterial bed of the rat, one occurring on the endothelium (apparently with a structural requirement for both disulphide bridges) and the other on the vascular smooth muscle. Secondly, that vasoconstriction to all the putative native peptides with 2 disulphide bridges is modulated by the endothelium and, finally, that the modulation by the endothelium of the responses to endothelin-1, at least, is the result of the production of nitric oxide.

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Pharmacological evidence for the existence of a local renin-angiotensin system in porcine interlobar renal arteries

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1 Changes in tension in response to cumulative additons of angiotensins (i.e., angiotensinogen, angiotensin I and angiotensin II), bradykinin and acetylcholine were monitored isometrically on ring preparations from porcine interlobar renal arteries.

2 Angiotensins consistently elicited contractile responses, whereas both bradykinin and acetylcholine produced relaxation of the arterial rings when active tone was induced by prostaglandin $F_{2\alpha}$.

3 Contractile responses to angiotensin II could be completely blocked by the combined action of the cyclo-oxygenase inhibitor, indomethacin (1 μM) and the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA, 10 μM).

4 Relaxant responses to bradykinin were unchanged during blockade of thromboxane A_2 synthesis by dazoxiben (30 μM) and proved to be largely resistant to blockade by indomethacin (1 μM) and the prostaglandin I_2 (prostacyclin) synthesis inhibitor, tranylcypromine (40 μM).

5 The angiotensin receptor blocker, saralasin (10 and 100 nM) antagonized responses to angiotensinogen, angiotensin I and angiotensin II effectively and with similar potency. Enalaprilic acid, the active metabolite of the converting enzyme inhibitor enalapril (300 nM), attenuated responses to angiotensin I but failed to inhibit those to angiotensinogen up to 1 μM. The serine protease kallikrein (0.001 to 1 u ml^{-1}) produced a dose-dependent shift to the left of the concentration-response curve for angiotensinogen.

6 It is suggested that the porcine interlobar renal artery possesses a local renin-angiotensin system with activatable angiotensin II forming enzyme(s) within the vessel wall.

Introduction

The conventional concept of the renin-angiotensin system is a circulating endocrine system, important for the maintenance of blood pressure and sodium and potassium homeostasis, especially during conditions of cardiovascular decompensation. However, during the last few years evidence has been accumulating that in addition to the well established circulating system, local renin-angiotensin system(s) are also present in many tissue sites (Dzau, 1988a,b). The existence of vascular renin-angiotensin system(s), which may contribute to the regional tonic control of vascular resistance, has been postulated in rat peripheral limb vessels (Oliver & Sciacca, 1984; Juul *et al.*, 1987b), in human resistance vessels from skin and subcutaneous fat (Bund *et al.*, 1989), in kidney and renal arteries (Carretero & Beierwaltes, 1984) and in canine saphenous veins (Müller-Schweinitzer, 1989). The results of the present studies add support to the contention that the porcine interlobar renal artery also possesses a local renin-angiotensin system that enables it to synthesize angiotensin II from angiotensinogen.

Methods

Porcine kidneys were obtained from a local slaughter house within 30 min after death, placed in Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) and transported to the laboratory, where interlobar renal arteries (outer diameter $\approx$600–800 μm) were excised and cut into rings of about 2 mm. The rings were mounted between two hooks of stainless steel wire (diameter 0.15 mm) and suspended in 10 ml organ baths containing Krebs-Henseleit solution at 37°C, gassed continuously with 5% CO_2 in oxygen. The tension of the rings was recorded isometrically under a resting tension of about 10 mN with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The preparations were allowed to equilibrate for about 3 h in the bathing medium. During this time the preparations were challenged twice with noradrenaline (10 μM) and the baseline tension of the rings was readjusted to about 10 mN. Concentration-response curves for agonists were determined by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. Responses to angiotensins were quick and tended to decline rapidly (Figure 1). In order to avoid tachyphylaxis in response to repeated stimulation with high concentrations of angiotensins (Juul *et al.*, 1987a,b; Bund *et al.*, 1989), in the present studies each preparation was exposed only once to these agonists and the effects were expressed in mN. When relaxant responses to acetylcholine or bradykinin were investigated, active tone was induced by the addition of 3 μM prostaglandin $F_{2\alpha}$ (a concentration producing about 80% of maximum response). Antagonists were added 30 min before

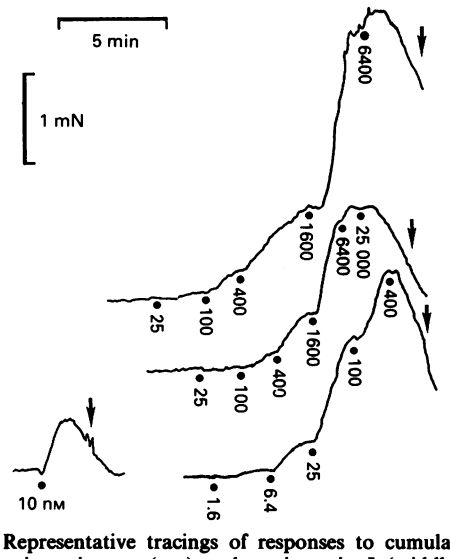

Figure 1 Representative tracings of responses to cumulative additions of angiotensinogen (top) and angiotensin I (middle), and to single and cumulative additions of angiotensin II (bottom). The numbers indicate final drug concentrations (nM), the arrows indicate washout of the drug from the bath solution.

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the agonist to the organ baths. When kallikrein was used, an incubation period of 10 min was applied.

Data analysis

Concentration-response curves were analysed with a linear computer programme and E_{max} (maximal effects) and pD_2 values (negative logarithms of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. For insurmountable antagonism apparent pD'_2 values were calculated according to the equation $pD'_2 = pD'_x + \log(x - 1)$, where x is the ratio of E_{max} of the agonist in the absence and presence of the antagonist and pD'_x is the negative logarithm of the molar concentration of the antagonist used (Van Rossum, 1963). Data are given as means ± s.e.mean, statistical analysis was performed by Student's unpaired *t* test.

Drugs

The following pharmacological agents were used: H-77 (H-His-Pro-Phe-His-Leu-D-Leu-Val-Tyr-OH, Bachem, Bubendorf, Switzerland), acetylcholine chloride (Fluka, Buchs, Switzerland), indomethacin (Merck, Darmstadt, F.R.G.), enalaprilic acid (Merck, Sharp & Dohme, Rahway, N.J., U.S.A.), dazoxiben hydrochloride (Pfizer, Central Research, Sandwich, U.K.), angiotensin II, angiotensin I, porcine angiotensinogen, saralasin ([Sar^1, Val^5, Ala^8]-angiotensin II acetate), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), nordihydroguaiaretic acid (NDGA), papaverine hydrochloride, tranylcypromine hydrochloride, kallikrein (Sigma, Munich, F.R.G.). H-77 was dissolved in dimethyl sulphoxide (DMSO, Merck-Schuchardt, Hohenbrunn, F.R.G.) and diluted in 5% glucose to give a 1 mM H-77 solution containing 2% (v/v) DMSO.

Results

Agonistic activities

Under control conditions angiotensinogen, angiotensin I and angiotensin II increased the tone of rings from porcine interlobar renal arteries in a concentration-dependent manner, eliciting maximal contractile effects of about 10 to 25% of that produced by $PGF_{2\alpha}$ or noradrenaline (Figure 2, upper traces). Both angiotensinogen (pD_2 value = 6.72 ± 0.05, $n = 45$) and angiotensin I (pD_2 value = 6.75 ± 0.09, $n = 25$) elicited vasoconstriction within the same concentration range, whereas angiotensin II (pD_2 value = 7.97 ± 0.09, $n = 30$) proved to be about 10 times more potent than the former. By contrast, both bradykinin and acetylcholine never elicited contractile effects but relaxed the arterial preparations when active tone was induced by 3 μM $PGF_{2\alpha}$, producing pD_2 values of 8.62 ± 0.12 ($n = 12$) and 7.60 ± 0.09 ($n = 6$) respectively (Figure 2, lower traces). In these experiments, only rings that relaxed in response to acetylcholine also responded with relaxation when exposed to bradykinin.

Antagonism of angiotensins

Preincubation of renal arteries with the cyclo-oxygenase inhibitor indomethacin (1 μM, $n = 6$) or with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10 μM, $n = 6$) reduced the maximal contractile responses to angiotensin II by 60 and 70% respectively, while the combined action of both drugs resulted in complete blockade of the angiotensin II effect ($n = 6$, Figure 3). Neither indomethacin, NDGA alone nor the combined action of both compounds elicited any noticeable change in baseline tension of the arterial preparations. The angiotensin II receptor blocker saralasin (10 and 100 nM), which induced no change in baseline tone by itself, attenuated contractile responses to angiotensinogen, angiotensin I and angiotensin II in a noncompetitive way and with similar potency, producing pD'_2 values of 7.58 ± 0.23, 8.10 ± 0.22 and 7.63 ± 0.27 (means ± s.e.mean, $n = 7$, Figure 4). Enalaprilic acid, the active metabolite of the converting

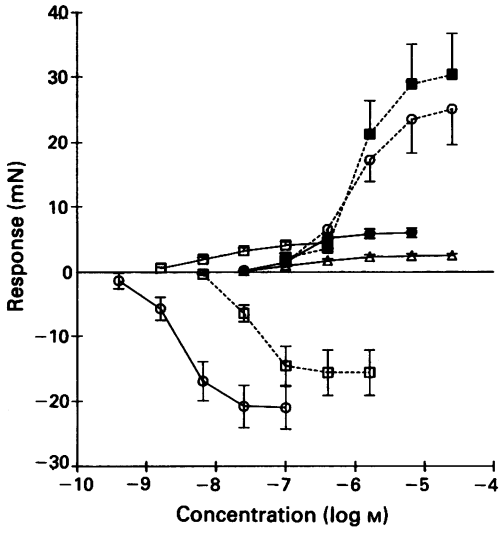

Figure 2 Cumulative concentration-response curves for various agonists on rings from porcine interlobar renal arteries. Upper traces: concentration-response curves for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, ■, $n = 5$), noradrenaline (○, $n = 10$), angiotensin II (□, $n = 30$), angiotensin I (△, $n = 25$) and porcine angiotensinogen (●, $n = 45$) on unstimulated preparations. Lower traces: concentration-response curves for bradykinin (○, $n = 12$) and acetylcholine (□, $n = 6$) on rings stimulated with 3 μM prostaglandin $F_{2\alpha}$. The points represent means and vertical lines show s.e.mean, the ordinate scale indicates changes in tension in mN.

enzyme inhibitor enalapril, attenuated the maximal responses to angiotensin I only when used at a concentration of 300 nM ($n = 6$, Figure 5) and failed to antagonize responses to angiotensinogen even when used at 1 μM ($n = 3$, Figure 5). Concentration-response curves to angiotensinogen were shifted to the left in a concentration-dependent way when kallikrein (0.001 to 1 u ml^{-1}) was present in the organ bath. Maximal enhancement of the activity of angiotensinogen was obtained with 1 u ml^{-1} kallikrein, which produced a 17 fold shift to the left of the concentration-response curve to angiotensinogen (Figure 6). During exposure to 1 u ml^{-1} kallikrein, the pD_2 value of angiotensinogen (7.83 ± 0.18, $n = 6$) was significantly higher ($P < 0.001$) than in the absence of the enzyme and not different from that of angiotensin II. The renin inhibitor H-77 (3 μM) failed to produce any inhibition of the angiotensinogen effect under control conditions ($n = 7$, Figure 7b), but tended to attenuate the maximal contractile response to angiotensinogen in the presence of 0.01 u ml^{-1} kallikrein, which increased the pD_2 value of angiotensinogen by a factor of 10 ($n = 7$, Figure 7a). Neither enalaprilic acid,

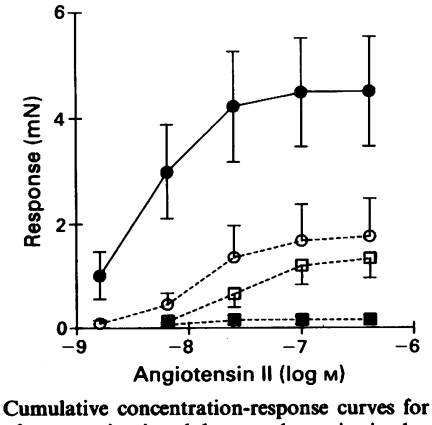

Figure 3 Cumulative concentration-response curves for angiotensin II on rings from porcine interlobar renal arteries in the absence (●) and presence of indomethacin (1 μM, ○), nordihydroguaiaretic acid (NDGA, 10 μM, □) and during the combined action of both antagonists (■). The ordinate scale indicates mN increase in tone. The points represent means and vertical lines show s.e.mean; for each point $n = 6$.

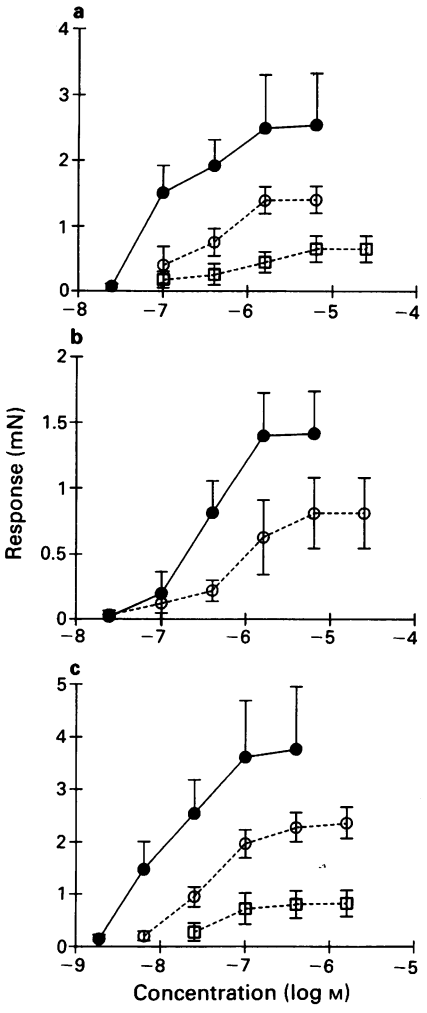

Figure 4 Cumulative concentration-response curves on rings from porcine interlobar renal arteries in the absence (●) and presence of saralasin (10 nM, ○ and 100 nM, □) for angiotensinogen (a), angiotensin I (b) and angiotensin II (c). The ordinates indicate mN increase in tone. The points represent means and vertical lines show s.e.mean; for each point $n = 3$–6.

kallikrein, nor H-77 induced any change in baseline tone of the arterial preparations.

Antagonism of bradykinin

Bradykinin proved to be a potent and efficacious relaxant agonist on rings from porcine interlobar renal arteries when active tone was induced by $PGF_{2\alpha}$ (3 μM, Figure 2, lower traces). The concentration-response curve to bradykinin was shifted to the left by a factor of 5 when renal arteries were preincubated with enalaprilic acid (10–100 nM, $n = 4$). Neither blockade of the prostaglandin synthesis by indomethacin (1 μM, $n = 5$), blockade of prostacyclin (prostaglandin I_2) synthesis by tranylcypromine (40 μM, $n = 5$) nor blockade of thromboxane A_2 synthesis by dazoxiben (30 μM, $n = 5$) modified the bradykinin-induced relaxation (not illustrated).

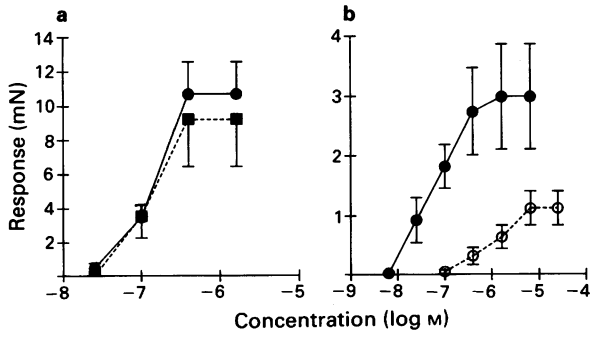

Figure 5 Cumulative concentration-response curves on rings from porcine interlobar renal arteries in the absence (●) and presence of enalaprilic acid (300 nM ○, and 1 μM ■) for angiotensinogen (a, for each point $n = 3$) and angiotensin I (b, for each point $n = 6$). The ordinates indicate mN increase in tone. The points represent means and vertical lines show s.e.mean.

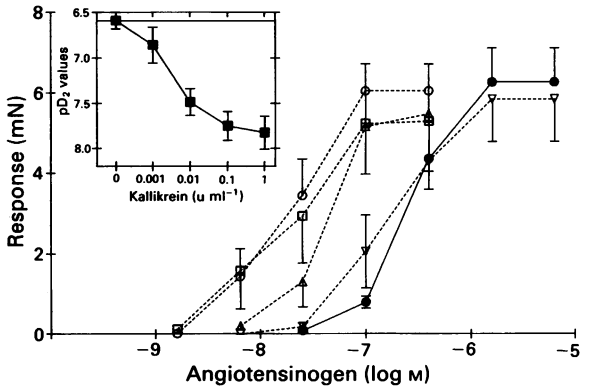

Figure 6 Cumulative concentration-response curve for angiotensinogen on rings from porcine interlobar renal arteries in the absence (●) and presence of kallikrein (0.001 u ml^{-1} ▽, 0.01 u ml^{-1} △, 0.1 u ml^{-1} ○, and 1 u ml^{-1} □). The ordinate scale indicates mN increase in tone. For each point $n = 6$. Inset: pD_2 values for angiotensinogen in the absence and presence of increasing concentrations of kallikrein. The points represent means and vertical lines show s.e.mean, for each point $n = 6$.

Discussion

Contractile responses of porcine interlobar renal arteries to angiotensin II could be completely blocked by the combined action of the cyclo-oxygenase inhibitor indomethacin (Vane, 1971) and the lipoxygenase inhibitor NDGA (Levine 1983; Gresele *et al.*, 1987; De Vries *et al.*, 1988). These observations suggested that the contractile response to angiotensin II was mediated through enhanced release of arachidonic acid and that end products of both the cyclo-oxygenase and lipoxygenase pathway contribute to the contractile responses of porcine interlobar renal arteries to angiotensin II. Hence, this artery shares the requirement of intact prostaglandin synthesis with renal arteries from dogs, which respond to stimulation by angiotensin II with relaxation that is also indomethacin-sensitive (Toda & Yamamoto, 1983; Toda, 1984; Toda *et al.*, 1987; Okamura *et al.*, 1990).

However, the main finding of the present experiments is the pharmacological evidence for the existence of a local renin-angiotensin system in porcine interlobar renal arteries. The inhibition of angiotensins (i.e., of angiotensinogen, angiotensin I and angiotensin II) by saralasin, was comparable to that

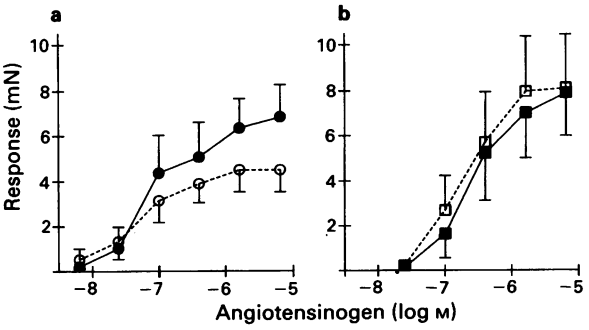

Figure 7 Cumulative concentration-response curves for angiotensinogen in the absence (closed symbols) and presence (open symbols) of H-77 (3 μM), performed without (b) and during incubation with kallikrein (0.01 u ml^{-1}, a). The ordinates indicate mN increase in tone. For each point $n = 7$.

described for rat (Juul *et al.*, 1987b) and human resistance vessels (Bund *et al.*, 1989) and for canine saphenous veins (Müller-Schweinitzer, 1989), suggesting (1) the existence of specific angiotensin receptors and (2) the presence of both renin and converting ezyme that may convert angiotensinogen as well as angiotensin I into its vasoactive form, angiotensin II. Furthermore, both a marked attenuation of the contractile activity of angiotensin I and a shift to the left of the bradykinin curve after blockade of converting enzyme by enalaprilic acid provided additional evidence for the presence of angiotensin converting enzyme in the renal artery. Kallikrein, a serine protease, known to convert inactive prorenin into active renin (Sealey *et al.*, 1980; Carretero & Beierwaltes, 1984), produced a marked and concentration-dependent shift to the left of the response curve to angiotensinogen, suggesting the existence of an inactive, but activatable form of renin in the vascular wall of porcine intrarenal arteries. This was supported by the observation that the renin inhibitor H-77 (Szelke *et al.*, 1982), tended to attenuate contractile responses to angiotensinogen in the presence of kallikrein, i.e., after activation of tissue renin, but not in the absence of the renin-activator. However, though blockade of angiotensin converting enzyme with enalaprilic acid effectively attenuated responses to angiotensin I, the same treatment produced no substantial inhibition of the angiotensinogen effects. This observation is consistent with findings on resistance vessels from rats (Juul *et al.*, 1987b) and man (Bund *et al.*, 1989), where no inhibition of tetradecapeptide renin substrate effects could be observed even after blockade of converting enzyme by 1 mM captopril (Bund *et al.*, 1989). It may be possible, therefore, that in addition to renin, other enzymes such as cathepsin G (Wintroub *et al.*, 1984; Dzau, 1987) or tonin (Boucher *et al.*, 1974), which may lead to direct formation of angiotensin II from angiotensinogen, are present in the vascular wall of porcine interlobar renal arteries.

Only arterial rings that relaxed in response to acetylcholine also responded to bradykinin with relaxation. The resistance to blockade of cyclo-oxygenase by indomethacin as well as to blockade of prostacyclin synthesis by tranylcypromine of this effect, suggested that the bradykinin-induced relaxation of porcine interlobar renal arteries, which are at least partially endothelium-dependent (Shimokawa & Vanhoutte, 1989), did not require intact prostaglandin synthesis. These findings are in agreement with data obtained on isolated renal arteries from dogs, which also respond with endothelium-dependent relaxation when exposed to either acetylcholine or bradykinin (Toda, 1977; Altura & Chand, 1981; Chand & Altura, 1981a,b; Cherry *et al.*, 1982; Toda *et al.*, 1987; Okamura *et al.*, 1990).

In conclusion, the present data suggest the existence of a local renin-angiotensin system with activatable angiotensin II forming enzyme(s) within the vessel wall of porcine interlobar renal arteries.

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Alterations in regional blood flow in rats following sensitization to the nematode *Nippostrongylus brasiliensis*: effects of PAF antagonists

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1 Changes in tissue and organ blood flow associated with sensitization of rats to the nematode parasite, *Nippostrongylus brasiliensis*, were studied 30 to 35 days after infection, a time when very few worms remain in the animal.

2 Neither active nor passive sensitization modified heart rate, mean arterial blood pressure, cardiac output or total peripheral resistance. Passive sensitization and administration of non-immune sera did not modify blood flow to any of the tissues studied.

3 Active sensitization increased hepatic arterial blood flow, but decreased blood flow to the stomach, duodenum, jejunum and the submandibular glands. These effects cannot be attributed to residual nematode infections as treatment with the anthelmintic, thiabendazole, did not alter blood flow relative to untreated, actively sensitized rats.

4 The effects of active sensitization on blood flow were probably due to an action of platelet-activating factor (PAF) since treatment of actively sensitized animals with the selective antagonists, WEB-2086 and BN 52021, reversed the decrease in flow seen to the intestinal regions. The PAF antagonists increased blood flow to the kidneys and the trachea of sensitized animals.

5 These results suggest that the PAF released from undetermined sources in nematode-sensitized rats, produces altered blood flow, primarily to the stomach and proximal small bowel.

Introduction

Helminths are common infectious agents in man and other animals. Even though many of the parasites do not establish long-term residency in the host, sensitization to the parasites frequently develops and this is often expressed by type I hypersensitivity reactions (Keller, 1970; Jarrett & Miller, 1982). Animal models of these infections, such as *Nippostrongylus brasiliensis* in the rat, have been useful in understanding IgE synthesis and function, as well as mast cell biology (Miller *et al.*, 1983; Heavey *et al.*, 1988). Although immune responses are frequently studied in the infected animals (Egwang *et al.*, 1984; McElroy & Befus, 1987; Ramaswamy & Befus, 1989), few attempts have been made to investigate pathophysiological alterations occurring in other organs consequent to sensitization, perhaps with the exception of some parameters in the intestine (King & Miller, 1984; Ramage *et al.*, 1988). In this study we examined systemic and microcirculatory changes that occur 30 to 35 days following active sensitization of rats with the nematode, *N. brasiliensis*. Although the rats are sensitized by the infection at this time, the majority of worms have been expelled within 3 weeks after infection. To ensure that the circulatory changes associated with infection could not be attributed to residual worms in the intestine, some rats were treated with an anthelmintic prior to investigations of blood flow.

Methods

Animals and sensitization

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Canada) of an initial weight of 200–250 g were maintained in filter top cages (2 to 3 per cage) to minimize the possibility of other infections. The rats were divided into three groups: untreated controls, infected (active sensitization) and passively sensitized.

For active sensitization, animals were infected in the scruff of the neck with 3000 third stage larvae of *N. brasiliensis*. The larvae were collected from faecal cultures and extensively washed prior to injection. The blood flow studies were performed 30 to 35 days following initial infection.

Passive sensitization was induced by intraperitoneal injection of 3 ml of 1:128 titre IgE-containing sera obtained from *N. brasiliensis*-infected rats. The IgE titres of the sensitizing sera were determined by passive cutaneous anaphylaxis (Befus *et al.*, 1982). Regional blood flows were also determined in a group of animals that were injected with 3 ml of non-immune sera obtained from normal (non-infected) rats. Forty-eight hours after intraperitoneal injection of the sera (immune or non-immune) regional blood flows were studied. To evaluate the effectiveness of passive sensitization and non-immune sera in sensitizing the animals, blood flows were also determined 5 min after an i.v. injection of 100 μl of antigen containing 150 worm equivalents of homogenized *N. brasiliensis*. This dose of antigen induces anaphylaxis in actively sensitized rats (Mathison *et al.*, 1990).

Deworming procedures

Even though previous observations (Woodbury *et al.*, 1984) have shown that most of the worms are expelled from the animal by 2 weeks after infection we treated 5 rats with the anthelmintic agent thiabendazole (Thizbenzole; Merck, Sharp & Dohme Ltd) to control for possible effects of residual parasitic infections on regional blood flows. The drug was given at 80 mg kg^{-1} in aqueous suspension by stomach tube (Jarrett & Stewart, 1973) and 7 days later blood flows were determined. In separate groups of rats (untreated and thiabendazole-treated) the number of worms present in the small bowel was determined.

Treatment with platelet-activating factor (PAF) antagonists

Actively sensitized rats were treated i.v. with PAF antagonists. Either 3 mg kg^{-1} of the antagonist, WEB-2086 (3-(4-(2-chlo-

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rophenyl)-9-methyl-6H-thieno(3,2-f)(1,2,4)-thiazolo-(4,3,α)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone; Boehringer-Ingelheim, Germany) or 20 mg kg^{-1} of BN 52021 (9H-1,7α-(epoxymethano)-1H,6αH- cyclopenta (c) furo-{2,3-β} furo-{3',2':3,4} cyclopenta {1,2-d} furan-5,9,12-{4H}-trione, 3-tert-butyl hexahydro-8-methyl; Institut Henri Beaufour, Paris, France) were administered 10 min before determining blood flows. Studies in our laboratory (Wallace *et al.*, 1990) have shown that these concentrations of WEB-2086 and BN 52021 inhibit haemoconcentration induced by PAF (10 min infusion of 100 ng kg^{-1} min^{-1}).

Microsphere experiments

Anaesthesia was induced prior to surgery with pentobarbitone sodium (30 mg kg^{-1} i.p.). To assess haemodynamic values and collect the reference blood sample the right femoral artery was cannulated with PE50 tubing. The left ventricle was cannulated through the right carotid artery with PE50 tubing. Verification of the ventricular cannula placement was obtained by monitoring the blood pressure until a characteristic left ventricular pressure tracing appeared and by post mortem examination. Mean arterial blood pressure (MABP) and mean left ventricular pressure were recorded with a Statham P23Db pressure transducer connected to a Beckman Dynograph and heart rate was derived from the pressure trace.

Regional blood flow and cardiac output were determined with radioactive microspheres (^{113}Sn or ^{95}Nb, 15 ± 3 μm diameter; New England Nuclear). The microspheres were suspended in 0.9% NaCl containing 0.01% Tween-80. The microspheres (approximately 80 000 to 100 000) were injected in a total volume of 0.5 ml followed by 0.8 ml saline to flush them into the left ventricle. Starting 15 s before the microsphere injection, the reference blood sample was drawn into a motor-driven syringe at a rate of 0.68 ml min^{-1} for 75 s.

Upon completion of the experiment the animals were killed with an overdose of urethane. Individual tissues were removed, weighed and counted on a dual channel LKB gamma counter. Data reductions were performed with the programme described by Flaim *et al.* (1984), adapted and modified to run on a VAX computer.

Several requirements, as defined by Hadengue *et al.* (1989), were met before using the data from an individual animal: firstly, a difference of less than 10% between left and right kidneys which is indicative of adequate microsphere mixing; secondly, stability in MABP and heart rate between the pre- and post-injection periods.

Blood flow was expressed as net flow g^{-1} of wet tissue. Cardiac output (ml min^{-1}) was calculated as radioactivity injected (c.p.m.)/reference sample radioactivity (c.p.m.) × 0.68 (ml min^{-1}). Total peripheral resistance (TPR) was calculated according to the formula (Hadengue *et al.*, 1989) where:

$$\text{TPR (dyn.s.cm}^{-5}) = \frac{\text{MABP (mmHg)} \times 80}{\text{cardiac output (ml min}^{-1})}.$$

Statistical analysis

Results are expressed as mean ± s.e.mean. In order to compare the variability between the groups of animals one-way analysis of variance was applied and the Student-Neuman-Keul procedure (Snedecor & Cochran, 1976) was used to determine significance at $P < 0.05$. Statistical analyses were performed with the package (SPSS-PC$^+$ SPSS Inc., Chicago, IL, U.S.A.).

Results

Macrovascular parameters including heart rate, MABP, cardiac output and TPR were not significantly different for the 7 groups of animals: unsensitized control rats ($n = 8$), unsensitized rats given non-immune serum ($n = 4$), passively sensitized rats ($n = 3$), actively sensitized rats ($n = 11$), and actively sensitized rats treated with thiabendazole ($n = 5$) or WEB-2086 ($n = 6$) or BN 52021 ($n = 6$). In unsensitized animals these parameters were: heart rate = 367 ± 9 beats min^{-1}; MABP = 111 ± 6 mmHg; cardiac output 121 ± 17 ml min^{-1}; TPR = 73.6 ± 16.0 dyn.s.cm^{-5}.

Passive sensitization or administration of non-immune sera, when compared to unsensitized rats, did not modify blood flow to the kidneys, liver, trachea, salivary glands and all regions of the small intestine (Table 1). Passive sensitization was effective in sensitizing rats to antigen as decreases in blood flow to the gastrointestinal tract were observed 5 min after administration of allergen to passively sensitized rats (Table 2). In contrast, administration of antigen to rats treated with non-immune sera did not alter blood flow to any of the tissues. The depressive effects of anaphylaxis on blood flow to various tissues in actively sensitized rats have been described previously (Mathison *et al.*, 1990).

Active sensitization decreased blood flows to the stomach and jejunum, but not the duodenum and ileum (Figure 1). Blood flow to the submandibular gland was also reduced in actively sensitized animals (Figure 2). Hepatic arterial blood flow was enhanced in the actively sensitized animals (Figure 3), whereas blood flows to all other tissues examined were not altered by the sensitization.

Blood flows in sensitized rats that had been treated 1 week previously with the deworming agent thiabendazole were similar to those observed in sensitized rats. Relative to the unsensitized controls, in anthelmintic treated and in untreated actively sensitized rats, blood flows were reduced in the stomach, jejunum (Figure 1), and submandibular gland (Figure 3), whereas hepatic arterial blood flow was enhanced (Figure 2). In 3 untreated rats a total of 4 nematodes were

Table 1 Blood flow in unsensitized, passively sensitized and non-immune serum treated rats

Tissue	*Unsensitized*	*Passive*	*Non-Immune serum*
Stomach	0.93 ± 0.16	0.77 ± 0.17	0.79 ± 0.24
Duodenum	2.04 ± 0.38	2.17 ± 0.33	1.96 ± 0.37
Jejunum	1.94 ± 0.33	1.62 ± 0.03	1.80 ± 0.17
Ileum	1.73 ± 0.42	1.74 ± 0.22	1.83 ± 0.41
Kidney	3.56 ± 0.61	3.95 ± 0.21	3.97 ± 0.25
Trachea	1.94 ± 0.47	2.43 ± 0.44	2.18 ± 0.38
Liver	0.09 ± 0.02	0.13 ± 0.03	0.14 ± 0.04
Submandibular	0.77 ± 0.12	0.62 ± 0.10	0.51 ± 0.10

Number of experiments: unsensitized ($n = 8$), passively sensitized ($n = 3$) and non-immune sera ($n = 4$).

Table 2 Effects of antigen challenge on blood flow to several tissues in unsensitized, passively sensitized rats and non-immune sera treated rats

Tissue	*Unsensitized*	*Passive*	*Non-immune sera*
Stomach	0.78 ± 0.11	0.42 ± 0.06*	0.77 ± 0.16
Duodenum	2.26 ± 0.38	1.02 ± 0.21*	1.96 ± 0.12
Jejunum	1.98 ± 0.35	0.58 ± 0.21*	1.80 ± 0.37
Ileum	2.36 ± 0.47	0.92 ± 0.36*	2.12 ± 0.33
Trachea	1.08 ± 0.28	1.29 ± 0.20	1.09 ± 0.29
Liver	0.13 ± 0.04	0.24 ± 0.03	0.15 ± 0.04
Submandibular	0.64 ± 0.12	0.69 ± 0.15	0.56 ± 0.09

* Significance ($P < 0.05$); passive < unsensitized and non-immune sera treated rats.
Number of experiments: unsensitized ($n = 8$), passively sensitized ($n = 3$) and non-immune sera ($n = 4$).

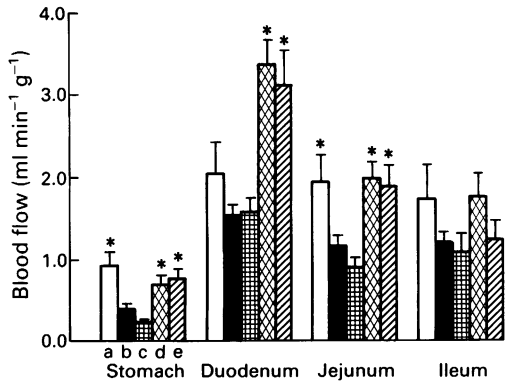

Figure 1 Blood flow to the stomach, duodenum, jejunum and ileum of unsensitized (□, a; $n = 8$), untreated actively sensitized (■, b; $n = 11$), and actively sensitized rats treated with thiabendazole (▦, c; $n = 5$), WEB-2086 (▩, d; $n = 6$) or BN 52021 (▤, e; $n = 6$). Values are presented as means with the bars showing s.e.mean. Significance ($P < 0.05$): * = unsensitized, WEB-2086 and BN 20521 > sensitized plus thiabendazole.

isolated from the small bowel, whereas none was found in 3 rats treated with the deworming agent.

Treatment with the PAF-antagonists, WEB-2086 and BN 52021, which on their own did not alter heart rate or MABP,

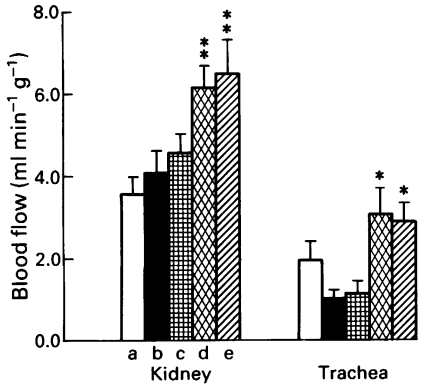

Figure 2 Blood flow to the kidney and trachea of unsensitized (□, a; $n = 8$), untreated actively sensitized (■, b; $n = 11$), and actively sensitized rats treated with either thiabendazole (▦, c; $n = 5$), WEB-2086 (▩, d; $n = 6$) or BN 52021 (▨, e; $n = 6$). Values are presented as means with the bars showing s.e.mean. Significance ($P < 0.05$): * = WEB-2086 and BN 52021 > unsensitized; ** = WEB-2086 and BN 52021 > sensitized and thiabendazole.

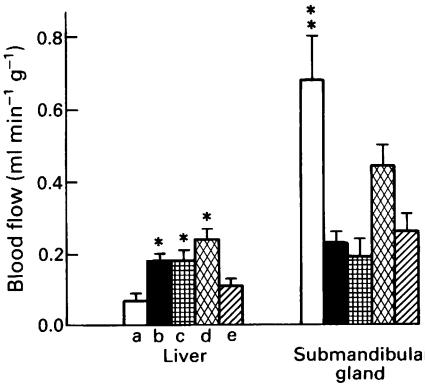

Figure 3 Blood flow to the liver and submandibular glands of unsensitized (□, a; $n = 8$), untreated actively sensitized (■, b; $n = 11$), and actively sensitized rats treated with either thiabendazole (▦, c; $n = 5$), WEB-2086 (▩, d; $n = 6$) or BN 52021 (▨, e; $n = 6$). Values are presented as means with the bars showing s.e.mean. Significance ($P < 0.05$): * = sensitized, thiabendazole and WEB-2086 > unsensitized and BN 52021; ** = unsensitized > sensitized, thiabendazole, WEB-2086 and BN 52021.

reversed the reduced blood flows noted with sensitization in several tissues, especially to the stomach, duodenum and jejunum (Figure 1) where flows were increased to levels comparable to those seen in unsensitized animals, or even enhanced as in the duodenum. Hepatic arterial flow remained high to the liver in the WEB-2086-treated groups, whereas BN 52021 tended to reduce blood flows to rates seen in the unsensitized rat (Figure 2). Neither antagonist reversed the reduction in blood flow to the submandibular gland (Figure 3) that was seen in the other sensitized groups of animals, but treatment with either of them resulted in enhanced blood flow to the kidneys (Figure 2).

Discussion

Acute parasitic infection increases blood flow to the intestine in the mouse infected with *Trichinella spiralis* (Ottaway *et al.*, 1980) and, in dogs infected with heart worm (*Dirofilaria immitis*) vascular reactivity to acetylcholine is altered (Kaiser *et al.*, 1987). The dirofilariasis infection involves two endothelium-derived relaxing factors, one a prostaglandin and the other a non-prostaglandin metabolite (Kaiser *et al.*, 1989). The hypothesis was put forward in the latter study that adult *D. immitis* release a factor that alters endothelial function.

The present study, however, describes changes in blood flow 30–35 days after active sensitization, when the parasites have been expelled from the intestine (Woodbury *et al.*, 1984). In fact, the changes in blood flow due to active sensitization cannot be due to residual infection as blood flows were similar in dewormed and untreated groups of animals. An intestinal mucosal mast cell hyperplasia is markedly developed and maintained for months after expulsion of the parasite *N. brasiliensis* from rats (Miller & Jarrett, 1971; Befus *et al.*, 1979). The alterations in blood flow seen with active sensitization, nevertheless, are not a generalized phenomena as many tissues and organs are not affected. Furthermore, the changes in blood flow are not transmitted passively to the rats and thus probably do not solely involve an IgE-mediated mechanism.

We found that the PAF antagonists, WEB-2086 and BN 52021, reversed the reduced blood flows to primarily gastrointestinal tissues in sensitized animals. Thus PAF appears to be a major contributor to sensitization-induced changes in vascular reactivity, although the role of arachidonic acid metabolites remains to be evaluated. Some of the other putative mediators of the vascular changes could be histamine (Church, 1975) and leukotrienes (Heavey *et al.*, 1988) and they may be involved in sensitization-induced alterations in blood flow to the submandibular gland.

Other studies have described effects of PAF on mesenteric and intestinal blood flow. A low dose of PAF (0.3 nmol kg^{-1}) increases mesenteric blood flow, whereas higher doses (1.0 and 3.0 nmol kg^{-1}) decrease blood flow to the mesentery of the rat (Siren & Feuerstein, 1989), the former effect being associated with the vasodilator effect of PAF on mesenteric blood vessels (Lagente *et al.*, 1989). Blood flow to the gastric mucosa is also reduced by PAF (Whittle *et al.*, 1986). In contrast, PAF has vasoconstrictor actions on hamster cheek-pouch blood vessels that are antagonized by dexamethasone, indomethacin and kadsurenone, a thromboxane A_2 and PAF-antagonist (Dillon *et al.*, 1988). PAF primarily causes stasis of flow, an action that might be related to the ability of PAF to stimulate platelet aggregation (Martins *et al.*, 1988). One interesting observation in this study was the enhanced blood flows to the duodenum and kidneys following treatment with PAF-antagonists. This enhanced blood flow to the kidney may be related to the ability of the rat kidney to synthesize and release PAF (Pirotsky *et al.*, 1984).

In conclusion, we have shown that sensitization of rats with *N. brasiliensis* results in reduced blood flow to several tissues, primarily those of the gastrointestinal tract 30 to 35 days post-infection, a time when the parasites have been largely eliminated from the animal. These effects are not caused by

residual infection with the nematode and appear to involve increased actions of PAF in the infected animals.

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Dihydropyridines alter adenosine sensitivity in the rat hippocampal slice

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1 The effects of adenosine and a range of adenosine analogues, which are resistant to uptake processes, were studied in the presence of dihydropyridines and verapamil on the population spike potential recorded from the CA1 area of the hippocampal slice.

2 Nifedipine and Bay K 8644, a calcium channel antagonist and activator respectively, enhanced the inhibitory action of adenosine in a concentration-dependent manner. This was in contrast to their effect on adenosine analogues where the inhibition of the population potential was significantly attenuated. Similiar interactions between the adenosine compounds and the dihydropyridines were also displayed in studies on spontaneous epileptiform activity in the CA3 region.

3 This effect of nifedipine and Bay K 8644 was not shown by the dihydropyridines, nimodipine or nitrendipine, or by the phenylalkylamine, verapamil.

4 Addition of the adenosine uptake blocker dipyridamole reversed the action of nifedipine on adenosine, so that inhibition by adenosine was now attenuated by nifedipine in a similar manner to that observed with the adenosine analogues.

5 These results can be explained with reference to binding studies that show displacement of adenosine analogues from the adenosine receptor by dihydropyridines. An action at the adenosine uptake site by the dihydropyridines explains the enhancement of adenosine inhibition.

6 The possible sites for this interaction are discussed.

Introduction

Binding sites exist in the central nervous system for several members of the dihydropyridine series of calcium channel activators, such as Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), and blockers such as nifedipine, nimodipine and nitrendipine (Gould *et al.*, 1982; Bellemann *et al.*, 1983). This binding exhibits well defined regional distributions within the rat brain (Skatteböl & Triggle, 1987), the highest density of receptors being found in the hippocampus and olfactory bulb. However, a functional effect of dihydropyridines on calcium channels in neuronal tissue has so far proved difficult to demonstrate. Middlemiss & Spedding (1985), when studying potassium-stimulated, calcium-dependent [^{3}H]-5-hydroxytryptamine release from rat cortical slices, showed that Bay K 8644 could augment 5-hydroxytryptamine release. Nifedipine reversed the augmentation but had no effect on potassium-stimulated release. A partial inhibition of potassium-stimulated ^{45}Ca uptake into cultured cerebellar granule cells by dihydropyridines has been demonstrated by Carboni *et al.* (1985), while Thayer *et al.* (1986) were able to show that nitrendipine could reduce, but not abolish, calcium influx into cultured neuronal cells from various regions of the brain, determined by use of fura 2. In electrophysiological studies, Docherty & Brown (1986), using voltage clamp techniques on hippocampal pyramidal cells in slices, have shown a partial inhibition of a persistent L-type calcium channel by nimodipine. However, the dihydropyridines appear to have no effect on synaptic transmission. Studies of ^{45}Ca uptake into rat brain synaptosomes have failed to show any significant action of the dihydropyridines on calcium uptake (Daniell *et al.*, 1983; Rampe *et al.*, 1984) and there are few accounts of dihydropyridine action on electrophysiological studies of synaptic transmission in the CNS.

Adenosine, on the other hand, is an endogenous neuromodulator that is capable of inhibiting synaptic transmission, particularly in the hippocampus (Dunwiddie & Hoffer, 1980). This effect appears to be mediated presynaptically and is probably due to a reduction of calcium influx into presynaptic terminals causing a reduced release of neurotransmitter (Stone, 1981; Schubert & Kreutzberg, 1987; Bartrup *et al.*, 1988).

Ligand binding studies have demonstrated that some dihydropyridines, most notably nifedipine and Bay K 8644, are able to reduce adenosine analogue binding to brain membranes, while having no effect on other receptors such as α- and β-adrenoceptors, muscarinic cholinoreceptors, opiate or γ-aminobutyric acid (GABA) receptors (Morgan *et al.*, 1987). These findings raise the possibility of a close functional relationship between adenosine receptors and dihydropyridine binding sites.

In contrast, the inhibitory action of adenosine on acetylcholine excited cortical neurones is enhanced by the addition of nifedipine (Phillis *et al.*, 1984). Binding studies have also revealed that the dihydropyridines can interact with the adenosine uptake system, indicating that the dihydropyridines may have a marked effect on adenosine metabolism which could explain the potentiation of adenosine.

So far, the existence of these interactions between the dihydropyridines and adenosine compounds, suggested by binding and uptake studies, has not been studied in detail in a functional model. As the hippocampus is rich in both dihydropyridine (Skatteböl & Triggle, 1987) and adenosine (Fastbom *et al.*, 1987) binding sites, the hippocampal slice was used in the present work to study the effect of adenosine and adenosine analogues on CA1-evoked field potentials, and the effect of the dihydropyridines on this action.

Methods

Male Wistar rats (150–250 g) were killed by stunning and cervical dislocation and the hippocampus was dissected out into ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (mM): KH_2PO_4 2.2, $MgSO_4$ 1.2, KCl 2, glucose 10, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, gassed with 95% O_2/5% CO_2. Five hundred μm transverse slices were prepared and incubated at room temperature for 1 h before trans-

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fer to the recording chamber. Individual slices were then transferred to a recording chamber where they were submerged in the ACSF and superfused at a rate of 4 ml min^{-1} at 30°C. A concentric bipolar stimulating electrode was placed in the stratum radiatum in the Schaffer collateral-commissural pathway. Evoked field potentials were recorded from the CA1 pyramidal cell body layer. Stimulation was at 250–350 μA, 0.1 ms, 0.1 Hz. The resulting field potential was recorded via a Grass amplifier on a digital storage oscilloscope, from which the population potential was plotted on a Grass chart recorder. The size of the negative going population potential was measured from the chart records. The effects of the drugs on the potential were determined as a percentage change with respect to potential size immediately before drug application.

All experiments were carried out under sodium lamp lighting to protect the light-sensitive dihydropyridines. The dihydropyridines were dissolved in ethanol as a 10 mM stock solution. The maximum final ethanol concentration added to the slice was never more than 0.1% and control ethanol concentrations of up to 1% had no effect on the population potential. Control responses to adenosine compounds were performed in triplicate before the addition of the dihydropyridines, which were then perfused for 10 min before the adenosine compounds were again added. A washout period of 20 min was allowed between each dihydropyridine concentration during preparation of the concentration-response curves.

Spontaneous epileptiform activity was induced by omitting magnesium from the ACSF or by increasing the potassium concentration to give a final level of 8.5 mM. The bursts were recorded via an electrode placed in the pyramidal cell layer of the CA3 region and recorded direct from the amplifier on a Grass chart recorder. The action of the drugs was measured as a percentage change in burst frequency, measured over a ten min period when the burst frequency had stabilized at its new level with respect to burst frequency prior to drug application.

All values were calculated as mean ± standard error of the mean (s.e.mean). Statistical significance was determined by a paired t test.

Results

The well documented action of adenosine compounds on hippocampal-evoked field potentials is to reduce synaptic transmission, as assessed by a reduction in the size of the population potential. The potency of adenosine in this system has been determined in previous experiments carried out in this laboratory (Bartrup & Stone, 1988). It was decided to use a single adenosine concentration of 25 μM throughout these experiments, as this gave a significant decrease in potential size that was not totally abolished in the presence of nifedipine. The addition of 25 μM adenosine inhibited population potential by 59 ± 3.4%, $n = 21$. The decrease in potential size was apparent within 1 min of the addition of adenosine and a maximum decrease was obtained within 2–3 min.

The addition of nifedipine alone to the perfusion medium had no detectable effect on the size of the population potential. In trial experiments, a range of nifedipine concentrations from 1 to 50 μM were perfused for up to 15 min with no change in potential size. When adenosine was added after nifedipine had been perfused for a minimum of 10 min, the inhibitory effect of adenosine on the population potential was increased. This effect was concentration-dependent (Figure 1).

The adenosine analogue 2-chloroadenosine also reduced the potential size, a stable depression of the potential being obtained within 10 min of application. However, in contrast to adenosine, the presence of nifedipine resulted in a significant reduction in the inhibitory action of 2-chloroadenosine (Figure 1).

These experiments were repeated with the dihydropyridine calcium channel activator Bay K 8644. This exerted a similar effect on the adenosine compounds to that observed with

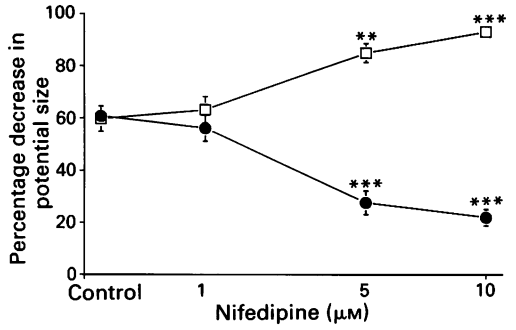


Figure 1 Percentage decrease in the CA1 evoked population potential size by adenosine and 2-chloroadenosine in the presence of increasing concentrations of nifedipine. Points are mean and vertical lines indicate s.e.mean of 8–14 experiments. (□) Adenosine 25 μM, (●) 2-chloroadenosine 0.5 μM. ** $P < 0.05$, *** $P < 0.01$, paired t test.

nifedipine, the inhibitory effect of adenosine being enhanced while that of 2-chloroadenosine was diminished at concentrations of 1–10 μM Bay K 8644 (Figure 2). With both nifedipine and Bay K 8644 the response to adenosine or 2-chloroadenosine was not restored to control levels even if the dihydropyridines were washed out for up to an hour. If, however, increasing concentrations of 2-chloroadenosine were added during this washout period, the action of nifedipine could be overcome. Once this potential size was restored, subsequent additions of 2-chloroadenosine at the original concentration (0.5 μM) elicited responses comparable to control values. Increasing the concentration of 2-chloroadenosine while nifedipine was present also increased the percentage inhibition of the population potential (Figure 3).

The adenosine analogues cyclohexyladenosine (CHA), N-ethylcarboxamidoadenosine (NECA) and R-phenylisopropyladenosine (R-PIA) were also affected by the addition of nifedipine in a similar manner to that observed with 2-chloroadenosine. Concentrations of the analogues were selected which produced an approximately 100% depression of the CA1 population potential. In the presence of 10 μM nifedipine these responses were then reduced by at least 50% (Figure 4).

In some experiments, a steady depression of the population potential by 25 μM adenosine was obtained, after which 10 μM nifedipine was added with the adenosine present. The inhibition of the potential then progressively increased to reach a new plateau in 28 ± 1.9 min ($n = 5$). At this time the potential had declined to 25 ± 4.9% of the size in adenosine alone. In similar experiments, nifedipine was added in the presence of 2-chloroadenosine. The inhibition of the potential gradually decreased to a new plateau over 43 ± 2.8 min ($n = 5$), at which time the response size was within 5% of the potential size prior to 2-chloroadenosine addition (Figure 5).

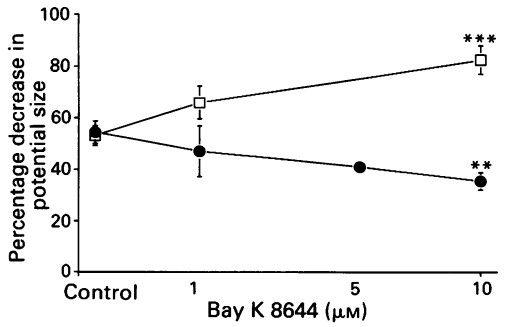


Figure 2 The action of adenosine and 2-chloroadenosine on the population potential in the presence of increasing concentrations of Bay K 8644. Points are mean and vertical lines indicate s.e.mean of 8–14 experiments. (□) Adenosine 25 μM, (●) 2-chloroadenosine 0.5 μM. ** $P < 0.05$, *** $P < 0.01$, paired t test.

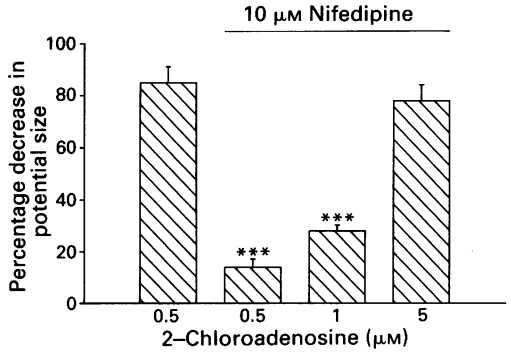


Figure 3 Percentage decrease in the CA1-evoked population potential size by 2-chloroadenosine in the presence of 10 μM nifedipine. Increasing the 2-chloroadenosine concentration overcame the block by nifedipine. Columns represent mean and bars show s.e.mean of 5 experiments. *** $P < 0.001$, paired t test.

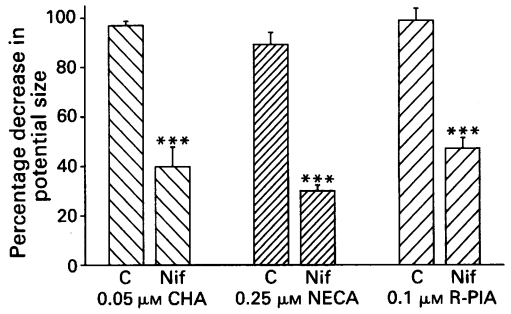


Figure 4 Columns represent the percentage inhibition of the population potential by three adenosine analogues, cyclohexyladenosine (CHA), N-ethylcarboxamidoadenosine (NECA) and R-phenylisopropyladenosine (R-PIA). C = control, Nif = 10 μM nifedipine. The addition of nifedipine significantly reduced the inhibition. Columns represent the mean and bars s.e.mean of experiments. *** $P < 0.01$, paired t test.

The dihydropyridines, nimodipine and nitrendipine and the phenylalkylamine, verapamil were also added to the hippocampal slices. These had little effect on the adenosine compounds at concentrations of 1–10 μM, with only nitrendipine showing a small but significant increase in the action of adenosine at a concentration of 50 μM (Table 1).

Dipyridamole

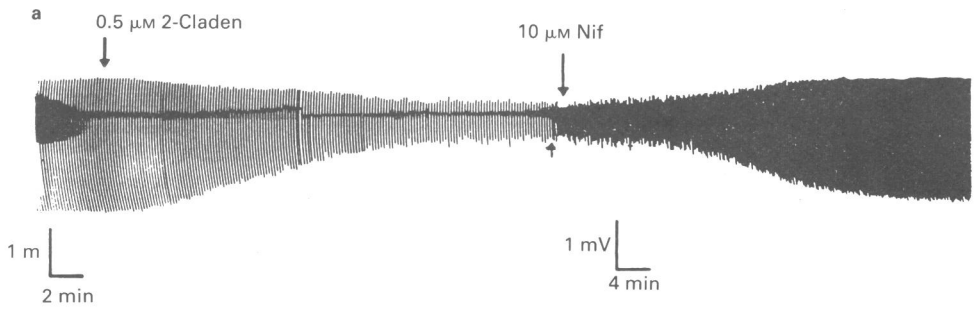
A final set of experiments was carried out to determine the contribution of the nucleoside transport system to the action of adenosine. Adenosine (10 μM) was added to the slice to give a stable depression of the population potential of around 10–20%. The adenosine uptake inhibitor, dipyridamole was then added at a concentration of 10 μM. This increased the level of inhibition by adenosine to approximately 50%. When nifedipine (10 μM) was added together with the adenosine and dipyridamole, the inhibition of the potential was attenuated, although the level of inhibition was not reduced to control levels (Figure 6).

Antiepileptiform activity

In addition to studies on the evoked population potentials, the effect of nifedipine on the antiepileptiform action of adenosine and 2-chloroadenosine was determined. Adenosine compounds are known to exhibit potent antiepileptiform properties in *in vitro* models of epileptiform activity (Ault & Wang, 1986). The action of adenosine and 2-chloroadenosine on spontaneous activity arising in the CA3 region as a result of raised potassium concentration (8.5 mM) or omission of magnesium from the ACSF, was studied in the absence or presence of nifedipine (Table 2). Nifedipine (10 μM) enhanced the antiepileptiform action of adenosine in both models of epileptiform activity, while reducing the action of 2-chloroadenosine. Nifedipine on its own had no effect on spontaneous activity at 10 μM.

Discussion

Nifedipine and Bay K 8644 appear to have opposite effects on adenosine and the uptake resistant adenosine analogues,



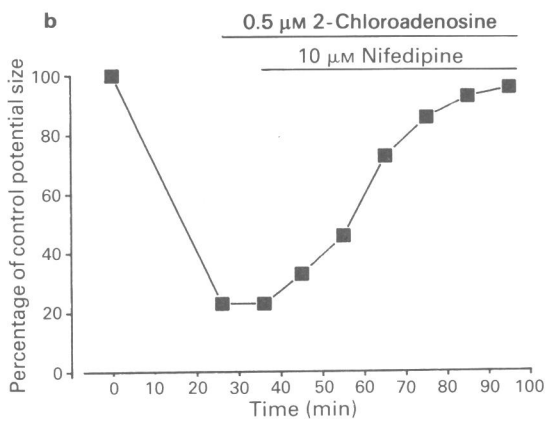


Figure 5 Inhibition of the CA1 population potential by 0.5 μM 2-chloroadenosine (2-Claden) could be overcome by the addition of 10 μM nifedipine (Nif). (a) Sample chart record. Each deflection represents the size of the evoked population potential at 0.1 Hz stimulation rate. 2-Chloroadenosine (0.5 μM) was present throughout the experiment. The time base has been reduced at the point indicated (↑). (b) Graphical representation of (a) showing the inhibition by 2-chloroadenosine over time in the presence of nifedipine. The points represent the size of the population potential as a percentage of the control response (100%).

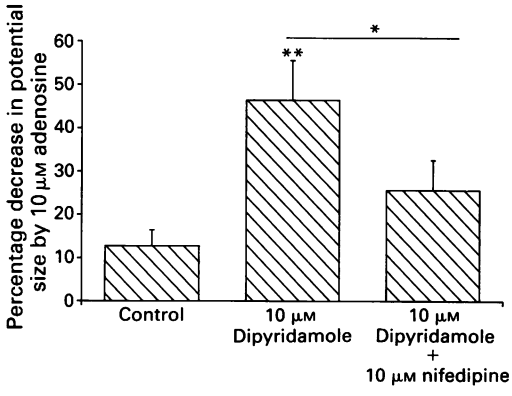


Figure 6 The addition of dipyridamole enhanced the percentage inhibition of the population potential by 10 μM adenosine. In the presence of dipyridamole, 10 μM nifedipine reduced the level of inhibition by adenosine in a similar manner to its effect on the adenosine analogues. Columns represent the mean and bars shown s.e.mean of 6 experiments. * $P < 0.1$, ** $P < 0.05$, paired t test.

enhancing the inhibitory action of adenosine while reducing that of the adenosine analogues. This effect was apparent on spontaneous activity induced by two sets of conditions as well as evoked potentials and appears to be specific to nifedipine and Bay K 8644, as the other calcium channel antagonists used, nitrendipine, nimodipine and verapamil, had no comparable activity.

The results obtained in these experiments may be explained by referring to binding studies which show the displacement of adenosine analogues by the dihydropyridines. Murphy & Snyder (1983) demonstrated that nifedipine could displace [^{3}H]-CHA from adenosine receptors in rat and bovine brain. Nisoldipine and nitrendipine exerted a partial effect while nimodipine and verapamil were virtually ineffective. Subsequently, more extensive studies have confirmed these initial findings (Morgan *et al.*, 1987; Hu *et al.*, 1987; Cheung *et al.*, 1987). Morgan *et al.* (1987) also found that Bay K 8644 was as potent as nifedipine in displacing [^{3}H]-CHA. An interaction of this nature would readily explain the effect of nifedipine and Bay K 8644 on the adenosine analogues, with the dihydropyridines directly displacing the analogues from the adenosine receptor, or possibly acting via an allosteric interaction to reduce the affinity of the receptor for the adenosine compounds.

In contrast, the enhancement of adenosine action by the dihydropyridines may involve an interaction at the nucleoside transport system. Marangos *et al.* (1984) showed that the dihydropyridines could potently displace [^{3}H]-NBI (nitrobenzylthioinosine), a nucleoside transport inhibitor, from binding sites in dog brain and heart. Similar results were obtained by Morgan *et al.* (1987) in rat brain. In this case, the dihydropyridines were all roughly equipotent in displacing [^{3}H]-NBI and Scatchard analysis indicated that binding was strictly competitive. In functional studies, the uptake of [^{3}H]-adenosine into rat brain synaptosomes has been shown directly to be reduced in the presence of the dihydropyridines (Phillis *et al.*, 1984; Morgan *et al.*, 1987). As with the adenosine receptor binding studies, verapamil and diltiazem had no significant effect on [^{3}H]-adenosine uptake or [^{3}H]-NBI binding.

The present studies also provide evidence that an action on adenosine uptake by nifedipine is involved in enhancing adenosine action. The addition of dipyridamole enhanced the inhibitory action of a low concentration of adenosine, as might be expected. However, the addition of nifedipine under these conditions reversed the inhibition of the population potential by adenosine, suggesting that nifedipine, unable to depress uptake further, was now acting to reduce the effects of adenosine.

Sites of interaction

The exact site at which the dihydropyridines bind to interact with adenosine receptors or uptake sites is not clear. As adenosine may act on presynaptic calcium channels to reduce neurotransmitter release (Stone, 1981), it is possible that the adenosine receptor and dihydropyridine binding site occur at a shared calcium channel. However, dihydropyridines have little or no effect on synaptic transmission, as is apparent in this study and by the inability of dihydropyridines to reduce calcium uptake into synaptosomes (Daniell *et al.*, 1983), while adenosine can completely inhibit synaptic transmission. It would therefore seem unlikely that there is a common site of action on synaptic calcium channels.

It is also possible that adenosine receptors exist on postsynaptic dendrites, acting to reduce synaptic transmission by activating potassium channels and hyperpolarizing the postsynaptic membrane (Gerber *et al.*, 1989). The interaction between adenosine compounds and nifedipine was also apparent on spontaneous epileptiform activity induced by both magnesium-free and high potassium ACSF. Adenosine inhi-

Table 1 Percentage inhibition of the CA1-evoked population potential by adenosine and 2-chloroadenosine (2-Claden) in the presence or absence of a calcium channel antagonist

	Nimodipine (50 μM)		*Nitrendipine* (50 μM)		*Verapamil* (10 μM)	
	Control	*Test*	*Control*	*Test*	*Control*	*Test*
Adenosine 25 μM	27.5 ± 4.3	37.5 ± 3.3	52.0 ± 3.1	60.5 ± 1.1*	ND	ND
2-Claden 0.5 μM	55.4 ± 2.2	64.8 ± 4.7	62.1 ± 4.3	72.3 ± 2.7	54.7 ± 4.7	63.0 ± 5.3

Each value is the mean ± s.e.mean of 4 experiments.
* $P < 0.1$, paired t test.
ND = not determined.

Table 2 Effect of 10 μM nifedipine on the antiepileptiform action of adenosine and 2-chloroadenosine (2-Claden)

	Magnesium-free ACSF		*High potassium ACSF*	
	Control	*Nifedipine*	*Control*	*Nifedipine*
Adenosine 5 μM	54.3 ± 3.8	83.3 ± 4.7***	40.0 ± 4.4	72.6 ± 3.0***
2-Claden 0.5 μM	66.3 ± 7.6	23.0 ± 2.1***	72.0 ± 4.8	28.0 ± 2.1***

Values are the percentage decrease in the burst frequency of spontaneous activity induced in the CA3 area by magnesium-free or high potassium (8.5 μM) artificial cerebrospinal fluid (ACSF)
Values are mean ± s.e.mean of 6 experiments.
*** $P < 0.01$, paired t test.

bits spontaneous activity at significantly lower concentrations than are required to reduce synaptic transmission in the hippocampus (Ault & Wang, 1986). It is therefore possible that the antiepileptiform action of adenosine is mediated by adenosine action at postsynaptic receptors linked to potassium channels. This is supported by data showing that adenosine can inhibit spontaneous depolarizations in low calcium ACSF, an effect that is independent of synaptic transmission (Lee *et al.*, 1984). The fact that nifedipine affected the antiepileptiform action of adenosine and 2-chloroadenosine suggests that the dihydropyridines may interact with the postsynaptic adenosine receptor.

The high density, high affinity dihydropyridine receptor is also unlikely to be the binding site for this particular interaction for a number of reasons. Nitrendipine and nimodipine have been shown to bind with high affintiy in the brain but have only weak effects on adenosine action, in comparison to Bay K 8644 and nifedipine. Secondly, the concentration of dihydropyridines required to have an effect on adenosine is several orders of magnitude greater than that required to saturate high affinity dihydropyridine binding sites (Belleman *et al.*, 1983) or to exert an action on calcium channels in smooth muscle. Finally, Morgan *et al.* (1987) showed that CHA or 2-chloroadenosine had no effect on [^{3}H]-nitrendipine binding, indicating that there was no common binding site for adenosine analogues on the high affinity nitrendipine binding site.

However, a separate low affinity dihydropyridine binding site may exist that interacts allosterically with the adenosine receptor. Bay K 8644 and nifedipine both exhibited a mixed competitive/non-competitive effect on ligand binding to the adenosine receptor (Morgan *et al.*, 1987). This suggests that Bay K 8644 and nifedipine are able to act at an additional binding site not available to other dihydropyridines. It is interesting that both nifedipine and Bay K 8644, a calcium channel antagonist and activator respectively, both exert the same effect on the adenosine compounds with a similar potency. There are only small structural differences between the two compounds and dihydropyridine activators can act as antagonists under certain conditions (Triggle & Rampe, 1989). For these compounds to act at a separate binding site, a structural characteristic between them might be expected that distinguishes them from the other dihydropyridines. Indeed, nifedipine and Bay K 8644, and also nisoldipine which can displace [^{3}H]-CHA from its binding site (Murphy & Snyder, 1983), possess 2 substituted phenyl moieties, while nimodipine and nitrendipine, that have little or no effect on binding, possess 3-nitrophenyl groups (Morgan *et al.*, 1987). It is possible, therefore, that a distinct low-affinity dihydropyridine binding site exists that preferentially binds the former molecules and is associated with the adenosine receptor. A low-affinity dihydropyridine binding site has been demonstrated in cardiac tissue (Sarmiento *et al.*, 1987). A study of [^{3}H]-nifedipine binding in brain, as opposed to the [^{3}H]-nitrendipine used by Morgan *et al.* (1987), may demonstrate the existence of a low-affinity receptor site from which ligands may be displaced by the presence of adenosine ligands.

The most straightforward interaction between the dihydropyridines and adenosine would be a simple competitive interaction at the adenosine receptor. In the present experiments, the action of nifedipine on 2-chloroadenosine could be overcome by increasing the concentration of 2-chloroadenosine suggesting that this may be the case.

The action of nifedipine and Bay K 8644 at the nucleotide transport system is also likely to be competitive (Morgan *et al.*, 1987), although in the present study nimodipine and nitrendipine were significantly less potent in enhancing the action of adenosine. This is in contrast to the binding studies where all the dihydropyridines studied were approximately equipotent in displacing [^{3}H]-NBI (Morgan *et al.*, 1987). The reason for this is unclear.

These results provide evidence from a functional approach to support the extensive ligand binding studies, highlighting the action of selective dihydropyridines on both the adenosine receptor and the nucleoside transport system. Because of the relatively high concentration of dihydropyridines required, it is unlikely that the clinical use of these compounds involves any action on endogenous adenosine. However, some of the side-effects, particularly of nifedipine, that have been observed during clinical use, may involve an interaction with adenosine (Swanson & Green, 1986). Similarly, some of the behavioural effects of the dihydropyridines, such as mild sedation and interference with sleep patterns (Hoffmeister *et al.*, 1982), or anticonvulsant effects (Meyer *et al.*, 1986), may be explained by an interaction with the endogenous adenosine system, since adenosine or its receptors may be involved in each of these behaviours (Stone, 1989).

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Characterization of the dopamine receptor mediating the hyperpolarization of cockroach salivary gland acinar cells *in vitro*

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1 Intracellular recordings have been made of the hyperpolarization of cockroach salivary gland cells induced by nerve stimulation and dopamine.

2 The relative potency of a number of dopamine antagonists in inhibiting the dopamine- and nerve-mediated hyperpolarization was studied. SCH23390 (10–50 μM), chlorpromazine (0.1–5 μM), haloperidol (10–100 μM) and metoclopramide (1 mM) inhibited the hyperpolarization.

3 In contrast, domperidone and (±)-sulpiride potentiated the hyperpolarization induced by both nerve stimulation and dopamine.

4 Apparent dissociation constants ($K_{D_{app}}$) were obtained for the blockade of the dopamine-induced hyperpolarization. The rank order of potency ($K_{D_{app}}$ in parentheses) was as follows: chlorpromazine (0.2 μM); haloperidol (3.3 μM); SCH23390 (4.1 μM); metoclopramide (265 μM); domperidone and (±)-sulpiride (inactive).

5 It is concluded that the receptor subserving the dopamine-induced hyperpolarization of the salivary gland acinar cells is the same as that mediating the secretory response to dopamine. In addition these data support our findings, which suggested that this receptor is similar to the D_1 dopamine receptor, but distinct from the D_2 receptor found in mammalian systems.

Introduction

Stimulation of the suboesophageal nerve evokes secretion from the cockroach (*Periplaneta americana* and *Nauphoeta cincerea* Olivier) salivary gland (Whitehead, 1971; Smith & House, 1977), which is accompanied by a hyperpolarization of the acinar cells (House, 1973). Previous studies have established that the neurotransmitter involved is dopamine (Bland *et al.*, 1973; Fry *et al.*, 1974; Kapoor *et al.*, 1983). Activation of the dopamine receptor leads to an increase in a calcium-dependent potassium conductance, which underlies the hyperpolarizing response (Ginsborg *et al.*, 1974; 1980), and an increase in cytosolic adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Grewe & Kebabian, 1982) which appears to be an intermediate in the secretory, but not the electrical response to dopamine (Gray *et al.*, 1984). More recently the rank order of potency of a number of dopamine antagonists was used to characterize the receptor mediating the secretory response of the salivary gland. It was concluded that the receptor was similar to the D_1 receptor found in the mammalian CNS and periphery (Evans & Green, 1990). It is therefore of some interest to establish whether or not the same order of potency is obtained for the receptor mediating the electrical response of the acinar cells to dopamine. To enable as direct a comparison as possible the present investigation follows closely the protocol used in the study of the secretory response. Thus the rank order of potency for the selective D_1 antagonist SCH23390, the selective D_2 antagonists domperidone, (±)-sulpiride, haloperidol and metoclopramide (for review see Clark & White, 1987), and the non-selective dopamine antagonist chlorpromazine (O'Boyle & Waddington, 1984) was obtained. The results suggest that the receptor mediating the dopamine-induced hyperpolarization is the same as that mediating the secretory response to dopamine, and is similar to the D_1 receptor found in mammalian systems.

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Methods

Paired salivary glands and associated ducts were dissected from adult cockroaches (*Nauphoeta cinerea* Olivier) as described previously (House, 1973). The preparation was pinned to the base of a shallow perspex chamber lined with silicone resin (Sylgard, Dow Corning). The main ducts of the gland were drawn into a suction electrode to allow stimulation of the encapsulated suboesophageal nerve. The preparation was kept at room temperature (18–25°C) and perfused at a rate of 5 ml min^{-1} with a solution of the following composition (mM): NaCl 160, $CaCl_2$ 5, KCl 1, glucose 20, Tris buffer 2.5, pH adjusted to between 7.70 and 7.80 with HCl. Recording microelectrodes were filled with 3M KCl and had resistances between 20 and 40 MΩ. Membrane potentials were recorded on a pen recorder (Gould, Brush 220) and on video tape (Sony SL-F 30; with a digital audio processor, Sony PCM-701 ES) via a pre-amplifier (Dagan 8900 patch clamp). Sub-maximal responses to nerve stimulation were obtained with a train of 1–10 impulses, 0.5 ms pulse width, 1–100 V, 20 Hz. Dopamine (500 nM) was applied to the preparation locally by pressure ejection from a broken microelectrode (pulse of 35 kPa, 0.003–5 s; Picospritzer II, General Valve Corporation). An interval of 90 s was allowed between any two stimuli. Antagonists were added to the perfusate after four consistent control responses had been obtained to each stimulus applied.

Estimation of apparent K_D *values*

When applied by pressure ejection the absolute concentration of the agonist at the acinus is, of course, unknown and a function of time. However, it was assumed that the effective concentration of agonist at the acinus was proportional to the duration of a pulse of fixed pressure (McCaman *et al.*, 1977; Sakai *et al.*, 1979). This assumption was supported by the fact that over a considerable range of pulse durations, the $\log_{10}$-duration response curve was linear (Figure 1). Therefore, throughout the present investigation the duration of the pressure pulse used for agonist application, and not agonist concentration, was used in the estimation of dose-ratios.

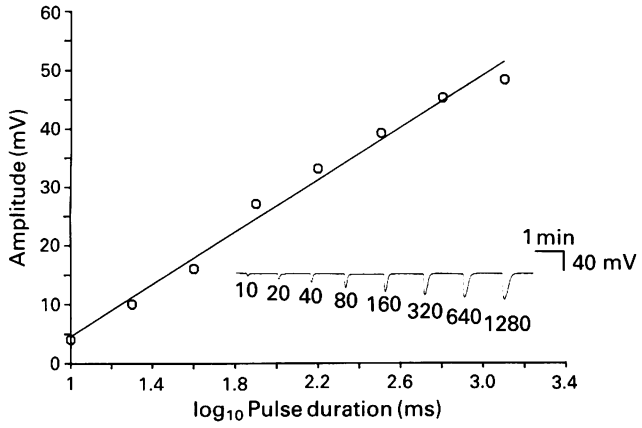

Figure 1 Dose–response curve for the dopamine-induced hyperpolarization of the cockroach salivary gland cells. The results are from a single experiment. Dopamine was applied by pressure ejection (35 kPa; duration of pulse as shown). The inset shows the experimental record, with the duration of the pulse marked below each response. Recordings of the membrane potential were made intracellularly. The initial resting potential was −55 mV. In this and all subsequent records the hyperpolarization is downwards.

The rate of equilibration of all active antagonists studied was inconsistent and often full equilibration was not obtained even during incubations of up to 1 h, with the dose-ratio increasing during this period. These considerations make it impossible to determine absolute values for the K_D. However, it seemed likely that useful estimates (at least for comparative purposes) would be obtained from the increase in stimulus required, after a fixed period of incubation in the presence of the antagonist, to match a response in antagonist-free solution. This assumption seems justified by the degree of agreement between the results in this paper and those obtained by more conventional methods in relation to the secretory response of this gland (Evans & Green, unpublished observation).

In practice, values for the apparent K_D ($K_{D_{app}}$) were obtained from 'dose-ratios' estimated in a 'three point assay' (Edinburgh Staff, 1968); the time of exposure to the antagonist was 30 min. The antagonists studied were: SCH23390 (1–100 μM); chlorpromazine (0.1–5 μM); haloperidol (1–100 μM); metoclopramide (0.1–1 mM); (±)-sulpiride (1–100 μM); domperidone (1–100 μM). Dose-ratios were estimated independently on separate preparations for three concentrations of each antagonist (except metaclopramide; see results). A separate value for the $\log_{10} K_{D_{app}}$ was estimated for each experiment (see Waud & Parker, 1971), and the geometric means of the $K_{D_{app}}$ together with confidence limits was calculated (Sokal & Rohlf, 1969).

Drugs and chemicals

Dopamine (3-hydroxytyramine HCl) and haloperidol (4-[4-(4-chlorophenyl) - 4 - hydroxypiperidino] - 4′ - fluorobutyro - phenone) were obtained from Sigma (Poole, Dorset). Chlorpromazine (2 - chloro - 10 - (3 - dimethylaminopropyl)pheno - thiazine HCl) was obtained from Aldrich Chemical Company (Gillingham, Dorset). (±)-Sulpiride (5-(aminosulphonyl)-n-[(1-ethyl - 2 - pyrrolidinyl)methyl] - 2 - methoxybenzamide) was obtained from Semat (St Albans, Herts). SCH23390 (8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate) was a gift from Dr V.G. Daniels, Kirby Warrick Pharmaceuticals (Bury St Edmunds, Suffolk). Domperidone (5 - chloro - 1 - {1 - [3 - (2 - oxobenzimidazolin - 1 - yl)propyl] - 4 - piperidyl}]benzimidazolin-2-one) was a gift from Janssen Pharmaceutical (Marlow, Bucks). Metoclopramide(4-amino-5-chloro-n-(2-diethylaminoethyl)-2-methoxybenzamide HCl) was a gift from Beecham Pharmaceuticals (Harlow, Essex).

Dopamine was dissolved in ascorbic acid 1 mg ml^{-1}, with the final dilution being made in physiological saline. The maximum concentration of ascorbic acid in the ejection pipette was 0.1 mg ml^{-1}, and when tested alone this had no effect on the preparation.

Domperidone, (±)-sulpiride and haloperidol were dissolved in tartaric acid, 5 mg ml^{-1}, and dilutions made from this in physiological saline. The pH of the solution was adjusted to control values (pH 7.7–7.8) with 1 M NaOH; in this condition tartaric acid was found to have no effect on the preparation.

Results

Stimulation of the suboesophageal nerve evokes a stimulus-dependent hyperpolarization of the cockroach (*Nauphoeta cinerea* Olivier) salivary gland acinar cells *in vitro*, which is occasionally followed by a depolarization (House, 1973). When applied locally by pressure ejection, dopamine mimicked the effect of nerve stimulation in a concentration-related manner (Figure 1). It should be noted that throughout this study the external potassium concentration was 1 mM. Thus it was not unusual for the hyperpolarization induced by dopamine to exceed a potential of 100 mV (see House, 1973; Ginsborg *et al.*, 1974).

The effects of SCH23390, haloperidol, chlorpromazine and metoclopramide

At high concentrations some of the antagonists had non-specific effects. The inhibition of the hyperpolarization by chlorpromazine at concentrations > 5 μM was insurmountable, while SCH23390 destabilized the acinar cells membrane at concentrations ≥ 100 μM. It was necessary therefore to study the effect of these compounds over a limited concentration range.

SCH23390 (10–50 μM), chlorpromazine (0.1–5 μM), haloperidol (10–100 μM) and metoclopramide (1 mM) blocked the hyperpolarization induced by both dopamine and nerve stimulation. The onset of inhibition of the response to dopamine was immediate, while in the case of nerve stimulation there was a delay of between 3–10 min. This is illustrated in Figure 2 which shows a typical record of the onset of blockade following addition of SCH23390 (50 μM) to the bath solution.

Nerve stimulation

Figure 3 illustrates the action of the antagonists on the response to nerve stimulation. Figure 3a (i) shows a control response; (ii) shows the response after addition of SCH23390 (20 μM) to the perfusate; (iii) shows the effect of increasing the number of impulses in the train on the response after the onset of blockade; and (iv) shows the degree of recovery on washing. Parts b, c and d of Figure 3 illustrate the corresponding results for chlorpromazine (5 μM), haloperidol (20 μM) and metoclopramide (1 mM). The inhibition of the

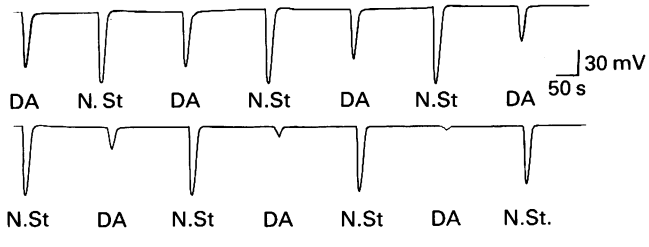

Figure 2 The time course for blockade of the hyperpolarization induced by locally applied dopamine (DA; 35 kPa; 10 ms) and nerve stimulation (NSt; 100 V; 20 Hz; 0.5 ms pulse width; 2 impulses in the train) respectively, following addition of SCH23390 (50 μM); (b) is continuous with (a). The initial resting potential was −42 mV.

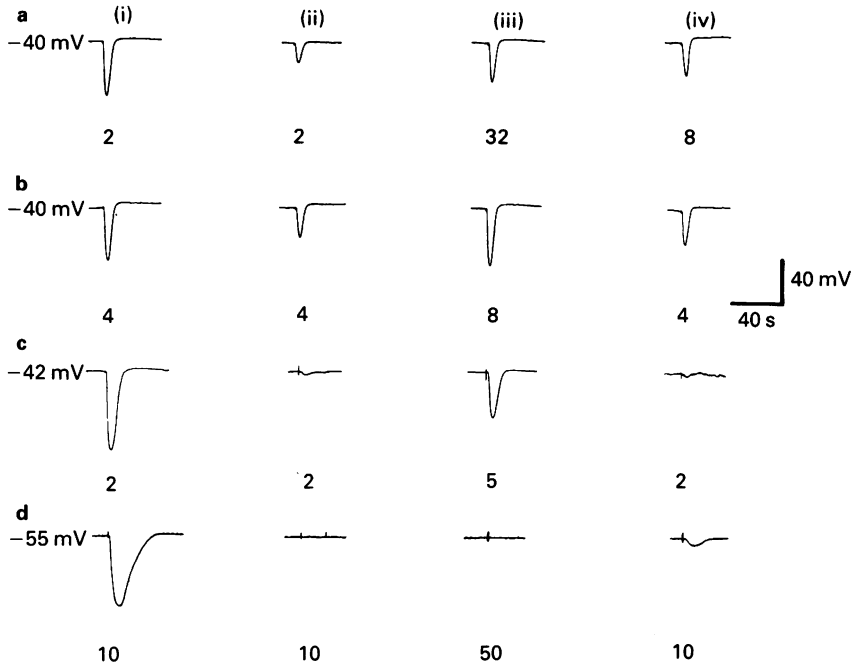

Figure 3 The blockade of the hyperpolarization induced by nerve stimulation following addition of (a) SCH23390 (20 μM), (b) chlorpromazine (5 μM), (c) haloperidol (20 μM) and (d) metoclopramide (1 μM). (i) Control response to nerve stimulation (100 V; 20 Hz; 0.5 ms pulse width; number of impulses in the train as indicated below each response), (ii) response following the addition of the antagonist, (iii) the effect of increasing the number of impulses in the train after 30 min incubation with the antagonist and (iv) recovery following a 30 min wash. The initial resting potential for each experiment was as shown.

response by SCH23390 (10–50 μM) and chlorpromazine (0.5–5 μM) was readily surmounted and was reversed on washing, although full recovery was not always obtained. In contrast, the blockade of the hyperpolarization by haloperidol (10–100 μM) and metoclopramide (1 mM) was insurmountable, and the inhibition by the former was not reversed on washing. The inhibition of the response to dopamine by these compounds was found to be surmountable and was reversed on washing (see below). Thus haloperidol and metoclopramide must have an additional presynaptic action.

Dopamine

As stated in the methods, a more quantitative analysis of the effects of the antagonists was undertaken with respect to the dopamine-induced hyperpolarization. Figure 4a illustrates the

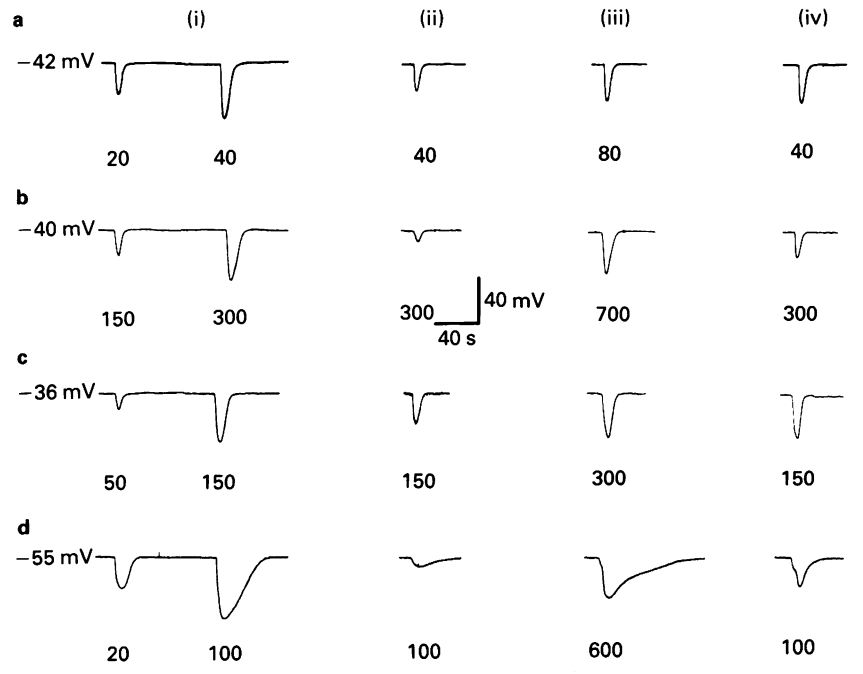

Figure 4 The blockade of the dopamine-induced hyperpolarization by (a) SCH23390 (20 μM), (b) chlorpromazine (5 μM), (c) haloperidol (20 μM) and (d) metoclopramide (1 mM). (i) Paired, submaximal control responses to dopamine (DA; 35 kPa; duration of pulse in ms as shown), (ii) the larger of the two control responses following addition of the antagonist, (iii) response to increased duration of dopamine application after a 30 min incubation, and (iv) the degree of recovery of the response following a 30 min wash. The initial resting potential for each experiment is shown.

Table 1 Dose-ratios estimated by 'three point assay' for each of the active dopamine antagonists together with the geometric mean $K_{D_{app}}$ and upper (L_2) and lower (L_1) confidence limits (95%)

Antagonist	*Concentration* (μM)	*Dose-ratio*	$K_{D_{app}}$ (μM) *Geometric mean*	*95% Confidence limits* (μM) L_1	L_2
Chlorpromazine	0.1	2.0			
	0.1	2.76			
	0.1	2.50			
	0.1	3.03			
	0.1	1.50			
	0.5	5.51			
	0.5	1.82	0.22	0.10	0.45
	0.5	2.08			
	0.5	2.15			
	5.0	43.37			
	5.0	7.61			
	5.0	3.49			
Haloperidol	10	5.61			
	10	4.76			
	10	2.20			
	10	2.10			
	20	10.48			
	20	2.08			
	20	50.35	3.77	0.51	28.05
	20	5.04			
	100	5.66			
	100	46.21			
	100	39.24			
	100	55.32			
SCH23390	10	1.45			
	10	3.48			
	10	1.70			
	10	4.80			
	10	1.45			
	20	6.19			
	20	14.27			
	20	2.35	4.06	0.49	33.67
	20	20.00			
	20	3.30			
	20	1.59			
	50	275.69			
	50	222.21			
	50	22.85			
	50	7.08			
Metaclopramide	1000	3.90			
	1000	3.50			
	1000	2.30	264.66	0.03	21.80
	1000	16.60			

'three point assay' for SCH23390 (20 μM): (i) shows two different sub-maximal responses to dopamine in the absence of the antagonist; (ii) shows the inhibition of the larger of the two control responses by SCH23390 (20 μM); (iii) demonstrates that increasing the duration of the pressure pulse surmounts the blockade; and (iv) shows the degree of recovery on washing. Figure 4 sections b, c and d illustrates the corresponding results for chlorpromazine (5 μM), haloperidol (20 μM) and metoclopramide (1 mM). In contrast to nerve stimulation, the inhibition of the dopamine-induced hyperpolarization by all of these antagonists could be surmounted and reversed on washing, although as with nerve stimulation full recovery was not always obtained. The dose-ratio for the antagonists was calculated in each experiment, and these, together with the geometric mean of the apparent K_D ($K_{D_{app}}$) with 95% confidence limits are shown in Table 1. The rank order of potency obtained from these data was as follows ($K_{D_{app}}$ in parentheses): chlorpromazine (0.2 μM) > haloperidol (3.3 μM) = SCH23390 (4.1 μM) > metoclopramide (265 μM).

The effect of (±)-sulpiride and domperidone

(±)-Sulpiride (1–100 μM) and domperidone (1–100 μM) failed to antagonize the hyperpolarization induced by either dopamine or nerve stimulation. On the contrary, higher concentrations of (±)-sulpiride (100 μM) and domperidone (50 and 100 μM) produced an immediate potentiation of the hyperpolarization induced by both dopamine and nerve stimulation, which was readily reversed on washing. A more detailed analysis of the effects of domperidone will be published elsewhere.

Discussion

Both exogenously applied dopamine and stimulation of the suboesophageal nerve induce a hyperpolarization of the cockroach salivary gland acinar cells, which is occasionally followed by a depolarization (House, 1973; Blackman *et al.*, 1979). Of the antagonists studied chlorpromazine, SCH23390, haloperidol and metoclopramide inhibited the hyperpolarization induced by both dopamine and nerve stimulation, while (±)-sulpiride and domperidone did not. For each of the effective antagonists the onset of the blockade of nerve stimulation was slower than that for exogenous dopamine. This has been reported previously in relation to the action of α-flupenthixol by House (1980). He concluded that the receptors located at the synapse are deeper within the acinus than those activated by the exogenously applied agonist. This may offer a further barrier to diffusion, resulting in the delayed onset of blockade.

The rank order of potency for the blockade of the hyperpolarizing response to locally applied dopamine was: chlor-

Table 2 Comparison of the rank order of potency of dopamine antagonists against the hyperpolarizing and the secretory response of the cockroach salivary gland to dopamine

Response to dopamine	*Antagonist rank order of potency*
Hyperpolarization	α-Flu ≧ Cpz > Phen > SCH = Halo ≫ Met ≫ (±)-Sulp = Domp (<500 nM) (215 nM) (1 μM) (4.1 μM) (3.8 μM) (265 μM) (>100 μM)
*Secretion	α-Flu > Cpz > Phen > SCH > Halo ≫ Met ≫ (±)-Sulp = Domp (3.3 nM) (0.2 μM) (1 μM) (2.2 μM) (17.5 μM) (1.2 mM) (>100 μM)

Values in parentheses are the K_{Dapp} except α-flupenthixol and phentolamine which represent the absolute K_D.
α-Flu = α-flupenthixol; Cpz = chlorpromazine; SCH = SCH23390; (±)-Sulp = (±)-sulpiride; Domp = domperidone; Halo = haloperidol; Phen = phentolamine; Met = metoclopramide.
* Evans & Green, (1990).

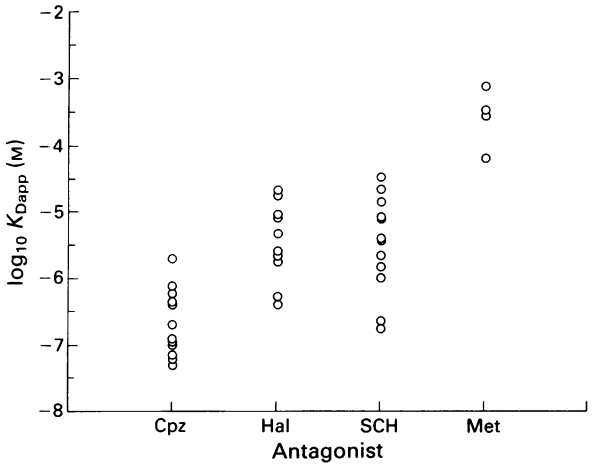

Figure 5 The range of $\log_{10} K_{Dapp}$ for each antagonist studied. Cpz = chlorpromazine; Hal = haloperidol; SCH = SCH23390; Met = metoclopramide.

promazine > haloperidol = SCH23390 ≫ metoclopramide ≫ (inactive) (±)-sulpiride and domperidone. As illustrated in Figure 5, there is a considerable scatter between K_Ds obtained from individual experiments, yet there is a clear separation between chlorpromazine, haloperidol and SCH23390, and metoclopramide. In Table 2 the rank order of potency obtained in this study, extended by the addition of phentolamine and α-flupenthixol (Bowser-Riley *et al.*, 1978; House & Ginsborg, 1976), is compared with that obtained for the secretory response to dopamine (Evans & Green, 1990). Thus, in contrast to the view of Gray and co-workers (1984), it would appear that a single receptor subserves both the hyperpolarization and the secretory response to dopamine. This receptor has an antagonist rank order of potency similar to the D_1 but distinct from the D_2 dopamine receptor found in the mammalian CNS and periphery (Hilditch & Drew, 1985; Anderson *et al.*, 1985; Seeman *et al.*, 1987). The similarity between the receptor on the cockroach salivary gland and the D_1 receptor in mammalian systems is most evident from the fact that the selective D_1 receptor antagonist SCH23390 inhibits the response to dopamine, while the selective D_2 antagonists domperidone and (±)-sulpiride do not. Although haloperidol and metoclopramide, which are also selective D_2 antagonists, inhibited the response of the salivary gland to dopamine, the potency of these compounds was similar to that found in studies of D_1 receptors in mammalian systems (Flaim *et al.*, 1985; Hilditch & Drew, 1985). Thus this inhibitory action is not inconsistent with the D_1 classification. This conclusion is supported by the results of a study of the interaction between selective agonists and antagonists. It was found that the rank order of agonist potency was fenoldopam > SKF38393 > LY163502 > quinpirole; the action of all four agonists was blocked by SCH23390, but not by (±)-sulpiride (Evans & Green, unpublished observations).

It must be noted that both in the present investigation and with respect to the dopamine-induced secretion of the salivary gland (Evans & Green, 1990), the K_{Dapp} for SCH23390 was between 2 and 5 orders of magnitude higher than that found in most functional studies of D_1 receptors in mammalian preparations (Hilditch & Drew, 1985; Anderson *et al.*, 1985). The pharmacology of all D_1 receptors is not however identical (see Plantje *et al.*, 1984; Niznik *et al.*, 1988; Ohlstein *et al.*, 1984; Anderson *et al.*, 1985; Fleminger *et al.*, 1983; Hilditch & Drew, 1985; Goldberg *et al.*, 1983) and the receptor on the cockroach salivary gland may thus represent a separate subtype.

As with D_1 receptors in other preparations, both invertebrate and mammalian (Lafon-Cazal Bockaert, 1984; Kebabian & Calne, 1979), the dopamine receptor in the cockroach salivary gland has been shown to be linked to a dopamine-sensitive adenylyl cyclase (Grewe & Kebabian, 1982). However, Gray and co-workers (1984) demonstrated that although cyclic AMP is an intermediate in the secretory response, it is not involved in the electrical response to dopamine. Thus there must be two distinct second messengers linked to this dopamine receptor, cyclic AMP mediating the secretory response and the other subserving the hyperpolarization.

In summary, it appears that the dopamine receptor mediating the hyperpolarizing response of the cockroach salivary gland acinar cells is the same as that mediating the secretory response. Both here and in the investigation of the secretory response (Evans & Green, 1990) we have established that this receptor is similar to the D_1 receptor found in mammalian systems.

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Endothelium modulation of the effects of nitroglycerin on blood vessels from dogs with pacing-induced heart failure

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1 The relaxant actions of nitroglycerin (previously considered to be an endothelium-independent relaxing agent) and acetylcholine (an endothelium-dependent relaxing agent) were compared on 4 vascular preparations (dorsal pedal artery, saphenous vein, left anterior descending coronary artery and circumflex coronary artery) from dogs with and without pacing-induced congestive heart failure (CHF).

2 Responses of the coronary arteries to acetylcholine were unaltered in endothelium-intact rings from dogs with and without heart failure. Similarly no such changes were observed in the peripheral vessels. The maximum relaxation produced by acetylcholine was always greater in the coronary vessels compared to the peripheral vessels.

3 Before heart failure, the coronary vessels were more sensitive and reactive to nitroglycerin compared to the peripheral vessels.

4 Removal of the endothelium in both the control (dogs without CHF) and experimental (dogs with CHF) rings enhanced the relaxant effects of nitroglycerin, such that the EC_{50} for nitroglycerin became significantly lower in all denuded rings, with the exception of the saphenous vein and the left anterior descending coronary artery, before the development of CHF.

5 When CHF was maximally developed, vascular sensitivity to nitroglycerin was increased in peripheral vessels with an intact endothelium, but not in the coronary vessels.

6 These findings indicate that relaxation produced by nitroglycerin cannot be considered as entirely endothelium-independent but should be considered endothelium-modulated.

Introduction

The pathophysiology of heart failure is poorly understood, which, in part, is due to the lack of suitable animal models. We have used a model of rapid ventricular pacing in dogs (Armstrong *et al.*, 1986; Forster *et al.*, 1989) which has low cardiac output and similar clinical manifestations to various heart failure syndromes seen in man (Franciosa & Cohn, 1981; Parmley, 1985). Vasodilators of the nitrate type are routinely used in the management of a number of cardiovascular disorders and are often used in the critically ill patient with severe symptoms of congestive heart failure (CHF: Gold *et al.*, 1972; Cottrell & Turndorf, 1978). However, the use of nitroglycerin is not without problems, for example, as much as a 10 fold variation in dose is often required to produce similar physiological effects among individual patients (Armstrong *et al.*, 1975). This finding suggests that there might either be enhanced metabolism or target organ resistance in patients who require higher doses.

It has been suggested that some manifestations of experimental CHF are a consequence of either altered sensitivity or responsiveness or both, of vascular smooth muscle to neurotransmitters and hormones (Karliner *et al.*, 1981; Conway *et al.*, 1987; Forster *et al.*, 1989). Most studies have concentrated on contractile function or binding characteristics of blood vessels in CHF, whereas vascular responses to vasodilators in heart failure have received little attention.

Evidence that the endothelium modulates the effect of nitroglycerin is controversial and ranges from findings of attenuation (White *et al.*, 1986) to potentiation (Shirasaki & Su, 1985) and of no response whatsoever (Furchgott *et al.*, 1981; Rapoport & Murad, 1983). Evaluation of these data is confounded by a number of ancillary factors which affect nitroglycerin responsiveness including the type and location of blood vessel, the species from which it is derived and the presence or absence of disease.

Since the original observations of Furchgott & Zawadzki (1980a), it has been demonstrated that the endothelium has an important function in mediating the effect of many vasodilators (Furchgott & Zawadzki, 1980b). We undertook the current study because of the continued used of nitroglycerin in the management of heart failure and the conflicting views on the role of the endothelium in mediating relaxation responses to nitroglycerin. The objectives were firstly, to evaluate the relaxant effects of nitroglycerin in coronary arteries and in a hindlimb artery and vein from dogs with pacing-induced CHF. Secondly, to compare the effects of nitroglycerin on peripheral vessels with those on coronary vessels and, finally, to assess the role of the endothelium in these responses.

Methods

Ten male mongrel dogs (weight range 19.2–26.0 kg) were randomly divided into 2 groups. All dogs were conditioned to the study environment and CHF was produced by rapid ventricular pacing according to the methods outlined by Armstrong *et al.* (1986). Briefly, under sodium thiopentone ($37.8 \pm 1.5\,mg\,kg^{-1}$; Abbott Laboratories Ltd, Montreal, Canada) anaesthesia, a pacemaker generator (Medtronic) and unipolar lead were inserted into a subcutaneous pocket and the apex of the right ventricle respectively. At the same time, segments of dorsal pedal artery and saphenous vein were removed in the region of the hind paw (Forster *et al.*, 1989). After a 5–7 day recovery period, the generator was programmed to deliver $250\,beats\,min^{-1}$ asynchronously for the remainder of the study. Bi-weekly clinical assessments were performed for signs of dyspnoea, pulmonary oedema and the presence of ascites. Animals having fulfilled the criteria for

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severe CHF, as defined by a 25% increase in heart size accompanied by the presence of oedema on a chest radiogram, were anaesthetized and the contralateral dorsal pedal artery and saphenous vein removed. Animals were then killed, the heart was excised and the left anterior descending and the circumflex coronary arteries were dissected.

A control group of 5 further animals was anaesthetized and pacemakers were implanted as described above. In this case, the pacemakers were not programmed 'on' and therefore this group of dogs behaved as sham-operated controls. These animals were monitored as above for 4–6 weeks. Previous data from sham-operated animals have suggested that both the dorsal pedal artery and the saphenous vein taken from the same animals, 8 weeks apart, behave similarly and therefore these vessels can justifiably be used as controls (Forster *et al.*, 1989).

Organ bath studies

All blood vessels were carefully dissected free of adherent tissue and placed in Krebs-Henseleit solution (at 4°C) of the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.0. In addition, indomethacin, 2.8×10^{-6} and 5.0×10^{-6} M, was incorporated into the Krebs-Henseleit solution in order to inhibit endogenous prostanoid production in peripheral and coronary vascular segments, respectively. The saphenous vein and the left anterior descending coronary artery were used immediately, whereas the dorsal pedal artery and the circumflex coronary artery were refrigerated overnight in Krebs-Henseleit solution since we, and others (Ito & Chiba, 1985), have shown that vascular preparations do not lose their activity when stored at 4°C.

Each vascular segment was cut into 6 rings (approximately 3 mm and 5 mm in length for peripheral and coronary vessels, respectively). Alternate rings from a particular segment were denuded of endothelium by gently rubbing the tip of a fine forcep against the lumen and rolling the ring back and forth on Krebs-Henseleit-moistened filter paper. Rings were carefully mounted on 2 platinum/nickel L-shaped hooks inserted through the lumen, the lower hook being anchored inside a 10 ml organ bath and the other connected to a force transducer (Grass FT03C) with surgical suture (4.0, silk; Ethicon). Changes in isometric tension were recorded and amplified by a Grass Polygraph (7D). Organ chambers contained Krebs-Henseleit solution, gassed with 95% O_2:5% CO_2 (pH 7.4) and maintained at 37°C with a circulating pump (Haake L; model D8).

The rings were progressively stretched to optimum resting tensions of 4.0 g and 2.5 g for the dorsal pedal artery and saphenous vein and 4.0 g and 6.0 g for the left anterior descending and circumflex coronary arteries, respectively. All rings were allowed to equilibrate for at least 1 h, during which time frequent washing with fresh Krebs-Henseleit solution occurred. In previous studies, we have found no change in the length-tension relationship of these vessels from dogs with CHF compared to control dogs. Following this equilibration period, all rings were challenged with KCl (20 mM) and the response was allowed to develop and stabilize. This concentration of KCl was chosen as in preliminary studies it was shown to represent an approximate EC_{50}. Selective removal of the endothelium was demonstrated by the absence of a relaxant effect to cumulative additions of acetylcholine (10^{-7} M–10^{-4} M). Another washout period of 60 min was allowed for each ring and all of the vessels were challenged with cumulative additions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in order to determine the EC_{50}. A further washout period of 60 min was allowed for each ring and all of the rings were challenged with a concentration equal to the EC_{50} of $PGF_{2\alpha}$. After stabilization of the contractile response, cumulative concentration-effect curves were generated, in at least one denuded ring and one intact ring, for the vasodilator nitroglycerin (10^{-9} M–10^{-5} M). In some instances, for both an intact ring and a denuded ring from a particular vessel, no relaxing agent was added to the precontracted rings and these served to determine any spontaneous decline in the response to $PGF_{2\alpha}$.

Drugs

The following were used: prostaglandin $F_{2\alpha}$ tris salt and acetylcholine iodide were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Nitroglycerin (Tridil, 50 mg 10 ml^{-1}) was obtained from AHS Division of McGaw Supply Ltd (Mississauga, Ontario), the vehicle of which contained 0.3 ml alcohol and 0.3 ml propylene glycol USP. Potassium chloride was purchased from Fisher Scientific (Nepean, Ontario).

All drugs, with the exception of $PGF_{2\alpha}$, were diluted in distilled water and stock solutions were made up daily. $PGF_{2\alpha}$ was made as a stock solution of 2×10^{-4} M and stored at 4°C for up to 1 month, further dilutions were made with distilled water on a daily basis.

Data analysis

Results are expressed as mean ± s.e.mean. For contractions evoked by $PGF_{2\alpha}$, the concentration-effect data were fitted to a sigmoidal curve by utilising a computer curve-fitting programme which was based on the standard logistic model described by Parker & Waud (1971) and modelled on the following equation:-

$$Y = \frac{a - d}{1 + (X/c)^b} + d$$

Where Y is the response; X, the arithmetic dose; a, the response when X = 0; d, the response for infinite dose; c is the EC_{50} and b the slope factor. The effective concentration producing 50% of the maximum response (EC_{50}) was thus determined. For relaxations, responses were expressed as percentage changes from the contraction given by the EC_{50} of $PGF_{2\alpha}$; the effective molar concentration of relaxing agent causing 50% of the maximum relaxation response to acetylcholine or nitroglycerin was calculated and termed the EC_{50}. Unless otherwise stated *n* refers to the number of vascular rings, where at least one ring was used from each of 5 animals. Statistical analysis was performed by Student's *t* test for either paired or unpaired observations. As appropriate, when more than two values were compared, a Bonferroni correction was applied. When EC_{50} values were compared, geometric means were used with 95% confidence limits and a Mann Whitney U test was used to assess statistical significance. Values were considered to be statistically significantly different when $P < 0.05$.

Results

The effect of KCl

Figure 1 shows the responses of the dorsal pedal artery, saphenous vein, and both the left anterior descending and circumflex coronary arteries (endothelium intact and endothelium denuded) to 20 mM KCl before development of and at maximally developed CHF. The response to KCl was not modified by removal of the endothelium, nor were there any differences between control vessels and those vessels obtained from dogs with CHF. However, the peripheral vessels developed a much lower tension to 20 mM KCl than the coronary vessels.

The effect of acetylcholine

Acetylcholine (10^{-7} M–10^{-5} M) produced concentration-dependent relaxations in all intact rings, but failed to elicit

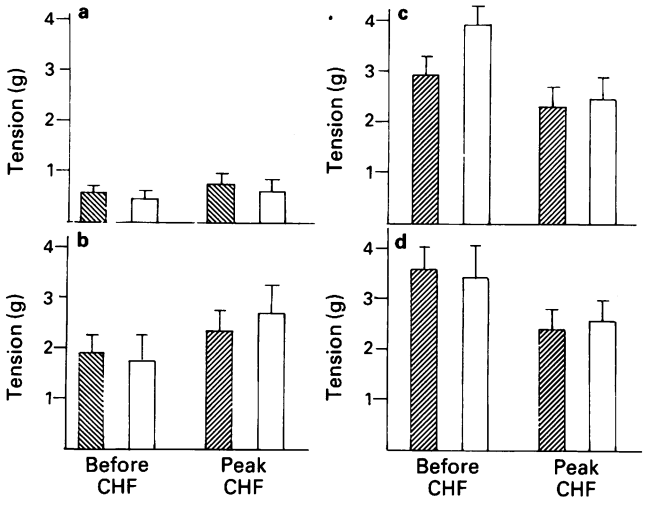

Figure 1 The effect of KCl (20 mM) on segments of (a) dorsal pedal artery, (b) saphenous vein, (c) left anterior descending coronary artery and (d) circumflex coronary artery before and at maximally developed congestive heart failure (peak CHF). Open columns represent the results obtained with the endothelium intact and hatched columns represent results from endothelium-denuded preparations. Results are given as mean with the bars showing s.e.mean of a total of 12–15 preparations obtained from 5 dogs in each group. Results are expressed as g tension developed to KCl.

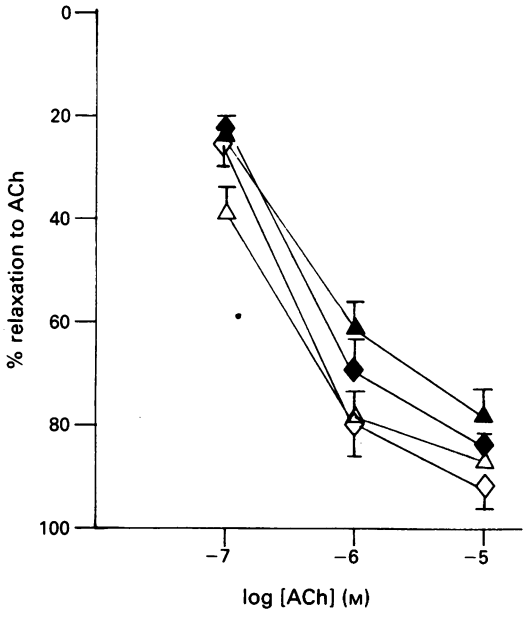

Figure 2 Cumulative concentration-effect curves for acetylcholine (ACh) in arteries from control dogs and dogs with congestive heart failure (CHF). Tone was induced with 20 mM KCl; 100% represents relaxation to baseline. All preparations were intact and (△) represent control/left anterior descending, (▲) CHF/left anterior descending, (◇) control/circumflex and (◆) CHF/circumflex. Data were derived from a total of 15 preparations obtained from 5 control and 5 CHF dogs. Each point is the mean and the vertical lines show s.e.mean.

any relaxation in denuded rings. The relaxation responses to acetylcholine in the coronary vessels from controls and dogs with severe CHF are shown in Figure 2. In contrast to the peripheral vessels (Forster *et al.*, 1989; see Discussion), almost 100% relaxation to acetylcholine could be demonstrated in the coronary vessels and there were no significant differences between control and CHF preparations.

Concentration-effect relationships of the blood vessels to prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ (10^{-8}–3×10^{-5} M) produced concentration-dependent increases in tension in all preparations tested. Table 1 shows the maximum response (which was obtained from the curve-fitting programme) and EC_{50} values for $PGF_{2\alpha}$ in intact and denuded rings from the 4 blood vessel groups in control dogs and dogs with CHF. There were no endothelium-dependent or CHF-dependent differences evident in the response of the coronary arteries to $PGF_{2\alpha}$. However, when CHF was maximally developed, responses to $PGF_{2\alpha}$ in both the dorsal pedal artery and the saphenous vein were significantly greater ($P < 0.05$). In addition, the EC_{50} for $PGF_{2\alpha}$ was significantly decreased in the dorsal pedal artery at peak CHF in both intact and denuded rings to a similar extent. In all cases when $PGF_{2\alpha}$ was used to precontract the vascular rings, there was no spontaneous decline in the tension developed in the control rings (i.e. those rings which did not receive any relaxing agent).

Nitroglycerin-induced relaxations

Nitroglycerin (10^{-9} M–5×10^{-5} M) produced concentration-dependent relaxations of all precontracted vascular preparations. Figure 3 shows cumulative concentration-effect curves constructed to nitroglycerin in the dorsal pedal artery (Figure 3a) and the saphenous vein (Figure 3b) before and when CHF was maximally developed. In the dorsal pedal artery, denuded rings produced greater relaxations to nitroglycerin than intact rings both before and at peak CHF

Table 1 Maximum responses and EC_{50} for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions of the dorsal pedal artery, saphenous vein, left anterior descending and circumflex coronary arteries with and without endothelium in control and dogs with congestive heart failure (CHF)

		Control		*CHF*	
		Intact	*Denuded*	*Intact*	*Denuded*
Dorsal pedal artery	Max	3.3 ± 0.8	4.2 ± 0.7	7.1 ± 1.1*	7.6 ± 0.6*
	EC_{50}	2.9×10^{-6} (7×10^{-6}–5.1×10^{-6})	1.9×10^{-6} (1.1×10^{-6}–4.0×10^{-6})	3.4×10^{-7}* (1.6×10^{-7}–7.0×10^{-7})	4.6×10^{-7}* (3.3×10^{-7}–8.4×10^{-7})
Saphenous vein	Max	4.6 ± 0.7	4.8 ± 0.7	9.9 ± 0.9*	9.8 ± 0.8*
	EC_{50}	6.1×10^{-7} (3.4×10^{-7}–10.8×10^{-7})	5.3×10^{-7} (3.4×10^{-7}–9.4×10^{-7})	5.3×10^{-7} 2.9×10^{-7}–9.3×10^{-7})	4.7×10^{-7} (3.7×10^{-7}–5.4×10^{-7})
Left anterior descending	Max	3.7 ± 0.6	4.6 ± 0.4	3.3 ± 0.4	3.7 ± 0.4
	EC_{50}	1.0×10^{-6} (0.9×10^{-6}–1.6×10^{-6}	2.0×10^{-6} (0.9×10^{-6}–3.3×10^{-6})	2.0×10^{-6} (1.1×10^{-6}–2.9×10^{-6})	1.2×10^{-6} (0.8×10^{-6}–2.8×10^{-6})
Circumflex	Max	4.1 ± 0.4	4.3 ± 0.4	3.8 ± 0.4	3.9 ± 0.4
	EC_{50}	8.0×10^{-7} (6.0×10^{-7}–11.1×10^{-7})	9.2×10^{-7} (6.6×10^{-7}–12.0×10^{-7})	1.3×10^{-6} (0.8×10^{-6}–1.9×10^{-6})	9.6×10^{-7} (6.6×10^{-7}–12.0×10^{-7})

Values represent means ± s.e.mean for maximum (Max) response in g and geometric means with 95% confidence limits in parentheses for EC_{50} (M) of 12–15 observations. Data were derived from 5 dogs in both groups of animals. * Represents statistical significance at $P < 0.05$ for CHF versus control.

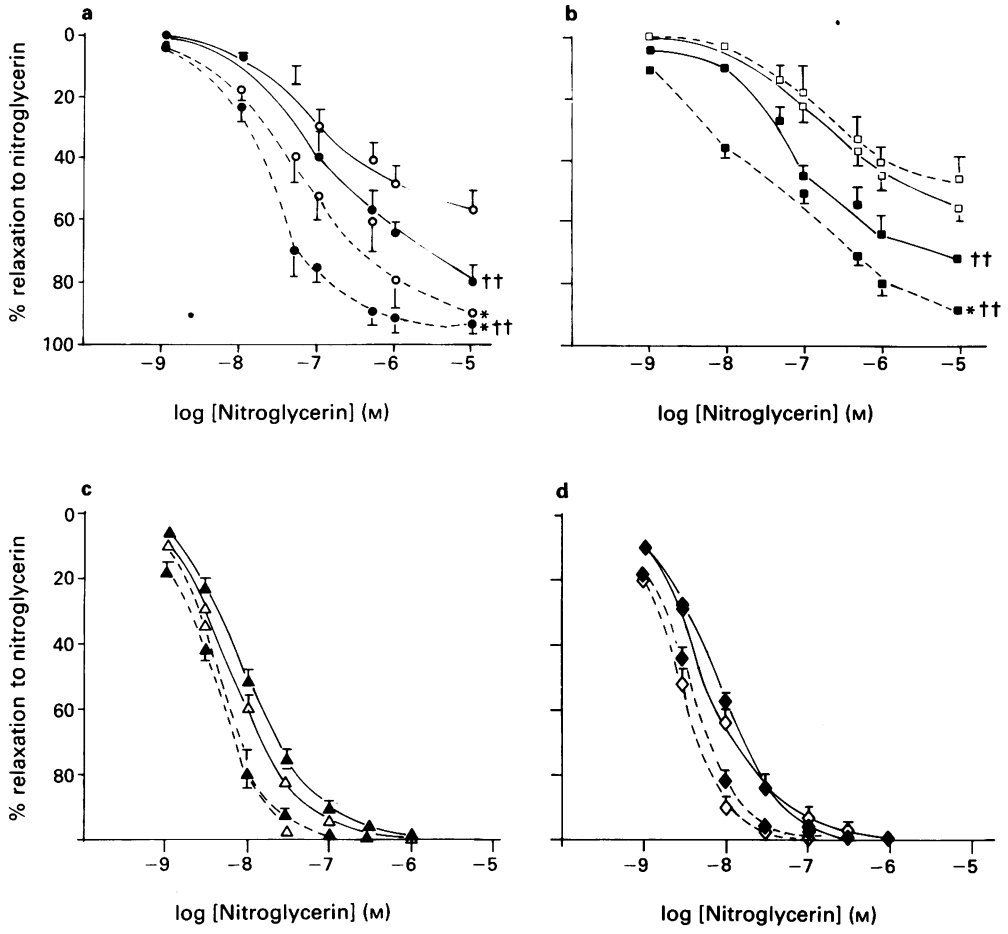

Figure 3 Cumulative relaxation concentration-effect curves constructed to nitroglycerin in (a) dorsal pedal arterial preparations, (b) saphenous vein preparations, (c) left anterior descending coronary artery and (d) circumflex coronary artery before and at maximally developed congestive heart failure (CHF). Tone was induced with prostaglandin $F_{2\alpha}$. Open symbols represent data obtained before induction of CHF and closed symbols results obtained when CHF was maximally developed. Broken lines were from endothelium denuded and solid lines from intact preparations. Each point is the mean and the vertical lines show s.e.mean for 5–15 preparations derived from 5 dogs. * Represents significant difference in maximum relaxation between endothelium-denuded and intact preparations ($P < 0.05$). †† Represents significant differences between control and heart failure.

(Figure 3a). In addition, denuded rings from these vessels were significantly more sensitive to nitroglycerin in both control and when CHF was maximal, as shown by a significantly lower EC_{50} value (Table 2). Before CHF, endothelium removal from the saphenous vein did not modify the vasodilatation observed with nitroglycerin (Figure 3b). However, nitroglycerin became a more potent relaxing agent of the saphenous vein at peak CHF as shown in Figure 3b.

In Figures 3(c and d) cumulative concentration-effect curves generated to nitroglycerin in the left anterior descending coronary artery and the circumflex coronary artery in control and CHF rings are shown. Nitroglycerin (10^{-9} M–10^{-6} M) produced concentration-dependent relaxations of all precontracted preparations, reaching a maximum relaxation of 100%. This figure shows that the concentration-effect curves constructed to nitroglycerin in denuded rings have a tendency to lie to the left of the curves generated in intact rings. In all cases, except in the left anterior descending coronary artery and the saphenous vein before the development of CHF, the potency of nitroglycerin was significantly increased by endothelium-removal (Table 2).

Table 2 EC_{50} (nM) values for nitroglycerin on intact and denuded blood vessels before and at maximally developed congestive heart failure (CHF)

	EC_{50}			
	Control		*CHF*	
	Intact	*Denuded*	*Intact*	*Denuded*
Dorsal pedal artery	350 (150–790)	36* (31–43)	88† (61–130)	36* (20–61)
Saphenous vein	500 (240–1020)	280 (130–610)	61† (42–88)	30†* (17–53)
Left anterior descending	8.3 (5.3 ± 12.8)	5.4 (4.2–6.9)	10.4 (7.9–14.6)	4.4* (3.1–6.3)
Circumflex	6.5 (4.3–9.9)	3.0* (2.4–3.8)	7.7 (6.1–9.6)	4.0* (3.1–5.2)

Values shown represent the geometric mean with 95% confidence limits in parentheses of $n = 5$–19 observations from 5 control and 5 CHF dogs.
* $P < 0.05$, endothelium-denuded versus intact preparations.
† $P < 0.05$, CHF versus control.

Discussion

This study clearly demonstrates that nitroglycerin produces concentration-dependent relaxations in blood vessels from control and dogs with pacing-induced heart failure. When CHF was maximally developed, the relaxant effects of nitroglycerin were enhanced, compared to control, in the dorsal pedal artery and the saphenous vein. In both control dogs and those with CHF the coronary arteries were much more reactive and sensitive to nitroglycerin compared to the peripheral blood vessels. However, in contrast to the peripheral vessels, the response of the coronary arteries to nitroglycerin was not enhanced in severe CHF. Furthermore, a most interesting and novel finding of the study was the discovery that the endothelium modulates the vasodilator effects of nitroglycerin in both peripheral and coronary blood vessels. All vessels with a functional endothelium were less sensitive, i.e. concentration-effect curves to nitroglycerin were displaced to the left in all prep-

arations denuded of endothelium relative to those from the same vessel except for the saphenous vein in CHF. In addition, the presence of the endothelium in the dorsal pedal artery and the saphenous vein enhanced the magnitude of the relaxant effects to nitroglycerin in pacing-induced CHF.

Following the development of CHF, neither $PGF_{2\alpha}$ nor KCl responses were changed in the coronary blood vessels, but responses to $PGF_{2\alpha}$ were enhanced in both the dorsal pedal artery and the saphenous vein (independently of endothelial removal). These data imply that, in the periphery, changes occur in the prostaglandin-receptor activation process, suggesting that the $PGF_{2\alpha}$ receptor mechanism in the peripheral vasculature differs from that in the coronary bed. We have previously demonstrated an increase in α_1-adrenoceptor activation in peripheral vessels from dogs with pacing-induced CHF (Forster *et al.*, 1989). It is possible that the $PGF_{2\alpha}$ and α_1-adrenoceptor-mediated effects are due to changes in phosphoinositol turnover since, in some situations, both have been shown to utilize this post-receptor activation process (Butcher & Putney, 1980; Aub & Putney, 1985).

In vessels from dogs with CHF, the response of endothelium to acetylcholine remained unaltered in both peripheral and coronary blood vessels, even though the response of the dorsal pedal artery differed from that of the saphenous vein. As shown previously, in the dorsal pedal artery, acetylcholine produced a maximum relaxation of 51% and 46% (before and at peak CHF respectively), and the relaxation response seen in the saphenous vein was even less (16% and 18%, before and at peak CHF respectively; Forster *et al.*, 1989). Above all, the responses to acetylcholine of the coronary blood vessels were greater in magnitude compared to the peripheral vessels. This difference can be assumed to be unrelated to the initial contractile tension developed in the tissue, since the dorsal pedal artery always showed a lower developed tension to KCl compared with the saphenous vein and the coronary arteries. In addition the concentrations of KCl and $PGF_{2\alpha}$ chosen always represented an approximate EC_{50}. That the maximum relaxant effect of acetylcholine was always close to 100% in coronary arteries is in agreement with results from other studies (Furchgott, 1983). The intermediate relaxant efficacy of acetylcholine in intact dorsal pedal artery segments is consistent with data concerning the activity of acetylcholine in the femoral artery (Rubanyi *et al.*, 1987). According to Furchgott (1983), veins generally relax less in response to acetylcholine compared to arteries, which is consistent with the current findings. Potential reasons for this include a masking of endothelium-dependent relaxations by a direct contracting effect of acetylcholine on venous muscle (De Mey & Vanhoutte, 1982), or the release of a specific contracting substance from the venous endothelium cells. In the absence of a contractile response, the current study suggests that this could not be the explanation.

It has been suggested that vascular relaxation is impaired in blood vessels which have been pretreated with nitroglycerin *in vivo* and *in vitro* (Axelsson & Andersson, 1983; Molina *et al.*, 1987; Abdollah *et al.*, 1987). In our experiments, endothelial removal increased the sensitivity of the vascular preparations to the relaxing effects of nitroglycerin. In the current study all vessels had been treated with acetylcholine before nitroglycerin but only those with an intact endothelium produced a relaxation to acetylcholine. It is therefore likely that endothelium-dependent relaxing agents and the nitrovasodilators produce relaxations through similar mechanisms i.e. the formation of nitric oxide and thus activation of soluble guanylate cyclase (Ignarro *et al.*, 1988). Although direct evidence is unavailable, one could speculate that, in preparations with an intact endothelium, desensitization to nitroglycerin-induced relaxation might occur as a result of acetylcholine pretreatment. When the endothelium is removed, no relaxation occurs to acetylcholine and the response to nitroglycerin, therefore, appears enhanced. Generally, maximal relaxation (100%) occurred in the coronary vessels, whereas, only 50% relaxation occurred in the dorsal pedal artery to acetylcholine and 50–70% relaxation occurred with nitroglycerin. This difference may imply that the guanylate cyclase activation processes may differ in the coronary as compared to the peripheral blood vessels. For instance, nitric oxide formation in the coronaries may be more efficient compared to the peripheral beds. Desensitization to nitroglycerin has been associated with reduced accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Axelsson & Andersson, 1983), suggesting that the tolerance to nitroglycerin involves two components: firstly, impaired metabolism and conversion to nitric oxide and secondly that a stable modification in the guanylate cyclase occurs rendering it inactive. Presumably the decreased relaxation to endothelium-independent vasodilators in nitroglycerin-tolerant vessels would involve the latter mechanism. Moreover, it remains possible that such endothelial-dependency could simply reflect more effective drug penetration to smooth muscle cells.

Having discussed differences in nitroglycerin responsiveness between coronary and peripheral blood vessels, and the role played by the endothelium, we must now consider the impact of pacing-induced CHF. Peripheral vessels with an intact endothelium were the least sensitive to nitroglycerin before CHF but became more sensitive when CHF was maximal. Even though the peripheral vessels became more sensitive to nitroglycerin when CHF was maximally developed, they were still not as sensitive as the coronary vessels. With the above in mind, it is interesting to consider the relative EC_{50} ratio of denuded: intact in each of the blood vessels studied. In all blood vessels, with the exception of the control dorsal pedal artery, this ratio is 1:2; the ratio for the control dorsal pedal artery being 1:10. Although there are anatomical differences between the coronary arteries and the peripheral blood vessels, this information implies that, during CHF, the factors governing sensitivity are identical to those in control saphenous vein and control coronary arteries. The fact that, before CHF, the dorsal pedal arterial endothelium attenuates the action of nitroglycerin to a much greater extent than the other blood vessels studied implies a unique action of the endothelium on the metabolic processes which control guanylate cyclase activation.

In CHF, relaxation responses to nitroglycerin were significantly enhanced in peripheral blood vessels with the endothelium intact. This may suggest that guanylate cyclase activation has a low threshold in intact peripheral vessels from dogs before the development of CHF. In addition, the log concentration-response curves for relaxation in all intact peripheral vessels were shifted to the right during CHF, implying the existence of a metabolic or diffusional barrier to the guanylate cyclase activation process. A further explanation of these findings may be that the endothelium acts as a physiological antagonist to excessive vasodilatation by releasing a contractile factor (Yanagisawa *et al.*, 1988).

Although this current study was peformed on isolated blood vessels from male mongrel dogs with pacing-induced CHF, it is interesting to speculate on the potential clinical significance of such findings. For instance, if the endothelium is damaged and increased likelihood of coronary vasospasm is prevalent (Cohen *et al.*, 1984), then the augmented relaxant effect of nitroglycerin would seem to favour its use as a coronary vasodilator. The fact that limited relaxation occurs in the peripheral vascular bed implies that, in a region which maintains vascular resistance, the ability to relax to both endothelium-dependent and -independent agents is limited. Finally, these findings indicate that relaxations due to nitroglycerin cannot be considered to be entirely as endothelium-independent as previously thought (Diamond & Chu, 1983; Rubanyi & Vanhoutte, 1985), but should be considered as endothelium-modulated.

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Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells

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1 The effects of phentolamine and yohimbine on adenosine 5'-triphosphate (ATP)-sensitive K^+ channels were studied in normal mouse β-cells.

2 In the presence of 3 mM glucose, many ATP-sensitive K^+ channels are open in the β-cell membrane. Under these conditions, phentolamine inhibited ^{86}Rb efflux from the islets. This inhibition was faster with 100 than with 20 μM phentolamine but its steady-state magnitude was similar with both concentrations. Yohimbine (20–100 μM) also inhibited the efflux rate but was not as potent as phentolamine.

3 In the presence of 6 mM glucose, most ATP-sensitive K^+ channels are closed in the β-cell membrane. Their opening by 100 μM diazoxide caused a marked acceleration of ^{86}Rb efflux from the islets. This acceleration was almost entirely prevented by 20 μM phentolamine. It was barely affected by 20 μM yohimbine and reduced by 50% by 100 μM yohimbine.

4 ATP-sensitive K^+ currents were studied in single β-cells by the whole cell patch-clamp technique. Phentolamine (20–100 μM) caused a progressive but almost complete and irreversible inhibition of the current. The effects of yohimbine were faster but smaller; the inhibition was still incomplete with 100 μM yohimbine.

5 The increase in ATP-sensitive K^+ current produced by 100 μM diazoxide was prevented by 100 μM phentolamine but only partially attenuated by 100 μM yohimbine.

6 It is concluded that phentolamine inhibits ATP-sensitive K^+ channels in pancreatic β-cells. This novel effect of phentolamine resembles that of hypoglycaemic sulphonylureas. It may account for previously unexplained effects of the drug. These observations also call for reinterpretation of many studies in which phentolamine was used as an allegedly specific blocker of α-adrenoceptors.

Introduction

Phentolamine is a classical blocker of α-adrenoceptors. However, its use has sometimes led to the erroneous conclusion that these receptors are involved in certain physiological or pharmacological events.

Because activation of α_2-adrenoceptors inhibits insulin release (Nakaki *et al.*, 1980), and because phentolamine reverses the inhibition of insulin release provoked by diazoxide (Blackard & Aprill, 1967; Burr *et al.*, 1971; Porter *et al.*, 1978), it was first thought that the latter drug activates α-adrenoceptors in β-cells. Subsequent studies showed that, among α-adrenoceptor antagonists, phentolamine (α_1, α_2), but not yohimbine (α_2) or dihydroergotamine (α_1, α_2) could prevent the acceleration of ^{86}Rb efflux and the inhibition of insulin release caused by diazoxide in rat islets (Henquin *et al.*, 1982). It was thus concluded that the effect of phentolamine was not mediated by α-adrenoceptors, but resulted from its ability to counteract the increase in K^+ permeability caused by diazoxide (Henquin & Meissner, 1982). The mechanism of action could not be further analyzed because it was only discovered later that diazoxide opens ATP-sensitive K^+ channels in β-cells (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988).

More recently it has been reported that phentolamine reverses the hyperpolarization and the relaxation of vascular and tracheal muscles caused by cromakalim (McPherson & Angus, 1989; Murray *et al.*, 1989). Since cromakalim opens ATP-sensitive K^+ channels in smooth muscle (Standen *et al.*, 1989), it is possible that phentolamine interferes with the action of cromakalim at the K^+ channel level.

In the present study we have investigated whether phentolamine has direct effects on ATP-sensitive K^+ channels in mouse pancreatic β-cells. The effects of phentolamine were also compared to those of yohimbine.

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Methods

All experiments were carried out on islets obtained by collagenase digestion of the pancreas of fed female NMRI mice. ^{86}Rb efflux experiments were performed at 37°C and patch-clamp experiments at room temperature (20–24°C).

For measurement of the efflux of ^{86}Rb (used as tracer for K), the islets were first loaded with the tracer for 90 min in a medium containing 15 mM glucose and supplemented with $^{86}RbCl$ (1.5 to 3 MBq ml^{-1}; sp.act. 7.4 to 18.5 TBq mol^{-1}). The concentration of Rb never exceeded 0.4 mM (Garrino & Henquin, 1988). The islets were then transferred to a dynamic system of perifusion, permitting ^{86}Rb efflux to be monitored (Henquin, 1978). The solutions used had the following ionic composition (in mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24. They were gassed with O_2/CO_2 (94:6) to maintain a pH of 7.4 and were supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany).

For patch-clamp measurements, islets were first dispersed into single cells which were then cultured for 1–2 days (Plant, 1988). Recordings were made in a bath solution containing (in mM): NaCl 135, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10, and titrated to pH 7.4 with NaOH. Pipettes were filled with a solution containing (in mM): KCl 135, $MgCl_2$ 4, $CaCl_2$ 2, EGTA 10, Na_2ATP 0.65, Na_2GTP 0.1, HEPES 20, titrated to pH 7.15 with KOH. This solution has a low free Ca^{2+} concentration (50 nM) and a lower than physiological concentration of ATP. The technique of whole-cell recording and the method used to isolate ATP-sensitive K^+ currents from other membrane currents have been described previously (Garrino

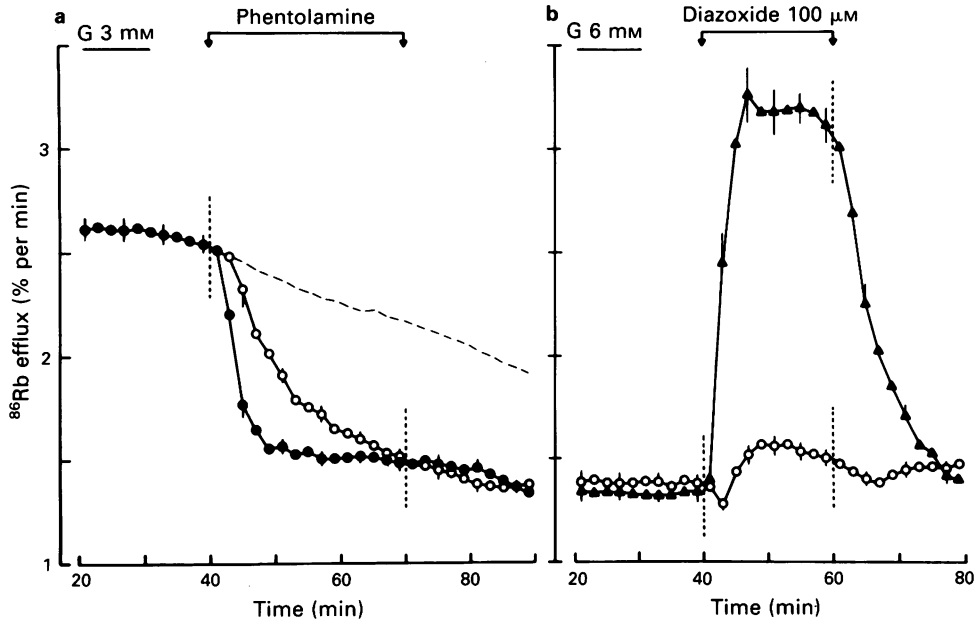

Figure 1 Effects of phentolamine on ^{86}Rb efflux from mouse islets. (a) Phentolamine was added at the concentration of 20 μM (○) or 100 μM (●) to a medium containing 3 mM glucose (G). Controls without phentolamine are shown by the broken line. (b) Diazoxide (100 μM) was added to a medium containing 6 mM glucose alone (▲) or with 20 μM (○) phentolamine. Values are means for 5 experiments with s.e.mean shown by vertical lines.

et al., 1989). Briefly, currents were measured at 15 s intervals at the holding potential (−70 mV), and in response to 100 ms voltage steps to −60 and −80 mV separated by 100 ms. At these potentials, which are more negative than the thresholds for the activation of voltage-dependent Ca^{2+} and K^+ channels, and with the intracellular medium used, the current which develops with time is almost entirely a current through ATP-sensitive K^+ channels. This is indicated by the near complete block of the current by 100 μM tolbutamide, a specific blocker of ATP-sensitive K^+ channels (Trube *et al.*, 1986).

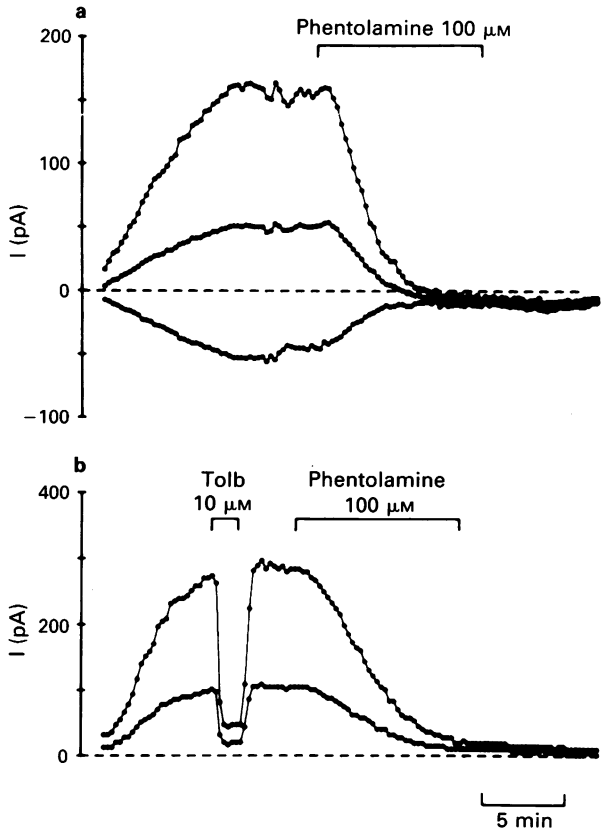

Figure 2 Inhibition of ATP-sensitive K^+ currents by 100 μM phentolamine in mouse β-cells. (a) The upper, middle and lower traces show currents recorded at −60, −70 and −80 mV respectively. (b) Comparison of the inhibition produced by 10 μM tolbutamide (Tolb) and 100 μM phentolamine. Currents were recorded at −60 mV (upper trace) and −70 mV (lower trace). The values shown correspond to currents measured at 15 s intervals.

To study the voltage-dependent K^+ currents in the absence of ATP-sensitive K^+ currents, 100 μM tolbutamide was added to the bathing solution. The solutions were otherwise the same as those used for the measurement of ATP-sensitive K^+ currents. Voltage-dependent K^+ currents activate at potentials above −40 mV (Rorsman & Trube, 1986). To monitor the effect of phentolamine, cells were held at a potential of −70 mV and hyperpolarized for 50 ms to −100 mV, to measure the leakage current. This pulse was followed after 100 ms by a 150 ms step to 0 mV which activates voltage-sensitive K^+ currents. The Ca^{2+}- and voltage-activated K^+ current, which is also present in these cells, is not activated when cells are dialysed with pipette solutions containing the high concentration of calcium buffer (10 mM EGTA, 2 mM Ca) which was used here (Satin *et al.*, 1989). Recordings were started 30 s after rupturing the membrane patch and entering the whole-cell configuration.

Phentolamine mesylate was obtained from Ciba Geigy (Basel, Switzerland), yohimbine hydrochloride was from Aldrich Chemie (Steinheim, F.R.G.), tolbutamide from Hoechst A.G. (Frankfurt, F.R.G.) and diazoxide was provided by Schering Corp. (Bloomfield, NJ, U.S.A.). Stock solutions of diazoxide (40 mM) were prepared in 0.1 M NaOH.

Results

Effects of phentolamine on ^{86}Rb efflux

In the presence of 3 mM glucose, many ATP-sensitive K^+ channels are open in the β-cell membrane (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988). Under these conditions, the rate of ^{86}Rb efflux is high and decreases slightly with time (Figure 1a). Addition of phentolamine to the medium inhibited ^{86}Rb efflux. The rate of this inhibition was higher with 100 than with 20 μM phentolamine, but its steady-state magnitude was similar with both concentrations of the drug.

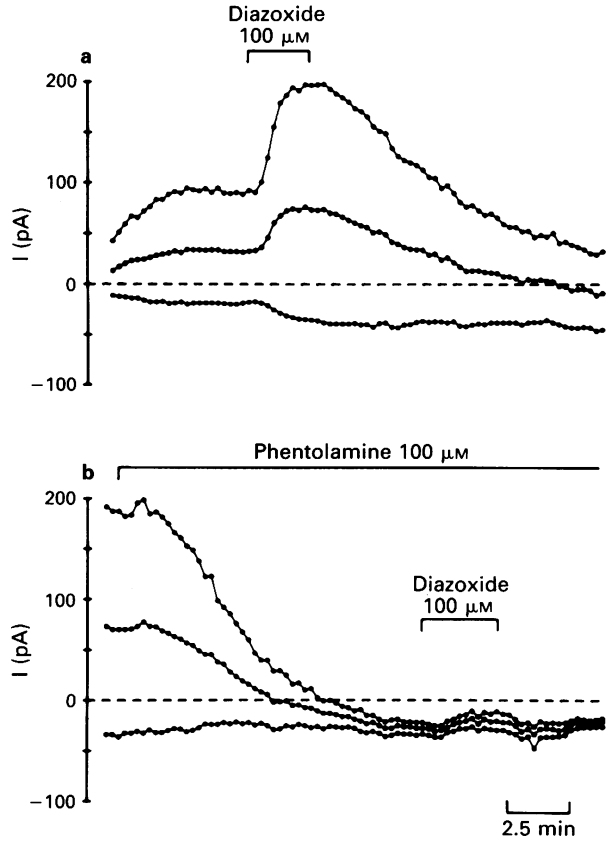

Figure 3 Effects of 100 μM diazoxide on ATP-sensitive K^+ currents in mouse β-cells, (a) under control conditions and (b) in the presence of 100 μM phentolamine. In both parts of the figure the upper, middle and lower traces are the currents recorded at intervals of 15 s at −60, −70 and −80 mV respectively.

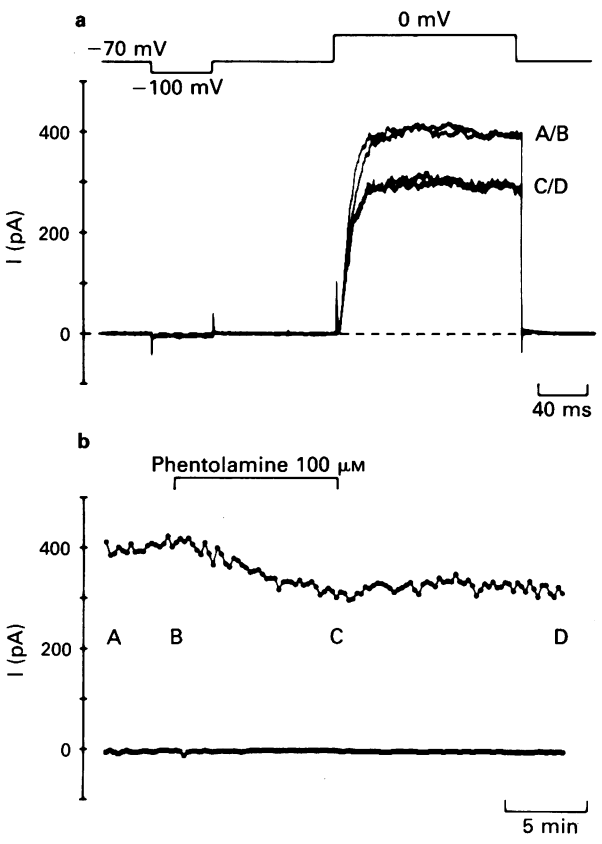

Figure 4 Effects of phentolamine on voltage-dependent K^+ currents in a mouse β-cell. (a) Currents recorded in response to the illustrated protocol at the beginning of the experiment (A), just before adding phentolamine (B), after 10 min of phentolamine application (C), and after washing for 14 min with a phentolamine-free solution (D). (b) Time-course of the effect of phentolamine: the times at which the currents shown in the upper part were recorded are indicated by capital letters. The lower trace near the 0 value shows that the leakage current measured during the hyperpolarizing pulse to −100 mV was not influenced by phentolamine.

In the presence of 6 mM glucose, most ATP-sensitive K^+ channels are closed in the β-cell membrane (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988), and the rate of ^{86}Rb efflux is lower and more stable than in 3 mM glucose (Figure 1b). Addition of 100 μM diazoxide markedly accelerated ^{86}Rb efflux. This acceleration was almost totally prevented by 20 μM phentolamine. This confirms our previous observation made in rat islets (Henquin *et al.*, 1982).

Effects of phentolamine on ATP-sensitive K^+ currents

At physiological ATP concentrations, only a small fraction of ATP-sensitive K^+ channels are active and whole cell currents are very small (Rorsman & Trube, 1985). As shown previously (Trube *et al.*, 1986; Garrino *et al.*, 1989), ATP-sensitive K^+ currents increase following breakage of the patch membrane and dialysis of the cell interior with a pipette solution containing less than the physiological concentration of ATP (Figure 2). In these experiments, with 0.65 mM ATP in the pipette solution, currents were not as large as those seen with lower ATP concentrations but were more stable, i.e. showed less run-down, allowing more accurate quantification of the changes produced by test agents.

After the currents had attained a steady level, addition of 100 μM phentolamine to the bathing solution resulted in a slow but very marked decrease in the ATP-sensitive K^+ current (Figure 2a). Currents were decreased by 94.3 ± 0.8% (mean ± s.e.mean, $n = 10$) compared to the current recorded before the addition of the drug. Figure 2b shows that 10 μM tolbutamide rapidly and reversibly blocked ATP-sensitive K^+ channels. This effect was much faster than that of phentolamine although the maximum block (88.3 ± 1.1%, $n = 6$) produced by this low concentration of the sulphonylurea was less than that produced by 100 μM phentolamine. Effects of similar magnitude, though slower than those observed with 100 μM, were observed with 20 and 50 μM phentolamine (results not shown). The effects of phentolamine were irreversible.

Since phentolamine inhibits the increase in ^{86}Rb efflux induced by diazoxide (Figure 1b), we tested the action of diazoxide on ATP-sensitive K^+ currents in the presence of phentolamine. Diazoxide increases ATP-sensitive K^+ currents in β-cells, the magnitude of its effect being dependent on the intracellular ATP concentration (Zünkler *et al.*, 1988). The effects of diazoxide under the conditions used in these experiments are shown in Figure 3a. On addition of diazoxide, the amplitude of the current change in response to a potential step from −70 to −60 mV increased by 65.9 ± 9.2 pA (10.5 ± 1.4 pA/pF; $n = 7$). Relative to the initial control current (100%), this corresponds to an increase to 268 ± 50%. Diazoxide had much less effect in the presence of 100 μM phentolamine (Figure 3b). It increased the evoked current by only 3.2 ± 0.4 pA (0.5 ± 0.1 pA/pF; $n = 5$), that is from 5.7 ± 0.8% to 9.1 ± 1.1% of the initial control current.

Effects of phentolamine on voltage-sensitive K^+ currents

To determine whether phentolamine specifically affects ATP-sensitive K^+ channels, we tested its effects on voltage-sensitive K^+ channels which are also present in the β-cell membrane. A 10 min application of 100 μM phentolamine decreased the voltage-sensitive K^+ current at 0 mV by 30.2 ± 3.3% ($n = 4$) compared to the control value. Neither the time-course of the voltage-sensitive current, nor the leakage current were affected by the treatment with phentolamine (Figure 4). As with the

ATP-sensitive K^+ current, the effects of phentolamine were irreversible.

Effects of yohimbine on ^{86}Rb efflux

In the presence of 3 mM glucose, yohimbine marginally decreased ^{86}Rb efflux from islet cells at the concentration of 20 μM, but caused a clear and reversible inhibition at 100 μM (Figure 5a). In the steady state this inhibition was less than that produced by 20 or 100 μM phentolamine. The acceleration of ^{86}Rb efflux brought about by 100 μM diazoxide in the presence of 6 mM glucose was only slightly attenuated by 20 μM yohimbine and reduced by half by 100 μM yohimbine (Figure 5b).

Effects of yohimbine on ATP-sensitive K^+ currents

Addition of 100 μM yohimbine to the medium resulted in an inhibition of the ATP-sensitive K^+ current which was more rapid than that observed with phentolamine. The inhibition was incomplete compared to the inhibition by 100 μM tolbutamide (Figure 6) and was, at least partially, reversible. In the steady-state, currents were decreased by 77.8 ± 1.3% (n = 5). Small effects of yohimbine were already detectable at a concentration of 10 μM.

On addition of 100 μM diazoxide to a medium containing 100 μM yohimbine, the amplitude of the current change in response to potential steps from −70 to −60 mV increased by 73.0 ± 12.2 pA (11.9 ± 2.2 pA/pF, n = 5). Relative to the initial control current (100%), this corresponds to an increase from 22.2 ± 1.3% in yohimbine alone to 97.9 ± 12.0% in yohimbine plus diazoxide.

Discussion

The present study conclusively shows that phentolamine inhibits ATP-sensitive K^+ channels in pancreatic β-cells. This conclusion is based on three sets of observations. Firstly, phentolamine decreased ^{86}Rb efflux from islets perifused with a low concentration of glucose. The only K^+ channels that are known to be open under these conditions are the ATP-sensitive K^+ channels (Rorsman & Trube, 1985; Ashcroft *et al.*, 1988; Misler *et al.*, 1986; Petersen & Findlay, 1987). More-

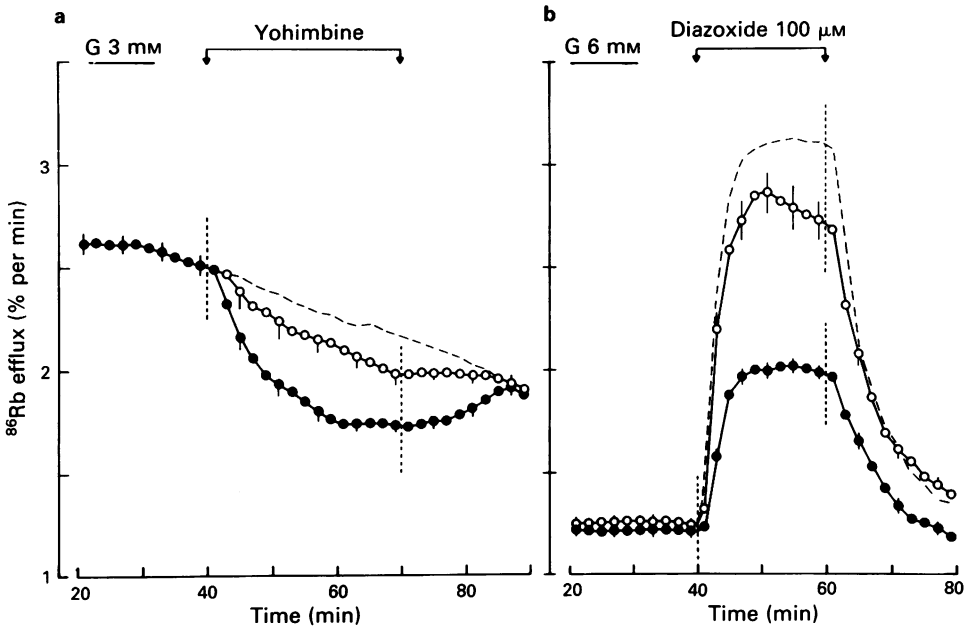

Figure 5 Effects of yohimbine on ^{86}Rb efflux from mouse islets. (a) Yohimbine was added at the concentration of 20 μM (○) or 100 μM (●) to a medium containing 3 mM glucose (G). (b) Diazoxide (100 μM) was added to a medium containing 6 mM glucose and 20 μM (○) or 100 μM (●) yohimbine. Controls without yohimbine are shown by the broken lines. Values are means for 4 experiments with s.e.mean shown by vertical lines.

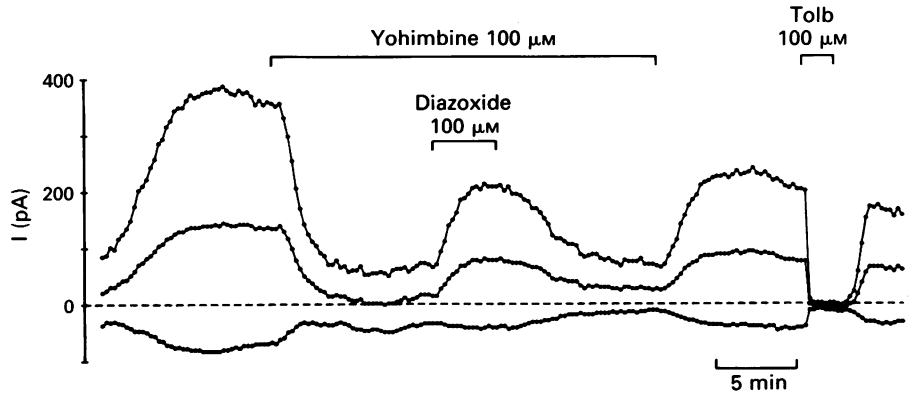

Figure 6 Inhibition of ATP-sensitive K^+ currents by 100 μM yohimbine in a mouse β-cell. Diazoxide (100 μM) was tested in the presence of yohimbine. Following washout of yohimbine, 100 μM tolbutamide (Tolb) was added to the bathing solution. The upper, middle and lower traces show currents recorded at −60, −70 and −80 mV respectively. The values shown correspond to currents measured at 15 s intervals.

over, the only drugs that are known to inhibit ^{86}Rb efflux in the presence of a low concentration of glucose are those which block ATP-sensitive K^+ channels. The best known of them are the hypoglycaemic sulphonylureas (Henquin & Meissner, 1982; Trube *et al.*, 1986). Secondly, ATP-sensitive K^+ currents measured in the whole cell configuration as described by Trube *et al.* (1986) were totally suppressed by phentolamine as they were by tolbutamide. Thirdly, phentolamine prevented the acceleration of ^{86}Rb efflux and the increase in ATP-sensitive K^+ currents otherwise produced by diazoxide, which is currently considered to be a selective activator of ATP-sensitive K^+ channels in β-cells (Trube *et al.*, 1986).

The present observations give direct support to previous presumptions that certain effects of phentolamine might be unrelated to blockade of α_2-adrenoceptors. An inhibition of ATP-sensitive K^+ channels may account for the ability of phentolamine to increase insulin release in the absence of an α-adrenoceptor agonist (Efendic *et al.*, 1975; Smith & Furman, 1988; Schulz & Hasselblatt, 1988; Garrino & Henquin, 1990) and to counteract the inhibition of release caused by diazoxide (Henquin *et al.*, 1982). On the other hand, it may now seem surprising that yohimbine does not share the properties of phentolamine on insulin release since it also inhibits ATP-sensitive K^+ channels. This is probably explained by the fact that yohimbine was generally tested at concentrations of 1–30 μM (Henquin *et al.*, 1982; Hillaire-Buys *et al.*, 1985; Schulz & Hasselblatt, 1988; Garrino & Henquin, 1990) and that the inhibition of ATP-sensitive K^+ channels is still incomplete at 100 μM.

A recent study has ascribed the insulin-releasing effect of phentolamine to its imidazoline structure rather than to its α-adrenoceptor blocking property (Schulz & Hasselblatt, 1989). This conclusion was based, firstly, on the evidence that the concentrations of phentolamine required to increase insulin release were higher than those required to counteract the inhibition by clonidine, and, secondly, on the insulin-releasing effects of imidazoline derivatives that do not block α-adrenoceptors. It is now well established that α-adrenoceptor agonists and antagonists which possess an imidazoline structure bind with high affinity to sites other than the α-adrenoceptor itself (Michel & Insel, 1989). The nature of these imidazoline binding sites is still unknown. The present study might suggest that ATP-sensitive K^+ channels are one of these sites, were not yohimbine, which is not an imidazoline, also able to inhibit these channels. One could even argue that the mere fact that both phentolamine and yohimbine inhibit K^+ channels indicates that this inhibition is related to blockade of the α-adrenoceptors. This problem is not easy to solve because all attempts to demonstrate directly that ATP-sensitive K^+ channels are opened by α_2-adrenoceptor agonists have been unsuccessful. Nevertheless, one observation does not support the above hypothesis: the concentrations of blockers that affect K^+ channels are much higher than those required to block the effects of α-agonists.

The mechanisms by which phentolamine blocks ATP-sensitive K^+ channels are not established, and their investigation was beyond the scope of this study. Some features of this inhibition deserve comment, however. The rate of inhibition by phentolamine was much lower than that by tolbutamide, and was dose-dependent, whereas the steady-state effect was not dose-dependent. These characteristics and the poor reversibility of the inhibition suggest that phentolamine has no direct or easy access to the blocking site of the channel. It is unlikely, however, that the effect is non-specific and the mere consequence of the accumulation of phentolamine in the membrane since voltage-dependent K^+ channels were only marginally affected. The inhibition of ATP-sensitive K^+ channels by yohimbine differed from that by phentolamine by its greater rapidity, reversibility and dose-dependency. Distinct mechanisms are thus likely to be involved.

The present findings have two important pathophysiological implications. Firstly, they call for reinterpretation of numerous human and animal studies in which phentolamine was used as an allegedly specific blocker of α-adrenoceptors. In particular, the role of the α-adrenergic control of insulin release in the absence of stress conditions may have been overestimated (Cerasi *et al.*, 1969; Buse *et al.*, 1970; Misbin *et al.*, 1970; Lundquist, 1972; Robertson & Porte, 1973). Secondly, our findings cast doubts on the hypothesis that an excessive α-adrenergic tone contributes to the impaired release of insulin in non-insulin-dependent diabetic patients (Robertson *et al.*, 1976). A cornerstone of this hypothesis was indeed the observation that phentolamine increased plasma insulin levels in these patients. A clinical study using idazoxan instead of phentolamine had already questioned this concept (Ostenson *et al.*, 1988).

In conclusion, both phentolamine and yohimbine are able to inhibit ATP-sensitive K^+ currents in pancreatic β-cells. Our findings suggest that care must be taken when interpreting results obtained with high concentrations of these blockers of α-adrenoceptors.

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Inhibition of endothelial-bound angiotensin converting enzyme, *in vivo*

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1 We determined apparent K_i constants of two inhibitors, captopril and CL242,817, for pulmonary endothelial-bound angiotensin converting enzyme (ACE) in anaesthetized rabbits. [^{3}H]-benzoyl-Phe-Ala-Pro was used as the substrate. The apparent kinetic parameters K_m and A_{max} (product of V_{max} and microvascular plasma volume) were measured, as was the ratio (A_{max}/K_m) (measured under first order reaction conditions) before and 30 s after the i.v. administration of captopril 10 nmol kg^{-1} or CL242,817, 35 nmol kg^{-1}.

2 Under mixed order reaction conditions, ([S] $\geqslant K_m$), apparent K_m values increased from 12.2 ± 1.9 μM to 32.9 ± 3.3 μM ($P < 0.05$) in the captopril-treated rabbits and from 9.3 ± 2.3 μM to 45.8 ± 9.8 μM ($P < 0.05$) in the CL242,817-treated rabbits, indicative of competitive inhibition. However, apparent A_{max} values decreased from 10.3 ± 2.1 to 4.5 ± 0.8 μmol min^{-1} ($P < 0.05$) and 8.9 ± 1.7 to 4.8 ± 0.5 μmol min^{-1} ($P < 0.05$), respectively.

3 Under first order reaction conditions ([S] $\ll K_m$), the A_{max}/K_m ratio decreased from 763 ± 100 to 125 ± 38 ml min^{-1} ($P < 0.05$) and 1009 ± 149 to 126 ± 44 ml min^{-1} ($P < 0.05$) in the captopril- and CL242,817-treated groups respectively.

4 When the single pass transpulmonary binding of 80 pmol [^{3}H]-RAC-X-65 (an ACE inhibitor) was measured in additional rabbits, a significant ($P < 0.05$) decrease in RAC-X-65 binding was observed 30 s after captopril (80% decrease) or CL242,817 (85% decrease), a result expected for a loss of catalytically active enzyme mass due to tightly bound captopril or CL242,817.

5 These results indicate that, *in vivo*, both captopril and CL242,817 are competitive, tight binding inhibitors of lung ACE. Furthermore, they suggest means for evaluating the interaction of other potential ACE inhibitors with the pulmonary endothelial membrane-bound enzyme, *in vivo*, possibly in phase I clinical trials.

Introduction

Angiotensin converting enzyme inhibitors (ACEI) are useful in the pharmacological management of moderate and severe hypertension (Gavras *et al.*, 1978; Mackaness, 1985). While it is sometimes postulated that ACEI exert their therapeutic action via complex mechanisms, a primary means of action is still believed to be through inhibition of angiotensin converting enzyme (ACE, kininase II, EC 3.4.15.1) (Ondetti & Cushman, 1984). ACE is located primarily on the cell membrane of endothelial cells of most organs of species studied thus far. However, most of the formation of angiotensin II from angiotensin I and degradation of bradykinin is believed to be catalyzed by pulmonary microvascular endothelial ACE, probably because of the vast enzyme concentration and higher substrate availability in the pulmonary microvasculature relative to other tissues (Ryan, 1983). Pre-clinical and clinical trials of the activities of new ACEI have mostly relied on comparing systemic blood pressure responses to i.v. angiotensin I before vs. after acute or chronic administration of ACEI (Antonaccio, 1983). If feasible, direct estimations of drug and membrane-bound enzyme interactions, *in vivo*, would provide important information on the mode of action of ACEI under various clinical conditions. *In vivo* monitoring of lung ACE inhibition by ACEI would be particularly desirable since correlation of antihypertensive activity with inhibition of plasma or serum ACE by various ACEI is at best problematic (Chen *et al.*, 1984). We and others have previously reported on a technique for measuring apparent Michaelis-Menten kinetics of lung ACE for a synthetic substrate, in anaesthetized rabbits (Catravas & White, 1984; Howell *et al.*, 1984). We now describe an application of this methodology for characterizing and quantifying the inhibitory activity of ACEI on pulmonary endothelium-bound ACE, as reflected by measurements of apparent K_i values of captopril (the first ACEI in clinical use) and CL242,817 (a new ACEI) in anaesthetized rabbits. The new methodology has potential for use in the clinic, and can be used to clarify modes of action of new ACEI, *in vivo*.

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Methods

Thirty seven New Zealand white male rabbits, each weighing approximately 3.5 kg, were anaesthetized with a mixture of urethane (200 mg ml^{-1}) and allobarbitone (50 mg ml^{-1}), artificially ventilated and equipped with carotid arterial and right atrial catheters and heparinized as described previously (Catravas & White, 1984; Toivonen & Catravas, 1986; 1987).

Measurements of apparent enzyme kinetics for lung ACE

For each of eleven animals, four determinations of single pass transpulmonary hydrolysis of the synthetic ACE substrate [^{3}H]-benzoyl-Phe-Ala-Pro ([^{3}H]-BPAP; 20 μCi nmol^{-1}) were performed by use of indicator dilution techniques (Catravas & White, 1984; Toivonen & Catravas, 1986; 1987). Saline solution (0.9 ml) containing 0.37 mg indocyanine green and 2 μCi of [^{3}H]-BPAP (0.1 nmol) was rapidly injected into the jugular vein catheter and was washed in with 1 ml saline. Simultaneously, blood was withdrawn from the carotid catheter by means of a peristaltic pump (28 ml min^{-1}) into a fraction collector (advancing at the rate of one tube every 0.7 s) until 15 blood samples were collected. Each collection tube contained 2 ml of 1 mM captopril in saline to stop further metabolism of [^{3}H]-BPAP by blood ACE. Immediately after collection of the last blood sample, and without stopping the pump or collector, a second injection of 0.9 ml of saline containing 0.37 mg indocyanine green, 2 μCi[^{3}H]-BPAP and 1622 nmol non-radioactive BPAP was given into the jugular vein catheter,

and arterial blood was collected into another set of 15 tubes. These are referred to as determinations 1 and 2. Another set of two such determinations (determinations 3 and 4) of transpulmonary BPAP metabolism were similarly obtained such that determination number 3 began exactly 30 s after the administration of 10 nmol captopril kg^{-1} body weight (a gift from the Squibb Institute for Medical Research, $n = 6$), or 35 nmol CL242,817 kg^{-1} body weight (CL 242,817 is [S-(R*S) [1-(3-acetylthio)-3-benzoyl-2-methylpropionyl]-L-proline]; it was a gift from Lederle Laboratories, $n = 8$), i.v. Determination 4 began 60 s after inhibitor administration. All sample tubes were centrifuged to separate cells from plasma. The amounts of unchanged [^{3}H]-BPAP and the radioactive product of ACE conversion ([^{3}H]-benzoyl-Phe; [^{3}H]-BP) were estimated by liquid scintillation counting after acidification and extraction of [^{3}H]-BP (in 0.5 ml of supernatant from each tube) into toluene. Thirty to forty-five min elapsed between determinations 2 and 3. During that time, the volume of blood previously removed was replaced with 3% dextran (70,000 mol. wt.) in saline. Arterial pH, P_{CO_2} and P_{O_2} were measured before and after each pair of determinations and remained within normal limits throughout. We have previously shown that [^{3}H]-BPAP and [^{3}H]-BP do not leave the circulation during a single transpulmonary passage, as reflected in congruent arterial outflow concentration curves of total ^{3}H and co-injected indocyanine green or ^{125}I-albumin, two known intravascular indicators (Catravas & Gillis, 1981). We have also shown that because of its much lower concentration, plasma ACE contributes minimally to the metabolism of [^{3}H]-BPAP thus measured (Catravas & Gillis, 1981; Catravas & White, 1984). Data were analyzed as follows, under either mixed order or first order reaction conditions.

Mixed-order method

We calculated the apparent parameters K_m and A_{max} (product of V_{max} and microvascular plasma volume; see Catravas & White, 1984) using data combined from determinations 1 and 2 (approximately 10–16 points) and apparent K'_m and A'_{max} using values from determinations 3 and 4 (after inhibitor administration). Data were fitted by linear regression to the Eadie-Scatchard transformation (equation 1) of the integrated Henri-Michaelis-Menten equation (Segel, 1975).

$$\ln([S_0]/[S]) = (-1/K_m)([S_0] - [S]) + A_{max}/(\dot{Q}_p K_m) \quad (1)$$

where $[S_0]$ is the initial substrate concentration (i.e., total ^{3}H concentration in each arterial blood sample), [S] is the final substrate concentration (i.e., [^{3}H]-BPAP concentration remaining in each arterial blood sample), $\dot{Q}_p$ is the pulmonary plasma flow calculated in each determination as previously described (Catravas & White, 1984) and

$$A_{max} \equiv Ek_{cat} = V_{max} nQ_{pc} \quad (2)$$

where Q_{pc} is capillary plasma volume (ml), n is the fraction of Q_{pc} in which E is distributed (assumed = 1), and E is enzyme mass (mol).

K_i values for captopril or CL242,817 were determined assuming that each compound could have behaved as either (a) a competitive or (b) a mixed inhibitor (Cornish-Bowden, 1979).

(a) *Competitive inhibitor*:

$$K'_m = K_m(1 + [I_f]/K_i) \quad (3)$$

$$A'_{max} = A_{max} \quad (4)$$

where $[I_f]$ is the concentration of free inhibitor (captopril or CL242,817) in plasma.

(b) *Mixed competitive-noncompetitive or competitive-irreversible inhibitor* (Cornish-Bowden, 1979):

$$K'_m = \frac{K_m(1 + [I_f]/K_i)}{1 + [I_f]/K'_i} \quad (5)$$

$$A'_{max} = A_{max}/(1 + [I_f]/K'_i) \quad (6)$$

in the case of mixed competitive-irreversible inhibition, $1/(1 + K'_i/[I_f])$ is the fraction of enzyme irreversibly bound by the inhibitor.

First-order method

Under first order reaction conditions, $[S] \ll K_m$, $K_m \simeq (K_m + [S])$ and the integrated Henri-Michaelis-Menten equation can be written (Segel, 1975) as:

$$A_{max}/K_m = \dot{Q}_p \ln([S_o]/[S]) \quad (7)$$

In the presence of an inhibitor, the apparent $(A_{max}/K_m)'$ ratio is reduced to:

$$(A_{max}/K_m)' = (A_{max}/K_m)/(1 + [I_f]/K_i) \quad (8)$$

This relationship holds true for competitive, mixed competitive-noncompetitive or mixed competitive-irreversible types inhibition, as can be seen from equations 3, 4, 5 and 6. The ratios (A_{max}/K_m) and $(A_{max}/K_m)'$, before and after the inhibitor, respectively, were computed as the integral of $\dot{Q}_p \ln([S_0]/[S])$ over the entire transpulmonary passage with data from determinations 1 or 3 only, using equation 7.

Determination of free inhibitor concentration in plasma

With both methods, $[I_f]$ is the concentration of free inhibitor in plasma at the time of the determination (i.e., 30 s after inhibitor administration). To calculate $[I_f]$, a separate group of 8 rabbits was studied. At time = 0, 1.2 μCi ^{3}H-captopril kg^{-1} body weight (10 nmol kg^{-1}; 0.1 μCi $nmol^{-1}$, New England Nuclear Corp., $n = 4$) or 0.16 μCi [^{14}C]-CL242,817 (4.5 mCi $mmol^{-1}$; 35 nmol kg^{-1}; $n = 4$; a gift from Dr P. Cervoni, Lederle Laboratories) was injected together with 0.2 mg Evans Blue dye (T-1824) into the right jugular catheter. At $t = 30$ s and 60 s, arterial blood (3 ml) was withdrawn into heparinized syringes. After centrifugation, radioactivity was determined in 0.5 ml plasma aliquot and in 0.5 ml fractions of eluant collected from Amicon disposable microconcentrators (>99% rejection of compounds with molecular weight >67,000). The latter sample provided estimates of the free (non-protein-bound) inhibitor levels in plasma. Dye concentrations were estimated spectrophotometrically (605 nm) in 0.5 ml plasma. Removal (R_t) of inhibitor from the intravascular space at $t = 30$ and 60 s was estimated as:

$$R_t = (FC_D - FC_I)/FC_D \quad (9)$$

where FC_D and FC_I are the fractional concentrations of the dye and inhibitor in plasma, respectively. Concentration of free inhibitor in plasma was calculated as the ratio of d.p.m. in 1 ml of the Amicon microconcentrator eluant to the specific activity (d.p.m. mol^{-1}) of radiolabelled inhibitor injected.

Determination of pulmonary endothelial 5'-nucleotidase activity

Since $A_{max} \equiv Ek_{cat}$, a decreased A_{max} could reflect reduced values of either E or k_{cat}. In order to distinguish between changes in A_{max} resulting from altered k_{cat} vs. changes due to altered perfused microvascular luminal surface area (i.e., E), we used 9 rabbits to measure the trans-pulmonary conversion of [^{14}C]-5'-AMP by endothelial-bound 5'-nucleotidase (NCT) and calculated A_{max}/K_m ratios together with those of ACE for BPAP. NCT is also an ectoenzyme distributed along the walls of the pulmonary microvascular lumen (Ryan & Smith, 1971) whose activity is not affected by the ACE inhibitor captopril (Catravas & Watkins, 1985).

Determination of pulmonary endothelial ACE mass

Measurement of transpulmonary binding of [^{3}H]-RAC-X-65 To investigate the possibility that either captopril or CL242,817 behaved as a competitive, but tight binding inhibitor (i.e. effectively irreversible for the 60 s of the experiment and thus causing an apparent reduction in the mass of the

'available' enzyme), we also studied the single pass transpulmonary binding (B) of trace amounts of the tight binding ACE inhibitor [^{3}H]-RAC-X-65 to lung ACE utilizing indicator dilution techniques with [^{14}C]-dextran (mol. wt. 70,000) as the intravascular indicator. In these experiments trace amounts (0.08 nmol) of [^{3}H]-RAC-X-65 were injected, representing less than 1% of the injected amounts of either captopril or CL242,817. Data were analyzed based on the differential equation describing the first order mass-action law of two interacting species (Rodbard, 1973). In the following equations, 10 to 13, 'I' refers to the radiolabelled RAC-X-65 and *not* to captopril or CL249,817 as in previous equations.

$$d[EI]/dt = k_1([I_0] - [EI])([E_t] - [EI]) - k_1[EI] \quad (10)$$

where [EI] is the concentration of [^{3}H]-RAC-X-65 bound to enzyme, $[I_0]$ is the total (initial) concentration of [^{3}H]-RAC-X-65, $[I_f]$ is the concentration of free [^{3}H]-RAC-X-65, $[E_t]$ is the concentration of total lung ACE, $[E_f]$ is the concentration of lung ACE not bound to inhibitor (i.e., $[E_f] = [E_t] - [EI]$), k_1 is the net forward association rate constant (which may represent more than one constant) of $[E_f]$ and $[I_f]$ and k_1 is the dissociation constant of [EI]. The (k_{-1}/k_1) ratio equals the K_i constant for an inhibitor-enzyme interaction.

For a tightly bound inhibitor which is essentially irreversibly bound during the time of incubation (i.e., during a single transpulmonary passage) as is the case for RAC-X-65, $k_{-1}[EI] \simeq 0$. Furthermore, in our studies, $[E_t] \gg [EI]$ (since I is injected at trace amounts, $I \ll E$) and thus $[E_t] - [EI] \simeq [E_t]$. Thus, equation 10 can be simplified:

$$d[EI]/dt = k_1([I_0] - [EI])[E_t] \quad (11)$$

Rearranging, integrating and substituting $[I_0] - [I_f]$ for [EI]:

$$\ln([I_0]/[I_f]) = k_1[E_t]t_c \quad (12)$$

Substituting E_t/Q_{pc} for $[E_t]$ and $\dot{Q}_p$ for Q_{pc}/t_c:

$$\dot{Q}_p \ln([I_0]/[I_t]) = k_1E_t \quad (13)$$

where E_t is the total mass of enzyme available to interact with the inhibitor.

Thus, we have calculated k_1E_t of [^{3}H]-RAC-X-65 with ACE before and 30 s after (k_1E_t') administration of captopril (10 nmol kg^{-1}; i.v., $n = 7$) or CL242,817 (35 nmol kg^{-1}; $n = 4$). We also calculated the total transpulmonary binding of [^{3}H]-RAC-X-65 during a single passage, (%B) as:

$$\%B = 100 \int_0^t (FC_{14C} - FC_{3H})/FC_{14C}$$

where FC_{14C} and FC_{3H} are the fractional concentrations of [^{14}C]-dextran and [^{3}H]-RAC-X-65 in the arterial outflow blood samples.

Synthesis of [^{3}*H*]*-RAC-X-65* [^{3}H]-RAC-X-65 (N-[1(S)-carboxy - 3 - carbox[^{3}H] - anilidopropyl] - L - Ala - L - Pro) (24 Ci mmol^{-1}) was prepared by catalytic dehalogenation in $^3H_2$-gas of N-[1(S)-carboxy-3-carbox[4'-iodo]anilidopropyl]-L-Ala-L-Pro. The latter compound was synthesized chirally as described previously (Chung & Ryan, 1986). RAC-X-65 is a tight binding ACEI the biochemical and pharmacological effects of which have been described (Ryan *et al.*, 1986).

Statistics

Statistical evaluations were performed with the aid of a DEC Rainbow or Zenith microcomputer and Student's *t* test for paired or independent samples was used as indicated.

Results

Effects of inhibitors on apparent K_m *and* A_{max} *values*

Thirty seconds after bolus administration of captopril or CL242,817, apparent K_m' values increased 3–4 fold, whereas apparent A_{max}' decreased to about half ($P < 0.05$; Table 1). These parameters were calculated in each animal using equation 1 from data, such as the ones depicted in Figures 1 and 2. Figure 1 shows that when [^{3}H]-BPAP is injected in trace amounts (0.1 nmol), fractional metabolism of BPAP is essentially constant and is independent of substrate concentration. Thus, first order enzyme kinetics are obeyed. However, when 1622 nmol BPAP was injected, BPAP metabolism is inversely proportional to BPAP concentration, reflecting saturation of the enzyme, as expected for mixed order kinetics. Thirty seconds after captopril or CL242,817, metabolism of [^{3}H]-BPAP under first or mixed order conditions was reduced. However, unlike the profile seen with the inhibitor, BPAP metabolism increased moderately over a single transpulmonary passage. Equation (7) was used to calculate the A_{max}/K_m ratios which decreased to about 15–20% of control values after either inhibitor (Table 1).

Plasma concentrations of unbound (free) inhibitors (captopril or CL242,817)

The plasma concentration of free captopril or CL242,817 ($[I_f]$) was estimated in additional animals 30 s and 60 s after receiving [^{3}H]-captopril ($n = 4$) or [^{14}C]-CL242,817 ($n = 3$). Both inhibitors were extensively removed from the circulation (captopril: 94–95%; CL242,817: 74–77%) with little binding to plasma proteins (1–2%). The calculated free plasma concentrations at 30 s (32.3 ± 2 nM and 190 ± 11 nM) were not significantly different from those at 60 s (29.7 ± 3 nM and 163 ± 12 nM for captopril and CL242,817, respectively;

Table 1 Apparent Michaelis-Menten constants of pulmonary endothelial angiotensin converting enzyme (ACE) for benzoyl-Phe-Ala-Pro (BPAP) in anaesthetized rabbits, *in vivo*

	Captopril ($n = 6$)	*CL242,817* ($n = 5$)
Mixed order method		
K_m (control)	12.2 ± 1.9 μM	9.3 ± 2.3 μM
K_m' (apparent)	32.9 ± 3.3 μM*	45.8 ± 9.8 μM*
A_{max} (control)	10.3 ± 2.1 μmol min^{-1}	8.9 ± 1.7 μmol min^{-1}
A_{max}' (apparent)	4.5 ± 0.8 μmol min^{-1}*	4.8 ± 0.5 μmol min^{-1}*
A_{max}/K_m (control)†	816 ± 87 ml min^{-1}	917 ± 128 ml min^{-1}
A_{max}'/K_m' (apparent)†	121 ± 29 ml min^{-1}*	115 ± 25 ml min^{-1}*
$[I_f]$	31.0 nM	177.5 nM
First order method		
A_{max}/K_m	763 ± 100 ml min^{-1}	1009 ± 149 ml min^{-1}
$(A_{max}/K_m)'$	125 ± 38 ml min^{-1}*	126 ± 44 ml min^{-1}*
$[I_f]$	32.3 nM	190.0 nM
$\dot{Q}_p$ (control)	293 ± 43 ml min^{-1}	324 ± 18 ml min^{-1}
$\dot{Q}_p$ (post-inhibitor)	248 ± 24 ml min^{-1}	268 ± 67 ml min^{-1}

Means ± 1 s.e.

* $P < 0.05$ from corresponding control with Student's *t* test for paired samples. $[I_f]$ is the concentration of free inhibitor in plasma at the time of the determination.

† Calculated from K_m, K_m', A_{max} or A_{max}' values.

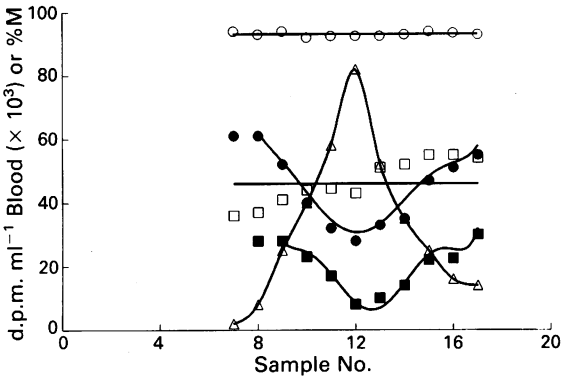

Figure 1 Arterial outflow curves of total tritium (△) and percentage metabolism (M; all other curves) of [^{3}H]-benzoyl-Phe-Ala-Pro ([^{3}H]-BPAP) (calculated as $\int_0^t (([S_0] - [S])/[S_0])$) by rabbit pulmonary endothelial angiotensin converting enzyme (ACE) *in vivo*. Data were collected from four successive indicator dilution determinations in one rabbit. The outflow curves of total tritium from the four determinations were congruent, so only the first one is presented. Amounts of BPAP injected were 0.1, 1622, 0.1 and 1622 nmol for determinations 1 (○), 2 (●), 3 (□) and 4 (■), respectively. Captopril was given 30 s before the third determination. Data from determinations 1 and 2 (before captopril) were used to calculate control apparent K_m and A_{max} values with equation 1, while data from determination 1 alone were used to calculate A_{max}/K_m ratios with equation 7. Data from determinations 3 and 4 were used to calculate apparent K'_m and A'_{max} values after captopril with equation 1, while data from determination 3 alone were used to calculate apparent $(A_{max}/K_m)'$ ratios with equation 7. Solid lines represent metabolism profile predicted by the model.

$P > 0.05$, Student's t test) and remained essentially constant over the subsequent 5 min. The $[I_f]$ value at 30 s was employed in K_i value calculations using data from determination 3. For mixed order kinetic determinations (i.e., utilizing data from both determinations 3 and 4), the average $[I_f]$ value between 30 and 60 s was used (i.e., 31 nM for captopril and 177.5 nM for CL242,817). This was done since sample collection for determination 3 started 30 s after inhibitor administration, and the last sample of determination 4 was obtained 28 s later.

K_i *determinations*

The observed increase in apparent K'_m values after either inhibitor can reflect either competitive or mixed type inhibition. The additional concomitant decreases in apparent A'_{max} values eliminate the case of pure competitive inhibition operating exclusively and suggest one of three possibilities: (a) mixed competitive-noncompetitive type inhibition, (b) simple competitive type inhibition with reduced amounts of available enzyme after inhibitor administration due to a reduction in perfused pulmonary endothelial surface area (derecruitment), or (c) mixed competitive and effectively irreversible type inhibition, where part of the mass of available enzyme is reduced after inhibitor administration by being occupied by tightly bound inhibitor, at least for the duration of the experiment. Situations (b) and (c) are similar since the end result is a reduction in available enzyme (E). We considered each of these three possibilities and calculated K_i values with the appropriate equations described under Methods. Mixed order and first order K_i calculations gave statistically similar results with both inhibitors. K_i values for captopril (6.3 ± 1.3 nM and 7.5 ± 1.2 nM for first and mixed order methods, respectively) were consistently lower than those for CL242,817 (26.8 ± 4.1 nM and 22.8 ± 3.8 nM for first and mixed order methods, respectively), indicating that captopril was the more potent inhibitor *in vivo*. However, CL242,817 is a racemic mixture; thus, the K_i values of the active isomer may be half those computed in the present study. Further, it is possible that CL242,817 must be deacylated to provide a free sulphydryl function for binding to the zinc atom of ACE (see Figure 6 and Lai *et al.*, 1983). If such is the case, a relatively low deacylation reaction *in vivo* could have the effect of yielding a falsely high K_1.

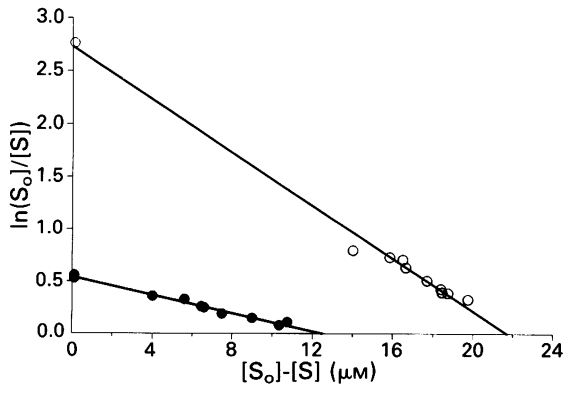

Figure 2 Plots of data shown in Figure 1 according to equation 1: (○) data from determinations 1 and 2 (before captopril); (●) data from determinations 3 and 4 (after captopril). Control, precaptopril values: $\dot{Q}_p = 320\,ml\,min^{-1}$, $K_m = 8.4\,\mu M$, $A_{max} = 7.4\,\mu mol\,min^{-1}$. After captopril: $\dot{Q}_p = 280\,ml\,min^{-1}$, $K'_m = 19.0\,\mu M$, $A'_{max} = 3.3\,\mu mol\,min^{-1}$. Decreasing slope reflects increasing apparent K'_m value while decreasing X-intercept reflects decreasing apparent $A_{max}/\dot{Q}_p$ values.

Effects of captopril or CL242,817 on endothelial luminal surface area

Control estimates of [^{14}C]-5'-AMP metabolism averaged 86 ± 3% and 85 ± 3% (in captopril, $n = 3$, and CL242,817, $n = 3$, treated groups, respectively). Percentage metabolism after administration of captopril or CL242,817 remained unchanged at 81 ± 3% and 82 ± 3%, respectively. Figure 3 shows arterial outflow blood concentrations of ^{14}C and percentage metabolism before and 30 s after the administration of captopril. Similar experiments were performed before and 30 s after the injection of CL242,817. The calculated A_{max}/K_m ratios for NCT also remained unaffected by either inhibitor as shown in Figure 4, even though the A_{max}/K_m ratio for ACE decreased dramatically. These results indicate that the reduction in A_{max}/K_m of BPAP for ACE was not due to pulmonary microvascular derecruitment.

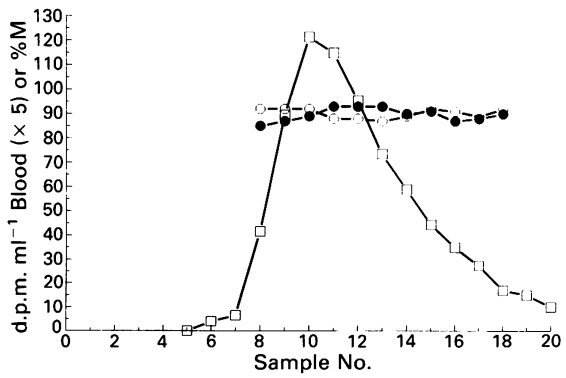

Figure 3 Arterial outflow curves of total ^{14}C (□) and percentage metabolism (M; all other symbols) of [^{14}C]-5'-AMP by rabbit pulmonary endothelial 5'-nucleotidase, *in vivo*. Data were collected from two indicator dilution determinations in one rabbit; 8 nmol [^{14}C]-5'-AMP were injected before (○) or 30 s after captopril (10 nmol kg^{-1}; ●). Outflow curves from the two determinations were congruent and for simplicity only the curve from determination 1 is presented. Data from determinations 1 and 2 were used to calculate apparent (A_{max}/K_m) and apparent $(A_{max}/K_m)'$ ratios, respectively, with equation 7.

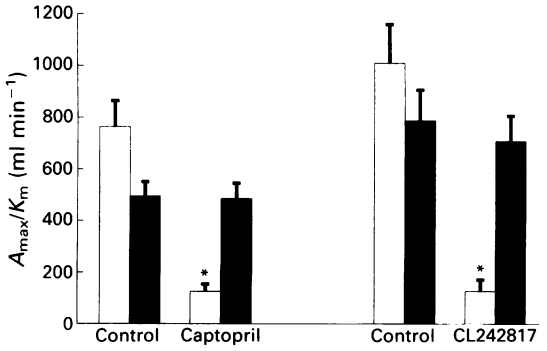

Figure 4 Effects of captopril or CL242,817 on A_{max}/K_m ratios of pulmonary endothelial ACE (open columns) and 5'-AMP, (solid columns) *in vivo*. A_{max}/K_m ratios were calculated from indicator dilution experiments (such as those in Figures 1 and 3) with equation 7. Inhibitors were administered 30 s prior to the apparent $(A_{max}/K_m)'$ ratio determinations. Means with ± s.e. shown by vertical bars. $P < 0.05$ compared to corresponding control values with paired t test.

Effects of inhibitors on amounts of active ACE

To investigate whether either inhibitor was tightly bound to ACE, we examined changes in the binding of trace amounts of the high affinity ACE inhibitor [^{3}H]-RAC-X-65. Figure 5 shows arterial blood outflow concentrations of [^{14}C]-dextran (intravascular indicator) and [^{3}H]-RAC-X-65 before captopril administration, as well as percent binding of the radiolabelled inhibitor before and after captopril. Captopril significantly reduced binding of [^{3}H]-RAC-X-65 from 65 ± 3% to 8 ± 1%; similarly, CL242,817 reduced percentage binding from 64 ± 3% to 6 ± 1%. From these data, Ek_1 products were calculated using equation 13. The Ek_1 product values of [^{3}H]-RAC-X-65 for ACE decreased significantly 30 s after captopril (from 360 ± 51 to 60 ± 16 ml min^{-1}) or CL242,817, (from 372 ± 54 to 66 ± 18 ml min^{-1}) suggesting that both captopril and CL242,817 effectively acted as irreversible inhibitors by remaining bound to a large fraction of ACE at 30 s after administration. If the k_1 value of [^{3}H]-RAC-X-65 for ACE *in vivo* is assumed to be similar to that for the captopril or enalapril, *in vitro* (approximately 10^6 M^{-1} s^{-1}, Shapiro & Riordan, 1984; this is a reasonable assumption considering the dissimilarity in structure between captopril and enalapril and the considerable similarity in structure between enalapril and RAC-X-65), the mass of active ACE before and after inhibitor administration can be calculated from equation 13. In 3–4 kg anaesthetized rabbits (with pulmonary blood flow of approximately 400 ml min^{-1}) there is 6 ± 0.5 nmol of ACE (capable of binding RAC-X-65) on the perfused microvascular luminal surface, which is reduced to about 1 ± 0.05 nmol, 30 s after captopril or CL242,817 administration.

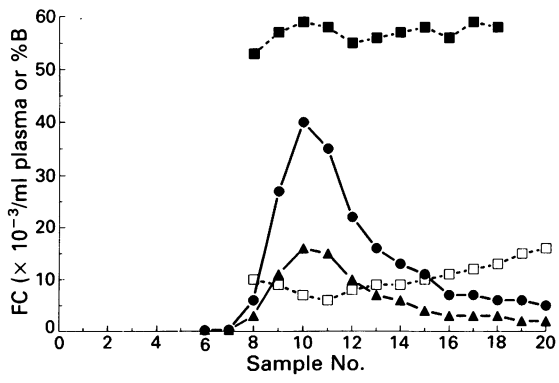

Figure 5 Arterial outflow curves of [^{14}C]-dextran (●) and [^{3}H]-RAC-X-65 (▲) and percentage binding (%B) of 0.08 nmol [^{3}H]-RAC-X-65 to pulmonary endothelial angiotensin converting enzyme (ACE), *in vivo*. Data were collected from two indicator dilution determinations in one rabbit, one before (■) and the other 30 s after captopril (□). For simplicity, outflow curves from determination 1 (pre-captopril) only are shown.

Discussion

The calculated parameters K_m and A_{max} have been termed 'apparent' because they represent approximations of true values which can be obtained only when the exact microvascular plasma concentrations of S_0 and S are known. In the present- and similar type-experiments, S_0 and S concentrations in arterial effluent plasma are utilized instead, with the inherent error of solute dilutions in the arterial blood. Nevertheless, over the last 10 years, these methods have been used to obtain relative estimates of Michaelis-Menten parameters, *in vivo* or in perfused organs (e.g. Catravas & White, 1984; Howell *et al.*, 1984; Pitt & Lister, 1984, Linehan *et al.*, 1989). Furthermore, dilution error appears to remain constant, at least within individual preparations, since repeated determinations of these parameters produce remarkably reproducible results (e.g. see Catravas *et al.*, 1988).

Control estimates of apparent K_m and A_{max} values for the reaction of ACE with BPAP, *in vivo*, agree with previously reported data in rabbits or lambs (Catravas & White, 1984; Howell *et al.*, 1984; Pitt & Lister, 1984; Catravas, 1986).

As with the aforementioned studies, these parameters were calculated without correcting for possible binding of BPAP to blood albumin (see Linehan *et al.*, 1989), which, in the present case, would be negligible given the very short exposure times (< 5 s) of the substrate to blood before it reached microvascular ACE.

This is confirmed by previous findings that in the rabbit, transpulmonary metabolism of BPAP is independent of blood proteins (e.g. see Gillis *et al.*, 1989 vs Catravas *et al.*, 1988). It is also supported by the good fit of the raw data, to the Michaelis-Menten model used (see Figure 1). An exception is present with the pattern of BPAP metabolism under first order conditions after captopril or CL242,817, which despite its predicted flat profile, increases moderately during the course of a single transpulmonary passage. Although this phenomenon has been observed by others (Moalli *et al.*, 1988) its cause remains unclear. Interestingly, despite their close structural similarity with BPAP, under the conditions of our experiment, neither of the two ACE inhibitors was bound significantly to plasma proteins as others have suggested (Linehan *et al.*, 1989). The affinity of [^{3}H]-RAC-X-65 for plasma albumin is unclear, as we did not test for it in the present studies.

The rise in apparent K'_m values after captopril or CL242,817 administration was not unexpected. Cushman *et al.* (1978) originally showed that captopril acts as a pure competitive inhibitor, *in vitro*, with a K_i of 2 nM. Similarly, Lai *et al.* (1983) found CL242,817 to be a pure competitive inhibitor of crude rabbit lung extract ACE with a K_i of 24 nM. In a previous study (Catravas & White, 1984), we also reported that captopril increased apparent K'_m values of rabbit lung ACE for BPAP, *in vivo*. However, then, as well as in the present study, A'_{max} decreased as well. A decrease in apparent A'_{max} of BPAP for lung ACE *in vivo* after captopril was also subsequently reported (Moalli *et al.*, 1985); however apparent K'_m values in that latter study were not altered and the authors postulated that captopril acts as a non-competitive inhibitor *in vivo* (Moalli *et al.*, 1985).

In these types of experiments, a special case of competitive inhibition can be conceived in which apparent A'_{max} could be reduced as a consequence of derecruitment of the pulmonary microvascular bed. Under these conditions, the mass of endothelial ACE available for reaction with substrate passing through patent vascular channels would be reduced. Consequently, a decrease in apparent A'_{max} (product of k_{cat} and enzyme mass, E) after administration of an ACE inhibitor may reflect a reduction in available enzyme mass, and cannot be considered to reflect by itself a noncompetitive type of inhibition. Moreover, there is the special case of a tightly bound

or irreversible inhibitor in which even though perfused enzyme mass may not change, the inhibitor occupies the active site in a fraction of the total enzyme, and thus the amount of *free* enzyme available to substrate (and hence A'_{max}) decreases even though k_{cat} remains unchanged.

Our results show that neither captopril nor CL242,817 reduced A_{max}/K_m for the reaction of 5'-AMP with NCT, thus competitive, readily reversible inhibition accompanied by loss of ACE mass consequent to derecruitment can be ruled out. The observed decrease in kinetic parameters is consistent with either competitive and effectively irreversible inhibition or mixed competitive-noncompetitive inhibition. The balance of published evidence, the data presented here and theoretical considerations strongly support the concept that captopril and CL242,817 act *in vivo* as competitive, tightly bound (effectively irreversible) inhibitors.

A competition for ACE binding sites between the substrate BPAP and the inhibitors captopril, CL242,817 and RAC-X-65 is to be expected. These four compounds are close structural analogues (see Figure 6), and each contains structural elements (either a C-terminal Ala-Pro or an analogue of Ala coupled to a C-terminal proline residue) selected for favourable binding to the S'_1 and S'_2 subsites of ACE (nomenclature of Schechter & Berger, 1967; see also Cushman *et al.*, 1978; Ondetti & Cushman, 1984). Further, each contains one or more functions capable of interacting with the catalytically essential zinc atom of ACE. Both captopril and CL242,817 contain a sulphur atom capable of binding at high affinity with ACE zinc (Cushman *et al.*, 1978; Ondetti & Cushman, 1984). BPAP and RAC-X-65 contain yet other structural elements capable of binding to the S_1 subside of ACE. Given the relatively large sizes of BPAP, captopril, CL242,817, and RAC-X-65 in respect to the catalytic site of ACE and the common modes of binding of these compounds to ACE, it is difficult to envisage formation of an enzyme: inhibitor:substrate complex, a requirement for either noncompetitive or mixed competitive-noncompetitive inhibition. Captopril behaves as a competitive inhibitor *in vitro* (Lai *et al.*, 1983; Ondetti & Cushman, 1984) and it is difficult to conceive how its mode of inhibition might be converted to a mixed competitive-noncompetitive inhibition *in vivo*. Nonetheless, we have no direct evidence that conclusively excludes formation of an enzyme:inhibitor:substrate complex.

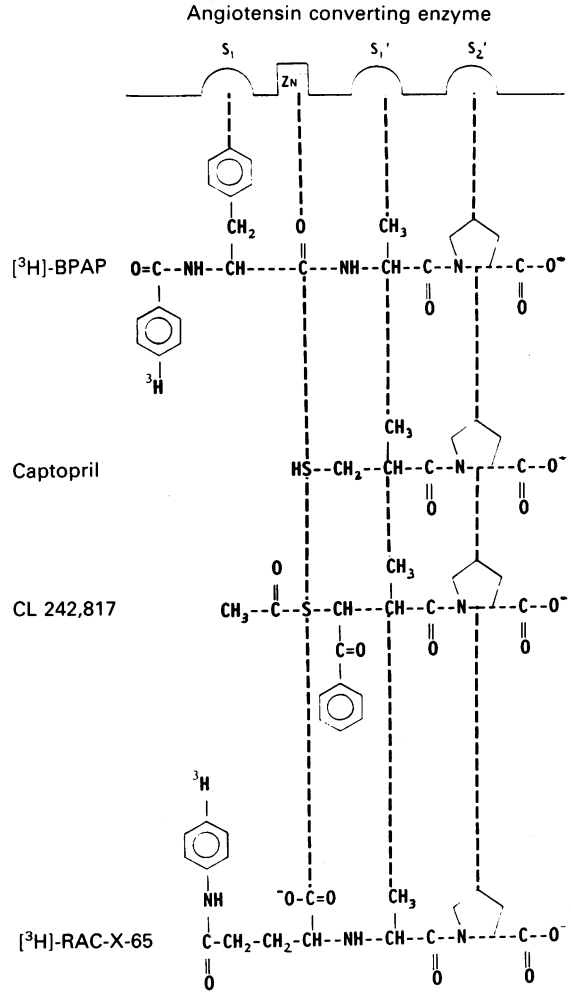

Figure 6 Chemical formulae of captopril, CL242,817, [^{3}H]-RAC-X 65 and [^{3}H]-benzoyl-Phe-Ala-Pro ([^{3}H]-BPAP) reflecting close structural similarities among the three inhibitors and substrate.

In our studies, because of the protocol used (i.v. injection of inhibitor followed by a nonsaturating dose of BPAP and thereafter by a saturating dose of BPAP), it is evident that an enzyme:inhibitor:substrate complex (if formed) would be formed by addition of substrate to preformed enzyme:inhibitor complex. Further, the observed 40–60% reductions in apparent A'_{max} would imply that 40–60% of the available enzyme was in the form of enzyme: inhibitor:substrate complexes immediately following injection of the saturating dose of BPAP. Such a possibility is refuted by the present data since it would have required at least a temporary 'loss' or 'uptake' of a significant portion of the circulating substrate under first order reaction conditions. On the contrary, all of the injected ^{3}H exited the pulmonary circulation (in the form of [^{3}H]-BPAP or product [^{3}H]-BPhe) in a pattern identical to that of the intravascular indicator (indocyanine green).

In contrast, the evidence that captopril can act as a competitive, tightly bound (slowly dissociable) inhibitor is strong. As noted above, captopril inhibits ACE competitively *in vitro*. Shapiro & Riordan (1984) have shown that captopril is a slow tight binding inhibitor of ACE and that the rate of dissociation of the tightly bound [EI] complex is remarkably slow ($4 \times 10^{-4} s^{-1}$; see also Bicknell *et al.* (1987) for evidence of binding of captopril to the cobalt form of ACE). Less is known of the properties of CL242,817, beyond the fact that it inhibits competitively *in vitro* (Lai *et al.*, 1983). Nonetheless, its close structural relationship with captopril (Figure 6) argues in favour of captopril-like properties.

Furthermore, our experiments indicate that the active site in a substantial fraction of the endothelium-associated enzyme was occupied by captopril or CL242,817, 30 s after the administration of the inhibitor. We have arrived at this conclusion by contrasting the changes in the transpulmonary binding of RAC-X-65 and those in the apparent kinetic parameters of BPAP before and after each inhibitor. When dealing with a substrate, the magnitude of inhibitor-induced changes in apparent K'_m, A'_{max} or $(A_{max}/K_m)'$ are similar whether mixed competitive-non-competitive type or competitive, irreversible type inhibition is present (Cornish-Bowden, 1979). This is because (a) apparent K'_m increases in both cases as a result of $[I_f]$ (that is, captopril or CL242,817) and [S] competing for [E] and (b) apparent A'_{max} decreases either due to lower dissociation of the formed [EIS] vs [ES] complexes (noncompetitive component) or due to the inability of [S] to combine with [EI] (irreversible type). In mixed competitive-noncompetitive type inhibition, [S] and $[I_f]$ have equal affinities for [E] and [EI], whereas in competitive, irreversible inhibition, [EI] assumes a form which is inaccessible to both [S] and $[I_f]$ (Segel, 1975). However, in enzyme-inhibitor interaction, product formation does not occur when the inhibitor (e.g., RAC-X-65) associates with [E], and consequently the magnitude of the decrease in the Ek_1 product will differ depending on the type of inhibition: for mixed competitive-noncompetitive type, the decrease in Ek_1 will be the result of competition between the trace amounts of RCA-X-65 and $[I_f]$ (captopril or CL242,817) for [E] and thus should be proportional to the post captopril or CL242,817 decrease in the apparent $(1/K_m)'$ of BPAP, whereas for competitive, irreversible inhibition, the decrease in Ek_1 will be the result of both the competition between RAC-X-65 and $[I_f]$ (captopril or CL242,817) for [E], as well as the decrease in available [E]

due to the formation of an inaccessible [EI] complex, and thus should be proportional to the post captopril or CL242,817 decrease in the apparent $(A_{max}/K_m)'$ ratio of BPAP. In our studies Ek_1 of RAC-X-65 decreased by 83% (after captopril) or 82% (after CL242,817); similarly apparent $(A_{max}/K_m)'$ ratios decreased by 84% and 89%, respectively, whereas apparent $(1/K_m)'$ only decreased by 63% and 78%, respectively. The similarity in the magnitude of changes between Ek_1 and $(A_{max}/K_m)'$ but not $(1/K_m)'$ strongly indicate that captopril behaves as competitive, tightly bound (i.e., effectively irreversible for at least 60 s) inhibitor, *in vivo*. Ek_1 values post CL242,817 fall in between those of $(1/K_m)'$ and $(A_{max}/K_m)'$, however the similarity of structure between captopril and CL242,817 would favour similar mechanisms of action.

We have described a process for calculating K_i values of ACEI for endothelial membrane-bound ACE *in vivo*. Our results suggest that both ACEI tested behave as competitive, tight binding inhibitors with apparent K_i values moderately higher than those reported for *in vitro* studies. Interestingly, both the mixed-order and the simpler first order method (i.e. one bolus injection containing trace amounts of substrate per determination) provide similar apparent K_i values for each drug. We have further simplified this first order approach recently (Toivonen *et al.*, 1988), so that a fraction collector is not needed and a single arterial blood sample suffices for %M and A_{max}/K_m calculation. We are encouraged that clinical applications of this technique are now feasible.

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Differential regulation of 5-lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism in rat peritoneal leukocytes

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1 Simultaneous activation of the 5-lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism in rat peritoneal mixed leukocytes in response to A23187, chemoattractant N-formyl-methionine-leucine-phenylalanine (FMLP) and arachidonic acid (AA) was studied by radioimmunoassay of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) respectively.

2 FMLP and AA preferentially activated cyclo-oxygenase and A23187 preferentially activated 5-lipoxygenase. Release of TXB_2 preceded that of LTB_4. A threshold amount of A23187 enhanced FMLP- and AA-induced LTB_4 production but not that of TXB_2.

3 Phorbol myristate acetate (PMA) abolished LTB_4 generation in response to FMLP with much less effect on TXB_2, but did not inhibit the formation of either eicosanoid caused by A23187 or AA. Instead, PMA caused a dose-dependent but modest stimulation of TXB_2 generation either on its own or when added with A23187 or AA.

4 These results show that the 5-lipoxygenase and cyclo-oxygenase pathways in rat peritoneal leukocytes are regulated differently and that functional compartmentalisation of the stimulus-generation sequence operates in these cells.

Introduction

It is not yet fully known how the enzymatic reactions which lead to the generation of eicosanoids are regulated in terms of the steps between receptor occupation, activation of coupling proteins, stimulation of substrate release and subsequent transformations of arachidonic acid (AA) via the lipoxygenase and cyclo-oxygenase pathways (Humes *et al.*, 1982; Burch & Axelrod, 1987; Nakashima *et al.*, 1988; Crooke *et al.*, 1989; Smith, 1989). Calcium is considered to be essential for the activation of the relevant phospholipases (Irvine, 1982), and the calcium ionophore A23187 is a powerful stimulant for eicosanoid generation in many cell systems. Agents such as phorbol myristate acetate (PMA) which cause a prolonged activation of protein kinase C have variable effects on eicosanoid generation, depending on cell type, eicosanoid studied and timing of addition (Humes *et al.*, 1982; Tripp *et al.*, 1985; Liles *et al.*, 1987; Slivka & Insel, 1988).

It is also possible that the two pathways might operate independently or be regulated differently, despite the reliance on arachidonate as a common substrate. Thus, there are several distinct isozymes of phospholipases A_2 and C (Irvine, 1982; Nakanishi *et al.*, 1988), distinct functional compartments of phospholipases (Irvine, 1982) which show differential sensitivity to inhibition by anti-inflammatory steroids and lipocortins (Robinson & Hoult, 1980; Cirino *et al.*, 1987), and there are stimuli known to activate one of the pathways differentially in cells capable of generating products derived from both (Humes *et al.*, 1982; Tripp *et al.*, 1985).

In this paper we provide several lines of evidence for the differential regulation of the two pathways in stimulated rat peritoneal leukocytes. This was obtained by activating the cells with A23187, chemoattractant N-formyl-methionine-leucine-phenylalanine (FMLP) and arachidonic acid in the absence and presence of varying concentrations of A23187 and PMA. Leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2, the hydrolysis product of thromboxane A_2) were taken as markers for the activity of 5-lipoxygenase and cyclo-oxygenase, respectively.

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Methods

Mixed peritoneal leukocytes containing approximately 75% polymorphonuclear neutrophil leukocytes (PMNs) and 25% mononuclear leukocytes were elicited from female Wistar rats by use of glycogen as described previously (Moroney *et al.*, 1988). The cells were resuspended in Hanks balanced salt solution containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} at a density of 2.5×10^6 cells ml^{-1}, incubated with stimulants, and the supernatants subjected to radioimmunoassay for LTB_4 and TXB_2 as described previously (see also legend to Table 1). A23187, AA, PMA and FMLP were from Sigma Chemical Company, Poole. Significance of differences between treatments was established by Student's unpaired *t* test.

Results

FMLP (10^{-8} M–10^{-5} M), A23187 (10^{-8} M–10^{-6} M) and AA (5×10^{-7} M–10^{-5} M) caused dose-dependent release of LTB_4 and TXB_2 from the rat mixed peritoneal leukocytes. Table 1 shows representative data, and Tables 2, 3 and 4 give results for a range of FMLP, A23187 and AA concentrations, respectively. The concentration-response curves were steep, with approximate EC_{50} values (given in the order LTB_4, TXB_2) of 2×10^{-7} M and 5×10^{-8} M for FMLP; 10^{-7} M and 3×10^{-8} M for A23187 and 7×10^{-6} M and 2×10^{-6} M for AA. Higher doses of the stimulants produced non-specific effects, with sharp discontinuities in the product yield and/or cell lysis. The time course of release of TXB_2 and LTB_4 in cells stimulated with A23187 and FMLP is shown in Figure 1. This indicates that TXB_2 is released more quickly from the cells than is LTB_4, and is substantially complete by 1–2 min, whereas levels of the leukotriene peak between 5–15 min. For this reason a standard incubation time of 10 min was used in most experiments.

The threshold doses of the three stimulants required for initiating TXB_2 generation were in all cases lower than for LTB_4. Moreover, the amounts of TXB_2 produced in response to FMLP and AA were larger than the amounts of LTB_4 (at the concentrations shown in Table 1), whereas the profile was

Table 1 Eicosanoid release by rat peritoneal leukocytes

	LTB_4 release (ng/2.5 × 10^6 cells)			TXB_2 release (ng/2.5 × 10^6 cells)			
Stimulant	Alone	+A23187 (10^{-8} M)	+PMA (10^{-6} M)	Alone	+A23187 (10^{-8} M)	+PMA (10^{-6} M)	n
FMLP (10^{-7} M)	5.4 ± 0.8**	11.3 ± 3.4†	0.5 ± 0.3††	13.1 ± 2.9**	8.9 ± 4.0	6.4 ± 3.5	18, 12, 12
FMLP (10^{-5} M)	19.7 ± 2.5**	27.5 ± 4.3	0.8 ± 0.3††	25.4 ± 5.0**	28.8 ± 9.8	10.2 ± 3.2††	18, 12, 12
AA (5 × 10^{-6} M)	1.4 ± 0.4*	7.7 ± 3.0†	2.2 ± 0.6	19.2 ± 4.0**	20.9 ± 2.1	33.0 ± 4.1†	12, 12, 12
AA (10^{-5} M)	8.6 ± 0.8**	12.4 ± 1.3†	9.6 ± 0.8	27.3 ± 3.5**	25.9 ± 3.2	30.8 ± 2.3	12, 12, 12
A23187 (10^{-7} M)	30.4 ± 2.8**	—	24.3 ± 2.1	25.4 ± 3.9**	—	25.4 ± 1.8	12, —, 12
A23187 (10^{-6} M)	108.1 ± 11.3**	—	75.5 ± 13.2	45.3 ± 5.8**	—	54.5 ± 6.4	12, —, 12

Results show mean ± s.e.mean for (n) tests on mixed peritoneal leukocytes prepared from (n/6) batches of female Wistar rats of 150–200 g, and are given as the amount of leukotriene B_4 (LTB_4) or thromboxane B_2 (TXB_2) released after stimulating the cells for 10 min at 37°C after deduction of spontaneous (basal) release. All values are shown as ng/2.5 × 10^6 cells. Basal values for unstimulated cells incubated similarly were: LTB_4 0.49 ± 0.08 (24) and TXB_2 5.41 ± 1.86 (18). A23187 alone at 10^{-8} M gave values of 0.7 ± 0.1 (LTB_4) and 2.6 ± 2.0 (TXB_2), and phorbol myristate acetate (PMA) at 10^{-6} M gave values of 0.1 ± 0.1 (LTB_4) and 1.5 ± 1.7 (TXB_2), after deduction of basal values (n = 18 for each). PMA was added 5 min before the stimulus; A23187 was added simultaneously with the stimulant. The symbols *, ** and †, †† indicate significant differences with respect to basal condition, or to stimulated cells lacking A23187 or PMA, respectively, at 0.05 and 0.001 levels.

reversed for A23187. This is also shown in Figure 1. Note also that the ionophore caused generation of much larger amounts of both metabolites in absolute terms (Table 1).

PMA (10^{-10} M–10^{-6} M) did not significantly activate LTB_4 generation by the rat leukocytes and caused a small but significant augmentation of TXB_2 release only at concentrations of 10^{-7}–10^{-6} M (Table 1 footnote and Tables 2 and 3). However, PMA pretreatment for 5 min completely abolished the ability of FMLP to cause LTB_4 generation in a dose-dependent manner with an approximate IC_{50} value of 10^{-9} M, but had much less inhibitory effect on TXB_2 production (Table 1 and Table 2). In contrast, PMA did not inhibit the formation of either eicosanoid caused by either A23187 or AA (Table 1). Table 3 shows that PMA dose-dependently augments eicosanoid production at low A23187 concentrations and has a modest inhibitory effect on LTB_4 generation induced by the higher concentrations of A23187. In the case of arachidonic acid-stimulated leukocytes, PMA had no effect on LTB_4 production but increased the generation of TXB_2 induced by the lower doses of AA, including those which on their own were not active (Table 4).

Co-incubation of a threshold amount of A23187 with FMLP or AA caused an increase in the generation of LTB_4 but did not alter that of TXB_2 (Table 1).

Discussion

Two main conclusions follow from these results: (a) that in rat peritoneal leukocytes there is differential regulation of the 5-lipoxygenase and cyclo-oxygenase pathways, and (b) that the phorbol ester PMA is an extremely powerful and selective inhibitor of the 5-lipoxygenase pathway when stimulated by FMLP. To our knowledge, this differential inhibitory effect of PMA on the two pathways has not been previously identified.

We do not yet know whether PMA inhibits LTB_4 production in response to activation by either phagocytosis or other receptor-active stimulants but we have found that complete inhibition of FMLP-induced LTB_4 release is also caused by phorbol dibutyrate (an active co-carcinogen) but not by inactive phorbol analogues (M.A. Moroney & J.R.S. Hoult, unpublished results). This suggests involvement of protein

Table 2 Effect of phorbol myristate acetate (PMA) on N-formyl-methionine-leucine-phenylalanine (FMLP)-induced generation of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) by rat peritoneal leukocytes

Stimulant: PMA in the presence and absence of FMLP

	LTB_4		TXB_2	
Stimulant (M)	(ng/2.5 × 10^6 cells)			
	Control	+FMLP 10^{-5} M	Control	+FMLP 10^{-5} M
Basal	1.8 ± 0.3	12.5 ± 1.5*	1.5 ± 0.5	15.1 ± 0.9*
PMA 10^{-10}	1.5 ± 0.2	8.0 ± 0.7*	2.2 ± 0.6	9.5 ± 1.1*
PMA 10^{-9}	1.4 ± 0.3	5.5 ± 0.9*	1.1 ± 0.5	11.1 ± 1.7*
PMA 10^{-8}	0.4 ± 0.2†	1.7 ± 0.4*	2.7 ± 0.6	8.3 ± 0.9*
PMA 10^{-7}	0.6 ± 0.1†	1.1 ± 0.3	5.8 ± 1.0†	9.0 ± 0.6*
PMA 10^{-6}	1.1 ± 0.3	1.4 ± 0.3	8.6 ± 0.8†	14.7 ± 0.8*

Stimulant: FMLP in the presence and absence of PMA

	LTB_4		TXB_2	
Stimulant (M)	(ng/2.5 × 10^6 cells)			
	Control	+PMA 10^{-6} M	Control	+PMA 10^{-6} M
Basal	0.4 ± 0.1	0.5 ± 0.1	4.7 ± 1.3	11.6 ± 0.9†
FMLP 10^{-9}	0.2 ± 0.1	0.3 ± 0.2	7.1 ± 0.6	13.6 ± 1.2*
FMLP 10^{-8}	0.6 ± 0.2	0.4 ± 0.2	7.8 ± 0.9	14.1 ± 2.1*
FMLP 10^{-7}	2.4 ± 0.4	0.4 ± 0.2*	22.2 ± 2.8	15.1 ± 1.8
FMLP 10^{-6}	7.7 ± 0.5	0.5 ± 0.1*	28.0 ± 1.7	19.1 ± 1.8*
FMLP 10^{-5}	8.6 ± 1.0	0.6 ± 0.2*	35.2 ± 2.6	25.0 ± 2.3*

Results show mean ± s.e.mean for 3 tests on pooled peritoneal mixed leukocytes assayed in duplicate. Similar results were obtained in a further experiment using mixed leukocytes, and in an additional experiment using a purified polymorphonuclear leukocyte preparation. The symbol * indicates a significant difference from the 'control' value, $P < 0.05$, and † indicates a significant effect of PMA on the release of eicosanoid in the absence of FMLP.

Table 3 Effect of phorbol myristate acetate (PMA) on A23187-induced generation of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) by rat peritoneal leukocytes

Stimulant: A23187 in the presence and absence of PMA

Stimulant (M)	LTB_4 (ng/2.5 × 10^6 cells)		TXB_2 (ng/2.5 × 10^6 cells)	
	Control	+PMA 10^{-6} M	Control	+PMA 10^{-6} M
Basal	0.6 ± 0.0	0.4 ± 0.1	3.5 ± 0.6	7.1 ± 0.7†
A23 10^{-9}	0.4 ± 0.1	3.4 ± 1.3*	3.5 ± 0.3	8.3 ± 1.4*
A23 5 × 10^{-9}	0.8 ± 0.7	1.1 ± 0.7	6.6 ± 0.3	7.3 ± 0.9
A23 10^{-8}	1.6 ± 0.3	6.3 ± 0.8*	9.1 ± 0.6	18.2 ± 2.6*
A23 2.5 × 10^{-8}	5.5 ± 0.5	8.6 ± 0.7*	18.0 ± 1.0	25.7 ± 1.2*
A23 5 × 10^{-8}	22.0 ± 3.9	17.3 ± 1.1	36.5 ± 3.7	27.0 ± 0.8*
A23 10^{-7}	36.6 ± 3.3	25.2 ± 2.3*	34.7 ± 4.5	30.3 ± 2.4
A23 10^{-6}	74.0 ± 3.3	62.6 ± 4.1	51.5 ± 5.7	58.1 ± 2.2

Stimulant: PMA in the presence and absence of A23187

Stimulant (M)	LTB_4 (ng/2.5 × 10^6 cells)		TXB_2 (ng/2.5 × 10^6 cells)	
	Control	+A23187 10^{-8} M	Control	+A23187 10^{-8} M
Basal	0.2 ± 0.1	0.8 ± 0.1*	3.5 ± 0.6	7.4 ± 1.0*
PMA 10^{-10}	0.1 ± 0.1	1.2 ± 0.6*	4.5 ± 0.6	13.5 ± 2.0*
PMA 10^{-9}	0.3 ± 0.2	3.3 ± 0.2*	1.9 ± 0.1	19.7 ± 3.4*
PMA 10^{-8}	0.2 ± 0.1	2.5 ± 0.2*	3.6 ± 0.7	24.8 ± 5.2*
PMA 10^{-7}	0.1 ± 0.4	3.2 ± 0.6*	3.7 ± 0.4	24.2 ± 6.6*
PMA 10^{-6}	0.2 ± 0.2	3.8 ± 0.8*	6.6 ± 1.7	30.2 ± 4.9*
PMA 10^{-5}	0.1 ± 0.1	5.0 ± 0.8*	7.9 ± 1.0†	33.5 ± 4.5*

Results show mean ± s.e.mean for 3 tests on pooled peritoneal mixed leukocytes assayed in duplicate. Similar results were obtained in a further experiment using mixed leukocytes, and in an additional experiment using a purified polymorphonuclear leukocyte preparation. The symbol * indicates a significant difference from the 'control' value, $P < 0.05$, and † indicates a significant effect of PMA on the release of eicosanoid in the absence of A23187. A23 indicates A23187.

kinase C. However, direct actions on the enzymes involved in LTB_4 generation or on LTB_4 metabolism can be excluded because the effects of A23187 and AA as stimulants for LTB_4 generation were both unimpaired by PMA and because ω-metabolites of LTB_4 are not formed by rat peritoneal leukocytes (Laughton *et al.*, 1989). Moreover, an effect of protein kinase C stimulation on the FMLP receptor seems unlikely as there was much less inhibition of TXB_2 generation observed in these and other experiments.

We can only rationalise these results by suggesting that there must be multiple mechanisms involved in stimulus-eicosanoid generation in these cells (including a protein kinase C-sensitive step following activation of the FMLP receptor in the pathway leading to leukotriene generation but not that which causes thromboxane generation). One possibility concerns the release of arachidonic acid from phospholipids by the sequential action of phospholipase C and diglyceride lipase, first suggested by Bell *et al.* (1979). Thus it is possible that rat leukocytes might contain several types of phospholipase C which are triggered differently by various stimulants and only some of which are inhibited by phorbol ester treatment. Inhibition of phospholipase C by phorbol esters has been well documented (see Smith *et al.*, 1987), and the role of diglyceride lipase in the rat cells, if any, could be investigated by use of the inhibitor RHC 80267 (Sutherland & Amin, 1982).

Another route known to lead to the synthesis of cyclo-oxygenase products depends upon arachidonate release catalysed by activation of phospholipase A_2: previous studies using leukocytes have suggested that this enzyme can be stimulated by PMA or via protein kinase C (e.g. Burch, 1987; Matsumoto *et al.*, 1988; Tao *et al.*, 1989). Provision of arachidonate for cyclo-oxygenase could then occur independently of the route(s) which supply 5-lipoxygenase, as suggested previously for macrophages (Humes *et al.*, 1982). Overall, this problem could be tackled by analysing the source(s) of arachidonate in terms of the phospholipid type(s) and phospholipase(s) involved in its release.

Table 4 Effect of phorbol myristate acetate (PMA) on arachidonic acid (AA)-induced generation of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) by rat peritoneal leukocytes

Stimulant: arachidonic acid in the presence and absence of PMA

Stimulant (M)	LTB_4 (ng/2.5 × 10^6 cells)		TXB_2 (ng/2.5 × 10^6 cells)	
	Control	+PMA 10^{-6} M	Control	+PMA 10^{-6} M
Basal	0.1 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	5.0 ± 0.7†
AA 5 × 10^{-8}	0.2 ± 0.1	0.5 ± 0.2	1.6 ± 0.2	4.9 ± 0.6*
AA 10^{-7}	0.2 ± 0.1	0.8 ± 0.3*	1.6 ± 0.4	7.1 ± 2.5
AA 5 × 10^{-7}	0.6 ± 0.2	0.3 ± 0.1	7.9 ± 0.4	30.6 ± 1.2*
AA 10^{-6}	0.5 ± 0.3	0.4 ± 0.2	17.4 ± 1.0	37.4 ± 2.0*
AA 5 × 10^{-6}	1.0 ± 0.3	1.2 ± 0.2	36.7 ± 2.6	47.1 ± 6.8
AA 10^{-5}	8.0 ± 0.9	7.5 ± 0.2	40.2 ± 2.6	41.2 ± 1.6

Results show mean ± s.e.mean for 3 tests on pooled peritoneal mixed leukocytes assayed in duplicate. Similar results were obtained in a further experiment using mixed leukocytes, and in an additional experiment using a purified polymorphonuclear leukocyte preparation. The symbol * indicates a significant difference from the 'control' value, $P < 0.05$, and † indicates a significant effect of PMA on the release of eicosanoid in the absence of arachidonic acid.

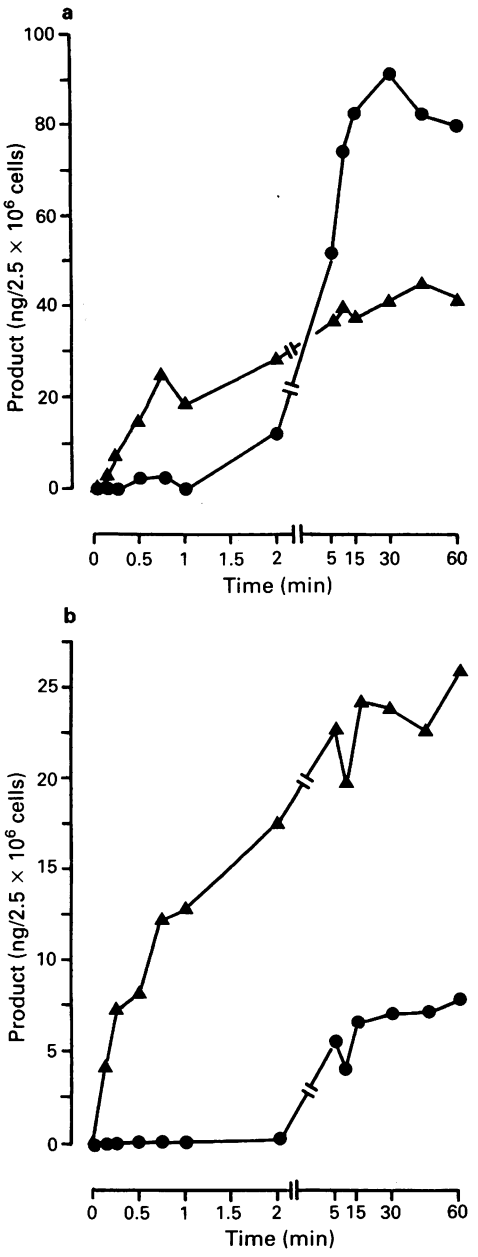

Figure 1 Time course of release of thromboxane B_2 (▲) and leukotriene B_4 (●) from rat peritoneal cells stimulated by 10^{-6} M A23187 (a) or 10^{-5} M N-formyl-methionine-leucine-phenylalanine (b). Results show mean for triplicate determinations at each time point. Note the change in calibration of the time scale after 2 min.

The conclusion that there is functional compartmentalisation of the 5-lipoxygenase and cyclo-oxygenase within rat peritoneal leukocytes is supported by several other lines of evidence. For example, the data show that the profile of metabolites formed differs between AA or FMLP as stimulants (levels of TXB_2 larger at all doses) and A23187 (levels of LTB_4 larger). Moreover, the concentration-response curves indicate that for each stimulant, TXB_2 is generated at lower concentrations (i.e. lower threshold concentrations and lower EC_{50} values, see Results). Further, a low concentration of calcium ionophore significantly augments LTB_4 generation, but not that of TXB_2 (Table 1). This is consistent with the known calcium dependency of 5-lipoxygenase (Rouzer & Samuelsson, 1985), and has been observed previously (Tripp *et al.*, 1985; Liles *et al.*, 1987). Finally, the time course of release of the two eicosanoids differs (Figure 1): TXB_2 is released from the cells much more rapidly than LTB_4, implying a spatial or functional difference between cyclo-oxygenase and 5-lipoxygenase pathways.

It was notable that the effects of PMA were monophasic, and did not show any trend towards biphasic or 'bell-shaped' effects (see Tables 2 and 3). It has been observed previously that in human polymorphonuclear neutrophils PMA has a biphasic effect on LTB_4 generation (Randall *et al.*, 1986). In those experiments, as in the results obtained here, PMA did not itself cause LTB_4 generation and lower PMA concentrations enhanced the effect of A23187. However, higher concentrations of PMA suppressed A23187-induced LTB_4 generation altogether, whereas this was clearly not the case in our experiments (Table 3, lower section). There is no obvious explanation for the difference, apart from the species, and indeed Randall *et al.* pointed out that their results were also at variance with the earlier findings of Tripp *et al.* (1985). However, biphasic effects of PMA on other human neutrophil functional responses have also been noted (e.g. O'Flaherty *et al.*, 1989).

These results for mixed rat peritoneal leukocytes have been replicated with purified (>95%) polymorphonuclear leukocytes, tested under identical conditions. Mononuclear cells also generate LTB_4 and TXB_2 in response to these three stimulants but in smaller amounts; however, in general their response to the described manipulations appears similar.

In conclusion, the 5-lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism in rat peritoneal leukocytes are functionally distinct and can be activated and modulated differentially by stimulants and agents which interact with cellular transduction mechanisms.

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Mode of inhibitory actions of acute and chronic chloroquine administration on the electrically stimulated mouse diaphragm *in vitro*

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1 The effects of bath applied chloroquine (Chlo) and of acute and chronic Chlo administration on skeletal muscle reactivity to electrical stimulation and to drugs have been studied on mouse hemidiaphragm preparations *in vitro*.

2 Chlo (0.15–150 μM) produced a concentration-dependent inhibition of twitch and tetanic contractions due to direct muscle stimulation (MS). Acute and chronic administration of Chlo (45 mg kg^{-1}, i.p. daily, for 3–28 days) progressively shifted the concentration-response curve to bath-applied Chlo to the right, with maximum effect occurring from day 14 of Chlo pretreatment.

3 Acute and chronic administration of Chlo decreased the twitch and tetanus tension, raised the minimal fusion frequency (MFR) for tetanic contraction to occur and did not alter the twitch/tetanus tension ratio. Tetanus tension unlike twitch tension was not significantly decreased on day 3.

4 Caffeine (5–500 μM) – and isoprenaline (0.001–0.8 μM)-induced potentiations of twitch contraction were attenuated in a concentration-dependent manner by bath-applied Chlo and by acute and chronic administration of Chlo. Higher concentrations of caffeine (0.1–5 μM) and KCl (10 mM–130 mM) produced contracture of the muscle which was sensitive to inhibition by Chlo (50–150 μM). Moreover, the spike contractions superimposed on caffeine contracture were more sensitive to the inhibitory effect of Chlo than the contracture.

5 The inhibitory effects of dantrolene sodium and (+)-tubocurarine on MS and on indirectly stimulated hemidiaphragm respectively were not significantly altered by acute and chronic administration of Chlo. In contrast, the inhibitory concentration-response curve to procaine was shifted to the right.

6 The inhibitory effect of bath-appled Chlo, or acute and chronic pretreatment on twitch tension (MS) was not significantly antagonized by stepwise increase in extracellular Ca^{2+} (0.05–2.5 mM). Sodium thiocyanate (1–5 mM) reversed in a concentration-dependent manner the inhibitory effects of Chlo.

7 Complete recovery of twitch contractions occurred after 3 days of stopping daily Chlo administration, with partial recovery to tetanic tension after 28 days and no recovery of MFR. The reactivity of the diaphragm to bath applied Chlo was progressively restored, whereas the tension curve area to caffeine and KCl was still attenuated even 28 days after stopping Chlo pretreatment.

8 It is concluded that acute and chronic Chlo administration results in changes in reactivity of the hemidiaphragm muscle to electrical stimulation and to drugs such that there is a decrease in muscle strength and tolerance to Chlo *in vitro*. These effects are dependent on its direct inhibitory action on skeletal muscle and may result from interference with Ca^{2+} mobilization within the muscle.

Introduction

Chloroquine (Chlo) is a 4-aminoquinoline used for the treatment and recently for prophylaxis of malaria (Rollo, 1980). In addition it has been used extensively for decades in the treatment of rheumatoid arthritis, systemic and lupus erythematosus and other collagen diseases (McChesney & Rothfield, 1964; Hughes *et al.*, 1974; Magnussen & Olivarius, 1977; Stillman, 1981). In such pathological conditions in man, high daily doses (250–500 mg, or more) have been found necessary and for prolonged periods (Bagnall, 1957; Hughes *et al.*, 1974; Magnussen & Olivarious, 1977). Such doses are much larger than those necessary for therapy of acute malarial attacks (Rollo, 1980). These regimens have frequently resulted in important side effects, particularly changes in the cardiovascular function manifested as congestive heart failure and cardiovascular collapse (Thompson & Werbel, 1972; Hughes *et al.*, 1974; Magnussen & Olivarius, 1977), retinopathy (Thompson & Werbel, 1972) and myopathy of the skeletal muscle (Lüllman-Rauch, 1979).

Skeletal muscle myopathy associated with chronic Chlo administration has been confirmed by electron microscopic studies (Stauber *et al.*, 1981; Ette & Essien, 1986) and may be the cause of decreased muscle strength following Chlo administration (Rollo, 1980). It has been reported that Chlo can accumulate in lysosomes, smooth endoplasmic reticulum and sarcoplasmic reticulum. Such an effect usually results in ultrastructural changes in some cells which appear as swellings, autophagic vacuoles, vacuolar myopathy and degeneration of myofibrils (Smith & O'Grady, 1966; MacDonald & Engel, 1970; Oberc & Engel, 1977; Ayitey-Smith & Gbewonyo, 1977; Ette & Essien, 1986). The present experiments were designed to determine the effects on skeletal muscle of acute and chronic administration of Chlo with special emphasis on the following: (i) responsiveness of the diaphragm muscle to electrical stimulation and drugs; (ii) mechanism of action of Chlo-induced decrease in muscle tension; (iii) reversibility of the effect of chronic administration of Chlo and (iv) reactivity of some skeletal muscle relaxants following acute and chronic administration of Chlo.

Methods

Swiss Webster mice (30–40 g) of either sex were stunned and killed by exsanguination. The diaphragm with the phrenic nerve was dissected out according to the method of Bülbring (1946) and placed under an initial load of 2 g in an organ bath

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containing double glucose Tyrodes solution of the following composition (mM): Na^+ 149.2, K^+ 2.7, Ca^{2+} 3.6, Mg^{2+} 2.1, Cl^- 145.3, $H_2PO_4^-$ 0.4, HCO_3^- 11.9 and glucose 10. The bath was maintained at room temperature (approximately 20°C). The pH of the solution was 7.3–7.4. Contractions of the hemidiaphragm were elicited either indirectly via the phrenic nerve (NS) or directly (MS) with rectangular pulses of 0.5 ms at a frequency of 1 Hz and supramaximal voltage. A Grass S 88 stimulator connected to a Grass SIU5 stimulus isolation unit pulse generator was used to deliver the rectangular pulses. Tetanizing stimuli were delivered at 20–60 Hz for 5 s. Isometric contraction of the muscle to electrical stimulation or to drugs was recorded with a force-displacement transducer (Grass FT.03) and displayed on a Grass ink writing curvilinear polygraph (Model 7D, Grass Instruments, Quincy, U.S.A., MA). The minimal fusion frequency (MFR) was obtained in any one experiment by stimulating the muscle directly (MS) for 1 s. The muscles were stimulated with trains of pulses 2 ms duration, with 5 Hz increases in frequency starting at 10 Hz every 5 min with supramaximal voltage until the fusion frequency was identified. The muscles were pre-equilibrated for 60–90 min before use. During that time, the physiological solution in the organ bath was replaced every 20 min and the passive tension re-adjusted when necessary.

Concentration-response curves and activity profile for chloroquine

Concentration-response curves to Chlor were obtained by cumulative addition of graded concentrations. Since the response to Chlo developed slowly each concentration of Chlo was allowed to remain in contact with the preparation until a plateau response had been obtained before the preparation was challenged with an additional concentration of Chlo. In experiments in which the time course of effect of Chlo was investigated, a pair of phrenic nerve-hemidiaphragm preparations from the same animal was used, with one acting as control, while the other was treated with the appropriate concentration of Chlo. Chloroquine was then added to the tissue bath 1–2 min after the start of electrical stimulation and allowed to act for 30–90 min or until maximum inhibition had been obtained. The effectiveness of NS and MS was assessed in the presence of (+)-tubocurarine (5 μM). Twitch tension to NS was usually abolished while MS was only slightly reduced (5–10%) and (+)-tubocurarine was present in experiments in which the effect of MS was studied.

Concentration-response curves for dantrolene sodium, procaine, (+)-tubocurarine, caffeine and potassium chloride

Concentration-response curves to the inhibitory effect of dantrolene sodium, procaine and (+)-tubocurarine and to the stimulatory effect of caffeine were obtained by cumulative addition of graded concentrations of each agent. The concentration-response curve for potassium chloride was established by sequential addition of an appropriate concentration which was left in contact with the tissue until a maximum response for that concentration had been obtained. IC_{50} and IC_{100} are concentrations of agents producing 50 and 100% inhibition of twitch contractions, respectively.

Chloroquine pretreatment

Preliminary studies indicated that tolerated daily dose of chloroquine diphosphate in mouse ranged from 45–50 mg kg^{-1}, i.p. In studies in which mice were pretreated with Chlo, the animals were divided into two groups with one group receiving Chlo and the other acting as control. The chloroquine pretreated group was given daily i.p. injections of 45 mg kg^{-1} of Chlo in isotonic saline (NaCl, 0.9% w/v). Chlo was administered daily for up to 4 weeks and animals were killed on day 0 (control), 3, 7, 14, 21 and 28. They were allowed free access to water and mouse pellets throughout the period of drug administration. In another series of experiments to study the effect of recovery from acute and chronic Chlo administration, it was administered for 4 weeks. The animals were then allowed to recover from the effect of Chlo and killed 3, 7, 14 and 28 days after the last Chlo administration. Muscle tension measurement was calculated in units of tension g^{-1} tissue mass which was estimated at the conclusion of each experiment.

Results are expressed as mean values ± s.e.mean, based on at least four experiments. Significance was determined by the Student's t test for paired data. A probability level of $P < 0.05$ was considered significant.

Materials

The following drugs were used: chloroquine diphosphate (Sigma), caffeine, sodium thiocyanate (BDH), dantrolene sodium (Norwich Eaton Pharmaceuticals, New York, U.S.A.), procaine HCl (Sigma). All drugs were made up in 0.9% w/v sodium chloride solution and concentration expressed as the salt weight.

Results

Effect of chlorquine on twitch contraction to electrical stimulation of mouse diaphragm

Chlo reversibly inhibited twitches in response to both nerve stimulation (NS) and to direct muscle stimulation (MS) in a concentration-dependent manner (Figure 1). Twitch contraction to NS was more sensitive to the inhibitory effect of Chlo (0.15–4.53 μM) with an IC_{50} of 1.73 ± 0.12 μM ($n = 5$) as compared to MS (0.45–50 μM) with an IC_{50} of 5.10 ± 0.16 μM ($n = 6$). In another series of experiments the activity profile of the inhibitory effect of Chlo at the IC_{50} and IC_{100} and their

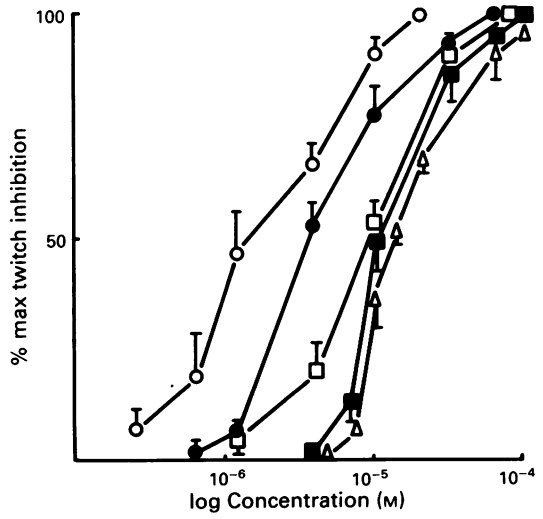

Figure 1 Effect of chlorquine (Chlo) on twitch tension due to direct muscle stimulation of the mouse diaphragm in normal non-treated and in acute and chronic Chlo pretreated preparations. Concentration-response curve to Chlo in non-treated diaphragm (day 0) (○); after pretreatment with Chlo for 3 days (●); 7 days (□); 14 days (■) and 28 days (△). The responses to Chlo are expressed as a percentage of maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines show s.e.mean.

reversal on washing were studied in order to identify any changes in muscle responsiveness to acute and chronic Chlo pretreatment. The response to MS was maximally inhibited at 2–3 min (IC_{50}) and 9–22 min (IC_{100}) while the response to NS was inhibited after 5–8 min (IC_{100}). Recovery from the effect of Chlo was faster for NS than MS and the data obtained (mS, day 0) from recovery of 5 muscle preparations following 10 min exposure to Chlo (50 μM) show that it required 25–30 min and 50–60 min to reverse the twitch inhibition to NS and MS to 100%, respectively, following washing with Chlo-free Tyrode solution.

Effect of acute and chronic chloroquine pretreatment on twitch response to electrical stimulation and on chloroquine concentration-response curve

Since Chlo is known to cause myopathy of the skeletal muscle which is associated with degenerative changes of the myofibrils, the effect of prolonged pretreatment with Chlo was studied to assess its effect on muscle strength. Chlo (0.75–100 μM) reversibly depressed the twitch height to NS and MS in preparations obtained from mice pretreated daily with Chlo (45 mg kg^{-1}, i.p.) for 3–28 days. Acute and chronic administration of Chlo shifted the concentration-response curve of Chlo to the right with maximum shift occurring from day 7 (Figure 1 and Table 1). Chlo pretreatment changed the concentration-response relations to Chlo, increasing the maximal, and minimal inhibitory concentrations for Chlo. The activity profile of Chlo was also altered being dependent on length of pretreatment with Chlo. The time to onset (Table 1) and maximum inhibition to MS was progressively increased with latent periods of 8.25 ± 2.70 and 62.4 ± 5.36 s on day 3 and day 28 pretreated diaphragms respectively, compared to control. Furthermore, the time to complete recovery of twitch amplitude (MS) after washout of Chlo was also dependent on the length of pretreatment taking 50–60 and 35–40 min for day 7 and day 28 pretreated diaphragms respectively. Recovery from the inhibitory effect of Chlo in these preparations was faster in NS than in MS. Moreover, recovery to NS and MS was faster in pretreated than in normal diaphragm.

Effect of acute and chronic chloroquine pretreatment on twitch and tetanus, and twitch/tetanus ratio

Chlo (3–15 μM) significantly depressed tetanus to NS and MS (Table 2) while higher concentrations (30–100 μM) abolished tetanic contractions. Chlo depressed to an equal extent the twitch and tetanic tension as indicated by the twitch/tetanus ratio of 0.10 ± 0.03 (Table 2). In diaphragms obtained from Chlo-pretreated animals, twitch tension and tetanus were significantly reduced by 15–70% and 20–65% respectively (Table 2), the effect being maximal from day 7 of pretreatment. Although Chlo-pretreatment depressed both twitch and tetanus tension to equal extent in day 7, 14 and 28 treated diaphragms, the decrease was larger in the twitch tension response in day 3 diaphragms as indicated by the decrease in the ratio of twitch/tension (Table 2).

Effect of acute and chronic chloroquine pretreatment on minimal fusion frequency

The minimal fusion frequency (MFR) was studied in non-Chlo-treated and in acute and chronic Chlo pretreated diaphragms. Throughout each experiment the muscles were stimulated with trains of pulses (15–18 Hz for 1 s) every 5 min so as to produce incomplete tetanic contraction (MFR). Chlo (5–10 μM) induced a concentration-dependent increase in the MFR of 40–50% from a mean control value of 18.2 ± 1.12 Hz in non-Chlo pretreated preparations. Acute and chronic Chlo pretreatment for 3–28 days also increased the MFR by 40–60%, an effect which was not dependent on the length of Chlo pretreatment (Table 2). In another series of experiments the effect of IC_{50} of Chlo (5.0 μM) on MFR was examined in muscles obtained from Chlo pretreated mice. Chlo did not increase further the MFR in these preparations (Table 2).

Effect of sodium thiocyanate and increase in extracellular calcium concentration on the inhibitory effect of chloroquine and acute and chronic chloroquine pretreatment on twitch tension

The depressant effect of Chlo (1.50–150 μM) on twitch tension (MS) was reversed in a concentration-dependent manner by

Table 1 Effect of chloroquine (IC_{50}) on twitch/tetanus tension ratio (Tw/Tt) and minimal fusion frequency (MFR) on chloroquine pretreated mouse diaphragm

Pretreatment day	*Tension (g)*				*In the presence of chloroquine (5.0 μM)* *Tension (g)*			
	Twitch	*Tetnus*	*Tw/Tt*	*MFR (Hz)*	*Twitch*	*Tetanus*	*Tw/Tt*	*MFR (Hz)*
Control	1.14 ± 0.62	13.5 ± 1.32	0.10 ± 0.03	18.5 ± 1.12	0.63 ± 0.17	5.33 ± 1.25	0.12 ± 0.03	26.7 ± 2.89
Day 3	0.77 ± 0.13*	11.7 ± 1.67	0.67 ± 0.01	27.2 ± 2.16*	0.77 ± 0.25	7.23 ± 2.54	0.11 ± 0.05	31.6 ± 1.92
Day 7	0.39 ± 0.12*	4.43 ± 0.44*	0.09 ± 0.03	28.5 ± 1.89*	0.42 ± 0.05	3.53 ± 1.66	0.12 ± 0.05	31.6 ± 2.01
Day 14	0.53 ± 0.06*	3.63 ± 0.73*	0.10 ± 0.04	27.1 ± 1.22*	0.55 ± 0.06	3.07 ± 1.28	0.14 ± 0.07	29.1 ± 1.86
Day 28	0.50 ± 0.11*	5.10 ± 0.12*	0.10 ± 0.02	26.4 ± 1.84*	0.62 ± 0.10	4.59 ± 1.44	0.14 ± 0.04	32.3 ± 2.12

* Significantly different from their respective controls $P < 0.05$.
Results are expressed as mean ± s.e.mean from at least 4 experiments.

Table 2 Chloroquine IC_{50} values and recovery profile on normal and chloroquine pretreated diaphragm

Pretreatment day	IC_{50} (μM)	*Time to inhibitory response (s)*	IC_{50} (μM)	*Recovery after 28 days pretreatment* *Tension (g)*			
				Twitch	*Tetanus*	*Tw/Tt*	*MFR (Hz)*
Control	5.10 ± 0.16	0.14 ± 0.05	25.8 ± 2.96	0.50 ± 0.11	5.10 ± 0.12	0.10 ± 0.03	26.4 ± 1.84
Day 3	8.13 ± 0.25	8.25 ± 2.70	26.3 ± 4.04	1.02 ± 0.07	8.99 ± 0.89	0.11 ± 0.01	26.7 ± 2.87
Day 7	10.3 ± 1.72	23.3 ± 1.92	18.7 ± 1.58	1.10 ± 0.08	8.67 ± 1.15*	0.12 ± 0.02	26.7 ± 2.35
Day 14	23.8 ± 2.82	27.8 ± 2.70	8.50 ± 0.62	1.01 ± 0.05*	8.73 ± 0.64*	0.12 ± 0.02	25.0 ± 3.01
Day 28	25.8 ± 2.96	62.4 ± 5.36	8.01 ± 0.30	1.06 ± 0.04*	9.01 ± 0.08*	0.11 ± 0.01	25.5 ± 29.6

* Significantly different from their respective controls $P < 0.05$;
Controls for recovery data are results obtained following Chlo pretreatment for 28 days;
Results are expressed as mean ± s.e.mean from at least 4 experiments. MFR – Minimal fusion frequency.

thiocyanate ions, SCN^{-1} (1–5 mM). The effects of Chlo (5–100 μM) were restored by 35–50% in the presence of 1 mM thiocyanate ion. The effect of SCN^- was rapid in onset (5–10 s) but tension returned to the original depressed level when the preparation was returned to Tyrode solution containing only Chlo. In Chlo pretreated diaphragm (day 28), the SCN^{-1} ion (1 mM) also restored the depressed twitch tension substantially to near control values of non-Chlo-treated diaphragms. When Chlo (100 μM) was added to Chlo pretreated preparations, the depressed twitch tension was also markedly reversed (30–40%) by the SCN^- ion (1 mM). In order to elucidate further the mechanism of the inhibitory action on the twitch tension, the effect of increase in $[Ca^{2+}]_o$ on the twitch tension was investigated. Stepwise increase in $[Ca^{2+}]_o$ of 0.05–2.5 mM did not affect the Chlo-induced inhibition in normal and Chlo (day 3 to day 28) pretreatment diaphragms. However, a Ca^{2+} concentration of 0.02 mM increased by 15–20% the twitch response to MS, the maximal effect occurring after 30 min of incubation.

Effect of chloroquine on caffeine- and isoprenaline-induced increase in twitch tension and on caffeine- and potassium-induced contracture

In order to elucidate further the possible effect of Chlo on Ca^{2+} sequestration, procedures known to interfere with Ca^{2+} mechanism were studied in normal and Chlo pretreated preparations. Caffeine (5–500 μM) and isoprenaline (0.001–0.8 μM) increased the twitch tension to direct muscle stimulation (MS) in a concentration-dependent manner (Figure 2). Chlo (2.5–150 μM) caused a concentration-dependent attenuation of the responses to caffeine (Figure 2a) and isoprenaline (Figure 2b). Propranolol (1.0 μM), reduced and/or abolished the twitch potentiation induced by isoprenaline but was without effect on the caffeine response. Caffeine (0.1–5 mM; Figure 3) and KCl (10 mM–130 mM) produced contracture of the mouse diaphragm in a concentration-dependent manner. The contracture produced by caffeine was superimposed with twitch contractions and Figure 4a shows the activity profile to caffeine. Potassium (20 mM–50 mM) induced a sustained contracture (lasting over 60 min) but higher concentrations (80 μM) produced a non-sustained contracture which usually declined to basal tension in 2–3 min in the presence of KCl (Figure 5a). Chlo (50–150 μM) produced a concentration-dependent attenuation of responses to caffeine and KCl. The twitch contraction to caffeine was more sensitive to inhibition by Chlo than was the contracture, being abolished at a Chlo concentration of 50 μM (Figure 4b). Chlo increased the latent period for caffeine- and KCl-induced contracture by 1–3 min, had no effect on the time for KCl contracture to fall to basal tension, but decreased that to caffeine by 10–20 min.

Effect of acute and chronic chloroquine pretreatment on caffeine, isoprenaline and KCl response

Chlo pretreatment daily for 3 to 28 days progressively shifted the concentration-response curve to caffeine and KCl to the right, the shift being maximum from day 14 (Figure 2a and b) but the maximum contraction to caffeine was not significantly depressed. Furthermore, the twitch contractions induced by caffeine (0.1–5 mM) were also attenuated and/or abolished by Chlo pretreatment. The decrease in the amplitude and frequency of twitch contraction was dependent on length of pretreatment with Chlo and was abolished from day 14 (Figure 4c–e). The twitch contraction was more sensitive to inhibition than was the contracture. The potentiation of twitch tension induced by caffeine (5–500 μM) was attenuated by pretreatment with Chlo for 7 to 28 days resulting in a significant shift ($P < 0.05$) to the right of the concentration-response curve. The shift was maximum after administration of Chlo for 14 days (Figure 2a). The effect of pretreatment with Chlo on isoprenaline-induced potentiation of twitch

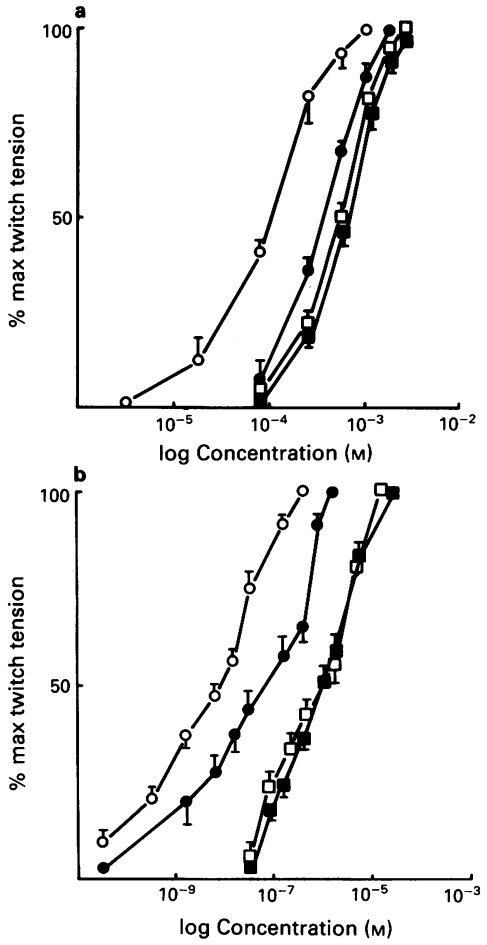

Figure 2 Effects of acute and chronic chloroquine (Chlo) administration on caffeine- and isoprenaline-induced potentiation of twitch contractions due to direct electrical stimulation of mouse diaphragm preparations. Concentration-response curve to caffeine (a) and isoprenaline (b) in non-treated diaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 7 days (●); 14 days (□) and 28 days (■). The responses to Chlo are expressed as a percentage of maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean. Note the progressive depression of the caffeine- and isoprenaline-induced twitch potentiation.

tension was qualitatively similar to that observed with caffeine (Figure 2b). The inhibitory effect of pretreatment with Chlo was dependent on the length of administration of Chlo with maximum shift of the concentration-curve occurring from day 14. Chlo pretreatment markedly altered the activity profile of KCl (130 mM; Figure 5a–d) on preparations obtained from animals treated with Chlo for 7–28 days. The duration of KCl contracture was prolonged, with the contracture being sustained for 8.5 ± 0.4 min compared to the control of 2.5 ± 0.2 min. Although the amplitude of the contracture was attenuated by $60 \pm 5.8\%$, the tension curve area (area under the contracture) was not significantly different from control of 550 ± 40.5 g s^{-1}.

Effect of acute and chronic chloroquine pretreatment on the inhibition of twitch tension by dantrolene sodium, procaine and (+)-tubocurarine

In order to evaluate the specificity of effect on Ca^{2+} sequestration of acute and chronic Chlo pretreatment on the diaphragm, drugs which are known to inhibit twitch and tetanus tension via different mechanisms on Ca^{2+} mobilization were examined in Chlo pretreated preparations. Dantrolene (0.1–10 μM) and procaine (1.0–100 μM; Figure 6) produced concentration-dependent inhibition of twitch tension due to

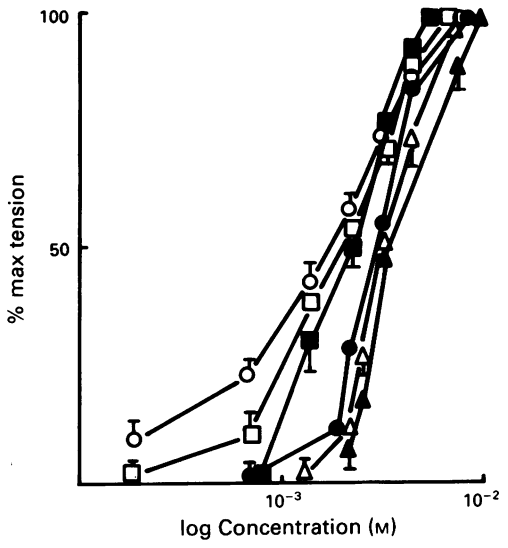

Figure 3 Effects of chloroquine added to the bath and of acute and chronic chloroquine (Chlo) administration on the contracture induced by caffeine in mouse diaphragm preparations. Concentration-response curves to caffeine in non-treated diaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 3 days (□); 7 days (■); 14 days (△) and 28 days (▲). The concentration-response curves to caffeine in the presence of Chlo (100 μM) are represented (●). The responses to caffeine are expressed as a percentage of maximal contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean.

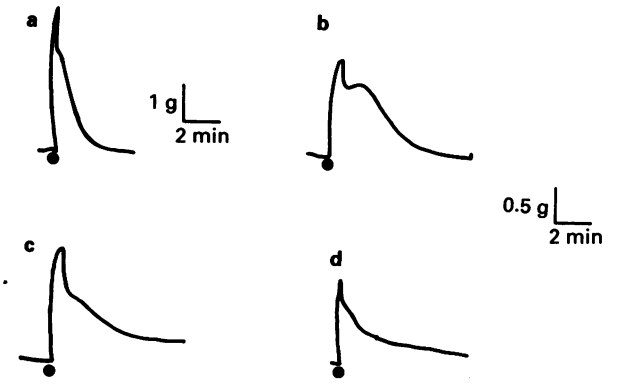

Figure 5 Effects of chloroquine (Chlo) added to the organ bath and of acute and chronic administration of Chlo on potassium-induced contraction in mouse hemidiaphragm preparation. Black dots indicate addition of 130 mM KCl. (a) Potassium contraction (130 mM) declined to basal tension in the presence of potassium in the bath. (b) Chlo (50 μM) decreased the contraction to potassium but prolonged the duration of the contraction. (c–d) Chlo administration (45 mg kg^{-1}, i.p.) daily for 7 days (c) and 28 days (d) on potassium-induced contraction. Chlo pretreatment decreased and prolonged the contraction to potassium.

MS. In Chlo pretreated preparations dantrolene inhibited the twitch contractions to MS in a concentration-dependent manner (Figure 6b) and pretreatment for 3–14 days did not alter the concentration-response relations i.e. the slope or the maximal or minimal effective concentrations for dantrolene. Although the IC_{50} values for dantrolene obtained before and after pretreatment with Chlo were not significantly different (day 3 to day 14), there was a shift to the right in day 28 pretreated diaphragms ($P < 0.05$). The time to onset and maximum inhibition of MS was not altered in any Chlo pretreated preprations (day 7 to day 28). Similarly procaine inhibited the twitch tension to MS following Chlo pretreatment for 3–28 days. However, the concentration-response curve to procaine was shifted significantly to the right ($P < 0.05$), an effect which was dependent on the length of pretreatment with Chlo (Figure 6a). Moreover, the rate of inhibition of twitch contraction by procaine was faster in the non-Chlo pretreated diaphragm (15–20 min) compared to pretreated preparations (30–40 min). (+)-Tubocurarine (0.1–5 μM) attenuated the twitch response to NS without significantly affecting that to MS. The concentration-effect relation was not altered by pretreatment with Chlo for 3–28 days (Figure 6c). Furthermore, pretreatment did not change the activity profile to (+)-tubocurarine; the time to maximum inhibition was not different from the control of 2.8 ± 0.35 min.

Effect of recovery from acute and chronic chloroquine pretreatment on tetanus, minimal fusion frequency, caffeine and KCl

In order to ascertain whether the effect of acute and chronic administration of Chlo observed in this study was reversible, a series of experiments was conducted on muscles obtained from

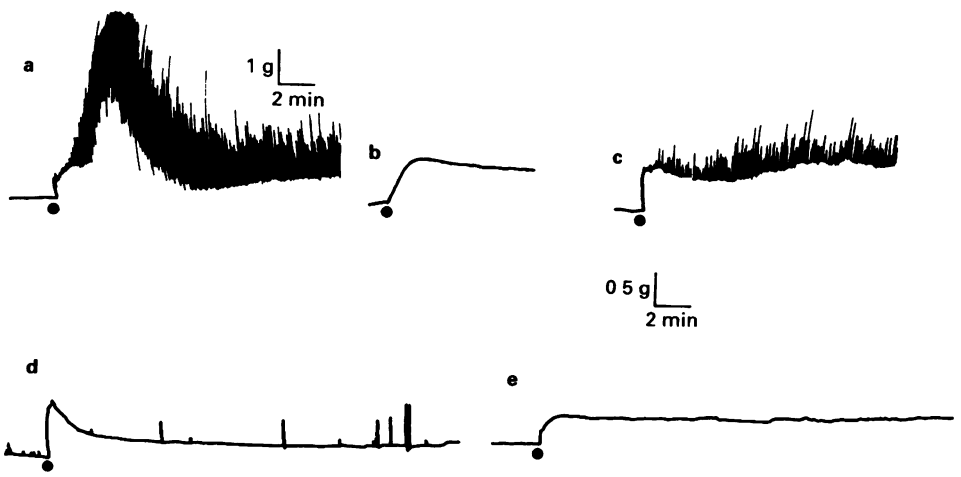

Figure 4 Effects of chloroquine (Chlo) added to the organ bath and of acute and chronic administration of chloroquine on caffeine-induced contracture in mouse hemidiaphragm preparation. (a) Caffeine-induced contracture (5 mM) was superimposed by spike contractions. (b) Chlo (50 μM) abolished the spike contractions and produced a sustained contraction to caffeine. (c–e) Chlo administration (45 mg kg^{-1}, i.p.) daily for 7 days (c), 14 days (d) and 28 days (e) on caffeine-induced contracture. Addition of 5 mM caffeine indicated by a black dot in each section. Chlo pretreatment produced a parallel decrease in the amplitude and frequency of spike contractions as well as a decrease in the contracture.

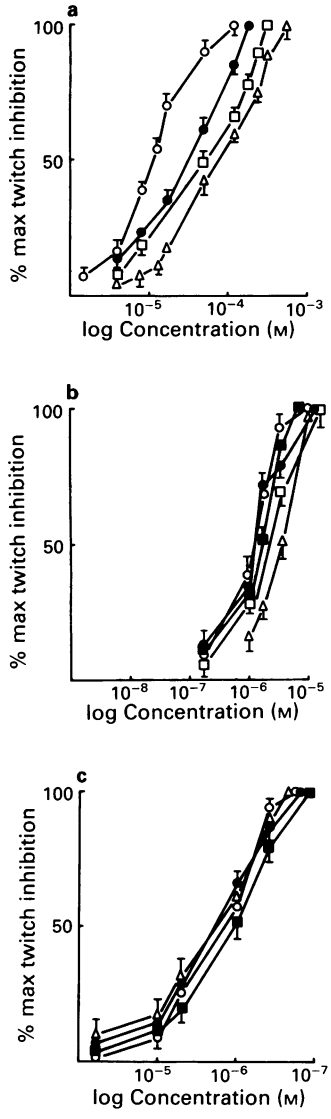

Figure 6 Effects of acute and chronic chloroquine (Chlo) administration on dantrolene sodium-, procaine- and (+)-tubocurarine-induced inhibition of twitch contraction due to electrical stimulation of mouse hemi-diaphragm preparations. Concentration-response curves to procaine (a), dantrolene (b) and (+)-tubocurarine (c) in non-treated hemidiaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 3 days (●); 7 days (□); 14 days (■) and 28 days (△). The responses to the inhibitory agonists are expressed as a percentage of the maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean. Experiments with (+)-tubocurarine were conducted in hemidiaphragm preparations stimulated indirectly via the phrenic nerve. Chlo pretreatment for 3–28 days selectively induced tolerance to the inhibitory effect of procaine.

mice pretreated with Chlo for 28 days and then allowed to recover from the effect of pretreatment. Reactivity of the muscle to the inhibitory effect of Chlo on twitch tension was gradually restored to normal as indicated by the progressive shift to the left of the concentration-response curve to Chlo (Table 2). Partial recovery was observed from day 7 with an IC_{50} of 18.7 ± 1.58 μM. The recovery profile to Chlo indicates that the time to onset and maximal inhibition of twitch contractions returned progressively to normal values with complete recovery occurring from day 14. Twitch and tetanus tension exhibited differential recovery. Twitch tension recovered to control tension after 3 days of stopping Chlo administration, whereas tetanus tension recovered only partially even after 28 days (Table 2). The MFR showed no recovery throughout the 28 days of observation (Table 2). The responsiveness of the diaphragm to the stimulatory effect of caffeine and KCl was partially restored with time after stopping the daily administration of Chlo. The contracture response to caffeine recovered faster than the twitch contractions with evidence of recovery being observed after 7 days of stopping pretreatment with Chlo. The tension curve area for caffeine after 14 and 28 days was 4.8 ± 0.25 and 5.2 ± 0.42 g min^{-1} respectively compared to a control of 24.5 ± 2.86 g min^{-1}. The tension curve area for KCl after 14 and 28 days was 6.34 ± 1.86 and 8.55 ± 1.98 g min^{-1} respectively compared to a control of 10.2 ± 1.58 g min^{-1}.

Discussion

The inhibitory effect of Chlo on twitch and tetanus tension and the increase in MFR is suggestive of an interference with Ca^{2+} sequestration normally necessary for excitation-contraction in skeletal muscle (Anderson, 1978; Frank, 1980). This effect of Chlo was not due to direct action on the contractile element e.g. troponin-tropomyosin complex and/or actomyosin adenosine triphosphatase since caffeine and KCl still produced contracture of the diaphragm in a concentration-dependent manner in the presence of Chlo and in pretreated preparations. Furthermore Na SCN which is known to potentiate twitch tension and K^+-induced contracture by increasing Ca^{2+} influx at the transverse tubular element and the terminal cisternal of the sarcoplasmic reticulum (Weiss & Bianchi, 1965; Bianchi & Bolton, 1966) also reversed Chlo-induced inhibition lending support to an intracellular site of action of Chlo. Since an increase in extracellular Ca^{2+} did not reverse Chlo-induced twitch inhibition, it seems unlikely that the main effect of Chlo is due to inhibition of extracellular Ca^{2+} influx.

The inhibitory effect of Chlo and Chlo-pretreatment (7–28 days) on K^+- and caffeine-induced contracture as well as on isoprenaline-induced twitch potentiation further support the contention that the site of action of Chlo is on intracellular Ca^{2+} sequestration. These agonists are known to produce their actions through interference at specific intracellular Ca^{2+} sites such as 'trigger' Ca^{2+} and SAR calcium (Bianchi, 1962; Weiss & Bianchi, 1965; Frank, 1980; Merican *et al.*, 1983). It is conceivable that this effect of Chlo is due to such intracellular Ca^{2+} inhibition.

In skeletal muscle, the MFR that cause a smooth tetanic contraction is considered to be a measure of the duration of the active state of the contractile elements which permit maximal tension development (Ritchie, 1954). In the present study, Chlo as well as acute and chronic Chlo administration increased the frequency required to fuse twitch response to a tetanus, suggesting that one of the results of Chlo treatment is a shortening in the duration of the active state. Since the intensity of the active state has been proposed to vary in proportion to a complex between Ca^{2+} and the contractile apparatus (Taylor, 1969), the duration of the active state would consequently correspond to the time course of the release and reuptake of Ca^{2+} within the muscle. The effect of Chlo on MFR is consistent therefore with an intracellular site of action, possibly Ca^{2+} stores of the SAR (Ayiteh-Smith & Vartanian, 1975).

The persistent reduction in muscle strength throughout the 4 weeks of Chlo administration is suggestive of the presence of Chlo in skeletal muscle contractile elements. It was to be expected that such muscle preparations would show greater reactivity to Chlo *in vitro*, but our results indicated a development of tolerance to Chlo. Since only the inhibitory effect of procaine on such skeletal muscle preparations was altered without any significant effect on (+)-tubocurarine and dantrolene sodium, it seems likely that tolerance to the effect of Chlo may in fact be a result of specific changes demonstrated to occur in muscles pretreated with Chlo (MacDonald & Engel, 1970; Oberc & Engel, 1977; Ayiteh-Smith & Gbewonyo, 1977; Lie & Schofield, 1978). Moreover, local anaes-

thetics such as procaine and dibucaine inhibit twitch and tetanic tension by an action on intracellular Ca^{2+} sequestration (Inoue & Frank, 1960; Franz & Perry, 1974). There is a structural similarity between Chlo, dibucaine and procaine (Chinyanga *et al.*, 1972; Kennedy & Knapp, 1982) which may explain the decreased responsiveness to Chlo and procaine reported in this study.

Acute and chronic administration of Chlo have been found to induce morphological changes in skeletal muscle (Ayiteh-Smith & Gbenwonyo, 1977; MacDonald & Engel, 1978; Lie & Scholfield, 1978). Since MFR contributes to the duration of active state during excitation-contraction coupling (Taylor, 1969; Anderson, 1978; Frank, 1980), the decreased muscle strength still observed after stopping Chlo pretreatment may be due in part to its effect on the active state in the contractile elements. In addition, reactivity of the muscle to caffeine, KCl and tetanus tension during recovery may be explained in part by these ultrastructural changes in response to Chlo reported by previous workers.

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Studies of receptor-mediated inhibition of ^{45}Ca accumulation into synaptosomes

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1 The effects of α_2-adrenoceptor and κ-opiate receptor activation on ^{45}Ca accumulation into rat cortical synaptosomes were examined.

2 Clonidine (1 μM) and U50488H (1 μM) significantly reduced ^{45}Ca accumulation under both resting (5 mM K^+) and depolarizing (15–30 mM K^+) conditions.

3 The inhibitory effects of the agonists on ^{45}Ca accumulation into synaptosomes were enhanced in the presence of the Na^+–Ca^{2+} exchange inhibitor sodium orthovanadate (vanadate, 2 mM), and were not present in mitochondrial preparations.

4 When the agonists were used together, their inhibitory effects were not additive but were, in fact, attenuated.

5 In the presence of the α_2-adrenoceptor antagonist idazoxan (1 μM), the inhibitory effect of U50488H on ^{45}Ca accumulation was enhanced. A similar increase in the inhibitory effectiveness of clonidine was observed in the presence of naloxone (20 μM).

6 When synaptosomes were pretreated with 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), dibutyryl-cyclic AMP (db-cyclic AMP, 10 μM) or 8-bromo-cyclic AMP (8Br-cyclic AMP, 10 μM), the inhibitory effects of clonidine and U50488H were abolished, suggesting that a decrease in cyclic AMP production is part of the receptor-effector coupling mechanism of both receptor systems.

7 The phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA, 0.05 μM) increased ^{45}Ca accumulation but did not alter the inhibitory effects of clonidine or U50488H, thus showing that the effects of the agonists are not mediated by protein kinase C.

8 We conclude that α_2-adrenoceptor and κ-opiate receptor activation dramatically reduce ^{45}Ca influx through Ca^{2+} channels (e.g., by 50%), that there is a functional antagonism between the two receptor systems and that in both cases, the receptor effector mechanism involves a decrease in cyclic AMP production.

Introduction

There is evidence that activation of presynaptic α_2-adrenoceptor or κ-opiate receptors inhibits neurotransmitter release from nerve terminals by mechanisms involving reductions in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Jackisch *et al.*, 1986; Schoffelmeer *et al.*, 1986; Adamson *et al.*, 1987). It has been proposed that this decrease in $[Ca^{2+}]_i$ may be due to closure of voltage-sensitive Ca^{2+} channels in the plasma membrane (Adamson *et al.*, 1989b; Attali *et al.*, 1989a; Lipscombe *et al.*, 1989), to an alteration in the binding of calcium to intracellular proteins or to Ca^{2+} sequestration into intracellular pools (Blaustein, 1988; Williamson & Monck, 1989). Although an interaction has been observed between κ-opiate and α_2-adrenoceptor agonist effects on $[Ca^{2+}]_i$ and on neurotransmitter release (Jackish *et al.*, 1986; Limberger *et al.*, 1986; Adamson *et al.*, 1988a; Allgaier *et al.*, 1989), it is unclear if this interaction is also responsible for changes in Ca^{2+} flux. GTP-binding proteins (G proteins), for example G_i, have been implicated in noradrenaline and κ-opiate-mediated closure of voltage-sensitive Ca^{2+} channels in several tissues (Holz *et al.*, 1986; Allgaier *et al.*, 1989; Attali *et al.*, 1989a). It has been suggested that adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and diacylglycerol act as intermediates between G proteins and channel events (Nichols *et al.*, 1987; Gross & MacDonald, 1989). While it is generally accepted that α_2-adrenoceptors are negatively coupled to a cyclic AMP generating system (Aghajanian & Wang, 1987; Adamson *et al.*, 1988b; Lipscombe *et al.*, 1989; Uhlen & Wikberg, 1989), the relationship between cyclic AMP generating systems and κ-opiate receptors remains to be defined. For example, κ-opiates reduce $[Ca^{2+}]_i$ by inhibiting adenylate cyclase in rat spinal cord (Attali *et al.*, 1989b), in locus coeruleus slices (Aghajanian & Wang, 1987) and in guinea-pig cortex (Werling *et al.*, 1989) but in rat cortical synaptosomes, the inhibitory effects of κ-opiate agonists on $[Ca^{2+}]_i$ are apparently potentiated by cyclic AMP analogues (Adamson *et al.*, 1988b). On the other hand, cyclic AMP analogues have been reported to reduce N type Ca^{2+} channel current in cultured mouse sensory neurones (Gross & MacDonald, 1989). Therefore, more investigations are necessary to explore the roles of these second messenger systems in the response to receptor activation and in the modulation of Ca^{2+} channels.

Our previous studies (Xiang *et al.*, 1990a,b) demonstrated that clonidine and U50488H decrease ^{45}Ca accumulation under physiological conditions mainly by closure of N type Ca^{2+} channels in the plasma membrane. In this study, we have compared the effects of α_2-adrenoceptor or κ-opiate receptor activation on ^{45}Ca accumulation in rat cortical synaptosomes under normal and depolarized conditions, and in the presence of the Na^+-Ca^{2+} exchange inhibitor vanadate (Varecka & Carafoli, 1982; Register & Wuthier, 1984). The effects on synaptosomes have been compared with those on mitochondria. Interactions between presynaptic α_2-adrenoceptors and κ-opiate receptors and the mechanism by which activation of these receptors leads to an inhibition of ^{45}Ca accumulation have also been examined.

Methods

Isolation of synaptosomes and mitochondria

Synaptosomes were prepared by a procedure based on that of Dunkley *et al.* (1988). Male Wistar rats (150–250 g) were killed by decapitation. Cerebral cortices were pooled on ice

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(subsequent procedures were performed at 4°C with pre-chilled reagents) and placed in approximately 1 g tissue per 9 ml gradient buffer (mM: sucrose 320, EDTA 1, dithiothreitol [as antioxidant] 0.25, pH 7.4) and homogenized (10 strokes at 700 r.p.m.) in an Aldridge glass homogenizer. Homogenates were centrifuged (11000 *g*, 10 min), the pellets were discarded, and the supernatants (S_1) were adjusted to 16 ml to give a protein concentration of approximately 5 mg ml^{-1}. Discontinuous Percoll gradient centrifugation (35,000 *g*, 10 min) was then used to obtain synaptosomal fractions (F3 + F4) which were resuspended and recentrifuged (7500 *g*, 5 min) in physiological buffer (mM: NaCl 143, KCl 4.7, $CaCl_2$ 1, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, Tris-base 20, glucose 10, pH 7.4, pre-gassed for 15 min with 95% O_2/5% CO_2) to produce well packed synaptosomal pellets (Adamson *et al.*, 1989b). To isolate mitochondria, the Percoll fraction F_5 (which contains mainly mitochondria, Dunkley *et al.*, 1988) was resuspended and recentrifuged (7500 *g*, 5 min) in 'intracellular' buffer (mM: KCl 126, $CaCl_2$ 1.5, $MgCl_2$ 1.2, Tris-base 20, glucose 10, pH 7.4, pregassed for 15 min with 95% O_2/5% CO_2) and resulting pellets were used in the experiments with mitochondria.

Studies of ^{45}Ca accumulation

Synaptosomal pellets were resuspended in physiological buffer to approximately 0.5 mg protein ml^{-1} in a glass mini-homogenizer, equilibrated at 37°C for 5 min and then aliquots (200 μl) transferred to tubes along with various drugs (normally in a volume of 10 μl): parallel controls contained drug vehicles. Precautions were taken against possible light sensitivities of the drugs by covering the tubes with foil. When the effects of depolarization on ^{45}Ca accumulation were measured, the K^+ concentrations in the buffer were increased from 5 mM to 15 or 30 mM and that of Na^+ reduced appropriately to maintain iso-osmolar conditions. Mitochondrial pellets were resuspended in intracellular buffer to 0.5 mg protein ml^{-1} and equilibrated and divided into aliquots as above. ^{45}Ca was diluted in the buffer and added to each tube (10 μl, specific activity: 0.9 mCi $mmol^{-1}$, final Ca^{2+} concentration = 1 mM, except when otherwise stated) and incubated for a further 2 min (except in the time course studies). Incubations were terminated by rapid vacuum filtration through pre-washed Whatman GF/B glass fibre filters using a Brandel Cell Harvester (Model M-24-R, Gaithersburg, MD). Filters were washed twice with 5 ml of ice-cold Ca^{2+}-free EGTA buffer (mM: NaCl 132, KCl 5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, Tris-HCl 20, glucose 10, EGTA 5, pH 7.4). In all experiments, duplicates were run at each time point. Blank filter values were subtracted to eliminate background counts: binding of ^{45}Ca to the filters was negligible (< 10%). Filters were dried and the radioactivity determined in 5 ml of toluene-based scintillation fluid (2,5-diphenyloxazole 1% w/v, 1,4-*bis*(5-phenyloxazole-2-yl)-benzene 0.02% w/v, Triton X-100 20% v/v) in a Packard Tricarb Scintillation Counter (efficiency for ^{45}Ca = 80%).

Biochemical assays

'Occluded' lactate dehydrogenase (LDH) activity was measured according to Marchbanks (1967): it was estimated by comparison of LDH activities of intact and Triton X-100 (0.1% w/v) solubilized synaptosomes. Only synaptosomes with LDH 'occluded' activities over 70% were used.

Proteins were determined by the method of Peterson (1977) with bovine serum albumin used as standard.

Data analysis

Data were converted from d.p.m. into nmol mg^{-1} protein and analysed by one-way or two-way analysis of variance (ANOVA), followed by Tukey's studentised range test for multiple comparisons. Values were presented as means ± s.e.mean. Differences were considered significant at $P < 0.05$.

Materials

Clonidine hydrochloride, 8-bromo-cyclic AMP (8Br-cyclic AMP), dibutyryl-cyclic AMP (db-cyclic AMP), 12-O-tetradecanoylphorbol 13-acetate (TPA) and Percoll (colloidal PVP coated silica) were obtained from the Sigma Chemical Co. (Poole, Dorset); idazoxan (RX781094) was obtained from Reckitt and Coleman Pharmaceuticals (Hull); U50488H {trans-(+)-3,4-dichloro-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide methansulphonate} was obtained from Upjohn (Kalamazoo, Michigan, U.S.A.); 3-isobutyl-1-methylxanthine (IBMX), naloxone hydrochloride and vanadate were from BDH (Poole, Dorset). [^{45}Ca]$CaCl_2$ (1 mCi 0.47 ml^{-1}, 147 g Ca ml^{-1}) was obtained from Amersham International (Bucks.). All other reagents were of at least analytical grade.

Results

Effects of clonidine and U50488H on ^{45}Ca accumulation into synaptosomes under normal and depolarized conditions

Under normal conditions (5 mM K^+), ^{45}Ca accumulation into synaptosomes was rapid and time-dependent (Table 1). Over 80% of the 15 min total accumulation of radioactivity occurred in the first 5 min. Thereafter, ^{45}Ca accumulation increased more slowly and reached equilibrium at 15 min. Clonidine (1 μM) and U50488H (1 μM) reduced ^{45}Ca accumulation, particularly within the first 5 min (reductions were 19% and 14% at 1 min, and 20% and 18% at 5 min, $P < 0.05$, $n = 6$, respectively). The inhibition by clonidine of ^{45}Ca accumulation persisted over 15 min while that by U50488H declined and returned to the control level.

When synaptosomes were depolarized with 15 or 30 mM K^+ for 1 min (Table 2), ^{45}Ca accumulation increased by 74% and 119% ($P < 0.05$, $n = 6$, respectively). Clonidine (1 μM) and

Table 1 Effect of clonidine and U50488H on ^{45}Ca accumulation into synaptosomes in the presence and absence of vanadate

	^{45}Ca *accumulation* (nmol mg^{-1} protein)				
Treatment	0 min	1 min	5 min	10 min	15 min
Control	—	3.6 ± 0.1	7.4 ± 0.2	8.5 ± 0.3	9.1 ± 0.4
Clonidine	—	2.9 ± 0.1[a]	5.9 ± 0.3[a]	7.2 ± 0.2[a]	7.6 ± 0.3[a]
U50488H	—	3.1 ± 0.2[a]	6.1 ± 0.3[a]	7.6 ± 0.4[a]	8.7 ± 0.5
Vanadate	—	7.8 ± 0.4[a]	10.2 ± 0.5[a]	14.8 ± 0.5[a]	15.5 ± 0.4[a]
Clonidine + vanadate	—	4.9 ± 0.2[b]	8.3 ± 0.3[b]	12.5 ± 0.4[b]	14.4 ± 0.6
U50488H + vanadate	—	4.6 ± 0.2[b]	7.4 ± 0.4[b]	12.3 ± 0.5[b]	14.8 ± 0.5

Synaptosomes were incubated in physiological buffer containing 5 mM K^+ in the presence and absence of vanadate (2 mM) at 37°C for 5 min before clonidine (1 μM) or U50488H (1 μM) and ^{45}Ca (specific activity: 0.9 mCi $mmol^{-1}$) were added. Incubation was terminated by vacuum filtration and ^{45}Ca accumulation was determined by scintillation counting (efficiency: 80%). Background counts were subtracted and d.p.m. were converted into nmol mg^{-1} protein. Measurements were made in duplicate. Data are shown as means ± s.e.mean of 6–8 determinations. [a] $P < 0.05$ versus control; [b] $P < 0.05$ versus vanadate alone, one-way ANOVA.

Table 2 Comparison of effects of clonidine and U50488H on ^{45}Ca accumulation into normal and depolarized synaptosomes in the presence and absence of vanadate

Treatment	^{45}Ca accumulation (nmol mg^{-1} protein, 1 min) 5 mM K^+	15 mM K^+	30 mM K^+
Control	4.2 ± 0.1	7.3 ± 0.2	9.2 ± 0.3
Clonidine	3.3 ± 0.1[a]	5.8 ± 0.2[a]	6.7 ± 0.3[a]
U50488H	3.5 ± 0.1[a]	6.0 ± 0.2[a]	7.3 ± 0.2[a]
Vanadate	10.5 ± 0.3[a]	20.0 ± 0.4[a]	21.3 ± 0.5[a]
Clonidine + vanadate	8.4 ± 0.2[b]	10.8 ± 0.3[b]	11.9 ± 0.3[b]
U50488H + vanadate	7.4 ± 0.3[b]	10.5 ± 0.2[b]	11.2 ± 0.3[b]

Synaptosomes were incubated in physiological buffers containing 5, 15 and 30 mM K^+ in the presence and absence of vanadate (2 mM) at 37°C for 5 min. Clonidine (1 μM) or U50488H (1 μM) and ^{45}Ca (specific activity: 0.9 mCi mmol^{-1}) were then added for a further 1 min incubation. Measurements were made as in Table 1. Data are shown as means ± s.e.mean of 6–8 determinations. [a]$P < 0.05$ versus control; [b]$P < 0.05$ versus vanadate alone, one-way ANOVA.

U50488H (1 μM) significantly decreased these K^+-evoked ^{45}Ca accumulation by 20% and 18% at 15 mM K^+, and by 27% and 21% at 30 mM K^+, ($P < 0.05$, $n = 6$, respectively). Although the percentage reductions caused by clonidine and U50488H under depolarized conditions were similar to those in physiological buffer, the absolute reductions in ^{45}Ca accumulation were obviously greater in the depolarized state.

Effects of vanadate on basal and K^+-evoked ^{45}Ca accumulation into synaptosomes and mitochondria

When synaptosomes were pretreated with vanadate (2 mM) in physiological buffer containing 5 mM K^+ for 5 min and then incubated with ^{45}Ca, the accumulation of ^{45}Ca into synaptosomes was increased (Table 1): it was doubled at 1 min period (116% increase, $P < 0.05$, $n = 6$) and this effect persisted to 15 min. The inhibitory effects of clonidine (1 μM) or U50488H (1 μM) were enhanced in the presence of vanadate: this was most apparent in the first minute when the percentage inhibition was 38% with clonidine and 41% with U50488H ($P < 0.05$, $n = 8$, respectively). Thereafter, the effects of the agonists declined and at 15 min, was almost back to the level seen with vanadate alone. The increase in synaptosomal ^{45}Ca accumulation in the presence of vanadate was even more remarkable under depolarizing conditions (Table 2). When synaptosomes were incubated with ^{45}Ca for 1 min, vanadate elevated ^{45}Ca accumulation by 174% at 15 mM K^+ and by 132% at 30 mM K^+ ($P < 0.05$, $n = 6$, respectively). Clonidine (1 μM) or U50488H (1 μM) strongly blocked K^+-evoked accumulation in the presence of vanadate, resulting in 46% and 47% decrease at 15 mM K^+ and 44% and 47% decrease at 30 mM K^+ ($P < 0.05$, $n = 6$, respectively).

The effect of vanadate on ^{45}Ca accumulation in mitochondria was also investigated (Figure 1). Uptake of ^{45}Ca into mitochondria was 1 nmol mg^{-1} protein after 1 min incubation, i.e., approximately 20% of the total ^{45}Ca accumulated into synaptosomes. It reached a maximum of 1.7 ± 0.2 nmol mg^{-1} protein at 5 min. Vanadate (2 mM) prevented ^{45}Ca uptake by mitochondria: the levels being reduced to 48% at 1 min and 49% at 5 min ($P < 0.05$, $n = 6$, respectively). Both clonidine (1 μM) and U50488H (1 μM) had no significant effect on uptake of ^{45}Ca into mitochondria.

Interaction between α_2-adrenoceptor and κ-opiate receptor agonists and antagonists on ^{45}Ca accumulation into depolarized synaptosomes

^{45}Ca accumulation was decreased when clonidine and U50488H were co-incubated at equal doses (0.01, 0.1, 1 and 10 μM) with depolarized (30 mM K^+) synaptosomes for 1 min

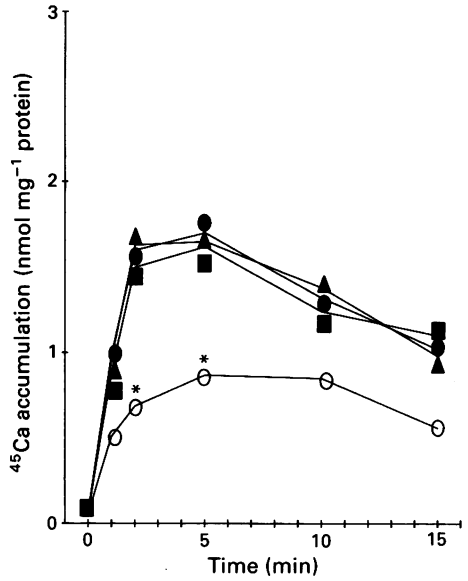

Figure 1 Effects of clonidine, U50488H and vanadate on ^{45}Ca uptake by mitochondria. Mitochondria were equilibrated in intracellular buffer at 37°C for 5 min before drugs and ^{45}Ca (specific activity: 0.9 mCi mmol^{-1}) were added. Measurements are made as in Table 1. (●) Control; (○) vanadate (2 mM); (■) clonidine (1 μM); (▲) U50488H (1 μM). Data are subtracted from controls and are shown as means of 6 determinations (all of the s.e.mean are omitted in the figure for clarity). *$P < 0.05$, one-way ANOVA.

(Figure 2). In the presence of both agonists (1 μM), ^{45}Ca accumulation was reduced by 13% ($P < 0.05$, $n = 8$). However, this reduction was much less than, and significantly different from, those of clonidine (28% reduction) or of U50488H (22% reduction) alone ($P < 0.05$, respectively).

In the presence of naloxone (1 and 10 μM for 1 min), the reductions in ^{45}Ca accumulation caused by clonidine (1 μM) were 24% and 32%, respectively. When synaptosomes were pretreated with 20 μM naloxone, the inhibitory effect of clonidine (1 μM) on ^{45}Ca accumulation was enhanced (39% reduction, $P < 0.05$, $n = 8$). However, in the presence of 100 μM naloxone, the reduction in ^{45}Ca accumulation was only 28%, which was not significantly different from clonidine alone ($P > 0.05$, $n = 8$). In the presence of idazoxan (0.01, 0.1 and 1 μM), the inhibitory effect of U50488H (1 μM) on ^{45}Ca

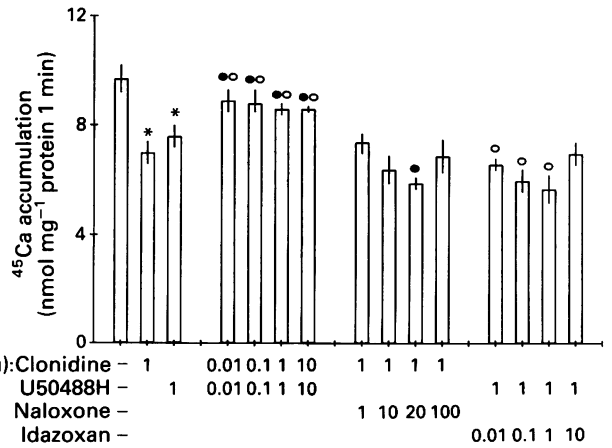

Figure 2 Effects of α_2-adrenoceptor and κ-opiate receptor agonists and antagonists on ^{45}Ca accumulation into depolarized synaptosomes. Synaptosomes were equilibrated in physiological buffer containing 30 mM K^+ at 37°C for 5 min and incubated with α_2-adrenoceptor and κ-opiate receptor agonists and/or antagonists and ^{45}Ca (specific activity: 0.9 mCi mmol^{-1}) for further 1 min before measurement. Data are shown as means of 6–8 determinations; s.e.mean shown by vertical lines; *$P < 0.05$ versus control; ●, ○, $P < 0.05$ versus clonidine and U50488H alone, respectively, two-way ANOVA.

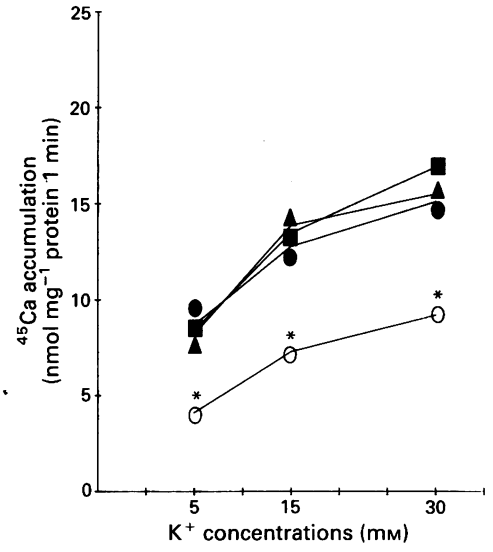

Figure 3 Effects of 3-isobutyl-1-methylxanthine (IBMX) on ^{45}Ca accumulation into normal and depolarized synaptosomes. Synaptosomes were incubated with IBMX in physiological buffer containing 5, 15 and 30 mM K^+ at 37°C for 5 min. Drugs and ^{45}Ca were added for a further 1 min incubation before measurement. (○) Control; (■) IBMX (0.5 mM); (●) IBMX plus clonidine (1 μM); (▲) IBMX plus U50488H (1 μM). Data are shown as means of 6–8 determinations. *$P < 0.05$, one-way ANOVA.

accumulation was larger (30%, 37% and 41%), significantly different from U50488H alone ($P < 0.05$). U50488H plus a higher dose (10 μM) of idazoxan decreased ^{45}Ca accumulation by 26%, i.e. not significantly different from U50488H alone ($P > 0.05$).

Effects of IBMX, db-cyclic AMP, 8Br-cyclic AMP and TPA on K^+-evoked ^{45}Ca accumulation into synaptosomes

The effects of IBMX on K^+-evoked ^{45}Ca accumulation into synaptosomes were examined (Figure 3). When synaptosomes were pretreated with IBMX (0.5 mM) in physiological buffer containing 5 mM K^+ for 5 min and then incubated with ^{45}Ca for 1 min, ^{45}Ca accumulation was doubled (104% increase, $P < 0.05$, $n = 8$). After the synaptosomes were depolarized with 15 or 30 mM K^+, ^{45}Ca accumulation was increased by 85% and 83% ($P < 0.05$, $n = 8$, respectively). In the presence of IBMX, the inhibitory effects of clonidine (1 μM) or U50488H (1 μM) on K^+-evoked ^{45}Ca accumulation were abolished.

The effects of cyclic AMP analogues and TPA on ^{45}Ca accumulation in K^+ (30 mM) – depolarized synaptosomes are shown in Figure 4. Incubation of depolarized synaptosomes with db-cyclic AMP (10 μM) for 5 min and then with ^{45}Ca for 1 min increased ^{45}Ca accumulation by 41% ($P < 0.05$, $n = 6$). This increase remained even when clonidine (1 μM) or U50488H (1 μM) were added, showing that the inhibitory effects of the agonists were completely reversed. 8Br-cyclic AMP (10 μM) had a similar effect on ^{45}Ca accumulation in K^+-depolarized synaptosomes (28% increase, $P < 0.05$, $n = 8$) and under these conditions, antagonized the inhibitory effects of clonidine (1 μM) and U50488H (1 μM). On the other hand, although TPA (0.05 μM) increased ^{45}Ca accumulation into the depolarized synaptosomes 18% ($P < 0.05$, $n = 6$), TPA did not antagonize the inhibitory effects of clonidine (1 μM) and U50488H (1 μM).

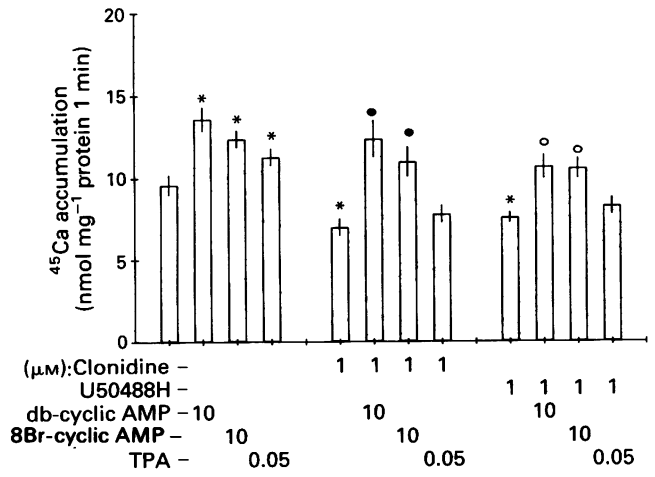

Figure 4 Effects of clonidine and U50488H on ^{45}Ca accumulation into depolarized synaptosomes in the presence of dibutyryl-cyclic AMP (db-cyclic AMP), 8-bromo-cyclic AMP (8Br-cyclic AMP) and 12-O-tetradecanoylphorbol 13-acetate (TPA). Synaptosomes were equilibrated in 30 mM K^+ at 37°C for 5 min. Clonidine (1 μM), U50488H (1 μM) and ^{45}Ca were incubated in the presence of db-cyclic AMP (10 μM), 8Br-cyclic AMP (10 μM) or TPA (0.05 μM) for a further 1 min before measurement. Data are shown as means of 6–8 determinations; s.e.mean shown by vertical lines. *$P < 0.05$ versus control; ●, ○ $P < 0.05$ versus clonidine and U50488H alone, respectively, two-way ANOVA.

Discussion

Ca^{2+} channels, the Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase are operational in synaptosomes and are involved in maintaining $[Ca^{2+}]_i$ at a constant level (Coutinho *et al.*, 1984). Ca^{2+} entry through Ca^{2+} channels is balanced by Ca^{2+} efflux facilitated by the Na^+-Ca^{2+} exchanger and by Ca^{2+} sequestration into intracellular stores (Nachshen *et al.*, 1986; Blaustein, 1988). In this study, we have shown that clonidine and U50488H significantly decrease ^{45}Ca accumulation into rat cortical synaptosomes but have no effect on uptake of ^{45}Ca into mitochondria. These findings, together with our previous studies (Xiang *et al.*, 1990a,b) which demonstrated that ω-conotoxin abolished the inhibitory effects of clonidine and U50488H on ^{45}Ca accumulation in synaptosomes, indicate that presynaptic α_2-adrenoceptor and κ-opiate receptor activation primarily inhibits Ca^{2+} entry rather than promotes intracellular Ca^{2+} sequestration.

Voltage-sensitive N and L type Ca^{2+} channels are functional in different voltage ranges (Bradford, 1986) and N but not L type Ca^{2+} channels can be closed by presynaptic α_2-adrenoceptor and κ-opiate receptor activation under resting membrane conditions (Xiang *et al.*, 1990a,b). The present study shows that the percentage decreases in ^{45}Ca accumulation caused by clonidine and U50488H are similar under normal and depolarized conditions. Thus, it appears that clonidine and U50488H inhibit Ca^{2+} influx throughout the voltage range in which N type Ca^{2+} channels can be opened. Vanadate increases both the rate and equilibrium level of ^{45}Ca accumulation into synaptosomes by blocking the Na^+-Ca^{2+} ATPase and hence reducing ^{45}Ca efflux. Because the efflux is reduced, the inhibitory effects of U50488H and clonidine on Ca^{2+} become more apparent in the presence of vanadate.

We have observed that some mutual antagonism occurs between the two receptor systems. Our results agree with other recent reports (Jackisch *et al.*, 1986; Limberger *et al.*, 1986; McWilliam & Campbell, 1987; Adamson *et al.*, 1988a) in which a functional antagonism has been demonstrated between presynaptic α_2-adrenoceptor and κ-opiate receptor systems and suggest that the mutual antagonism between α_2-adrenoceptor and κ-opiate receptor activation may be a general phenomenon in the presynaptic control of neurotransmitter release. This may prevent excessive inhibition of neuronal systems which might interfere with synaptic processing of information.

The decreases in accumulation which occur when clonidine or U50488H are added to TPA-treated samples suggest that the effects of these agonists are not related to activation of protein kinase C and to changes at L type Ca^{2+} channels (Nichols *et al.*, 1987; Adamson *et al.*, 1989a). The observation that the inhibitory effects of clonidine and U50488H are abolished by IBMX, db-cyclic AMP and 8Br-cyclic AMP under resting and depolarized conditions suggests that clonidine and U50488H reduce Ca^{2+} influx by inhibiting cyclic AMP formation. This supports the involvement of G-proteins and cyclic AMP in the inhibition of Ca^{2+} currents by α_2-adrenoceptor, κ-opiate and $GABA_B$ receptor agonists (Holz *et al.*, 1986; Aghajanian & Wang, 1987; Adamson *et al.*, 1988b; Freissmuth *et al.*, 1989). In this way, neurotransmitters may act to reduce the number of functional calcium channels in the cell (Dunlap & Fischbach, 1981) or to induce change in the voltage-dependence with which channels are opened (Bean, 1989).

In conclusion, our results show that (a) the inhibitory effects of α_2-adrenoceptor and κ-opiate receptor activation on ^{45}Ca accumulation into rat cortical synaptosomes occur by blocking Ca^{2+} entry through the plasma membrane rather than by promoting Ca^{2+} sequestration into intracellular stores; (b) the existence of a functional antagonism between these two receptor systems may be useful as a 'fail-safe' mechanism to prevent excessive signal input and (c) that cyclic AMP is necessary to couple the activation of presynaptic α_2-adrenoceptor and κ-opiate receptors with voltage-sensitive N type Ca^{2+} channels.

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L-Arginine and arginine analogues: effects on isolated blood vessels and cultured endothelial cells

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1 The present study examined effects of arginine (Arg) and various Arg analogues on the vascular tone of rabbit and rat aortic rings, the release of nitrite from cultured bovine aortic endothelial cells and the metabolism of L-Arg in bovine and porcine endothelial cell homogenates. The respective D-enantiomers or N-α-benzoyl-L-arginine ethyl ester did not substitute for L-Arg.

2 In bovine aortic endothelial cells, the release of nitrite was only observed in the presence of L-Arg or L-Arg methyl ester in the cell culture medium.

3 In dialyzed homogenates of porcine and bovine aortic endothelial cells, L-Arg was metabolized independently of NADPH and Ca^{2+} to yield L-ornithine (L-Orn) and L-citrulline (L-Cit). No concomitant nitrite formation was detected.

4 Pretreatment of rabbit and rat aortic rings with L-canavanine (L-Can) or N^G-monomethyl-L-Arg (L-NMMA) inhibited ATP- and acetylcholine-induced relaxations (endothelium-dependent) but not glyceryltrinitrate-induced relaxations (endothelium-independent).

5 In rabbit aortic rings, Arg and monomeric Arg analogues induced endothelium-independent relaxations. L-Arg methyl ester induced an endothelium-independent contraction, and L-NMMA induced a relaxation in the absence of endothelium and a contraction in the presence of endothelium. Polymeric basic amino acids such as poly L-Arg induced endothelium-dependent relaxations (inhibited by L-Can), a subsequent refractoriness to endothelium-dependent vasodilatators (not prevented by L-Can) and endothelial cell death.

6 We suggest that extracellular L-Arg is essential for the formation of endothelium-derived nitrogen oxides (EDNO). However, Arg and Arg analogues do not exert endothelium-dependent relaxation. L-Can and L-NMMA inhibit endothelium-dependent relaxation, consistent with an inhibition of EDNO formation from L-Arg, but also exert endothelium-independent effects on vascular tone.

Introduction

Vascular endothelial cells synthesize and release one or more labile substances referred to as endothelium derived relaxing factor(s) (EDRF; Furchgott & Zawadzki, 1980) which induce a relaxation of the adjacent vascular smooth muscle and an inhibition of platelet aggregation. The molecular mechanism of action of at least one EDRF (Förstermann *et al.*, 1986; Ignarro *et al.*, 1986) is similar to that of nitric oxide (NO) and various NO-containing compounds such as sodium nitroprusside, i.e. direct stimulation of soluble guanylyl cyclase (Kimura *et al.*, 1975; Böhme *et al.*, 1978). The release of endothelium-derived NO or NO-containing compounds (EDNO) has been observed in several vascular tissue preparations (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Schmidt *et al.*, 1988b; Kelm & Schrader, 1988) and EDNO accounts, at least in part, for the biological activity of EDRF.

Experiments with guanido ^{15}N-labelled L-arginine revealed that the nitrogen atom of EDNO is derived from one or both of the terminal guanidino nitrogens of L-arginine (L-Arg; Schmidt *et al.*, 1988b). Endothelium-dependent relaxation is inhibited by various structural analogues of L-Arg, e.g. L-canavanine (L-Can; Schmidt *et al.*, 1988a; Ignarro *et al.*, 1989b; Gold *et al.*, 1989), N^G-monomethyl-L-Arg (L-NMMA; Sakuma *et al.*, 1988) and succinyl-L-Arg (Gold *et al.*, 1989). Furthermore, it has been suggested that peptide-bound rather than free L-Arg (Thomas & Ramwell, 1988a; Thomas *et al.*, 1989b), polymeric basic amino acids such as poly-L-Arg (L-pArg), poly-L-lysine (L-pLys) and poly-L-ornithine (L-pOrn) and naturally occurring peptides rich in L-Arg, L-lysine or L-ornithine (L-Orn) moieties (Ignarro *et al.*, 1989a) serve as physiological precursors for EDNO. The purpose of the current study was to determine the metabolites of L-Arg in endothelial cell homogenates, the stereospecificity of the utilization of L-Arg in intact endothelial cells with regard to EDNO formation, and the endothelium-dependent and independent effects of L-Arg and various Arg analogues on the tone of isolated blood vessels.

Methods

Porcine and bovine aortic endothelial cell culture

Endothelial cells were harvested from male bovine or porcine thoracic aortae obtained from a local slaughterhouse. The lumen was incubated with collagenase (type IV; 0.5% w/v; Sigma, Deisenhofen, F.R.G.) for 20 min at 37°C. After centrifugation of the cell suspension ($20 \cdot g_{av}$; 15 min), the endothelial cells were re-suspended in Eagle's Minimal Essential Medium (MEM) containing penicillin G sodium salt ($100\,u\,ml^{-1}$), streptomycin sulphate ($0.1\,mg\,ml^{-1}$) and foetal calf serum (10% v/v) and were transferred into $25\,cm^2$ cell culture flasks (Falcon Primaria, Becton and Dickinson, Heidelberg, F.R.G.). After 1 h at 37°C under an atmosphere of 95% air and 5% CO_2, non-adherent cells were removed. The adherent cells were then allowed to spread for another four days until a confluent monolayer of about 2×10^6 cells/flask was obtained and were passaged (1:6; trypsin/EDTA, 0.05/0.02%, w/v, 1 min, 37°C) up to 9 times. For each experiment, modifications of MEM were prepared by adding various Arg analogues to L-Arg free MEM. Prior to some of the experiments, standard MEM was exchanged for 4 h by MEM without L-Arg. For nitrite accumulation experiments (see below), cells were incubated in modified MEM for 1 to 4 h. Morphological changes

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of the endothelial cells (visible by light microscopy) induced by Arg or various Arg analogues were documented.

Determination of nitrite

To investigate the effect of Arg and various Arg analogues on EDNO release, the accumulation of nitrite in the culture medium of the endothelial cell monolayers was determined by a chemiluminescence method. A 0.5 ml aliquot of the culture medium was injected into a solution of KI (1%, w/v; in glacial acetic acid). Under these conditions, nitrite is reduced to NO. NO was extracted from this reaction mixture by a constant flow of nitrogen into an NO gas analyser as previously described (Schmidt *et al.*, 1988b). The nitrite accumulation in the culture medium of endothelial cell monolayers (mol/10^6 cells) was expressed as the arithmetic mean ± s.d. Significant differences in nitrite accumulation between groups were tested by Student's unpaired, two-tailed *t*-test ($P < 0.05$ accepted as significant).

Metabolism of L-arginine

The metabolism of L-Arg was determined in porcine and bovine aortic endothelial cells: 10^8 cells were homogenized in 5 ml triethanolamine/HCl-buffer (2×10^{-2} M; pH 7.5; 4°C) containing NaCl (5×10^{-2} M), phenylmethylsulphonyl fluoride (5×10^{-4} M), pepstatin A (10^{-6} M), benzamidine (2×10^{-4} M), penicillin G (60 u l^{-1}) and streptomycin (60 mg l^{-1}). The homogenates were dialyzed twice for 3 h at 4°C against 200 volumes of the homogenization buffer. The dialysates (adjusted to 2.0 mg protein ml^{-1}; Bradford, 1976) were incubated immediately after dialysis or were stored at −80°C for up to 2 months without loss of activity. Aliquots of 0.04 ml of the dialysates were incubated at 37°C for 60 min in the presence of triethanolamine/HCl-buffer (10^{-4} M; pH 7.5) containing [U-^{14}C]-L-Arg (2.7×10^{-7} to 10^{-4} M) in a final volume of 0.1 ml. In some of the experiments, NADPH (up to 10^{-3} M) was added to the incubates and/or the free Ca^{2+} concentration (up to 10^{-3} M) was adjusted with EDTA (10^{-3} M). The reaction was stopped by addition of 0.01 ml trichloroacetic acid (100% w/v). ^{14}C-labelled L-Arg and ^{14}C-labelled metabolites of L-Arg in the incubates were separated by thin layer chromatography (solvent system $CHCl_3$:CH_3OH:NH_4OH:H_2O, 1:4:2:1, v/v). Under these conditions, the R_F values for L-Arg, L-Orn and L-citrulline (L-Cit) were 0.38, 0.58 and 0.88 as detected with ninhydrin (0.2% w/v; in butane-(2)ol:acetic acid, 199:1 v/v). The formation of [^{14}C]-Orn and [^{14}C]-Cit in the dialyzed homogenates of endothelial cells were expressed as the arithmetic mean ± s.d. ($n = 3$–6).

Ring preparations of rabbit and rat aortae

Male Wistar rats (Han: WIST) or New Zealand White rabbits were killed by decapitation. The thoracic aorta was quickly and carefully removed, cleaned of fat and connective tissue and cut into rings, 4 mm in width. Care was taken to avoid destroying the endothelium. The intimal surface of some of the rings was gently rubbed with filter paper to remove the endothelium. Each ring was mounted between two stainless steel hooks in a 25 ml organ bath containing a modified Krebs-Henseleit bicarbonate solution of the following composition (in mM): NaCl 119.7, KCl 5.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.6, continually gassed with 95% O_2 and 5% CO_2 (pH 7.4; 37°C). Tension was recorded isometrically by attachment of the upper hook to a TF6V50 force transducer (Fleck, Mainz, F.R.G.). The ring preparations were allowed to equilibrate for 2 h at a resting tension of 1.0 g (rat aortic rings) or 1.5 g (rabbit aortic rings). This resting tension was defined as zero baseline expressed as 0.0 relative tension. In some experiments, the rings were exposed to L-Can (sulphate; 2×10^{-3} M: 2 h), L-NMMA (acetate; 3×10^{-4} M; 15 min), A23187 (10^{-6} M; 45 min) or D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK; 10^{-6} M; 1 h) during that equilibration time. Phenylephrine was then added at a concentration of 3×10^{-7} M which produced a sustained contraction of about 80% of maximum. The phenylephrine-induced contraction was defined as 1.0 relative tension. Cumulative concentration-response curves were then obtained for various compounds. The effects of these compounds were expressed in relation to the phenylephrine-induced contraction (1.0) with values < 1.0 representing relaxations and values > 1.0 representing contractions. Changes in tension are given as the median of $n = 3$ to 15 experiments. Each compound was tested on ring preparations of at least 2 different aortae. None of the tested compounds affected the pH of the Krebs-Henseleit bicarbonate solution. Statistical differences between controls and drug treatment were assessed by analysis of variance followed by the Fisher protected-least-significant-difference test (ANOVA/PLSD). Significance was accepted at $P < 0.05$.

Materials

All compounds were of analytical grade: D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) and N^G-monomethyl-L-arginine methyl ester acetate (L-NMMA) (both Calbiochem, Frankfurt, F.R.G.), [U-^{14}C]-L-arginine (Amersham Buchler, Braunschweig, F.R.G.), MEM and foetal calf serum (both Biochrom, Berlin, F.R.G.). L- and D-arginine methyl ester·2 HCl (L- and D-AME) were kind gifts of Dr P. Del Soldato (Italfarmaco Research Center, Milan, Italy). L-Canavanine sulphate (L-Can), N-α-benzoyl-L-arginine ethyl ester·HCl (L-BAEE), N-α-tosyl-L-arginine methyl ester·HCl (L-TAME), poly L-arginine·HCl (L-pArg), poly L-ornithine·HBr (L-pOrn), poly L-lysine·HBr (L-pLys), glyceryl trinitrate (GTN) and all other compounds were from Sigma (Deisenhofen, F.R.G.).

Results

EDNO release and endothelial cell morphology

In bovine aortic endothelial cells, ATP-induced release of nitrite, a stable degradation product of EDNO, was detected when L-Arg or L-AME (both at a concentration of 10^{-3} M) were added to the cell culture medium (Table 1). Omission of L-Arg from the culture medium or replacement with D-Arg, D-AME or N-α-benzoyl-L-Arg ethyl ester (L-BAEE) resulted in a markedly reduced ATP-induced release of nitrite. L-pArg

Table 1 Effects of L-arginine (L-Arg) and various arginine analogues on the ATP-induced release of nitrite from cultured bovine aortic endothelial cells

Substrate	Concentration [M]	Nitrite accumulation [10^{-8} mol/10^6 cells] after 1 h	after 4 h
None	10^{-3}	10 ± 5	8 ± 1
L-Arginine	10^{-3}	53 ± 4	52 ± 11
D-Arginine	10^{-3}	14 ± 3	ND
L-Arg methyl ester	10^{-3}	21 ± 2	45 ± 9
D-Arg methyl ester	10^{-3}	6 ± 2	ND
N-α-benzoyl-L-Arg ethyl ester	10^{-3}	<1	ND
poly-L-Arg (12 kDa)	10^{-6}	cell death	

Cultured bovine aortic endothelial cells (see Methods) were exposed for 4 h to medium which contained no L-Arg. Thereafter, the accumulation of nitrite in fresh culture medium induced by the purinergic agonist ATP (1 mM) was determined for 1 or 4 h in the absence and presence of L-Arg or various Arg analogues. Values represent mean ± s.d. ($n = 3$–6). ND, not determined.

Table 2 Formation of L-ornithine (L-Orn) and L-citrulline (L-Cit) from L-arginine (L-Arg) in dialyzed homogenates of bovine aortic endothelial cells

L-Arginine [M] added	*L-Ornithine* [10^{-8} M] formed	*L-Citrulline* [10^{-8} M] formed
2.7×10^{-7}	0.24 ± 0.05	0.00 ± 0.02
10^{-6}	2.4 ± 0.1	3.3 ± 0.3
10^{-5}	59 ± 5	60 ± 2
10^{-4}	160 ± 10	240 ± 30

Bovine aortic endothelial cells were homogenized and dialyzed (see Methods). An aliquot of the dialysate was incubated in the presence of [^{14}C]-L-Arg at various concentrations of L-Arg. ^{14}C-labelled metabolites of L-Arg were identified as L-Orn and L-Cit by thin layer chromatography. Values represent mean concentrations of L-Orn and L-Cit ± s.d. ($n = 3$).

(12 kDa; 10^{-6} M), within 30 min, induced cell lysis and other morphological changes of endothelial cell monolayers that were consistent with endothelial cell death (not shown).

Metabolism of L-arginine

[U-^{14}C]-L-Arg (10^{-5} M) was metabolized in dialyzed homogenates of porcine or bovine endothelial cells. In both cell types, two metabolites of L-Arg were detected by thin layer chromatography with R_F values identical with those for L-Cit and L-Orn (see Methods). In homogenates of dialyzed bovine aortic endothelial cells, L-Arg increased the formation of L-Orn and L-Cit in a concentration-dependent manner (Table 2). In dialyzed homogenates of porcine aortic endothelial cells, alteration of the free Ca^{2+} concentration in the incubation buffer within a range of 10^{-9}–10^{-3} M (not shown) or addition of non-labelled L-Orn, to inhibit arginase activity, or NADPH (both up to 10^{-3} M; Table 3) to the incubates did not affect the formation of L-Orn or L-Cit. No nitrite formation was detected under any of these conditions.

L- and D-arginine

In rabbit aortic rings, L-Arg and D-Arg at an exceedingly high threshold concentration of 3×10^{-3} M ($P < 0.05$) induced a concentration-dependent endothelium-independent submaximal relaxation (Figure 1). When rabbit aortic rings were pretreated with A23187 (10^{-6} M) for 45 min to stimulate maximally EDRF release, the L- and D-Arg-induced relaxations were not affected (data not shown).

Table 3 Effects of L-ornithine (L-Orn) and NADPH on the formation of L-Orn and L-citrulline (L-Cit) from L-arginine (L-Arg) in dialyzed homogenates of porcine aortic endothelial cells

NADPH [M] added	*L-Orn* [M]	*L-Orn* [10^{-8} M] formed	*L-Cit* [10^{-8} M] formed
0	0	46 ± 22	5 ± 3
0	10^{-3}	31 ± 2	11 ± 8
10^{-3}	0	32 ± 7	6 ± 1
10^{-3}	10^{-3}	36 ± 1	5 ± 1

Porcine aortic endothelial cells were homogenized and dialyzed (see Methods). An aliquot of the dialysate was incubated in the presence of [^{14}C]-L-Arg (10^{-5} M), NADPH (10^{3} M) and/or L-Orn (10^{-3} M). Metabolites of L-Arg were identified as L-Orn and L-Cit by thin layer chromatography. Values represent mean concentrations of L-Orn and L-Cit ± s.d. ($n = 3$).

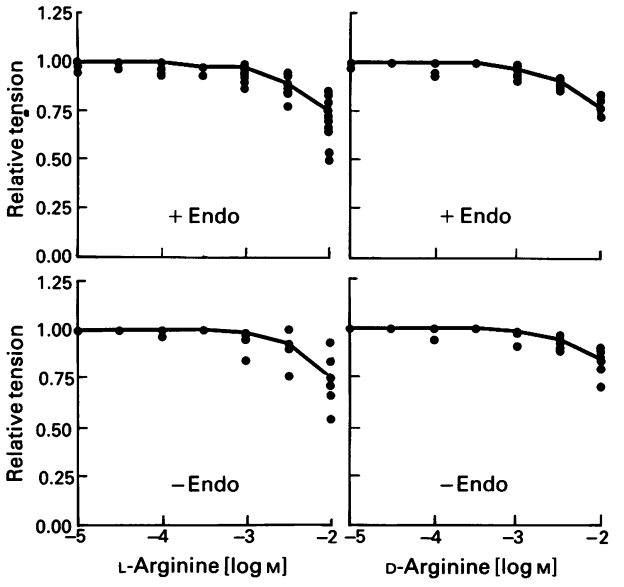

Figure 1 Effects of L- and D-arginine on the tension of rabbit aortic rings with (+Endo) and without (−Endo) an intact endothelium, precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 9$–15 experiments.

Monomeric arginine analogues

In rabbit aortic rings the methyl esters of Arg (AME), induced endothelium-independent responses that appeared to be stereospecific (Figure 2). L-AME induced marked contractions (threshold concentration 10^{-3} M; $P < 0.05$) while D-AME induced relaxation (threshold concentration 10^{-2} M; $P < 0.05$).

L-BAEE was the most potent monomeric analogue of Arg to induce a relaxation of rabbit aortic rings (Figure 3; threshold concentration 3×10^{-5} M; $P < 0.05$). The L-BAEE-induced relaxation was endothelium-independent and was not affected by pretreatment of the rings with PPACK (10^{-6} M;

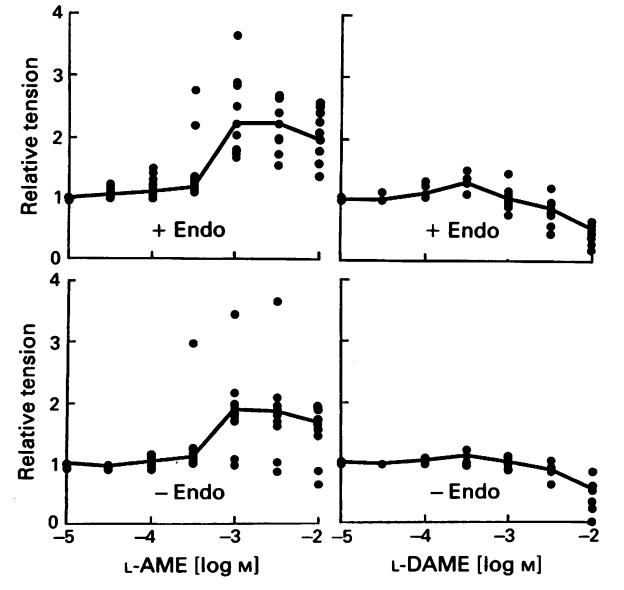

Figure 2 Effects of L- and D-arginine methyl ester (L- and D-AME) on the tension of rabbit aortic rings with (+Endo) and without (−Endo) an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 6$–9 experiments.

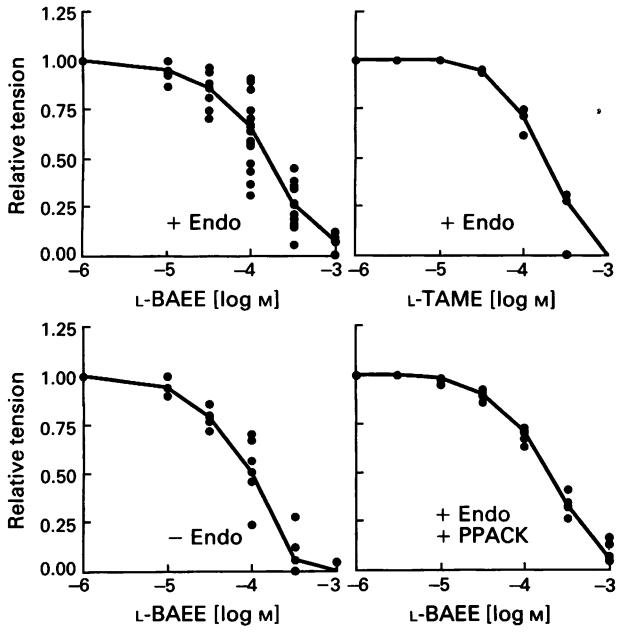

Figure 3 Effects of N-α-benzoyl-L-arginine ethyl ester (L-BAEE) and N-α-tosyl-L-arginine methyl ester (L-TAME) on the tension of rabbit aortic rings with (+Endo) and without (−Endo) an intact endothelium precontracted with phenylephrine (3×10^{-7} M). In some of the experiments with L-BAEE, rings were pretreated with D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK; 10^{-6} M; 1 h). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 3$–12 experiments.

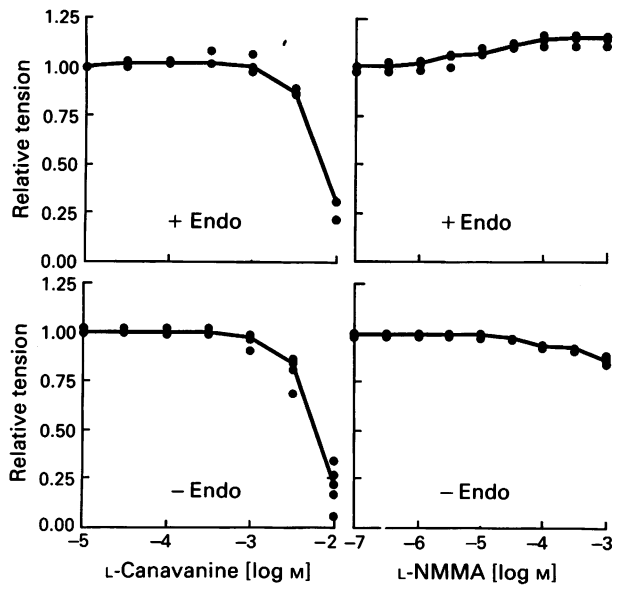

Figure 4 Effects of L-canavanine and N^G-monomethyl-L-arginine (L-NMMA) on the tension of rabbit aortic rings with (+Endo) and without (−Endo) an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 6$ experiments.

1 h), an inhibitor of thrombin, trypsin and related proteases that cleave Arg peptide bonds or hydrolyze the amide bond of L-BAEE. Various other Arg analogues were tested and all produced endothelium-independent relaxations at threshold concentrations between those for L-BAEE and L-Arg as shown for L-TAME (Figure 3; threshold concentration 3×10^{-5} M; $P < 0.05$).

L-Can induced endothelium-independent relaxations of rat aortic rings (threshold concentration 3×10^{-3} M, $P < 0.05$; Figure 4). L-NMMA induced endothelium-dependent effects on precontracted rabbit aortic rings, i.e. relaxations in rings without an intact endothelium (threshold concentration 10^{-4} M; $P < 0.05$) and contractions in rings with an intact endothelium (threshold concentration 10^{-4} M; $P < 0.05$).

The GTN-induced endothelium-independent relaxation of rabbit and rat aortic rings was not affected by pretreatment of rings with L-Can (2×10^{-3} M; 2 h) or L-NMMA (3×10^{-4} M; 15 min; data not shown). Pretreatment of rabbit (Figure 5) or rat (Figure 6) aortic rings with L-Can or L-NMMA, partially inhibited the endothelium-dependent relaxation induced by acetylcholine (ACh) ($\geq 10^{-7}$ M; $P < 0.05$) or ATP ($\geq 10^{-4}$ M; $P < 0.05$).

Polymeric basic amino acids

L-pArg was the only Arg analogue which induced strictly endothelium-dependent responses in rabbit aortic rings (12 kDa; Figure 7). L-pArg induced a relaxation (threshold concentration 3×10^{-7} M; $P > 0.05$) which was reversed at higher L-pArg concentrations. L-pArg was without effect in rings without an intact endothelium. Rings that were once exposed to a maximally effective concentration of L-pArg (10^{-6} M; 45 min; not shown) became refractory to a second exposure to L-pArg or to ACh (endothelium-dependent) but not to GTN (endothelium-independent). Pretreatment of rings with L-Can (2×10^{-3} M; 2 h) inhibited the effectiveness ($P < 0.05$ for $\geq 10^{-6}$ M, L-pArg) but not the potency (threshold concentration 3×10^{-7} M; $P > 0.05$) of L-pArg to induce endothelium-dependent relaxation (see Figure 7), and did not prevent the L-pArg-induced refractoriness (not shown). Moreover, the L-pArg-induced refractoriness was neither prevented by preincubation with L-Arg (10^{-3} M), nor reversed by washout of L-pArg in the absence or presence of L-Arg

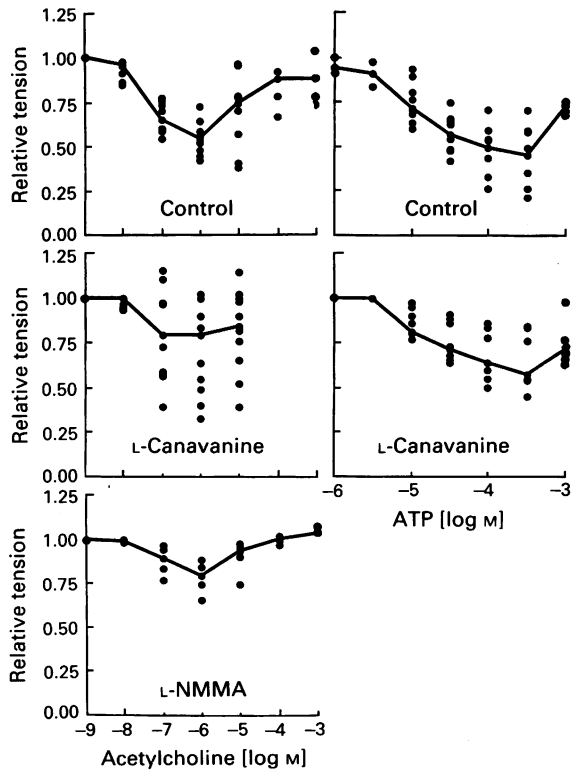

Figure 5 Effects of pretreatment with L-canavanine (2×10^{-3} M; 2 h) or N^G-monomethyl-L-arginine (L-NMMA; 3×10^{-4} M; 15 min) on the acetylcholine- and ATP-induced endothelium-dependent relaxation of rabbit aortic rings with an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 6$–12 experiments.

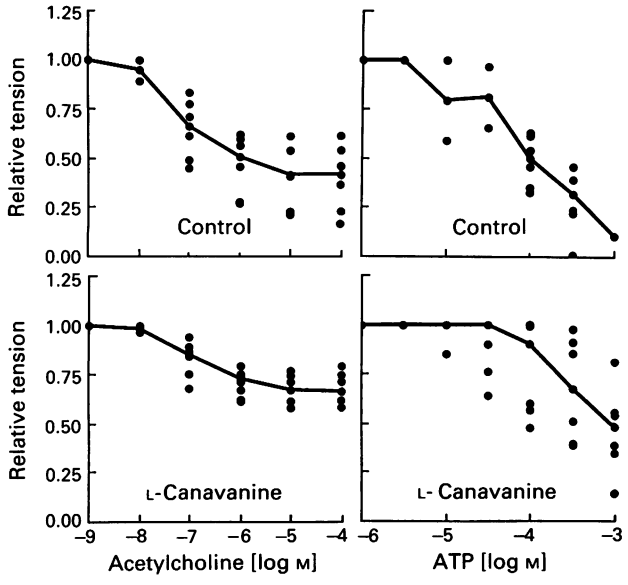

Figure 6 Effects of pretreatment with L-canavanine (L-Can; 2×10^{-3} M; 2 h) on the acetylcholine- and ATP-induced endothelium-dependent relaxation of rat aortic rings with an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 6$–9 experiments.

(10^{-3} M; not shown). In addition, the effects of L-pArg were not specific for L-Arg-containing polymeric basic amino acids. L-pOrn (6.2 kDa) and L-pLys (12.0 and 100.5 kDa) also induced endothelium-dependent relaxations. Irrespective of

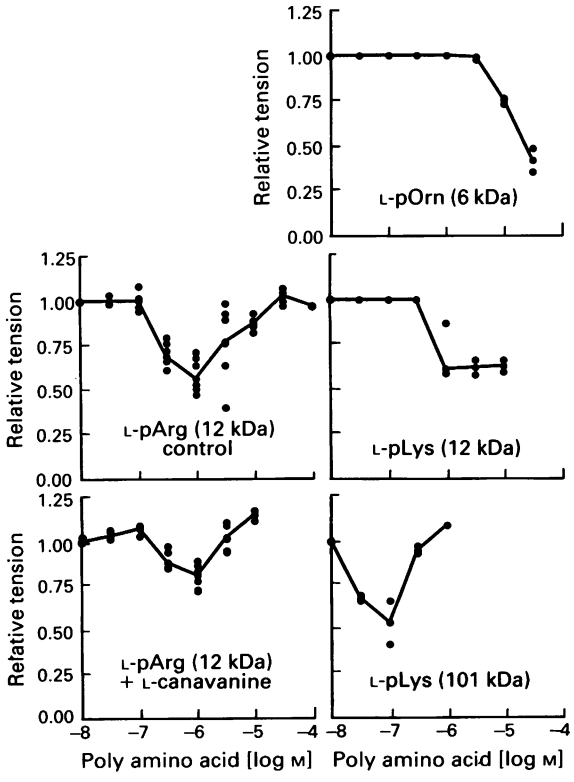

Figure 7 Effects of poly L-arginine (L-pArg), poly-L-ornithine (L-pOrn) and poly-L-lysine (L-pLys) of molecular masses between 6 and 101 kDa on the tension of rabbit aortic rings with an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 3$–9 experiments.

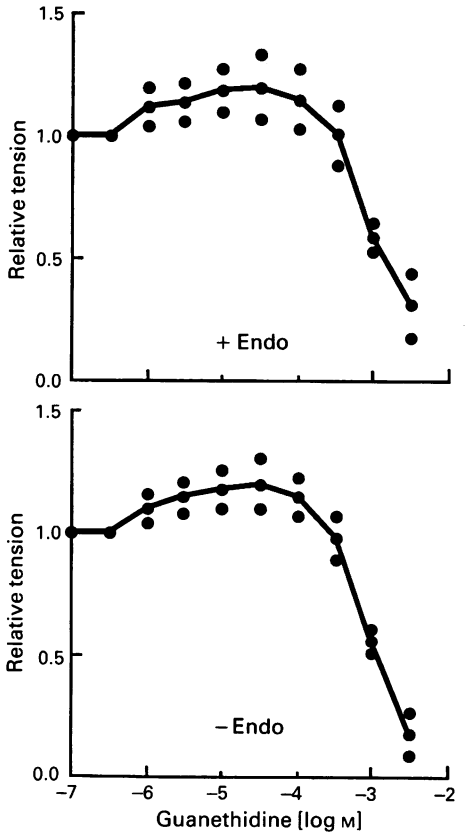

Figure 8 Effects of guanethidine on the tension of rabbit aortic rings with (+Endo) and without (−Endo) an intact endothelium precontracted with phenylephrine (3×10^{-7} M). Points represent original data of relative tension, i.e. in relation to the phenylephrine-induced tension (1.0). Lines are drawn through the median of $n = 3$ experiments.

the polymerized amino acid (L-pArg, L-pOrn or L-pLys), the potency of these polymeric basic amino acids to induce relaxations increased with their molecular masses. The respective threshold concentrations for the 6.2 kDa, 12.0 kDa, and 100.5 kDa polyamino acids were 3×10^{-6} M, 3×10^{-7} M and 10^{-8} M ($P < 0.05$).

Guanethidine

Guanethidine, a compound that contains a non-substituted guanidino moiety, induced a biphasic endothelium-independent response in rabbit aortic rings (Figure 8). The threshold concentrations for the guanethidine-induced contraction and relaxation were 3×10^{-7} M and 10^{-3} M ($P < 0.05$).

Discussion

L-Arg or L-AME were essential constituents of the cell culture medium when the ATP-induced release of nitrite, one possible degradation product of EDNO, from cultured endothelial cells was determined. L-Arg was not replaceable by D-Arg, D-AME or by L-BAEE, a substrate for enzymes utilizing peptidyl L-Arg. These data suggest that the utilization of Arg for EDNO formation shows stereospecificity for the L-enantiomer and that L-BAEE-utilizing mechanisms, e.g. enzymes utilizing protein-bound L-Arg, are apparently not involved.

L-Arg was metabolized in porcine and bovine aortic endothelial cell homogenates independently of NADPH, free Ca^{2+} and L-Orn-inhibitible arginase activity to yield both L-Cit and L-Orn. The ratio of these metabolites, i.e. Cit versus Orn, varied between species and no concomitant nitrite formation was detected. In contrast, L-Arg was reported to be NADPH-dependently metabolized in cytosols of porcine endothelial

cells to yield L-Cit but not L-Orn (Palmer & Moncada, 1989). It cannot be ruled out, in the present study, that (i) small NADPH- or Ca^{2+}-induced increases in L-Cit formation from L-Arg which might be related to EDNO formation were masked or (ii) other metabolites of L-Arg, e.g. N^G-hydroxy-L-Arg, were below the detection limit (10^{-10} M). L-Arg exerted neither endothelium-dependent nor stereoselective effects on the tension of precontracted rabbit aortic rings. Only at exceedingly high concentrations of 10^{-3} M did L-Arg induce relaxations, which was possibly due to non-specific interaction with the vascular smooth muscle. These findings are consistent with earlier studies which showed that L-Arg exerts similar non-specific effects on rat aortic rings (Thomas et al., 1989a) and that pretreatment of rat aortic rings with L-Arg does not potentiate the ATP-induced endothelium-dependent relaxation (Schmidt et al., 1988a). Moreover, it is questionable whether the reported stimulation of EDRF release from porcine aortic endothelial cells (Palmer et al., 1988; De Nucci et al., 1988) and from rat mesenterium (Thomas et al., 1989a) by L-Arg free base is related to its precursor role for EDNO as D-Arg or alkaline buffers had the same effect (De Nucci et al., 1988). Assuming that the formation of EDNO is an intracellular event as in macrophages (Marletta et al., 1988; Stuehr et al., 1989), the intracellular concentration and uptake (Sneddon et al., 1989) of L-Arg in endothelial cells are critical when the effects of extracellular Arg or Arg analogues on the tension of isolated blood vessels are studied. In bovine intrapulmonary artery, in the absence of extracellular L-Arg, an 'L-Arg depletion' induced by either a prolonged basal (Ignarro et al., 1989b) or A23187-stimulated (Gold et al., 1989) EDRF release which leads to a subsequent augmentation of the vasorelaxant effects of extracellular L-Arg has been postulated. Our findings in A23187-pretreated rabbit aortic rings did not support this proposed mechanism, at least in this tissue.

In contrast to these effects of Arg, AME exerted stereospecific but endothelium-independent effects on the tension of rabbit aortic rings. These effects may be explained by a stereospecific, direct action on the vascular smooth muscle plasma membrane.

We confirmed the potent vasorelaxant effect of L-BAEE (Thomas & Ramwell, 1988a) and L-TAME (White, 1988) in rabbit aortic rings. These compounds are N-α-substituted analogues of L-Arg and are utilized by enzymes hydrolysing L-Arg peptide bonds, e.g. thrombin and trypsin. Thrombin or trypsin alone induce endothelium-dependent relaxation, which is inhibited by PPACK (Ku, 1986). However, the L-BAEE- and L-TAME-induced relaxations of rabbit aortic rings were not dependent on an intact endothelium and were not inhibited by PPACK. When L-Arg in endothelial cell culture medium was replaced by L-BAEE, the ATP-induced release of nitrite was markedly decreased. Our findings suggest that it is unlikely that L-BAEE is a substrate for EDNO synthesis in the rabbit aorta or bovine aortic endothelial cells. However, we cannot eliminate this possibility in other vascular beds (Thomas & Ramwell, 1988a).

Furthermore, we confirmed the endothelium-dependent vasorelaxant effect of polymeric basic amino acids, e.g. L-pArg, L-pOrn and L-pLys, in rabbit aortic rings. The higher the molecular weight of these basic polyamino acids, the more potent the compounds were to induce relaxation. Ring preparations that were once exposed to maximally effective concentrations of L-pArg were thereafter refractory to either a second exposure to L-pArg or to ACh (also refer to Ignarro et al., 1989a). L-Can inhibited the effectiveness of L-pArg to induce a relaxation of rabbit aortic rings but did not affect the observed refractoriness to L-pArg. At a concentration and exposure time that induced refractoriness in rabbit aortic rings, L-pArg caused endothelial cell death (refer to Morgan et al., 1988; Rapoport et al., 1989). Thus, the endothelium-dependent effects of polymeric basic amino acids, i.e. relaxation and refractoriness, apparently involve non-specific mechanisms (Thomas et al., 1989a). A precursor role of basic poly-amino acids for the formation of EDNO is unlikely.

L-Can and L-NMMA partially inhibited the endothelium-dependent relaxation in aorta of two different species, rabbit and rat, induced by two different intercellular signal molecules, ACh and ATP. However, in rat kidney (Bhardwaj & Moore, 1989) and rabbit aorta (Rees et al., 1989), L-Can has been reported to be without effect on endothelium-dependent relaxation. In these studies the pretreatment with L-Can lasted for less than 2 h or the chosen concentration of L-Can was less than 2×10^{-3} M. Moreover, L-Can was also reported to inhibit the sodium nitroprusside-induced, endothelium-independent relaxation in the rat kidney (Bhardwaj & Moore, 1989). However, we observed that L-Can does not inhibit the GTN-induced endothelium-independent relaxation of rat and rabbit aortic rings. These discrepancies suggest that L-Can inhibits endothelium-dependent relaxation less specifically than L-NMMA and, in an intact organ, mechanisms other than a singular action on the blood vessel wall are involved. Both inhibitors, L-Can or L-NMMA, exerted dilator effects on endothelium-denuded rabbit aortic rings. In the case of L-Can, this effect also occurred in rings with an intact endothelium; a condition in which L-NMMA contracts. The latter effect of L-NMMA may be interpreted as an inhibition of basal EDRF release but, in the light of the multiple non-specific effects of Arg analogues, e.g. L-AME, that have so far been reported (Thomas & Ramwell, 1988b; Thomas et al., 1986, 1989a), an alternative non-specific mechanism cannot be ruled out.

In rabbit aortic rings, the biphasic response to guanethidine was endothelium-independent and is, therefore, unlikely to be mediated through a metabolism of the guanidino moiety in endothelial cells and subsequent EDNO release.

In conclusion, L-Arg was an essential constituent of cell culture medium with respect to the formation and/or the release of nitrite and could not be substituted by D-Arg or L-BAEE, supporting our earlier proposal that one or both of the terminal guanidino nitrogens of L-Arg are physiological precursors of EDNO (Schmidt et al., 1988b). L-Arg was metabolized in homogenates of endothelial cells generating L-Orn and L-Cit. However, there is no evidence at the present time that these metabolic pathways are involved in the biosynthesis of EDNO from L-Arg. Arg and Arg analogues exerted non-specific, possibly plasma membrane effects on isolated blood vessels. In the case of AME, these effects appeared to be stereospecific. In the case of L-pArg and other basic polyamino acids, these effects appeared to involve only the endothelial cell plasma membrane, leading to EDRF release and subsequent endothelial cell death. Direct and endothelium-dependent vascular effects, which were clearly related to the precursor role of L-Arg for EDNO, were not found with any of the Arg analogues investigated. Findings from other groups on specific endothelium-dependent effects of L-Arg, L-NMMA and L-BAEE on isolated blood vessels could not be confirmed. Our results do not support a precursor role of peptide-bound L-Arg for the formation of EDNO.

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Stimulation of cyclic GMP production in cultured endothelial cells of the pig by bradykinin, adenosine diphosphate, calcium ionophore A23187 and nitric oxide

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1 The effects of bradykinin, adenosine diphosphate, calcium ionophore A23187 and nitric oxide on the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) were investigated in cultured aortic endothelial cells of the pig.

2 Bradykinin (10^{-7} M), adenosine diphosphate (3×10^{-5} M), nitric oxide (2×10^{-6} M) and A23187 (10^{-6} M) stimulated the production of cyclic GMP. This stimulation reached a maximum within 1 min and declined rapidly with the first three agonists whereas that induced by A23187 was long-lasting.

3 These concentrations of bradykinin, A23187 and nitric oxide had no effect on cyclic AMP production. However, adenosine diphosphate (3×10^{-5} M) slightly but significantly enhanced its production by about 1.7 fold.

4 The basal content of cyclic GMP in endothelial cells was significantly reduced by haemoglobin (10^{-5} M, a scavenger of endothelium-derived relaxing factor(s)) and methylene blue (10^{-5} M, an inhibitor of the activation of soluble guanylate cyclase) and was significantly enhanced by superoxide dismutase (500 u ml^{-1}, a scavenger of superoxide anions). The basal content of cyclic GMP was not affected by N^G-monomethyl-L-arginine (10^{-5} M, a specific inhibitor of the formation of nitric oxide from L-arginine) and was slightly but significantly increased by its D-enantiomer, N^G-monomethyl-D-arginine.

5 The production of cyclic GMP stimulated by bradykinin, adenosine diphosphate, A23187 and nitric oxide was inhibited by haemoglobin (10^{-5} M) and methylene blue (10^{-5} M) but was unaffected by superoxide dismutase (500 u ml^{-1}).

6 The production of cyclic GMP stimulated by bradykinin, adenosine diphosphate or A23187, but not that stimulated by nitric oxide, was significantly reduced by N^G-monomethyl-L-arginine (10^{-5} M). The production of cyclic GMP evoked by nitric oxide, but not that induced by the other three agents, was enhanced significantly by N^G-monomethyl-D-arginine by about 1.5 fold.

7 These data indicate that the endothelium-dependent vasodilators bradykinin, adenosine diphosphate and A23187 activate the production of cyclic GMP in endothelial cells via the synthesis of nitric oxide, which in turn stimulates the soluble guanylate cyclase.

Introduction

Nitric oxide is produced and released from endothelial cells under basal conditions and in response to bradykinin, adenosine triphosphate and the calcium ionophore A23187 (Palmer *et al.*, 1987, 1988a,b; Kelm *et al.*, 1988). Endothelial cells are able to synthesize nitric oxide from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine (Palmer *et al.*, 1988a), suggesting that L-arginine is the physiological precursor for the synthesis of nitric oxide in endothelial cells (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988). Biochemical measurements have shown that endothelium-derived relaxing factor(s), like nitric oxide, enhances directly the activity of soluble guanylate cyclase and hence induces the production of guanosine 3':5'-cyclic monophosphate (cyclic GMP, Förstermann *et al.*, 1986; Ignarro *et al.*, 1986). The production of cyclic GMP mediates, at least partially, the relaxation of vascular tissues, as well as the inhibition of adhesion and aggregation and the disaggregation of platelets which have been observed in response to endothelium-derived relaxing factor(s) (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983; Murray *et al.*, 1986; Azuma *et al.*, 1986; Radomski *et al.*, 1987; Sneddon & Vane, 1988).

The present experiments were designed to investigate the effects of agents such as bradykinin, adenosine diphosphate and A23187 which enhance the production of endothelium-derived relaxing factor(s) in comparison to exogenous nitric oxide on the production of both cyclic GMP and adenosine 3':5'-cyclic monophosphate (cyclic AMP) in cultured aortic endothelial cells of the pig.

Methods

Cell culture

Segments (15 to 20 cm in length) were cut from freshly excised porcine thoracic aortae obtained from a nearby slaughterhouse and placed in ice-cold physiological salt solution (composition in mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, EDTA 0.026 and glucose 11.1) containing 1% of an antibiotic-antimycotic solution (penicillin 10,000 u ml^{-1}; streptomycin 10,000 u ml^{-1} and fungizone 25 μg ml^{-1}). Under aseptic conditions, the aortae were cleaned of connective tissue and washed in 700 ml of Earle's balanced salt solution containing 1% of the antibiotic-antimycotic solution. Each culture was obtained by scraping the intimal surface of four aortae with a sterile scalpel. The cells were collected in 15 ml of Earle's balanced salt solution and then centrifuged at 120 *g* for 7 min. The pellet was resuspended in 5 ml of complete tissue culture medium [v/v medium 199 supplemented with 10% Ryan growth supplement, 1% of the antibiotic-antimycotic solution and

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40 $\mu g\,ml^{-1}$ heparin] and the collected sheets of endothelial cells were dissociated mechanically, by passing the cell suspension six times through a needle with a diameter of 0.7 mm. The suspension, composed of single and small clumps of cells, was then transferred to ten T-25 flasks. After 24 h of incubation at 37°C in an incubator (5% CO_2 in air, humidified), the culture medium was replaced with fresh medium which was subsequently changed every two to three days. The cells were subcultured without using enzymes. The cultures of endothelial cells were characterized by the presence of Factor VIII-related antigen and by the absorption of a fluorescent acetylated low density lipoprotein (DiI-Ac-LDL; Boulanger *et al.*, 1989).

Measurement of cyclic nucleotides

Endothelial cells (first and second passages) were cultured in 35 mm Petri dishes and were used when they had reached confluence and showed a typical cobblestone pattern. The culture medium was removed by aspiration and replaced twice with 2 ml of warm (37°C) physiological salt solution. After a 30 min equilibration period in the incubator, the incubation medium was replaced with physiological salt solution containing indomethacin (10^{-5} M) and 3-isobutyl-1-methyl xanthine (IBMX, 10^{-4} M) and the cells were then equilibrated for at least 30 min before the addition of a test compound or solvent. The incubation medium was removed by aspiration and the cells were extracted immediately with 0.6 ml of 6% trichloroacetic acid. The cells were then scraped off with a rubber policeman and the suspension collected in an Eppendorf tube. The cells remaining on the Petri dish were collected following a second addition of 0.4 ml of trichloroacetic acid and added to the first extract. The cell suspension was sonicated for 5 s before being centrifuged for 10 min at 2000 *g*. The supernatant was extracted with four volumes of water-saturated ether (anaesthetic grade) and lyophilized. Each sample was resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2) and the content of cyclic AMP and of cyclic GMP were determined following their acetylation by a cyclic AMP and a cyclic GMP kit from Biomedical Technologies Inc., (Stoughton, U.S.A.), respectively. The content of cyclic nucleotide was expressed as pmol/10^6 cells. The number of cells per dish for each culture was determined by counting with a haemocytometer single cell suspensions obtained after trypsin-EDTA digestion from 6 different dishes.

Preparation of oxyhaemoglobin and nitric oxide

Stock solutions of oxyhaemoglobin and nitric oxide (NO) were prepared as previously reported (Martin *et al.*, 1985; Palmer *et al.*, 1987).

Materials and drugs

Bradykinin, adenosine diphosphate (ADP), A23187, IBMX, methylene blue, superoxide dismutase, haemoglobin and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO). N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (D-NMMA) were synthesized at the Wellcome Research Laboratories, Langley Court, Beckenham, Kent. Indomethacin was prepared daily in equimolar (10^{-3} M) concentrations of Na_2CO_3. A23187 (10^{-3} M) and IBMX (10^{-1} M) were dissolved in pure dimethylsulphoxide and further diluted in distilled water. All drug concentrations are expressed in terms of the base. Media 199, Earle's balanced salt solution and the antibiotic-antimycotic solution were obtained from M.A. Bioproducts, Walkesville, MD, U.S.A.; Ryan growth supplement was purchased from Hyclone, Logan, UT, U.S.A.; all plastic flasks and dishes for cell culture were purchased from Falcon Lincoln Park, NJ, U.S.A.

Statistics

Data are presented as mean ± s.e.mean of two or three experiments performed on triplicated dishes; n represents the number of 35 mm Petri dishes studied. Statistical comparisons were made by Student's paired t test. P values less than 0.05 were considered to be statistically significant.

Results

Basal production of cyclic GMP

Treatment of endothelial cells for 5 min wih haemoglobin (10^{-5} M) reduced significantly, by 27.5%, the content of cyclic GMP in endothelial cells from 0.080 ± 0.003 to 0.058 ± 0.003 pmol/10^6 cells ($n = 5$; $P < 0.005$). Incubation of endothelial cells for 30 min with methylene blue (10^{-5} M) also reduced significantly, by 35%, the content of cyclic GMP (from 0.080 ± 0.003 to 0.055 ± 0.002 pmol/10^6 cells, $n = 6$, $P < 0.001$). A 5 min incubation period with superoxide dismutase (500 u ml^{-1}) enhanced significantly the content of cyclic GMP by 1.75 fold (from 0.080 ± 0.003 to 0.140 ± 0.011 pmol/10^6 cells, $n = 6$, $P < 0.005$). Treatment of endothelial cells with L-NMMA (10^{-5} M, for 30 min) did not affect the basal content of cyclic GMP (0.19 ± 0.03 and 0.15 ± 0.02 pmol/10^6 cells, $n = 9$ in the absence or the presence of the compound, respectively) while a 30 min incubation period of the cells with D-NMMA (10^{-5} M) enhanced significantly the production of cyclic GMP by 1.7 fold (from 0.19 ± 0.03 to 0.33 ± 0.04 pmol/10^6 cells, $n = 9$, $P < 0.01$).

Stimulated production of cyclic GMP

Kinetic studies Bradykinin (10^{-7} M) stimulated the production of cyclic GMP from 0.26 ± 0.03 to 11.38 ± 1.99 pmol/10^6 cells ($n = 9$, $P < 0.001$) within 1 min; after 1 min, the effect of bradykinin on cyclic GMP accumulation declined with time. ADP (3×10^{-5} M), A23187 (10^{-6} M) and NO (2×10^{-6} M) also stimulated the production of cyclic GMP in cultured endothelial cells of the pig (Figure 1); this accumulation of cyclic GMP reached a maximum within less than

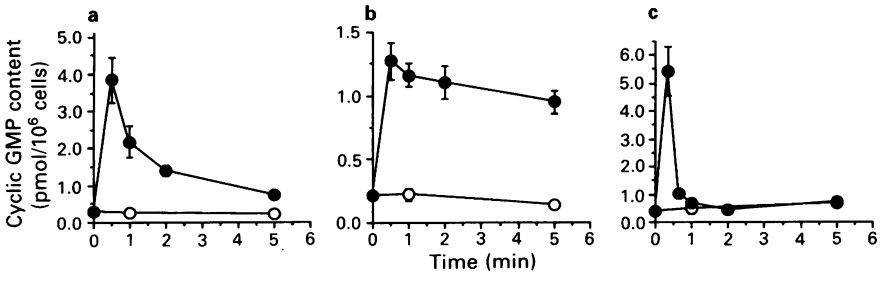

Figure 1 Effects of adenosine diphosphate (3×10^{-5} M; a), the calcium ionophore A23187 (10^{-6} M; b) and nitric oxide (2×10^{-6} M; c) on the accumulation of cyclic GMP in cultured porcine aortic endothelial cells as a function of time. The experiments were performed in the presence of indomethacin (10^{-5} M) and isobutyl-1-methylxanthine (10^{-4} M). The results are expressed as the mean of two (c) or three (a,b) different experiments performed on three dishes; s.e.mean shown by vertical bars.

Table 1 Effect of bradykinin, adenosine diphosphate (ADP), the calcium ionophore A23187 and nitric oxide on the accumulation of cyclic AMP in cultured porcine aortic endothelial cells

	Cyclic AMP content (pmol/10^6 cells)						
Time (s)	0	20	30	40	60	120	300
Control	2.63 ± 0.34	ND	ND	ND	2.79 ± 0.38	ND	2.74 ± 0.26
Bradykinin (10^{-7} M)	—	ND	2.87 ± 0.25	ND	2.84 ± 0.33	2.88 ± 0.34	2.64 ± 0.21
Control	2.63 ± 0.34	ND	ND	2.79 ± 0.38	ND	2.74 ± 0.26	
ADP (3×10^{-5} M)	—	ND	4.28 ± 0.62*	ND	4.51 ± 0.67*	4.84 ± 0.60*	4.51 ± 0.63*
Control	1.07 ± 0.11	ND	ND	0.95 ± 0.08	ND	0.99 ± 0.10	
A23187 (10^{-6} M)	—	ND	0.97 ± 0.10	ND	0.97 ± 0.08	0.98 ± 0.07	0.92 ± 0.07
Control	2.76 ± 0.16	ND	ND	ND	3.17 ± 0.18	ND	3.65 ± 0.28
Nitric oxide (2×10^{-6} M)	—	3.05 ± 0.16	ND	2.99 ± 0.22	3.16 ± 0.20	2.75 ± 0.51	3.04 ± 0.17

The experiments were performed in the presence of indomethacin (10^{-5} M) and isobutyl-1-methyl xanthine (10^{-4} M). The results are expressed as mean ± s.e.mean of two (nitric oxide) or three (ADP, A23187) different experiments performed on three dishes. The asterisks denote a significant difference between the cyclic AMP content of control (0 s) and agonist-treated endothelial cells.
ND = not determined.

1 min of stimulation and declined rapidly thereafter with ADP and NO (Figure 1a,c). The production of cyclic GMP evoked by A23187 was long-lasting, declining to about 75% of the maximal accumulation of cyclic GMP after 5 min of observation (Figure 1b).

Bradykinin, A23187 or NO did not affect the content of cyclic AMP in endothelial cells (Table 1). ADP (3×10^{-5} M) significantly increased the content of cyclic AMP to a plateau level of 1.7 fold basal content after 30 s of stimulation ($P < 0.001$, Table 1).

Role of endothelium-derived relaxing factor(s) Treatment of endothelial cells with haemoglobin (10^{-5} M) for 5 min markedly inhibited the production of cyclic GMP stimulated by bradykinin (10^{-7} M, 1 min, $P < 0.001$), by A23187 (10^{-6} M, 1 min, $P < 0.005$), by ADP (3×10^{-5} M, 1 min, $P < 0.005$) and also that measured in response to a 20 s incubation period with NO (2×10^{-6} M, $P < 0.001$) (Figure 2). The production of cyclic GMP in response to an activator of the particulate guanylate cyclase, α-human atrial natriuretic peptide (10^{-7} M, 1 min), was not affected by the incubation of endothelial cells with haemoglobin (10^{-5} M for 5 min, data not shown).

Incubation of endothelial cells with methylene blue (10^{-5} M) reduced markedly the production of cyclic GMP detected in response to a 1 min stimulation period with bradykinin (10^{-7} M, $P < 0.001$), ADP (3×10^{-5} M, $P < 0.01$), A23187 (10^{-6} M, $P < 0.01$) and also that evoked by NO (2×10^{-6} M for 20 s, $P < 0.001$) (Figure 2). Methylene blue (10^{-5} M) also reduced, but to a lesser extent, the production of cyclic GMP stimulated by α-human atrial natriuretic peptide (10^{-7} M for 1 min) from 1.01 ± 0.05 to 0.68 ± 0.02 pmol/10^6 cells ($n = 6$, $P < 0.001$).

A 5 min incubation period with superoxide dismutase (500 u ml^{-1}) did not affect the production of cyclic GMP in response to bradykinin (10^{-7} M, 1 min), ADP (3×10^{-5} M, 1 min), A23187 (10^{-6} M, 1 min) or NO (2×10^{-6} M, 20 s) (Figure 2).

Treatment of endothelial cells with L-NMMA (10^{-5} M, 30 min) significantly reduced the production of cyclic GMP stimulated by 1 min of exposure to bradykinin (10^{-7} M, $P < 0.001$), ADP (3×10^{-5} M, $P < 0.001$) and also to A23187 (10^{-6} M, $P < 0.01$) (Figure 3). The production of cyclic GMP stimulated by NO (2×10^{-6} M, 20 s) was not affected by the treatment of endothelial cells with L-NMMA (10^{-5} M) (Figure 3). Incubation of endothelial cells with D-NMMA (10^{-5} M, 30 min) did not affect the production of cyclic GMP stimulated by a 1 min incubation of endothelial cells with bradykinin (10^{-7} M), ADP (3×10^{-5} M) or with A23187 (10^{-6} M)

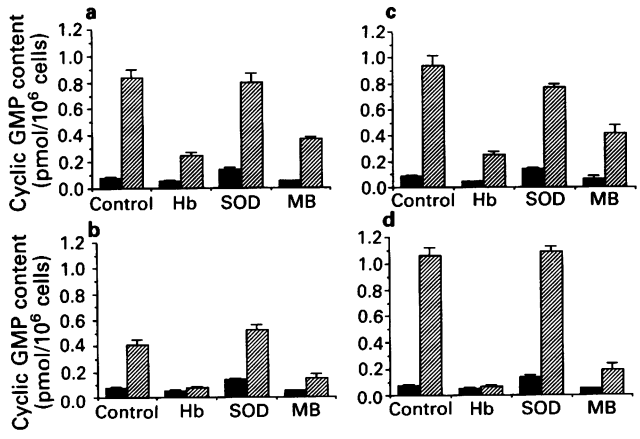

Figure 2 Effects of modulators of the biological action of endothelium-derived relaxing factor(s) on basal cyclic GMP content (solid columns) and stimulated cyclic GMP production (hatched columns) in the presence of (a) bradykinin (10^{-7} M, 1 min); (b) adenosine diphosphate (3×10^{-5} M, 1 min); (c) A23187 (10^{-6} M, 1 min) and (d) nitric oxide (2×10^{-6} M, 20 s). The cells were incubated with haemoglobin (Hb, 10^{-5} M) or with superoxide dismutase (SOD, 500 u ml^{-1}) for 5 min or with methylene blue (MB, 10^{-5} M) for 30 min before the addition of a test compound or drug-vehicle. All experiments were performed in the presence of indomethacin (10^{-5} M) and isobutyl-1-methyl xanthine (10^{-4} M). The results are expressed as the mean of two different experiments performed on three dishes; s.e.mean shown by vertical bars.

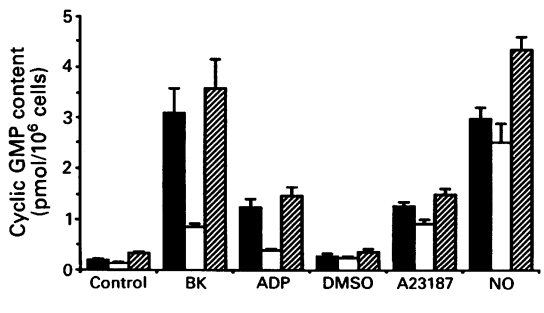

Figure 3 Effect of N^G-monomethyl-L-arginine (L-NMMA, 10^{-5} M) and N^G-monomethyl-D-arginine (D-NMMA, 10^{-5} M) on basal and on bradykinin (BK, 10^{-7} M, 1 min), adenosine diphosphate (ADP, 3×10^{-5} M, 1 min), nitric oxide (NO, 2×10^{-6} M, 20 s), A23187 (10^{-6} M, 1 min) or dimethylsulphoxide (DMSO, 0.1%, the solvent of the calcium ionophore, 1 min)-stimulated production of cyclic GMP (solid columns). The cells were incubated with L-NMMA (open columns) or D-NMMA (hatched columns) for 30 min before the addition of a test compound or solvent. All experiments were performed in the presence of indomethacin (10^{-5} M) and isobutyl-1-methyl xanthine (10^{-4} M). The results are expressed as the mean of three different experiments performed on three dishes; s.e.mean shown by vertical bars.

while that stimulated by NO (2×10^{-6} M, 20 s) was enhanced significantly by 1.5 fold (from 2.97 ± 0.24 to 4.35 ± 0.26 pmol/10^6 cells, $n = 9$, $P < 0.01$) (Figure 3).

Discussion

The present study indicates that bradykinin, ADP and A23187 markedly enhanced the production of cyclic GMP in cultured aortic endothelial cells of the pig. The enhanced production of cyclic GMP was almost abolished by treatment of endothelial cells with haemoglobin, a compound which binds endothelium-derived relaxing factor(s) (Martin *et al.*, 1985; Ignarro *et al.*, 1987); it was inhibited following the treatment of the cells with methylene blue, an inhibitor of the activation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986). Treatment of endothelial cells with superoxide dismutase, a compound which protects endothelium-derived relaxing factor(s) from degradation probably by scavenging superoxide anions (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986), did not affect the production of cyclic GMP stimulated by bradykinin, A23187 and ADP. These observations indicate that under our experimental conditions, the agonist-stimulated production of endothelium-derived relaxing factor is not affected by superoxide anions. Nevertheless, taken together the data suggest that the enhanced production of cyclic GMP detected in response to bradykinin, ADP and to A23187 is mediated by an increased production of endothelium-derived relaxing factor(s) which in turn enhances the activity of endothelial soluble guanylate cyclase. The production of cyclic GMP in response to agents which stimulate the release of endothelium-derived relaxing factor(s) has also been observed in cultured human umbilical vein and porcine aortic endothelial cells (Brotherton, 1986; Martin *et al.*, 1988) although not in bovine aortic endothelial cells (Ganz *et al.*, 1986; Loeb *et al.*, 1987). The lack of agonist-stimulated accumulation of cyclic GMP may be related to the species, to the culture conditions (e.g., high versus low passages) and/or to the experimental conditions (e.g., presence or absence of a phosphodiesterase inhibitor). It is noteworthy that with increasing times in culture, vascular cells lose the ability to induce biological responses (Goldsmith *et al.*, 1984; Loeb *et al.*, 1987). In the case of cultured vascular smooth muscle cells, the production of cyclic GMP in response to sodium nitroprusside was completely lost after five passages indicating that the activity of soluble guanylate cyclase is labile during long-term culture (Loeb *et al.*, 1987). Furthermore, cultured endothelial cells possess phosphodiesterase activities that are likely to participate in the regulation of their cyclic nucleotide content (Lugnier & Schini, 1988) and which may not allow the estimation of the production of cyclic nucleotide when experiments are performed without the presence of a phosphodiesterase inhibitor (Hopkins & Gorman, 1981; Makarski, 1981; Brotherton, 1986).

The content of cyclic GMP in unstimulated porcine endothelial cells was augmented by treatment of the cells with superoxide dismutase and reduced following incubation of the cells with either haemoglobin or methylene blue. These observations indicate the existence of a tonic release of endothelium-derived relaxing factor(s) from unstimulated endothelial cells. The fact that superoxide dismutase increases the accumulation of cyclic GMP in unstimulated but not that in agonist-stimulated cells may be due to the presence of a small amount of superoxide anions which are able to reduce significantly the limited basal production of endothelium-derived relaxing factor without affecting that evoked by the agonists. Treatment of endothelial cells with L-NMMA, a specific inhibitor of the formation of NO from L-arginine (Rees *et al.*, 1989), did not affect the content of cyclic GMP of unstimulated endothelial cells. A possible explanation for the lack of effect of the antagonist is that, under basal conditions, the enzyme which synthesizes NO from L-arginine preferentially utilizes endogenous L-arginine rather than the L-arginine analogue. A basal release of endothelium-derived relaxation factor(s) from cultured endothelial cells has also been obtained under bioassay conditions (Gryglewski *et al.*, 1986; De Nucci *et al.*, 1988; Boulanger *et al.*, 1989).

The production of cyclic GMP evoked by bradykinin, ADP and A23187 is impaired by L-NMMA but not by its D-enantiomer, D-NMMA. This inhibition of the production of cyclic GMP does not seem to be due to a non-specific effect of the L-NMMA as the production of the cyclic nucleotide detected in response to exogenous NO (this study) or to an atrial natriuretic peptide (unpublished observations) were not affected. These observations suggest that bradykinin, ADP and A23187 stimulate the production and/or release of NO or of NO-containing substances from endothelial cells which then enhances the activity of soluble guanylate cyclase and hence induces the accumulation of cyclic GMP.

D-NMMA increased the basal production of cyclic GMP and that evoked by nitric oxide. One possible explanation would be that the compound prolongs the half-life of endothelium-derived relaxing factor NO, possibly by scavenging superoxide anions; the fact that both basal and NO-stimulated production are augmented, whereas only the former is augmented by superoxide dismutase, argues against this hypothesis. Alternatively, D-NMMA may have a weak stimulating effect on guanylate cyclase, or possess some inhibitory action on cyclic GMP phosphodiesterases; these interpretations fail to explain why the compound did not affect the response to bradykinin, ADP and A23187. The present observations do not allow further speculation on the mechanism underlying this surprising effect.

Nitric oxide, a direct activator of soluble guanylate cyclase (Katsuki *et al.*, 1977; Arnold *et al.*, 1977), also enhanced markedly the content of cyclic GMP, further indicating that after a limited number of passages, porcine aortic endothelial cells in culture retain a functional soluble guanylate cyclase. In the case of NO, the production of cyclic GMP was short lasting, a response which may be explained by the oxidation of nitric oxide into the biologically inactive NO_2. Bradykinin and ADP also enhanced the production of cyclic GMP in a transient manner, suggesting a short-lasting release of NO-containing factor(s) and hence activation of soluble guanylate cyclase. In the case of bradykinin, this may be due to the desensitization of the kinin-receptors on the cell membrane (Luckhoff *et al.*, 1988). Alternatively, bradykinin and ADP may be degraded by ectoenzymes such as carboxypeptidases, angiotensin-converting enzymes and nucleoside diphosphatases into inactive or less active metabolites with regard to their ability to enhance the activity of soluble guanylate cyclase. However, when the release of endothelium-derived relaxing factor(s) is stimulated by a non-receptor operating agent such as A23187, the content of cyclic GMP is increased maximally after 30 s of stimulation and declined only slightly thereafter. Thus, the calcium ionophore, in contrast to bradykinin and ADP, may induce a long lasting production of NO-containing factor(s) by endothelial cells.

Neither bradykinin, A23187 nor NO affect the content of cyclic AMP in endothelial cells, indicating that cyclic AMP does not play an important role in the biological responses induced by these substances in cultured endothelial cells. However, ADP slightly but significantly increased the production of cyclic AMP. Such a response may possibly be related to the metabolism of adenosine diphosphate into adenosine, a substance which could stimulate the production of cyclic AMP in cultured endothelial cells from swine aorta (Goldman *et al.*, 1983).

In conclusion, substances that increase the production of endothelium-derived relaxing factor(s) markedly augment the content of cyclic GMP in porcine cultured aortic endothelial cells. The production of cyclic GMP probably is mediated by an enhanced production of NO or of NO-containing substance(s), which increases the activity of soluble guanylate cyclase and hence the production of cyclic GMP. Cyclic GMP may play an important second messenger role in the bio-

logical responses induced by various hormones and pharmacological agents in endothelial cells.

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Cytoplasmic calcium and the relaxation of canine coronary arterial smooth muscle produced by cromakalim, pinacidil and nicorandil

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1 In order to investigate the vasodilator mechanisms of the K^+ channel openers, cromakalim, pinacidil and nicorandil, we measured changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) simultaneously with force by a microfluorimetric method using fura-2, a calcium indicator, in canine coronary arterial smooth muscle cells.

2 The three K^+ channel openers all produced a concentration-dependent reduction of $[Ca^{2+}]_i$ in 5 and 30 mM KCl physiological salt solution (PSS) but failed to affect $[Ca^{2+}]_i$ in 45 and 90 mM KCl-PSS.

3 Cromakalim only partly inhibited (−45%) the 30 mM KCl-induced contractures, whereas pinacidil and nicorandil nearly abolished contractions produced by 45 mM, 90 mM and 30 mM KCl-PSS.

4 Tetrabutylammonium (TBA), a nonselective K^+ channel blocker, or glibenclamide, a supposed adenosine 5'-triphosphate (ATP)-sensitive K^+ channel blocker, abolished the reduction of $[Ca^{2+}]_i$ caused by the three K^+ channel openers and the relaxant effect of cromakalim, whereas they only slightly attenuated the relaxant effects of pinacidil and nicorandil.

5 The increase in $[Ca^{2+}]_i$ produced by 45 or 90 mM KCl-PSS in the presence of pinacidil or nicorandil was abolished by 10^{-5} M verapamil, indicating that the increase in $[Ca^{2+}]_i$ was caused by the influx of extracellular Ca^{2+} and that pinacidil and nicorandil did not affect the voltage-dependent Ca^{2+} channel directly.

6 The $[Ca^{2+}]_i$-force relationship in the presence of cromakalim was not distinguishable from that of control.

7 The $[Ca^{2+}]_i$-force curve was shifted to the right by pinacidil and nicorandil.

8 These results show that cromakalim is a more specific K^+ channel opener than pinacidil and nicorandil, and that vasodilatation produced by cromakalim in this study is predominantly a result of a reduction of $[Ca^{2+}]_i$ due to the closure of voltage-dependent Ca^{2+} channels by hyperpolarization. In contrast, additional mechanisms are involved in the vasodilator actions of pinacidil and nicorandil. One of these is related to a reduction in the sensitivity of contractile proteins to Ca^{2+}. The latter mechanism of nicorandil is akin to that of nitroglycerin. K^+ channels opened by these K^+ channel openers may be ATP-sensitive ones which are blocked by glibenclamide.

Introduction

Increasing attention has recently been paid to vasodilator drugs which activate or open K^+ channels in vascular smooth muscle and which eventually increase coronary blood flow or lower blood pressure. These drugs have been called 'K^+ channel activators or openers' and include nicorandil (Taira *et al.*, 1979; Yanagisawa *et al.*, 1979), pinacidil (Arrigoni-Martelli *et al.*, 1980; Bray *et al.*, 1987) and cromakalim (BRL 34915; Hamilton *et al.*, 1986; Weir & Weston, 1986a,b), although nicorandil also has a nitrate-like action (Taira, 1987; 1989). In canine atrial muscle, these three K^+ channel openers reduce the force of contraction exclusively by opening K^+ channels, because their negative inotropic effects are totally blocked by the quaternary ammonium K^+ channel blockers, tetraethylammonium (TEA) or tetrabutylammonium (TBA) (Yanagisawa *et al.*, 1988; 1989a).

In vascular smooth muscle cells the resting membrane potential is less negative than the K^+ equilibrium potential (Furukawa *et al.*, 1981). When the membrane is hyperpolarized towards the K^+ equilibrium potential by K^+ channel openers, excitation-induced Ca^{2+} influx via voltage-dependent Ca^{2+} channels is thought to decrease (Cook, 1988; Weston, 1989). However, no direct measurement of the effects of K^+ channel openers on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has yet been obtained. Furthermore, as K^+ channel openers are structurally different, it is possible that some possess other vasodilator mechanisms.

Although $[Ca^{2+}]_i$ is one of the main determinants of mechanical activity of vascular smooth muscle, various studies have shown that it is not the only determinant of this activity (Kamm & Stull, 1989; Karaki, 1989). By improving the methods of Ozaki *et al.* (1987) and Bruschi *et al.* (1988) we have recently developed a microfluorimetric method, using the Ca^{2+} indicator, fura-2 (Grynkiewicz *et al.*, 1985), to measure simultaneously $[Ca^{2+}]_i$ with force in isolated vascular rings (Yanagisawa *et al.*, 1989b). In the present study we used this method to investigate the effects of K^+ channel openers on $[Ca^{2+}]_i$ and tension of canine isolated coronary arterial rings. We further studied how their effects were modified by the nonselective K^+ channel blocker, TBA (Stanfield, 1983) and the supposed adenosine 5'-triphosphate (ATP)-sensitive K^+ channel blocker, glibenclamide (Cook, 1988; Castle *et al.*, 1989), to identify what kind of K^+ channels are involved.

Methods

Tissue preparation

Hearts were obtained from mongrel dogs of either sex, weighing 5 to 13 kg, anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.). Coronary arterial rings (0.5–1.5 mm in diameter, about 1 mm in width) were dissected and connective

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tissues were carefully removed in a dissecting chamber under a binocular microscope. The endothelium was removed by gentle rubbing and the lumenal side was turned to face outwards.

Fluorescence measurements

Coronary arterial rings were exposed to 10 μM fura-2 acetoxymethyl ester (fura-2 AM) for about 2 h at 37°C. The non-cytotoxic detergent, pluoronic F-127 (0.1% w/v), was premixed in the loading physiological salt solution (PSS) to help dissolve fura-2 AM in PSS (Poenie *et al.*, 1986). After the fura-2 loading, muscles were rinsed with normal PSS for more than 1 h and used for experiments. Fluorescence was measured in a fluorimeter equipped with a dual wavelength excitation device (CAM-200, Japan Spectroscopic, Tokyo, Japan) connected to an inverted microscope (TMD, Nikon, Tokyo, Japan). For further details see Yanagisawa *et al.* (1989b). The fluorescence image was obtained by focusing on the smooth muscle cells in the medial layer with a Nikon CF UV (Fluor) 10× objective lens. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml min^{-1}. The muscle ring was stretched to a resting tension of about 5 mN between two tungsten needles, one of which was glued to a transducer element (AE801, AME, Horten, Norway). The photosignals and the mechanical activity were measured simultaneously and both recorded on a chart recorder (Recti-horiz-8k, NEC-Sanei, Tokyo, Japan). They were also digitized by A/D converters and fed into a personal computer for further calculation and graphical analysis (Himpens & Somlyo, 1988) by a microcomputer (PC-9801, NEC, Tokyo, Japan).

Every experiment commenced with perfusion of 90 mM KCl-PSS for 10 min. We subtracted the F_{340} and F_{380} values due to autofluorescence from the corresponding values of F_{340} and F_{380} obtained under conditions of fura-2 loading to derive a recalculated ratio$_{rc}$. Changes in the ratio$_{rc}$ (changes in $[Ca^{2+}]_i$) and those in force were expressed as percentages of the differences between basal values and those obtained with a 10 min perfusion with 90 mM KCl-PSS. None of the drugs and chemicals used in the present study, with the exception of fura-2 and $MnCl_2$, affected fluorescence signals at the concentrations used.

Drugs and solutions

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

Drugs and chemicals were obtained from the following sources: cromakalim (BRL 34915; Beecham, Harlow, U.K.), nicorandil (Chugai, Tokyo, Japan), pinacidil monohydrate (Shionogi, Osaka, Japan), verapamil hydrochloride (Eisai, Tokyo, Japan), glibenclamide (Hoechst Japan, Tokyo, Japan and Yamanouchi, Tokyo, Japan), EGTA (glycol-etherdiaminetetraacetic acid), fura-2 AM, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and pluronic F-127 (Dojin, Kumamoto, Japan), TBA (tetrabutylammonium chloride), DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). Fura-2 AM was dissolved in DMSO at a concentration of 1 mM. Pluronic F-127 was dissolved in 25% w/v in DMSO. Glibenclamide was dissolved in DMSO to give a concentration of 10 mmol l^{-1}. Cromakalim was dissolved in 70% ethanol to give a concentration of 10 mmol l^{-1}. Pinacidil was dissolved in 0.1 N HCl to give a concentration of 200 mmol l^{-1}. Other drugs were dissolved in distilled water to the desired concentrations. From these stock solutions the appropriate concentrations were obtained by diluting with PSS.

Evaluation of responses

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

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

where E is the normalized resonse, M is the maximum response, A is the drug concentration, K is the EC_{50} value of each drug and p is the slope parameter (Parker & Waud, 1971). When the maximum response of each drug was not obtainable because of its limited solubility, M and p were fixed at 100 and 1, respectively. The concentration-effect curves for K^+ channel openers in reducing $[Ca^{2+}]_i$ below the basal level in 5 mM KCl-PSS were also computer fitted to equation (1). The maximum response, M, however, was the maximum reduction of $[Ca^{2+}]_i$ produced on perfusion with Ca^{2+}-free-PSS containing 1 mM EGTA which was taken as 100%.

The concentration-effect curves for extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) were also computer fitted to a logistic equation:

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

where E is the response normalized by the differences between basal values and those of 90 mM KCl at 10 min, A is $[Ca^{2+}]_o$, K is EC_{50} value of $[Ca^{2+}]_o$ and p is slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$).

Experimental values are given as means or means ± s.e.mean. Statistical significance of differences between mean values was estimated by Student's t test. A t test for paired comparison was used when it was applicable. A P value smaller than 0.05 was considered to be significant.

Results

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

In canine coronary artery loaded with fura-2, perfusion with 30 mM KCl-PSS increased $[Ca^{2+}]_i$ and elicited a contracture. Cromakalim (10^{-7}–3×10^{-5} M) reduced both $[Ca^{2+}]_i$ and contracture in a concentration-dependent manner (Figure 1a). These effects of cromakalim could be reversed by washing. The summarized data from 7 muscles are shown in Figure 1b and the results of the analysis of the concentration-effect curves for cromakalim to reduce the KCl-induced contracture and increase in $[Ca^{2+}]_i$ are summarized in Table 1. The pD_2 ($-\log EC_{50}$) values of cromakalim required to reduce the increase in $[Ca^{2+}]_i$ were not significantly higher than those needed to inhibit contracture.

The effects of cromakalim on KCl-induced changes were dependent on the concentration of KCl. When the arterial rings were perfused with 45 mM KCl-PSS, cromakalim had no effect on either the force of contracture or the increase in $[Ca^{2+}]_i$ (Figure 2a). In 5 mM KCl-PSS, cromakalim reduced $[Ca^{2+}]_i$ from the basal level, whereas the resting tone was not affected. When extracellular Ca^{2+} was removed by perfusion with Ca^{2+}-free-PSS containing 1 mM EGTA, $[Ca^{2+}]_i$ was reduced further (Figure 2b). There was no difference between the respective pD_2 values for cromakalim-induced changes in both $[Ca^{2+}]_i$ and force observed in the presence of 5 mM and 30 mM KCl, although the membrane potentials under these conditions should have been different.

Modification by glibenclamide and TBA of the effects of cromakalim

Figure 3a shows the modification by glibenclamide of the effects of 10^{-5} M cromakalim on the force of contracture and

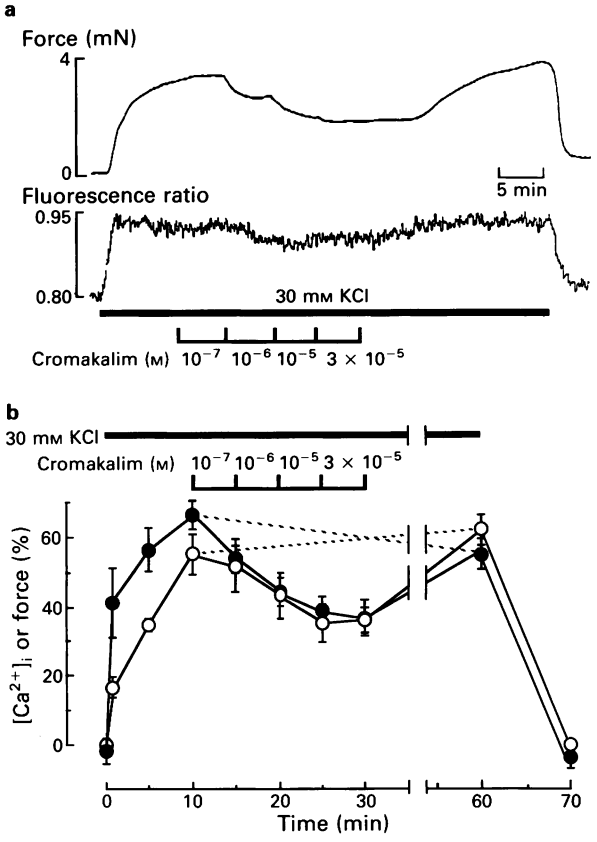

Figure 1 (a) Effects of cromakalim (10^{-7}–3 × 10^{-5} M) on the force of contracture and increase in $[Ca^{2+}]_i$ (measured as fluorescence ratio) produced by 30 mM KCl-PSS in a single experiment in canine coronary artery. (b) The summarized data obtained from 7 arterial rings. The force of contracture (○) and the increase in $[Ca^{2+}]_i$ (●) are expressed as a percentage of the 10 min responses to 90 mM KCl-PSS in this and subsequent figures; vertical lines show s.e.mean.

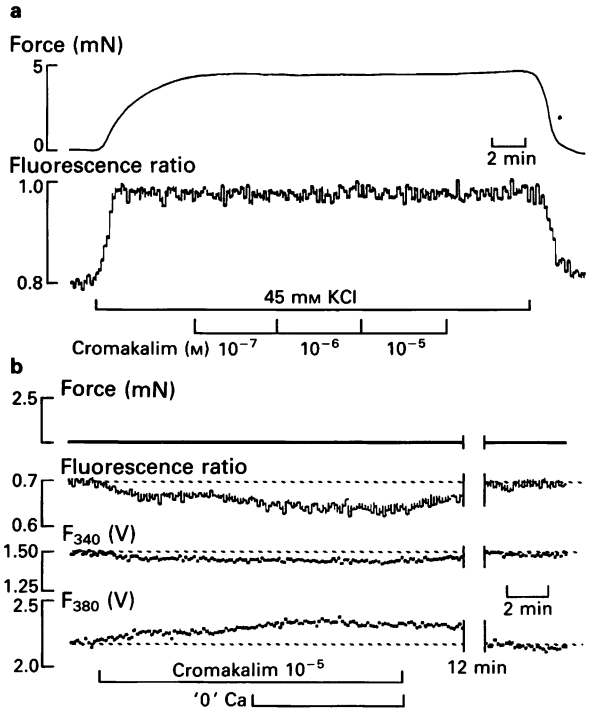

Figure 2 (a) Effects of cromakalim on force of contracture and the increase in $[Ca^{2+}]_i$ (measured as fluorescence ratio) of a canine coronary artery perfused with 45 mM KCl-PSS. (b) Effects of cromakalim on the resting force and $[Ca^{2+}]_i$ derived from fluorescence measurements in 5 mM KCl-PSS and Ca^{2+}-free PSS containing 1 mM EGTA.

the increase in $[Ca^{2+}]_i$ in canine coronary artery perfused with 30 mM KCl-PSS. Cromakalim (10^{-5} M) reduced both $[Ca^{2+}]_i$ and the force of contracture. When glibenclamide (10^{-6} and 10^{-5} M) was applied 10 min after perfusion with 30 mM KCl-PSS, no change in the increase in $[Ca^{2+}]_i$ or force was observed. In contrast the ability of cromakalim to reduce the increase in $[Ca^{2+}]_i$ and force were greatly attenuated by exposure to glibenclamide at 10^{-6} M and abolished at 10^{-5} M (Figure 3b). The effects of cromakalim were also blocked by 10^{-3} M TBA (Figure 4). Although TBA *per se* had little effect on the force of contracture and the increase in $[Ca^{2+}]_i$ in 30 mM KCl-PSS, its effects persisted after washout, since $[Ca^{2+}]_i$ after washout in 5 mM KCl-PSS was higher than the basal level (Figure 4a,b). In 5 mM KCl-PSS glibenclamide affected neither $[Ca^{2+}]_i$ nor the resting tension, whereas TBA slightly increased $[Ca^{2+}]_i$ by about 10% without changing the resting tension (data not shown).

Effects of pinacidil on KCl-induced contractures and increases in $[Ca^{2+}]_i$

In 5 mM KCl-PSS, pinacidil reduced $[Ca^{2+}]_i$ below the basal level without changing the resting tension (Table 1). The reduction of $[Ca^{2+}]_i$ produced by 10^{-5} M pinacidil was abolished by 10^{-3} M TBA ($n = 3$). When the coronary arterial rings were perfused with 30 mM KCl-PSS, pinacidil, like cromakalim, reduced the increase in $[Ca^{2+}]_i$ with a pD_2 value of

Table 1 Effects of cromakalim, pinacidil and nicorandil on the force of contracture and $[Ca^{2+}]_i$

		Reduction of force			*Reduction of $[Ca^{2+}]_i$*		
KCl (mM)	(n)	*Maximum response* (%)	pD_2	p	*Maximum response* (%)	pD_2	p
Cromakalim							
5	(7)	no effect (< 10^{-5} M)			60.1 ± 10.5	6.66 ± 0.17	1.07 ± 0.04
30	(7)	45.1 ± 6.4	6.04 ± 0.20	1.11 ± 0.13	40.1 ± 5.5	6.55 ± 0.11	0.94 ± 0.18
45	(6)	no effect (< 10^{-5} M)			no effect (< 10^{-5} M)		
Pinacidil							
5	(7)	no effect (< 3 × 10^{-4} M)			79.5 ± 8.6	5.40 ± 0.11	1.05 ± 0.10
30	(6)	100[a]	5.11 ± 0.08	0.79 ± 0.06	44.8 ± 6.7	4.94 ± 0.16	1[a]
45	(7)	100[a]	4.47 ± 0.04	1.07 ± 0.04	no effect (< 3 × 10^{-4} M)		
90	(5)	100[a]	4.16 ± 0.33	1.21 ± 0.26	no effect (< 10^{-4} M)		
Nicorandil							
30	(7)	100[a]	4.33 ± 0.18	1.04 ± 0.17	35.9 ± 4.0	4.57 ± 0.9	1[a]
45	(6)	100[a]	3.72 ± 0.04	1.29 ± 0.19	no effect (< 10^{-3} M)		
90	(8)	100[a]	3.64 ± 0.12	1.02 ± 0.13	no effect (< 10^{-3} M)		

The concentration-effect curves for cromakalim, pinacidil and nicorandil were analysed by computer fitting to a logistic equation: $E = 100 - M \times A^p/(A^p + K^p)$ where E is normalized response (basal value = 100%). M is maximum response, A is drug concentration. K is EC_{50} value of each drug and p is slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$). [a] M and p were fixed at 100 and 1, respectively.

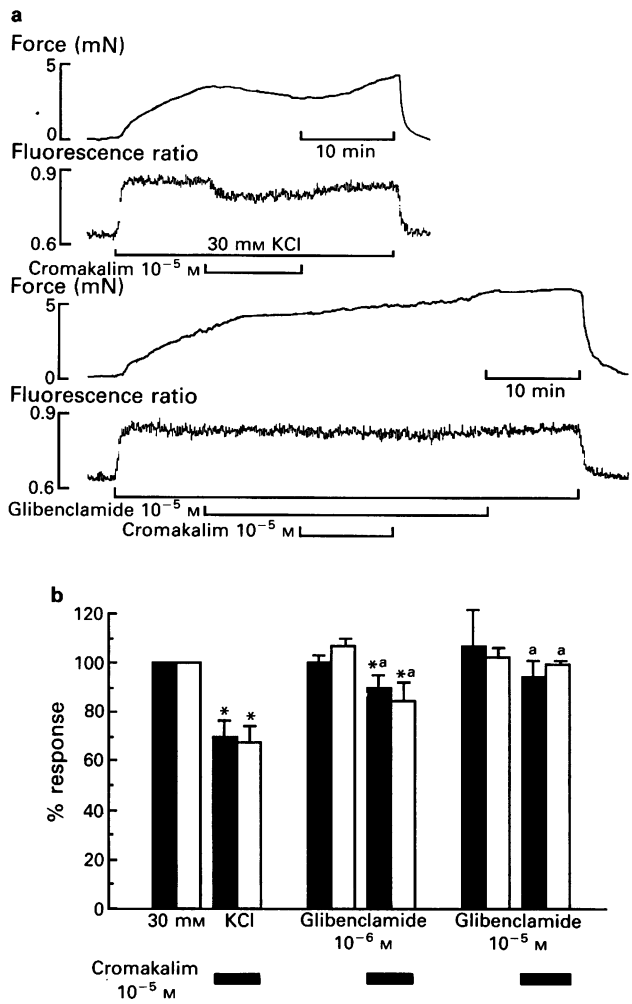

Figure 3 (a) Modification by glibenclamide (10^{-5} M) of the effects of cromakalim (10^{-5} M) on the force of contracture and the increase in $[Ca^{2+}]_i$ in a single experiment in canine coronary artery depolarized by 30 mM KCl-PSS. (b) Summarized data, expressed as a percentage of the values obtained just before the administration of cromakalim or glibenclamide ($n = 5$–9). * $P < 0.05$ compared with control values. *$P < 0.05$ compared with values in the presence of cromakalim 10^{-5} M alone. Open columns, force of contracture; solid columns, $[Ca^{2+}]_i$.

4.94 ± 0.16. The 30 mM KCl-induced contracture was, however, nearly abolished by 10^{-4} M pinacidil with a pD_2 value of 5.11 ± 0.08 (Figure 5a; Table 1). Thus, although the pD_2 value of pinacidil for mechanical inhibition was slightly but not significantly greater than that for reduction of $[Ca^{2+}]_i$, the maximum effect on the former was much larger than that on the latter. In 45 mM KCl-PSS, pinacidil, in contrast to cromakalim, inhibited the force of contracture in a concentration-dependent manner, whereas the increase in $[Ca^{2+}]_i$ was not changed by pinacidil at all. Even during the washout of pinacidil the increase in $[Ca^{2+}]_i$ did not change (Figure 5b; Table 1). In 90 mM KCl-PSS, pinacidil inhibited the force of contracture without modifying the increase in $[Ca^{2+}]_i$. These results suggest that pinacidil, in addition to its K^+ channel opening action, is able to inhibit the high KCl-induced contracture without reducing the increase in $[Ca^{2+}]_i$. The results of the analysis of the concentration-effect curves for pinacidil are summarized in Table 1.

Effects of nicorandil on KCl-induced contractures and increases in $[Ca^{2+}]_i$

Without changing the resting tension in 5 mM KCl-PSS, 10^{-4} M nicorandil reduced $[Ca^{2+}]_i$ below the basal level, an effect which was blocked by 10^{-3} M TBA. When arterial rings were perfused with 30 mM KCl-PSS, nicorandil, like cromakalim and pinacidil, reduced the increase in $[Ca^{2+}]_i$ with a pD_2 value of 4.57 ± 0.19. The KCl-induced contracture was, however, nearly abolished by 10^{-3} M nicorandil. When the maximum effect was assumed to be 100%, its pD_2 value for inhibition of contracture was 4.33 ± 0.18. Thus, although this pD_2 value for inhibition of contracture was slightly but not significantly smaller than that associated with a reduction of the increase in $[Ca^{2+}]_i$, the maximum effect was much larger on the former than on the latter (Table 1). In 45 mM KCl-PSS, nicorandil inhibited the force of contracture in a concentration-dependent manner, whereas the increase in $[Ca^{2+}]_i$ was not changed by nicorandil. Even during the washout of nicorandil, there was no change in the increase in $[Ca^{2+}]_i$, and in 90 mM KCl-PSS, nicorandil inhibited the force of contracture without changing $[Ca^{2+}]_i$ (Table 1). These results indicate that nicorandil also has an effect additional to that of K^+ channel opening in inhibiting the high KCl-induced contracture without reducing the increase in $[Ca^{2+}]_i$. The relaxant potency of nicorandil was dependent on KCl concentration; with lower pD_2 values the greater the presumed muscle depolarization.

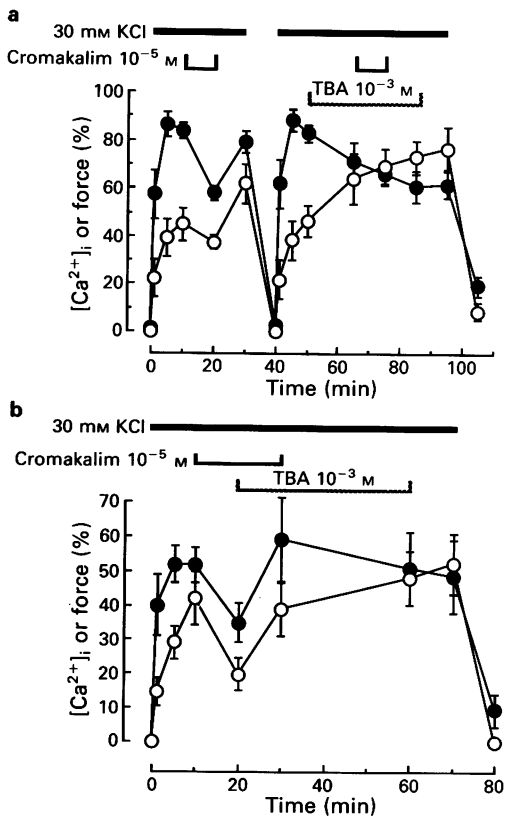

Figure 4 Modification by tetrabutylammonium (TBA, 10^{-3} M) of the effects of cromakalim (10^{-5} M) in canine coronary arteries pefused with 30 mM KCl-PSS. (a) Cromakalim was applied in the presence of TBA ($n = 5$). (b) TBA was applied after the effects of cromakalim had become steady ($n = 6$). (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

Modification by TBA or glibenclamide of the effects of pinacidil and nicorandil

TBA (10^{-3} M) or glibenclamide (10^{-5} M) antagonized the ability of pinacidil (10^{-4} M and 3×10^{-4} M) to reduce $[Ca^{2+}]_i$ following exposure to 30 mM KCl-PSS, but had little effect on pinacidil-induced inhibition of contracture (Figure 6). Glibenclamide (10^{-5} M) also abolished the ability of nicorandil (10^{-4} and 10^{-3} M) to reduce $[Ca^{2+}]_i$ and only slightly attenuated its inhibition of contracture (Figure 7).

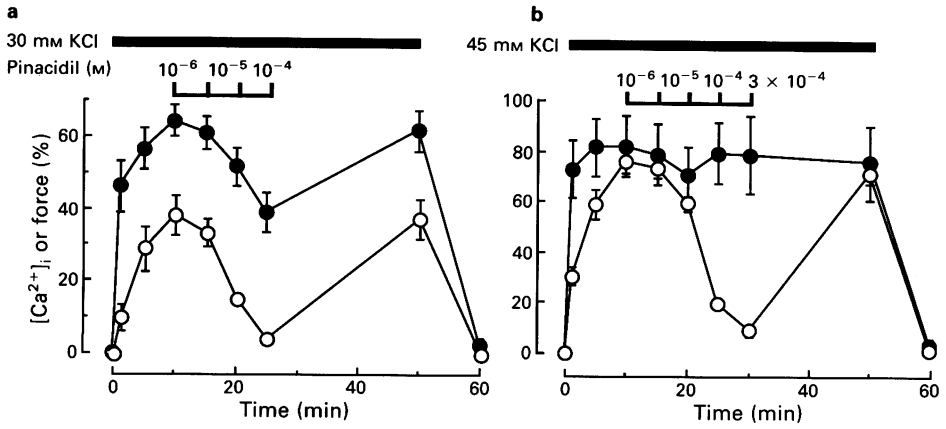

Figure 5 (a) The summarized data of the effects of pinacidil (10^{-6}–3×10^{-4} M) on the force of contracture (○) and increase in $[Ca^{2+}]_i$ (●) in 6 canine coronary arteries depolarized by 30 mM KCl-PSS. (b) Summarized data obtained from 8 muscles perfused with 45 mM KCl.

Effect of verapamil on the increase in $[Ca^{2+}]_i$ induced by high KCl-PSS in the presence of pinacidil or nicorandil

The observation that pinacidil or nicorandil relaxed coronary artery contracted by 45 or 90 mM KCl-PSS without changing the increase in $[Ca^{2+}]_i$ prompted a series of experiments to determine whether the change in $[Ca^{2+}]_i$ was verapamil-sensitive. Figure 8 shows the results of such experiments for pinacidil or nicorandil in arterial rings exposed to 45 mM KCl-PSS. Without changing the increase in $[Ca^{2+}]_i$, pinacidil (10^{-4} M) reduced the force of contracture induced by 45 mM and 90 mM KCl-PSS to 22.4 ± 6.3% ($n = 7$) and 47.9 ± 11.6% ($n = 6$) (force produced by 45 mM or 90 mM KCl-PSS at 10 min = 100%), respectively. In the presence of 45 mM KCl-PSS containing 10^{-4} M pinacidil, addition of 10^{-5} M verapamil abolished the remaining force and the increase in $[Ca^{2+}]_i$ (Figure 8a,b). Nicorandil 10^{-3} M reduced the force of contracture induced by 45 mM or 90 mM KCl-PSS to 15.9 ± 5.8% ($n = 6$) and 24.9 ± 2.2% ($n = 8$), respectively, with no effect on the increase in $[Ca^{2+}]_i$. Exposure to 10^{-5} M verapamil in the presence of 10^{-3} M nicorandil abolished the remaining force and the increase in Ca^{2+} (Figure 8c,d).

Effect of pinacidil or nicorandil on extracellular Ca^{2+}-induced changes in mechanical activity and $[Ca^{2+}]_i$ in KCl-depolarized coronary artery

As previously described, the relaxant potency of both pinacidil and nicorandil was dependent on the KCl concentration (Table 1). To clarify whether the extent of depolarization or the increase in $[Ca^{2+}]_i$ has the major influence on the relaxant effects of piacidil and nicorandil, coronary muscle was at first perfused with Ca^{2+}-free PSS for 10 min and then with Ca-free 90 mM KCl-PSS. Removal of extracellular Ca^{2+} produced a reduction of $[Ca^{2+}]_i$ below the basal level (−27 ± 2%; expressed as a % of the increase following 10 min exposure to 90 mM KCl-PSS), whereas the force was not changed. After removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free PSS, exposure of the muscle to 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ (−31 ± 2%) or any change in force. During exposure to 90 mM KCl-PSS, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 0.03 to 15 mM with a consequent increase in both $[Ca^{2+}]_i$ and force (Figure 9, left panel). The results of computer fitting of the curve to the logistic equation (2) are summarized in Table 2. The maximum is the difference between values at Ca^{2+}-free and 15 mM $[Ca^{2+}]_o$. The pD_2 values for $[Ca^{2+}]_o$ of 3.05 ± 0.23 indicate the apparent K_D values of $[Ca^{2+}]_o$ for the binding constant at voltage-dependent Ca^{2+} channels presumed to be opened by depolarization. The relationship between $[Ca^{2+}]_o$ and force of contracture was also analysed and is summarized in Table 2. The maximum force produced by 90 mM KCl was 239 ± 41%, the pD_2 value for $[Ca^{2+}]_o$ was 2.62 ± 0.14, and the slope parameter was 1.22 ± 0.12. The difference between pD_2 values for the influence of $[Ca^{2+}]_o$ on the increase in $[Ca^{2+}]_i$ and the increase in force are a consequence of the steepness of $[Ca^{2+}]_i$-force relationship (Figure 10, control).

When pinacidil (10^{-4} M) or nicorandil (3×10^{-4} M) was applied to coronary arterial muscle perfused with Ca^{2+}-free 90 mM KCl-PSS, $[Ca^{2+}]_i$ was not changed. Although increases in $[Ca^{2+}]_o$ produced the same increases in $[Ca^{2+}]_i$ in the presence of pinacidil or nicorandil as in control, the force produced by the increased $[Ca^{2+}]_i$ was much smaller than that of control and the threshold values for $[Ca^{2+}]_o$ and

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

	Increase in force			*Increase in $[Ca^{2+}]_i$*		
	Maximum response (%)	pD_2	p	*Maximum response* (%)	pD_2	p
Control	239 ± 41	2.62 ± 0.14	1.22 ± 0.12	160 ± 23	3.05 ± 0.23	0.96 ± 0.14
Pinacidil 10^{-4} M	82 ± 29*	2.05 ± 0.15*	1.39 ± 0.17	146 ± 24	2.86 ± 0.18	0.93 ± 0.08
Washout	217 ± 41	2.71 ± 0.07	1.30 ± 0.09	150 ± 20	3.17 ± 0.28	0.96 ± 0.15
Control	309 ± 85	2.53 ± 0.06	1.39 ± 0.07	174 ± 25	2.99 ± 0.07	0.88 ± 0.11
Nicorandil 3×10^{-4} M	138 ± 24*	1.97 ± 0.13*	1.29 ± 0.07	163 ± 19	2.93 ± 0.14	0.90 ± 0.11
Washout	285 ± 63	2.59 ± 0.06	1.37 ± 0.23	160 ± 30	3.11 ± 0.10	0.93 ± 0.09

Increase in $[Ca^{2+}]_i$ was calculated from the difference between the value obtained in Ca^{2+}-free 90 mM KCl-PSS and the maximum value of $[Ca^{2+}]_i$ in each experiment (usually at 15 mM $[Ca^{2+}]_o$). * $P < 0.05$ compared with control values; p = slope parameter.

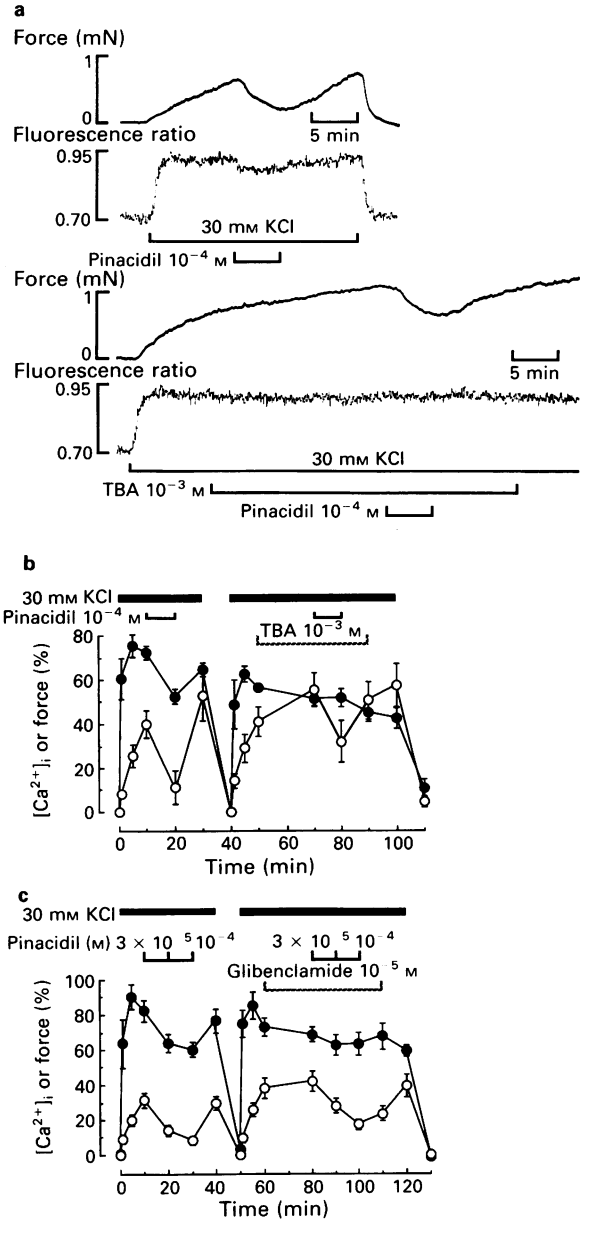

Figure 6 (a) Modification by tetrabutylammonium (TBA, 10^{-3} M) of the effects of pinacidil (10^{-4} M) in a single experiment in canine coronary artery perfused with 30 mM KCl-PSS. (b) Summarized data obtained from 4 muscles. (c) Summarized data of the influence of glibenclamide (10^{-5} M) on the effects of pinacidil (3×10^{-5}–10^{-4} M) in 6 canine coronary arterial muscles perfused with 30 mM KCl-PSS. (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

$[Ca^{2+}]_i$ required to generate force were increased (Figure 9). Furthermore, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly reduced in the presence of pinacidil or nicorandil. The inhibitory effects of pinacidil or nicorandil were reversible (Figure 9 and Table 2).

Effects of pinacidil or nicorandil on the relationship between $[Ca^{2+}]_i$ and force in KCl-depolarized canine coronary artery

Figure 10 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and presence of pinacidil (10^{-4} M) or nicorandil (3×10^{-4} M) in depolarized canine coronary artery. The curve in the presence of pinacidil and nicorandil was different from that of the control and was shifted to the right and downward. These results obtained from the analysis of $[Ca^{2+}]_i$-tension relationship in the presence of pinacidil or nicorandil suggest

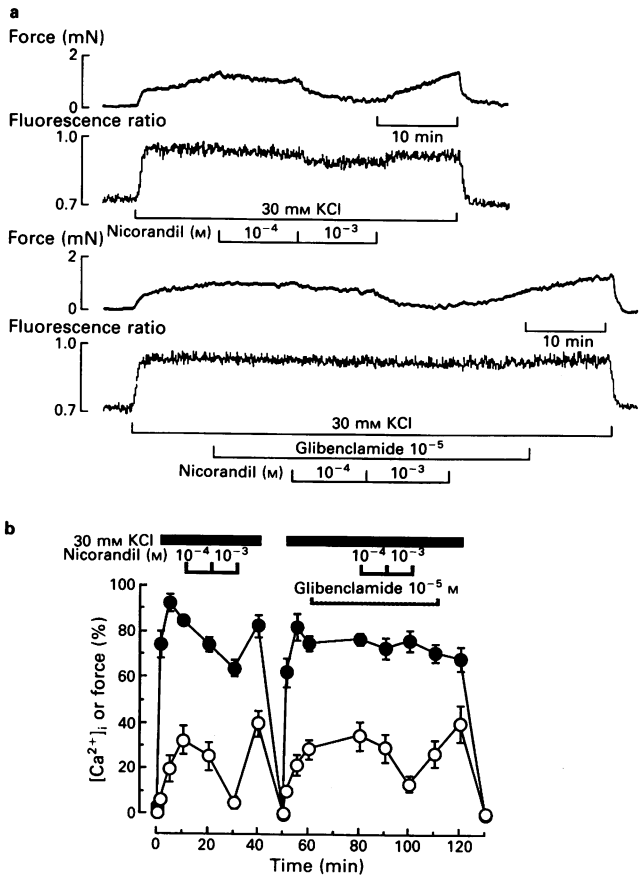

Figure 7 Modification by glibenclamide (10^{-5} M) of the effects of nicorandil (10^{-4}–10^{-3} M) in a single experiment in canine coronary artery perfused with 30 mM KCl-PSS. (b) Summarized data obtained from 6 muscles. (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

that both agents reduce the Ca^{2+}-sensitivity of the contractile proteins.

Discussion

In the present study, presumed depolarization of canine coronary artery by 30–90 mM KCl-PSS produced a concentration-dependent increase in $[Ca^{2+}]_i$ and force. A reduction of $[Ca^{2+}]_i$ below the basal level did not affect the tone while the threshold increase in $[Ca^{2+}]_i$ necessary to generate tension was about 10% of basal level. The observed increase in $[Ca^{2+}]_i$ and tension was verapamil-sensitive and thus was almost certainly associated with an increase in Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Godfraind *et al.*, 1986; Yanagisawa *et al.*, 1989).

When tissues were exposed to PSS containing KCl ($\leqslant$30 mM), the three K^+ channel openers, cromakalim, pinacidil and nicorandil, each reduced the increase in $[Ca^{2+}]_i$, whereas when the KCl concentration was 45 mM or greater the increase in $[Ca^{2+}]_i$ was unaffected. Thus, the effects of K^+ channel openers in reducing the increase in $[Ca^{2+}]_i$ may only be achieved when the resultant membrane potential associated with the presence of a raised KCl concentration is less negative than the equilibrium potential for K^+ (Yanagisawa & Taira, 1980; Furukawa *et al.*, 1981), and when the K^+ channel openers can hyperpolarize the membrane potential to reduce the opening probability of voltage-dependent Ca^{2+} channels (Weston, 1987). In normal (5 mM KCl) PSS, cromakalim, pinacidil and nicorandil reduced $[Ca^{2+}]_i$ below the basal level and this effect of pinacidil and nicorandil was blocked by TBA. This suggests that certain Ca^{2+} channels are open in normal PSS and that a presumed hyperpolarization of the plasma

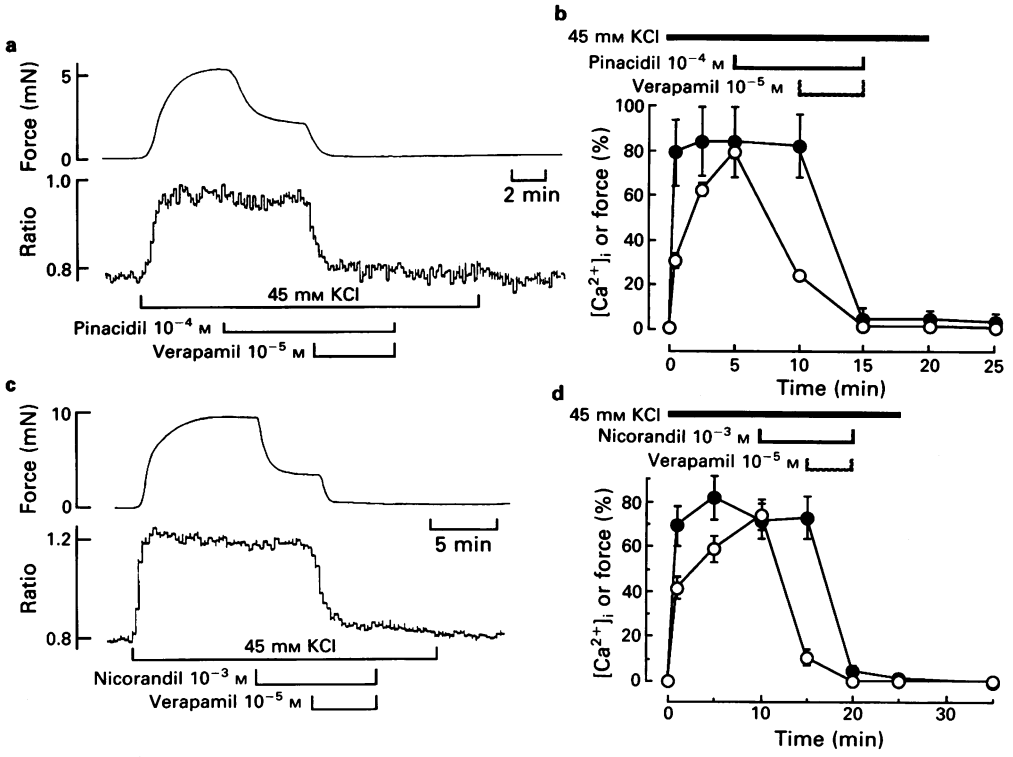

Figure 8 Effects of 10^{-4} M pinacidil (a,b) and 10^{-3} M nicorandil (c,d) and 10^{-5} M verapamil on changes in the increase in $[Ca^{2+}]_i$ (●) and force of contracture (○) produced by 45 mM KCl-PSS. Summarized data obtained from 7 muscles for pinacidil (b) and from 6 muscles for nicorandil (d) are also shown.

membrane by a K^+ channel opener is capable of closing this Ca^{2+}-entry pathway.

Although the vasodilator effect of K^+ channel openers is blocked by several nonselective K^+ channel blockers such as procaine, 4-aminopyridine and tetraethylammonium (TEA) (Wilson *et al.*, 1988), the identity of the K^+ channels involved is uncertain. Recently, sulphonylureas like glibenclamide have been shown to block cromakalim-induced changes in cardiac (Fosset *et al.*, 1988; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) and vascular smooth muscle (Buckingham *et al.*, 1989; Quast & Cook, 1989; Winquist *et al.*, 1989; Eltze, 1989), suggesting a role for ATP-sensitive K^+ channels (Standen *et al.*, 1989). In the present study glibenclamide blocked the effects of the three K^+ channel openers in reducing $[Ca^{2+}]_i$, although rather high concentrations ($\geqslant 10^{-6}$ M) were needed. The finding that glibenclamide affected neither $[Ca^{2+}]_i$ nor force

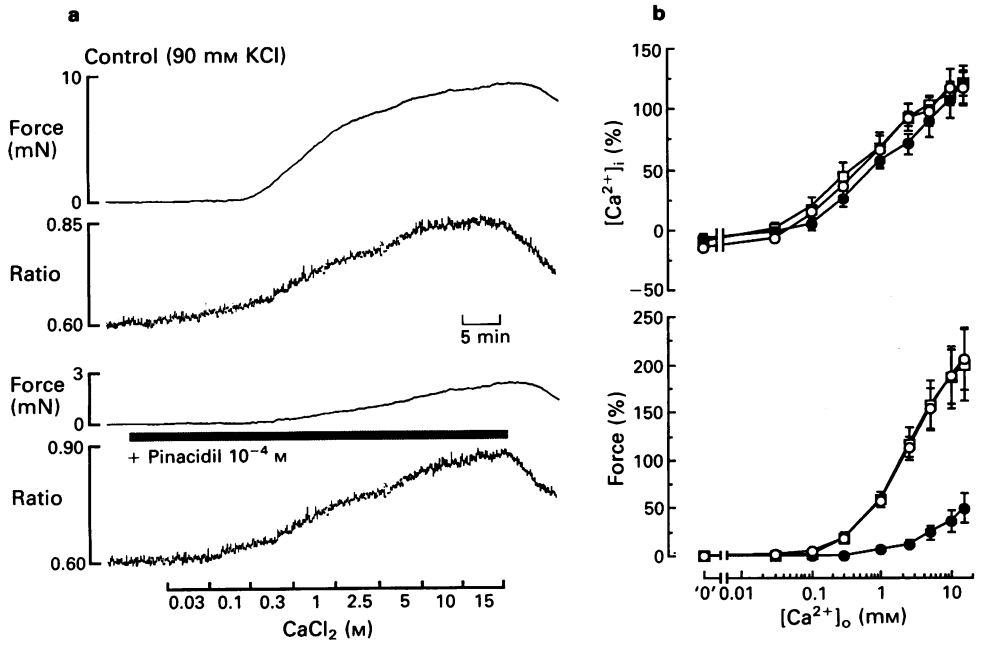

Figure 9 (a) Changes in $[Ca^{2+}]_i$ and force produced by increasing $[Ca^{2+}]_o$ in a canine coronary arterial muscle depolarized with 90 mM KCl-PSS and the effects of pinacidil on these changes. (b) Summarized data obtained from 6 experiments. Control (○), in the presence of 10^{-4} M pinacidil (●), washout (□).

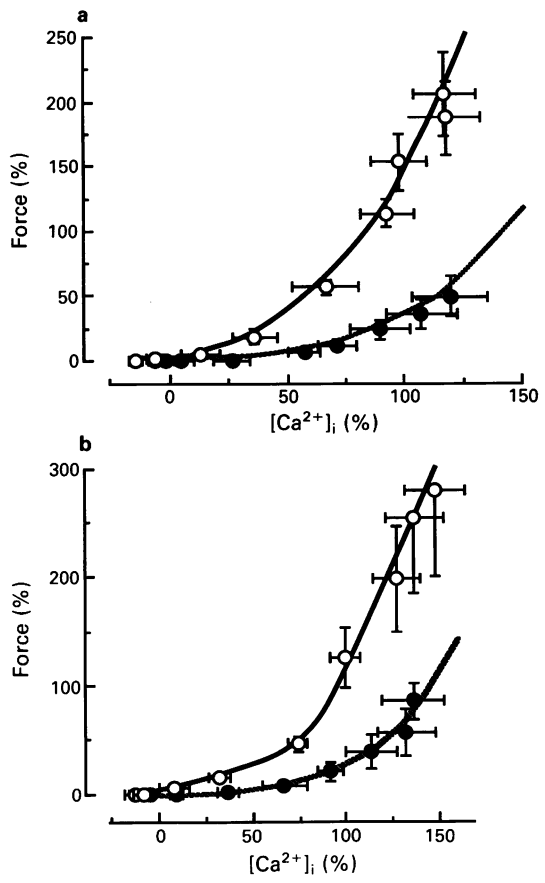

Figure 10 Relationship between $[Ca^{2+}]_i$ and force in the absence (control, ○), and the presence of 10^{-4} M pinacidil (a, ●) or 3×10^{-4} M nicorandil (b, ●). Vertical and horizontal bars are s.e.mean. The curves were fitted by eye.

in both normal and high KCl-PSS indicates that glibenclamide-sensitive K^+ channels are closed under these conditions.

In 30 mM KCl-PSS in which the coronary artery generated force, all three K^+ channel openers reduced both the force of contraction and the increase in $[Ca^{2+}]_i$. However, cromakalim reduced tension development by only 45%, whereas pinacidil and nicorandil abolished the force of contracture. The maximum % reduction of the increase in $[Ca^{2+}]_i$ by the three K^+ channel openers was in the range 36–45%. Although the reduction of the increase in $[Ca^{2+}]_i$ by the three K^+ channel openers and the relaxant effect of cromakalim were abolished by 10^{-3} M TBA or 10^{-5} M glibenclamide, these K^+ channel blockers only partly attenuated the relaxant effects of pinacidil and nicorandil. In 45 and 90 mM KCl-PSS, cromakalim failed to affect the increase in $[Ca^{2+}]_i$ and force as has been shown in rat blood vessels (Weir & Weston, 1986b), whereas pinacidil and nicorandil relaxed the tissues without reducing the increase in $[Ca^{2+}]_i$. However, this increased $[Ca^{2+}]_i$ was reduced by 10^{-5} M verapamil to basal levels (Figure 8), suggesting that it was due to an influx of extracellular Ca^{2+}.

Nicorandil contains a nitroxy moiety within its structure and is known to increase intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) by activating soluble guanylate cyclase in various vascular smooth muscle tissues (Endoh & Taira, 1983; Holzmann, 1983) and in cardiac muscle (Yanagisawa *et al.*, 1988). The resultant activation of cyclic GMP-dependent protein kinase is thought to be responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). Thus, it is likely that nicorandil partly relaxes smooth muscle cells without reducing the increase in $[Ca^{2+}]_i$ following Ca^{2+} influx by depolarization, via mechanisms like those of nitroglycerin (Yanagisawa *et al.*, 1989). Unlike nicorandil, pinacidil does not change cyclic GMP levels in either vascular smooth muscle (Kauffman *et al.*, 1986) or in cardiac muscle (Yanagisawa *et al.*, 1988). Thus, pinacidil exerts some of its relaxing effects by a mechanism independent of the involvement of cyclic nucleotides but which remains to be determined.

The relaxant potencies of pinacidil and nicorandil in PSS containing KCl in concentrations of 45 mM or greater were reduced at the upper end of the KCl concentration range (45–90 mM) (Table 1). This phenomenon was due to the changed relationship between $[Ca^{2+}]_i$ and force observed in the presence of pinacidil or nicorandil (Figure 10). A similar rightward shift of the $[Ca^{2+}]_i$-tension relationship by cyclic GMP has previously been obtained (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The mechanism involved is uncertain, but in the case of nicorandil the effects of an increase in $[Ca^{2+}]_i$ in generating force may have been counteracted by a cyclic GMP-mediated inhibition of myosin light chain phosphorylation.

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Inhibitory role of endothelium-derived relaxing factor in rat and human pulmonary arteries

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1 The inhibitory role of endothelium-derived relaxing factor was studied in both rat and human pulmonary arteries *in vitro* by inhibiting its synthesis with the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA).

2 In rat pulmonary arteries, L-NMMA pretreatment (10–300 μM) dose-dependently inhibited acetylcholine-induced relaxation (which is endothelium-dependent). N^G-monomethyl-D-arginine (D-NMMA, 100 μM) was without effect. L-Arginine, but not D-arginine, dose-dependently reversed this inhibition. L-NMMA had no effect on relaxation induced by sodium nitroprusside.

3 In human small pulmonary arteries L-NMMA (100 μM) pretreatment similarly inhibited the acetylcholine-induced relaxation but had no effect on the sodium nitroprusside-induced relaxation.

4 In both rat and human pulmonary arteries, L-NMMA, but not D-NMMA, always caused contraction of preconstricted tissues whereas it had no effect on baseline tone. In the rat this contraction was completely prevented by prior treatment with L-arginine.

5 L-NMMA (100 μM) pretreatment mimicked the effect of endothelium removal on phenylephrine-induced vasoconstriction, both resulting in an increase in tension development at each concentration of phenylephrine. This enhancement was greatest at low concentrations of phenylephrine but was still present even at the highest concentrations. Pretreatment with L-NMMA (100 μM) also significantly increased the responses to single doses of phenylephrine.

6 These results suggest that endothelium-derived relaxing factor from endothelial cells both mediates the relaxation response to acetylcholine and also acts as a physiological brake against vasoconstriction in pulmonary vessels.

Introduction

Since Furchgott & Zawadzki (1980) first demonstrated that vascular relaxation by acetylcholine (ACh) results from the release of relaxant factor from the endothelium, the identity and physiology of this endothelium-derived relaxing factor (EDRF) has been the target for much research. EDRF has now been identified as nitric oxide (NO) (Palmer *et al.*, 1987), and the source of NO has recently been elucidated. Infusions of L-arginine through a column of porcine aortic endothelial cells result in an increased bradykinin-induced release of NO only in those cells deprived of L-arginine for 24 h. This effect is stereospecific as the D-isomer is without effect (Palmer *et al.*, 1988a). In bovine aortic endothelial cells incubation with L-[guanidino-$^{15}N_2$] arginine results in the formation of ^{15}NO, $^{15}NO_2$, and $^{15}NO_3$, indicating that the terminal guanidino nitrogens of L-arginine are the physiological precursors of NO (Schmidt *et al.*, 1988).

The L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA) inhibits NO release and also inhibits endothelium-dependent relaxation *in vitro* (Rees *et al.*, 1989a). *In vivo* it causes marked hypertension in anaesthetized rabbits (Rees *et al.*, 1988b); an effect reversed by L-arginine, indicating that L-NMMA restricts the availability of L-arginine for the synthesis of NO. In rabbit aortic rings *in vitro*, L-NMMA inhibits endothelium-dependent relaxation and this effect can be reversed by L-arginine but not D-arginine, indicating stereospecificity of NO formation from L-arginine (Palmer *et al.*, 1988b). However, it has been demonstrated that L-arginine, N-α-benzoyl-L-arginine, D-arginine and L-histidine produce endothelium-independent relaxation, and that L-arginine *per se* cannot be the immediate precursor of NO (Thomas & Ramwell, 1988; Thomas *et al.*, 1989b).

Endothelial-dependent relaxation has been demonstrated in pulmonary arteries of several species, including dogs (Chand & Altura, 1981) and man (Greenberg *et al.*, 1987). We have studied the effect of L-NMMA on the endothelium-dependent vasodilatation induced by ACh in rat and human pulmonary arteries *in vitro*. We have also used L-NMMA to determine whether the release of EDRF has an effect on vasoconstriction in these vessels.

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Methods

Tissue preparation

The heart and major blood vessels of 250 g male Wistar rats were placed into Krebs-Henseleit (Krebs) solution containing the following (in mM): NaCl 118, KCl 5.9, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 6H_2O$ 2.5, $NaH_2PO_4 \cdot H_2O$ 1.2, glucose 5.6, $NaHCO_3$ 25.5. The right and left pulmonary arteries were then dissected free of surrounding tissue and each cut into two rings, approximately 2 mm in length. Care was taken not to touch or damage the intimal surface.

Human small pulmonary arteries (0.7–1.5 mm internal diameter) were obtained from surgical lobectomy specimens from patients with lung carcinoma. Tissues were always used within 12 h of surgery. Pulmonary arteries were identified at the cut surface of the lung and then traced peripherally. The specimens were placed in Krebs solution at 4°C and oxygenated with 95% O_2 and 5% CO_2. Following removal of the surrounding connective tissue, the vessels were cut into rings (2–3 mm in length).

Experimental procedure

The rings were mounted over two parallel wires, one being fixed and the other attached to a force-displacement transducer (FT.03 Grass, Quincy, U.S.A.). Measurements of tension were recorded on a polygraph (Grass model 7). Resting tensions of 0.5 g (rat) and 0.5–0.7 g (human) were applied to the vessels, in order to give optimal response characteristics. After the vessels had been mounted, the wires were lowered into a 2 ml organ bath filled with Krebs solution (37°C) and bubbled

with 95% O_2 and 5% CO_2. The vessels were then left to stabilize for one hour and were washed with fresh Krebs every 15 min. Endothelium-dependent and endothelium-independent relaxation were studied by preconstriction with phenylephrine (PE, 1 μM) in rat or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 30 μM) in human vessels, and then a cumulative concentration-response curve to ACh (1 nM–10 μM), which acts via the endothelium, or to sodium nitroprusside (1 nM–10 μM), which acts directly on vascular smooth muscle, constructed. Contractile responses were studied in rat vessels either by the construction of a cumulative concentration-response curve to PE (1 nM–10 μM) or by exposing the vessels to a single concentration of PE. In experiments involving single concentrations of PE, a single concentration of PE was applied to the vessels until a consistent constrictor response was obtained. Following a washout/recovery period, the tissues were then exposed to a similar concentration of PE, some tissues having been pretreated with L-NMMA. In experiments involving endothelium-denuded vessels, the endothelium was removed by lightly rubbing the vessel lumen with a small steel probe and removal confirmed by the loss of the relaxation response to ACh in preconstricted tissues. In all experiments involving pretreatment, the drug(s) was added 10 min before the experiment.

The effects of L-NMMA, D-NMMA, L-arginine and D-arginine on both relaxant and constrictor responses were investigated. The interactions of L-histidine, N-α-benzoyl-L-arginine and N-α-L-histidine with L-NMMA were also investigated.

Drugs and chemicals

Drugs were obtained from the following sources: phenylephrine, acetylcholine, sodium nitroprusside, L-histidine, L-arginine, D-arginine, N-α-benzoyl-L-arginine, and N-α-benzoyl-L-histidine (Sigma, Poole, U.K.). Propranolol hydrochloride (ICI, Cheshire, U.K.). $PGF_{2\alpha}$ Upjohn Ltd, Crawley, U.K.). N^G-monomethyl-L-arginine and N^G-monomethyl-D-arginine were kindly supplied by Dr S. Moncada (Wellcome, Beckenham, U.K.).

Statistics

The results were statistically analysed by either Student's paired t test (probability values <0.05 being considered significant), or a one-way analysis of variance followed by modified t-statistics using the Bonferroni correction for multiple comparisons.

Results

Relaxation studies

Rat pulmonary artery ACh (1 nM–30 μM) caused a dose-dependent relaxation of the rat isolated pulmonary artery rings (mean $EC_{50} = 97 \pm 12$ nM). This relaxation was unaffected by pretreatment with L-arginine (100 μM), but was inhibited dose-dependently by pretreatment with L-NMMA. The tension generated by 1 μM PE together with 3 μM L-NMMA was 400 ± 30 mg. ACh reduced this tension to 43 ± 7 mg at the highest concentration of ACh tested (10 μM). This is compared to control values of 375 ± 48 mg (1 μM PE alone) versus 62 ± 20 mg (10 μM ACh). By increasing the concentration of L-NMMA, the lowering of tension by 10 μM ACh was reduced such that at 10 μM L-NMMA, ACh reduced the tension in the tissues from 342 ± 30 mg to 120 ± 10 mg; at 30 μM L-NMMA, ACh reduced the tension from 366 ± 11 mg to 125 ± 21 mg; at 100 μM L-NMMA, from 380 ± 40 mg to 178 ± 9 mg and at 300 μM L-NMMA from 430 ± 35 mg to 215 ± 11 mg (Figure 1). Concentrations of L-NMMA up to 1 mM did not increase the inhibition of the ACh-induced relaxation beyond that seen with 100 μM and 300 μM. By contrast, D-NMMA had no effect. This inhibition was not overcome by ACh, but was dose-dependently reversed by L-arginine pretreatment. L-Arginine 10 μM had no significant effect on the reduction in tension by 10 μM ACh (final tension 193 ± 10 mg). However, both 100 μM and 1 mM L-arginine significantly reduced the effect of 100 μM L-NMMA, final tensions in the tissues being 138 ± 21 mg and 73 ± 8 mg respectively (Figure 2). D-Arginine was without effect. L-NMMA did not significantly affect the sodium nitroprusside-induced relaxation (mean EC_{50} for untreated vessels = 44 ± 5 nM compared to mean EC_{50} for treated

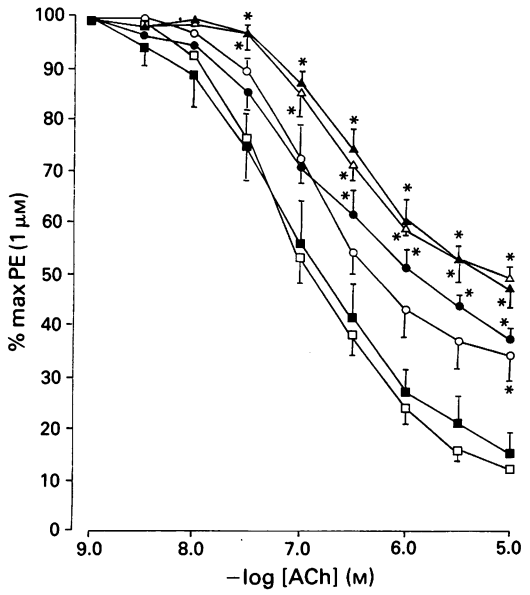

Figure 1 Concentration-response curves for the relaxation (expressed as a % of the contraction induced by 1 μM phenylephrine, PE) in rat pulmonary artery induced by acetylcholine (ACh, 1 nM–30 μM) in the absence (■) and presence of 3 μM (□), 10 μM (●), 30 μM (○), 100 μM (▲) and 300 μM (△) N^G-monomethyl-L-arginine. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean. * $P < 0.01$ compared to control values ($n = 6$).

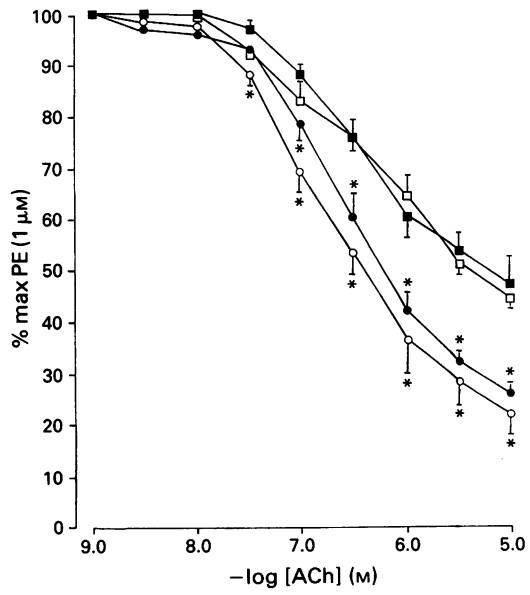

Figure 2 Reversal by L-arginine of the inhibition by 100 μM N^G-monomethyl-L-arginine (L-NMMA) of the acetylcholine (ACh)-dependent relaxation (expressed as a % of the contraction induced by 1 μM phenylephrine, PE) in rat pulmonary artery at 10 μM (□), 100 μM (●), and 1 mM (○). Relaxation to ACh in the presence of 100 μM L-NMMA alone (■). * $P < 0.05$ as compared to control values. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean.

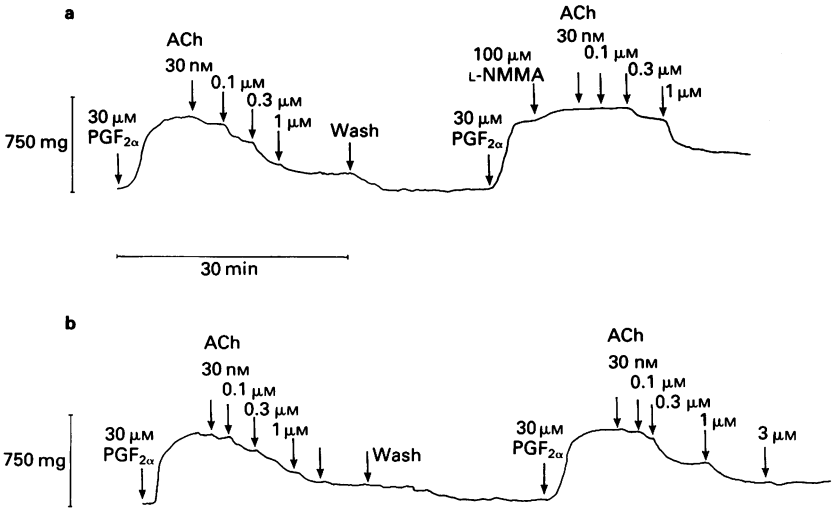

Figure 3 Traces showning the effect of N^G-monomethyl-L-arginine (L-NMMA, 100 μM) pretreatment on the acetylcholine-induced relaxation in human pulmonary arteries (a) preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), compared to a time-matched control vessel (b).

vessels = 42 ± 7 nM). L-NMMA caused a substantial contraction of preconstricted tissues at all concentrations used for pretreatment (>100% increase above baseline in some tissues), whereas D-NMMA (100 μM) was without effect. L-Arginine (100 μM) pretreatment prevented this contraction to L-NMMA completely, and exposure of vessels contracted by L-NMMA to L-arginine (100 μM) resulted in complete reversal of the contraction. D-Arginine (100 μM) was ineffective. By contrast L-NMMA had no effect on baseline tone at any concentration tested. Neither L-arginine nor L-histidine induced a relaxation of preconstricted tissues even at concentrations as high as 1 mM. Their benzoyl derivatives caused substantial relaxation (40% and 30% respectively at 0.3 mM) in both endothelium-intact and denuded vessel rings. Unlike L-arginine, both N-α-benzoyl-L-arginine and N-α-benzoyl-L-histidine caused relaxations independent of the presence of an L-NMMA contraction. L-Histidine was without effect in endothelium-intact and denuded vessels, and did not reverse contractions to L-NMMA. This indicates that both compounds cause a relaxation by mechanisms unrelated to the action of L-arginine and L-NMMA.

Endothelium removal completely abolished both the relaxant response to ACh and the contraction by L-NMMA in preconstricted tissues.

Human small pulmonary artery ACh (10 nM–10 μM) caused a dose-dependent relaxation of human small pulmonary artery rings; EC_{50} = 0.28 μM (Figure 3). Pretreatment with L-NMMA (100 μM) caused a contraction of all preconstricted tissues (mean increase = 46 ± 11%, n = 5), whereas baseline tensions were unaffected. Figure 4 shows the inhibition by 100 μM L-NMMA of the acetylcholine-induced relaxation (n = 3). D-NMMA (100 μM) failed either to inhibit the acetylcholine-induced relaxation or to cause a further contraction in preconstricted vessel rings, demonstrating the stereoselectivity of the L-NMMA effect on human small pulmonary arteries. As in the rat pulmonary artery, the relaxation to sodium nitroprusside was unaffected by L-NMMA.

Contraction studies

In all contractile studies involving PE, rat pulmonary vessels were pretreated with propranolol (10 μM) to block β-adrenoceptor-mediated vasodilatation.

Pretreatment of the tissues with L-NMMA (100 μM) significantly decreased the EC_{50} of PE (85 ± 23 nM vs 24 ± 5 nM after L-NMMA pretreatment), and resulted in a significant increase in maximum tension generation (355 ± 35 mg vs 425 ± 25 mg after L-NMMA, n = 5, $P < 0.05$) and a significant increase in tension generation at the lowest effective PE concentration (30 ± 10 mg vs 130 ± 40 mg, $P < 0.05$ after treatment). The increased contraction was greatest at low concentrations but was maintained at higher PE concentrations. Endothelium removal mimicked this effect of L-NMMA. The tensions generated by 55 mM KCl did not significantly differ between control and L-NMMA-pretreated tissues (Figure 5).

Pretreatment with L-NMMA (100 μM) significantly increased the response to a single dose of PE at all concentrations tested. When expressed as a % of the initial consistent response to PE, the potentiation was greater at the lowest PE concentration tested with an increase of 212 ± 33% at 30 nM compared to 143 ± 9% at 1 μM PE. Pretreatment with L-arginine (100 μM) had no significant effect on the contractile response.

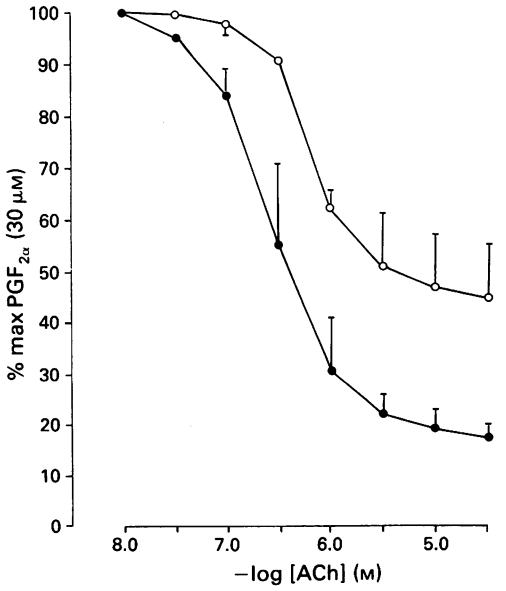

Figure 4 Concentration-response curves for the relaxation induced by acetylcholine (ACh, expressed as a % of the contraction induced by 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in human pulmonary artery rings; control rings (●), and rings pretreated with 100 μM N^G-monomethyl-L-arginine (○) are shown. Each point represents the mean (n = 3) and vertical lines show s.e.mean.

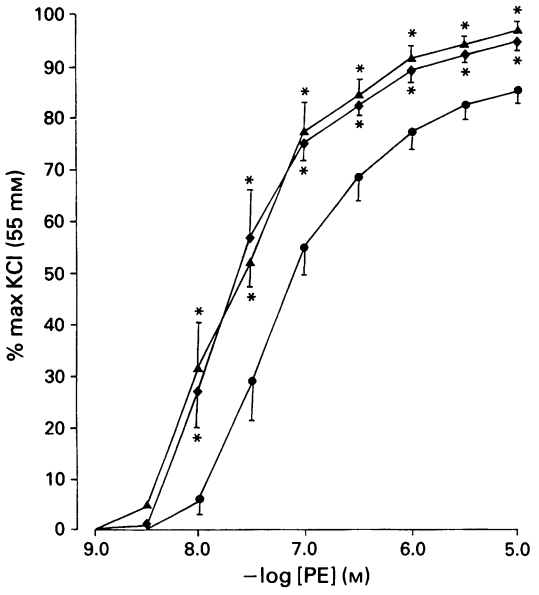

Figure 5 Concentration-response curves for the contraction induced by phenylephrine (PE) in rat pulmonary artery rings; control vessels (●), vessels pretreated with 100 μM N^G-monomethyl-L-arginine (◆), and vessels denuded of endothelium (▲). * $P < 0.05$, as compared to control values. Mean observations are shown ($n = 6$) and vertical lines indicate s.e.mean. Tensions generated by KCl did not differ significantly between the groups.

Discussion

We have demonstrated that inhibition of EDRF synthesis with L-NMMA reduces the endothelium-dependent relaxation in rat and human pulmonary arteries, indicating that EDRF is involved in this response. We have also shown that EDRF modulates the contractility of pulmonary arteries. The inhibition of ACh relaxation by L-NMMA but not D-NMMA, and the reversal of this inhibition by L-arginine but not D-arginine provides evidence that EDRF in rat and human pulmonary arteries is likely to be EDRF derived from L-arginine. The lack of effect of L-NMMA on sodium nitroprusside-induced relaxation in both species demonstrates that the site of action of L-NMMA is the endothelium.

It is of interest that L-NMMA did not completely abolish the ACh-induced relaxation in either rat or human vessels. There are several possible explanations for this. Perhaps relaxant factors other than EDRF are released from pulmonary endothelial cells, or EDRF could be produced from a source unaffected by L-NMMA. The most likely explanation, however, is that L-NMMA is not a highly potent inhibitor of EDRF production from L-arginine.

It has been suggested that L-arginine does not uniquely elicit endothelium-dependent relaxation and that the basic amino acids such as arginine, lysine, glutamine and histidine induce a non-specific relaxation at high concentrations (Thomas *et al.*, 1989b). In our study, only L-arginine reversed the contraction caused by L-NMMA, whereas histidine and its benzoyl derivatives were without effect. Furthermore the effect of arginine was stereoselective since D-arginine was without effect. Similarly, only L-arginine was effective in reversing the inhibitory effect of L-NMMA on ACh-induced relaxation. Unlike with L-arginine, increasing the concentration of ACh failed to overcome the inhibition of endothelium-dependent relaxation by L-NMMA. All of our conclusions rely on the assumption that L-NMMA does indeed specifically inhibit EDRF production in rat and human pulmonary arteries. In the rabbit aorta, the release of NO by ACh infusions (detected by chemiluminescence) is inhibited dose-dependently by 3–300 μM L-NMMA (IC_{50} = 15 μM) (Rees *et al.*, 1989a). Also, the release of NO from bradykinin-stimulated porcine aortic endothelial cells is inhibited by 0.3–10 μM L-NMMA (IC_{50} = 3 μM) (Palmer *et al.*, 1988b). We have shown that L-NMMA, but not D-NMMA, only caused a contraction in tissues which were preconstricted, and never caused a contraction at baseline tensions in any experiment in either rat or human pulmonary arteries. This is consistent with the observation that increments in flow through arteries *in vitro* cause vasodilatation due to the release of EDRF (Rubanyi *et al.*, 1986), in that when the tissue is at baseline tension *in vitro*, no EDRF is released. Once tension is increased with the α-adrenoceptor agonist PE, EDRF is released and thus L-NMMA causes a further contraction by removing the EDRF relaxant brake. It has been suggested that L-NMMA is a non-specific inhibitor of vascular relaxation (Thomas *et al.*, 1989a). However, this is based on the observation that L-NMMA reversed a 30–50% vasodilatation of a number of unrelated compounds. Because none of the tissues were at baseline tension when L-NMMA was applied, the shear stress resulting from the remaining tension would itself generate EDRF release and hence L-NMMA would cause contraction by removing the EDRF brake, as in our preparations.

L-NMMA may also be useful in demonstrating the nature of EDRF release from pulmonary arteries *in vitro*. L-NMMA pretreatment did not alter the lowest effective concentration of PE as expected, because EDRF is only released (and thus removed) when tension is generated by the agonist. At all concentrations of PE tested, the tension generated was significantly increased by L-NMMA pretreatment. This increase in tension was greater at low concentrations but was seen over the whole range of the dose-response curve. This indicates that either EDRF release rises with increased tension, or that a fixed amount of EDRF is released which is more effective as a functional antagonist at lower tensions. Single dose applications of PE support this finding in that the potentiation (as a % of the original contraction) is greater at lower PE concentrations (and hence low tensions). At high PE concentrations the potentiation is less but significantly and consistently maintained. Since stretch-activated ion channels exist in porcine endothelial cells as mechanotransducers (Lansman *et al.*, 1987), it seems more probable that EDRF release rises with increases in tension. Both L-NMMA pretreatment and endothelium removal significantly increased the response attained at any given PE concentration. Responses obtained after endothelium removal were identical to those seen after L-NMMA pretreatment, indicating that EDRF is probably the only relaxing factor released under these conditions from endothelial cells of rat pulmonary artery.

Physiologically we have demonstrated that EDRF can act as a brake which is most effective at lower tensions and, perhaps, provides a protective mechanism against inappropriate vasoconstriction. It would be interesting to investigate the efficacy of this brake in pulmonary vascular disease. Any impairment in EDRF generation would predispose to excessive pulmonary vasoconstriction and thus pulmonary hypertension.

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Actions of 5-HT_1 ligands on excitatory synaptic transmission in the hippocampus of alert rats

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1 The effects of 5-hydroxytryptamine$_1$ (5-HT_1) ligands on excitatory synaptic transmission were examined in the stratum radiatum of the CA1 region of the dorsal hippocampus of alert, gently restrained, rats.

2 5-HT produced a dose-dependent reduction in the amplitude of the electrically evoked population excitatory postsynaptic potential (e.p.s.p.) when injected directly into the hippocampus via a cannula (dose producing 50% maximum inhibition, ED_{50} = 0.46 μg).

3 Direct intrahippocampal (i.h.) application of buspirone (ED_{50} = 0.29 μg), gepirone (1 μg), ipsapirone (1 μg), BMY 7378 (0.1 μg) and 5-carboxamidotryptamine (5-CT, 0.02 μg) mimicked the inhibitory effect of 5-HT.

4 Systemic injection of buspirone (ED_{50} = 0.88 mg kg^{-1}, i.p.), BMY 7378 (0.01 mg kg^{-1}, i.p.) and RU 24969 (1 mg kg^{-1}, s.c.) also had an inhibitory effect on the amplitude of the e.p.s.p.

5 Injection of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 2 μg) and spiroxatrine (1 μg) i.h. alone had no effect on the e.p.s.p. amplitude but prevented the inhibitory effect of 5-HT.

6 Systemic injection (i.p.) of methysergide (5 mg kg^{-1}) and spiroxatrine (1 mg kg^{-1}) antagonized the inhibitory effect of buspirone whereas pretreatment with ketanserin (1 mg kg^{-1}), trifluoperazine (1 mg kg^{-1}) and idazoxan (1 mg kg^{-1}) had no effect on the response to buspirone.

7 It is concluded that hippocampal synaptic transmission is highly sensitive to the agonist and antagonist properties of 5-HT_1 ligands in the alert rat.

Introduction

The discovery of the diversity of 5-hydroxytryptamine (5-HT) receptors in the brain has been largely due to the development of a number of new compounds that are relatively selective for the different receptor subtypes (Peroutka, 1988). In the case of 5-HT_1 recognition sites much of the early research was based on ligand binding studies and it is only recently that attention has been given to the functional significance of these sites (Hoyer, 1988).

Neurophysiological studies of the relatively selective 5-HT_{1A} ligands 8-hydroxy-2-(di-n-propylamino) tetralin (8-OP-DPAT), buspirone and its analogues such as gepirone and ipsapirone show that these compounds can mimic or antagonize the inhibitory effect of 5-HT. *In vitro* experiments and *in vivo* recordings from anaesthetized animals indicate that these agents can both decrease the output of the 5-hydroxytryptaminergic system by inhibiting the firing of dorsal raphe neurones (Van der Maelen *et al.*, 1986; Sprouse & Aghajanian, 1986) and can mimic or antagonize 5-HT at 5-hydroxytryptaminergic effector sites in the hippocampus (Beck *et al.*, 1985; Colino & Halliwell, 1986; Rowan & Anwyl, 1986; Andrade & Nicoll, 1987a; Martin & Mason, 1987). Given the potentially opposing nature of these effects it is not clear which actions would be predominate at the effector sites in the hippocampus of conscious animals *in vivo*. This is of particular interest since the anxiolytic effect of some 5-HT_{1A} ligands may be due to an action in the dorsal or median raphe nuclei (Higgins *et al.*, 1988; Carli *et al.*, 1989) or the hippocampus (Kostowski *et al.*, 1989).

The present study examined the effects of a range of agents which show a relatively high affinity for 5-HT_1 sites on electrically evoked synaptic responses at the dendrites of pyramidal cells in the CA1 region of the hippocampus of alert, gently restrained, rats. Emphasis was placed on the determination of possible agonist and antagonist activity at these sites when the drugs were applied either systemically or locally into the region. The neurophysiological actions of the putative 5-HT_{1A} antagonists spiroxatrine (Nelson & Taylor, 1986) and BMY 7378 (Yocca *et al.*, 1987) were of particular interest, given the present need for selective antagonists at this site. A preliminary account of some of this work was presented to the British Pharmacological Society (O'Connor *et al.*, 1988).

Methods

Animals and surgery

Experiments were carried out on male Wistar rats weighing 200–250 g at the time of surgery. Surgery was carried out under sodium pentobarbitone (40 mg kg^{-1}, i.p.) anaesthesia. Two Teflon-coated tungsten wire extracellular recording electrodes (0.075 mm outer diameter and 0.05 mm inner diameter) glued to a guide cannula (0.7 mm outer diameter) and a pair of bipolar stimulating tungsten wire electrodes, similar to the recording electrodes, were lowered into the stratum radiatum of the CA_1 region of the hippocampus. The recording electrodes were staggered and extended 0.3 mm and 0.7 mm beyond the tip of the guide cannula. Stereotactic coordinates, relative to bregma with the skull level were: 2.8 mm lateral and 3 mm posterior and 2.4 mm below the surface of the dura. The stainless steel internal cannula tip (0.36 mm outer diameter) projected 0.1 mm beyond the end of the guide cannula. Stainless steel wire (0.36 mm outer diameter) which served as a dummy inner cannula between injection sessions, projected 0.1 mm below the guide cannula. The stimulating electrodes were placed 3.8 mm lateral and 4.0 mm posterior to bregma, and 2.8 mm below the dura. The correct placement of electrodes was verified by electrophysiological criteria during implantation (Leung, 1980) and by histological evidence with post-mortem examination (see below). Two brass screws (8 BA) were inserted into the skull (8 mm posterior and 4 mm

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lateral; 3 mm anterior and 3 mm lateral to bregma) just above the dura and were used as the ground and reference electrodes respectively. The electrode socket assembly was fixed onto the skull with dental cement.

Five animals had an intrahippocampal injection of 5-HT while still anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.). Stimulation and recording parameters were the same as used in alert rats (see next section).

At least 5 days recovery from surgery were allowed before recordings were taken from alert rats. Animals were handled regularly for familiarisation before and after surgery and were housed individually. The rats were in good health and put on weight as normal.

Recordings from alert rats

Recordings were made from alert rats while they were gently restrained in a canvas hammock to which they were habituated and from which they could easily escape. During recording a flexible cable was inserted into the rat's electrode socket. The stratum radiatum was stimulated with single square wave pulses of 0.1 ms duration generated at a rate of 0.02 Hz by a constant current isolation unit. The response was fed into an amplifier with a broad band setting of 4 Hz to 6 kHz. Stimulus intensity (2–6 mA) was adjusted to evoke a population excitatory postsynaptic potential (e.p.s.p.) which was approximately 60% of the maximum amplitude (0.6 to 1.8 mV) and which was below the threshold for eliciting any observable behavioural effect. The amplitude of the population e.p.s.p. was measured as the voltage between the point of its initiation to the peak negativity. There was a linear relationship between the slope of the e.p.s.p. and its amplitude. The average of 3–5 e.p.s.ps was used.

Drug treatment

The following drugs were used: buspirone hydrochloride (Bristol-Myers Co., Evansville), 5-HT creatinine sulphate (Sigma), 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]8-azaspirol[4.5]-decane-7,9-dione hydrochloride (BMY 7378; Bristol-Myers), creatinine hemisulphate (Sigma), 5-carboxamidotryptamine maleate (5-CT; Glaxo), gepirone hydrochloride (Bristol-Myers), 8-hydroxy-2-(di-n- propylamino)tetralin hydrobromide (8-OH-DPAT); Research Biochemicals Inc.), idazoxan hydrochloride (Reckitt & Coleman), ipsapirone hydrochloride (Tropenwerke), methysergide hydrogen maleate (Sandoz), 5-methoxy-3(1,2,3,6- tetrahydro-4-pyridinyl)-1H-indole (RU 24969; Rousell-Uclaf), spiroxatrine (Janssen Pharmaceutica), ketanserin tartrate (Janssen Pharmaceutica), tartaric acid (British Drug House) and trifluoperazine hydrochloride (Smith, Kline & French).

Drugs and vehicles were applied via the i.p. route (1 ml kg^{-1}) or directly into the hippocampus (i.h.; dissolved in 0.5 μl volume). Water control injections had no significant effect on the e.p.s.p. amplitude (n = 5) nor did doses of up to 8 μg of creatinine sulphate (control for 5-HT; n = 4) or tartaric acid (0.05 M; control for spiroxatrine; n = 4). Control experiments in which Indian ink (0.5 μl) was injected from the internal cannula tip indicated that chemicals applied in this way did not diffuse out of the hippocampus to any appreciable extent. RU 24969 was administered s.c. in order to be comparable with other studies (Tricklebank *et al.*, 1986).

Histology

At the completion of data acquisition each animal was deeply anaesthetized with pentobarbitone. The animal's head was then excised for wax fixation after its dental cement cap had been removed. The position of the cannula and microelectrode tracts was verified by light microscopy in thionin-stained sections.

Statistical analysis

The statistical significance of the difference between the mean values was determined by paired or unpaired Student's two-tail t test. Values are expressed as the mean % control e.p.s.p. amplitude ± s.e.mean.

Results

Effect of local injection into the hippocampus (i.h.) of 5-HT and 5-HT$_1$ ligands on the amplitude of the e.p.s.p.

The direct injection of 5-HT into the hippocampus of an alert rat produced a transient reduction in the amplitude of the e.p.s.p. (Figure 1a, Table 1) which was dose-dependent in the range 0.1 to 50 μg (dose producing 50% inhibition, ED_{50} = 0.46 μg, Figure 1b). Similar experiments were carried out while animals were under anaesthesia. The response to 5-HT (10 μg) was significantly less (peak effect was 85 ± 9% baseline control under pentobarbitone, 40 mg kg^{-1}, i.p., n = 5, compared to 53 ± 7% in alert animals, n = 8; $P < 0.01$).

Local application of buspirone also produced an inhibitory effect on the e.p.s.p. amplitude with a similar time course to that of 5-HT (Table 1). This response was dose-dependent in the range 0.1 to 10 μg (ED_{50} = 0.29 μg, Figure 1b).

The selective 5-HT$_1$ receptor agonist 5-CT (Hoyer, 1988; Zgombick *et al.*, 1989) had an inhibitory effect on the e.p.s.p. amplitude when applied at a relatively low dose (Table 1). Both ipsapirone and gepirone had reversible inhibitory effects

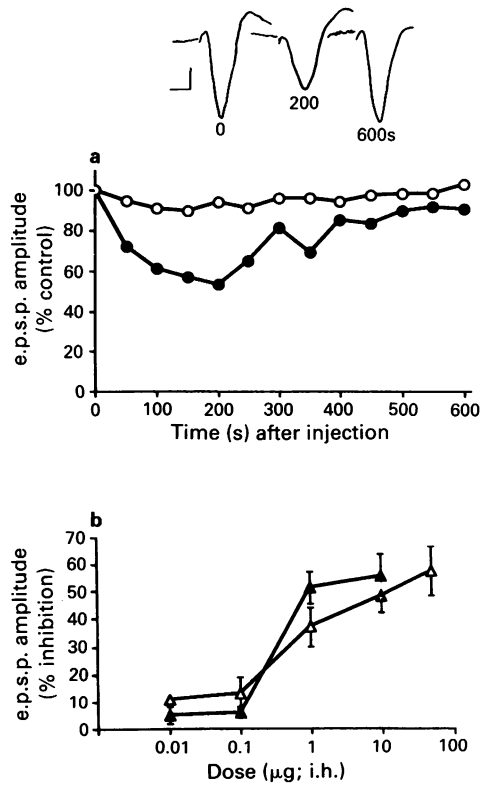

Figure 1 The effect of direct injection of 5-hydroxytryptamine (5-HT) and buspirone into the rat hippocampus on the amplitude of the e.p.s.p. (a) Typical time course for the effect of 5-HT (●, 10 μg in 0.5 μl water) compared to the changes produced by the application of an equivalent volume of water (○). Values are expressed as a % baseline control. Inset shows e.p.s.ps at 0, 200 and 600 s after 5-HT injection; calibration bars: vertical, 0.25 mV; horizontal, 10 ms. (b) Log dose-response curves for the inhibitory effects of 5-HT (△) and buspirone (▲) injected directly into the hippocampus. Values are expressed as the mean % inhibition of the pre-injection baseline e.p.s.p. amplitude (n = 3–8); vertical bars show s.e.mean.

Table 1 The effect of 5-hydroxytryptamine (5-HT) receptor ligands on the amplitude of the e.p.s.p. in the rat hippocampus

Compound	Dose (μg)	n	e.p.s.p. amplitude (mean ± s.e.mean, % baseline control)	Time to peak (s)	Time to recover (s)
5-HT	10.0	8	53.0 ± 7.0***	50–250	400–550
Buspirone	1.0	6	57.00 ± 8.0***	100–250	350–600
5-CT	0.02	3	80.0 ± 6.0*	100	450–500
	0.1	3	68.0 ± 8.0**	50	550–600
Ipsapirone	1.0	3	71.0 ± 10.0*	150–200	350–400
Gepirone	1.0	3	70.5 ± 7.0*	100–200	400–450
BMY 7378	0.1	4	64.0 ± 8.0*	100–200	500–600
8-OH-DPAT	0.2	3	82.0 ± 5.0	—	—
	2.0	4	90.0 ± 6.0	—	—
Spiroxatrine	1.0	3	94.5 ± 5.5	—	—

5-CT = 5-carboxamidotryptamine maleate; BMY 7378 = 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]8-azaspiro[4.5]-decane-7,9-dione hydrochloride; 8-OH-DPAT = 8-hydroxy-2-(di-n- propylamino)tetralin hydrobromide.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to pre-injection baseline controls.

on the e.p.s.p. amplitude at 1 μg (Table 1). Somewhat surprisingly local injection of the putative 5-HT_{1A} antagonist, BMY 7378 (0.1 μg; Yocca *et al.*, 1987) into the hippocampus was also found to have a marked inhibitory effect on the e.p.s.p. amplitude. The time course of the effects of these compounds was similar to that of the response to local injection of 5-HT.

The prototypical selective 5-HT_{1A} ligand 8-OH-DPAT (0.2 and 2 μg) had no significant effect on the e.p.s.p. amplitude when directly injected into the hippocampus. In fact it was found to antagonize the response to 5-HT. Application of 5-HT (10 μg, i.h.) caused no change in the e.p.s.p. amplitude after 10 min pretreatment with 2 μg 8-OH-DPAT (Figure 2b, $n = 4$; $P < 0.05$, compared to 5-HT alone). Administration of the putative selective 5-HT_{1A} antagonist, spiroxatrine (1 μg) directly into the hippocampus did not have any significant effect on the e.p.s.p. amplitude over a 20 min observation period ($n = 4$). Pretreatment with spiroxatrine (1 μg) for 10 min significantly reduced the effect of 10 μg 5-HT ($n = 4$; $P < 0.05$, compared to 5-HT alone, Figure 2c). This blockade of the inhibitory effect of 5-HT was still apparent 100 min after spiroxatrine treatment (97 ± 5% baseline control, $n = 4$; $P < 0.05$, compared to 5-HT alone).

Effect of systemic injection of 5-HT_1 ligands on the amplitude of the e.p.s.p.

Acute systemic injection of buspirone (3 mg kg^{-1}, i.p.) produced a marked reduction in the amplitude of the e.p.s.p. which was maximal between 30 and 60 min (Figure 3a; $n = 6$; $P < 0.05$) and recovered at 90 min. This response was dose-dependent in the range 0.5 to 3.0 mg kg^{-1}, i.p. (ED_{50} = 0.88 mg kg^{-1}). Similarly injection of BMY 7378 (0.01 mg kg^{-1}, i.p.) produced a transient reduction in the e.p.s.p. amplitude with a maximal response at 30 min (Figure 3b; $n = 4$; $P < 0.05$) and recovery at 90 min. The 5-HT_1 ligand RU 24969 (1 mg kg^{-1}, s.c., Tricklebank *et al.*, 1986) also caused an inhibitory effect on the e.p.s.p. amplitude (Figure 3c). The maximum effect was reached at 15 min post injection ($n = 4$; $P < 0.05$) and full recovery was noted at 60 min.

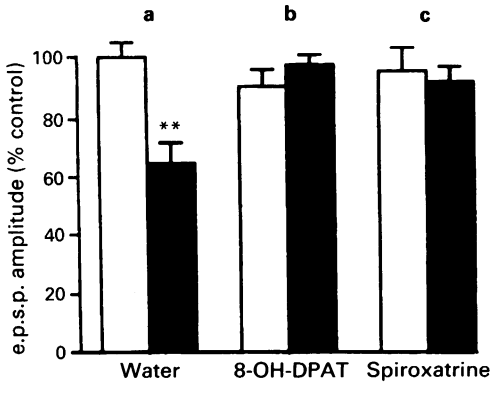

Figure 2 The effect of 5-hydroxytryptamine (5-HT, 10 μg, i.h.) on the e.p.s.p. amplitude in animals pretreated with an intra-hippocampal injection of (a) water (0.5 μl), (b) 8-hydroxy-2-(di-n- propylamino)-tetralin (8-OH-DPAT, 2.0 μg) and (c) spiroxatrine (1.0 μg). The open columns show the e.p.s.p. amplitude 10 min after pretreatment with the compounds and the solid columns show the peak change in e.p.s.p. amplitude after the 5-HT injection. Values are the mean % baseline control e.p.s.p. amplitude ($n = 4$–6); vertical bars show s.e.mean. ** $P < 0.01$ compared to pre-5-HT e.p.s.p. amplitude.

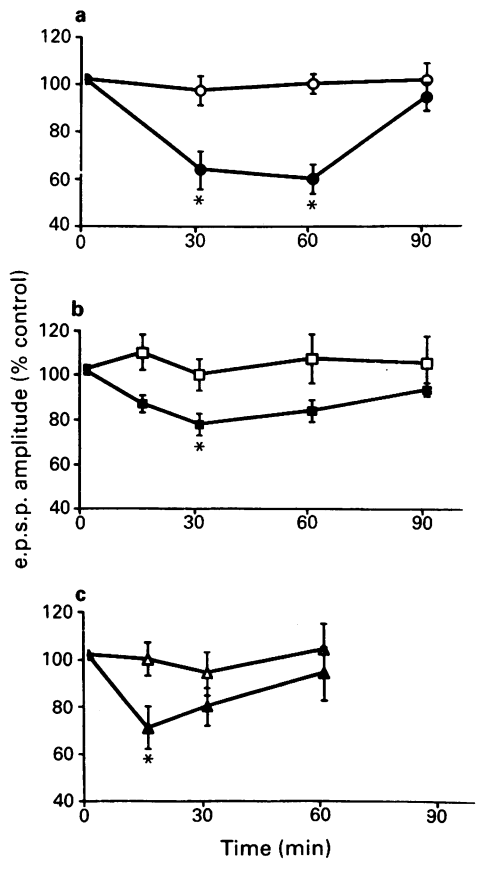

Figure 3 The effect of systemic injection of 5-hydroxytryptamine (5-HT) receptor ligands on the amplitude of the e.p.s.p. Time course for the acute effects of (a) buspirone (3 mg kg^{-1}, i.p.), (b) BMY 7378 (0.01 mg kg^{-1} i.p.) and (c) RU 24969 (1 mg kg^{-1} s.c.) on the e.p.s.p. amplitude are shown. Values are the mean % baseline control e.p.s.p. amplitude ($n = 4$–8). Vertical bars show s.e.mean. * $P < 0.05$ comparing the effect of the drug (closed symbols) to water injected controls (open symbols).

Effect of local injection of 5-HT and buspirone following systemic buspirone administration

The effect of i.h. buspirone on the response to systemic buspirone was determined in order to assess whether or not similar mechanisms were involved in producing the effects of the drug when given by the different routes of administration. Application of buspirone (i.p.). There was a significant effect of buspirone (i.h.) in the 0.5 mg kg^{-1} buspirone-treated animals ($n = 4$; $P < 0.01$, compared to pre-buspirone injection) but there was no further change in the 2.5 mg kg^{-1} buspirone group ($P > 0.05$, pre- versus post-injection of buspirone i.h.; Figure 4a).

In order to investigate possible actions of buspirone on 5-HT receptors in the hippocampus, the response to 5-HT was studied after systemic buspirone administration. The effect of an acute application of 5-HT (10 μg, i.h.) was measured in animals pretreated for 30 min with either 0.5 or 2.5 mg kg^{-1} buspirone (i.p.). There was a significant effect of 5-HT in the 0.5 mg kg^{-1} buspirone group ($n = 4$; $P < 0.01$, pre- versus post-injection of 5-HT) but there was an apparent lack of change in the 2.5 mg kg^{-1} buspirone group ($P > 0.05$, pre- versus post-injection of 5-HT; $n = 4$; Figure 4b). A similar apparent absence of effect was observed when 5-HT (10 μg, i.h.) was given after 3 mg kg^{-1} i.p. buspirone ($n = 4$, $P > 0.05$).

Effect of systemic application of receptor antagonists on the response to local injection of buspirone

Pretreatment for 30 min with either the non-selective 5-HT receptor antagonist, methysergide (5 mg kg^{-1}, i.p.) or spiroxatrine (1 mg kg^{-1}, i.p.) blocked the inhibitory effect of buspirone (i.h.; Table 2). Neither of these antagonists when administered on their own had any significant effect on the e.p.s.p. amplitude ($P > 0.05$). Ketanserin (1 mg kg^{-1}, i.p.), a 5-HT$_2$ receptor antagonist had no effect alone or on the response to buspirone (Table 2).

Administration of the dopamine D_2 receptor antagonist trifluoperazine (1.0 mg kg^{-1}, i.p.) or the α_2-adrenoceptor antagonist, idazoxan (1.0 mg kg^{-1}, i.p.) had no observable effect on the e.p.s.p. amplitude or on the response to buspirone (1 μg, i.h.; Table 2).

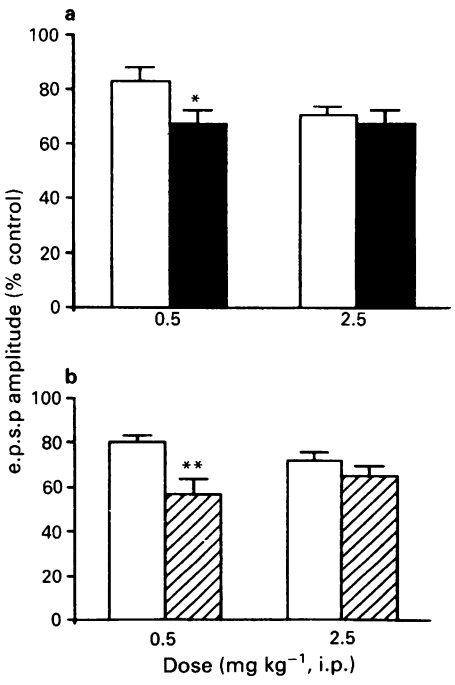

Figure 4 The effect of local application of buspirone and 5-hydroxytryptamine (5-HT) into the hippocampus on the response to systemically injected buspirone (0.5 and 2.5 mg kg^{-1}, i.p.). The peak effect of intra-hippocampal application of (a) buspirone (1 μg, solid columns) and (b) 5-HT (10 μg, hatched columns) on the amplitude of the e.p.s.p. 30 min after pretreatment with systemic buspirone. The open columns show the e.p.s.p. amplitude 30 min after the systemic buspirone injection. Values are the mean % baseline control e.p.s.p. amplitude ($n = 4$). Vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$.

Table 2 The effect of pretreatment with 5-HT receptor antagonists (methysergide, 5 mg kg^{-1}, i.p.; spiroxatrine, 1 mg kg^{-1}, i.p.; ketanserin, 1 mg kg^{-1}, i.p.) trifluoperazine (1 mg kg^{-1}, i.p.), idazoxan (1 mg kg^{-1}, i.p.) or water, (1 ml kg^{-1}, i.p.) on the response to locally applied buspirone (1.0 μg, i.h.)

Treatment	n	*e.p.s.p. amplitude* (mean ± s.e.mean % baseline control)
Water	6	88 ± 7
Water + buspirone	6	54 ± 9*
Methysergide	3	96 ± 11
Methysergide + buspirone	4	105 ± 13
Spiroxatrine	3	105 ± 10
Spiroxatrine + buspirone	4	98 ± 12
Ketanserin	4	108 ± 11
Ketanserin + buspirone	3	69 ± 7*
Trifluoperazine	4	98 ± 4
Trifluoperazine + buspirone	4	63 ± 7*
Idazoxan	3	95 ± 7
Idazoxan + buspirone	3	66 ± 15*

* $P < 0.05$ compared to water alone or antagonist alone.

Effect of the systemic application of 5-HT receptor antagonists on the response to systemic injection of buspirone

Pretreatment with either spiroxatrine (1 mg kg^{-1}, i.p.) or methysergide (5 mg kg^{-1}, i.p.), blocked the inhibitory effect of systemic injection of buspirone (3 mg kg^{-1}, i.p.), whereas ketanserin (1 mg kg^{-1}, i.p.) had no effect (Table 3).

Discussion

The 5-HT-induced decrease in the amplitude of the electrically evoked population e.p.s.p. is consistent with the reported preponderance of inhibition of hippocampal pyramidal cells when 5-HT was applied by iontophoresis, droplets or superfusion in both *in vivo* and *in vitro* preparations (Segal, 1976;

Table 3 The effect of pretreatment with 5-HT receptor antagonists (methysergide, 5 mg kg^{-1}, i.p.; spiroxatrine, 1 mg kg^{-1}, i.p.; ketanserin, 1 mg kg^{-1}, i.p.) on the response to systemic buspirone (3 mg kg^{-1}, i.p.)

Treatment	n	*e.p.s.p. amplitude* (mean ± s.e.mean % baseline control)
Water	8	95 ± 5
Water + buspirone	8	68 ± 6*
Methysergide	6	107 ± 4
Methysergide + buspirone	6	90 ± 13
Spiroxatrine	5	98 ± 6
Spiroxatrine + buspirone	5	96 ± 9
Ketanserin	3	89 ± 10
Ketanserin + buspirone	3	65 ± 9*

* $P < 0.05$ compared to water alone or antagonist alone.

1981; Jahnsen, 1980). Jahnsen (1980), Beck & Goldfarb (1985) and Rowan & Anwyl (1985) found that 5-HT could inhibit excitatory synaptic responses of pyramidal cells although Mauk *et al.* (1988) and Segal *et al.* (1989) did not detect any effect. Unlike our previous report of *in vitro* studies (Rowan & Anwyl, 1985), we failed to see evidence of an initial transient increase in the amplitude of the e.p.s.p. The importance of carrying out experiments in non-anaesthetized animals is underlined by the observation of a greatly attenuated response to 5-HT in non-conscious animals. The animals were lightly restrained in a hammock to which they had previously been habituated in order to reduce the range of behaviours during recording sessions. These conditions were considered to be relatively non-stressful in the sense that the rat could easily escape, if it wished to.

Previous intracellular studies by Segal (1981) and Jahnsen (1980) have shown that 5-HT has both hyperpolarizing and depolarizing actions on the hippocampal pyramidal cells. A hyperpolarizing action of 5-HT on the dendrites of the pyramidal cells due to a postsynaptic opening of K^+ channels (Andrade *et al.*, 1986; Colino & Halliwell, 1987; Yakel *et al.*, 1988) would seem the most likely explanation of the inhibitory effect of 5-HT on the e.p.s.p. Evidence for a postsynaptic site of action is the observation by Sprouse & Aghajanian (1988) that 5-HT can inhibit the effect of the presumed excitatory transmitter, glutamate, on pyramidal cell firing rate, although clearly a presynaptic effect cannot be ruled out.

Local or systemic injection of the 5-HT_{1A} ligand buspirone also caused a decrease in the population e.p.s.p. Local application of buspirone after pretreatment with a maximum dose of systemic buspirone had no further significant effect. It would seem that buspirone, when given by either the i.p. or i.h. route, was acting through a similar mechanism. In order to investigate whether or not buspirone was acting through 5-HT receptors, the response to 5-HT after systemic buspirone pretreatment was examined. The effect was not a summation of individual effects. This finding is indicative of shared mechanisms for the compounds. The lack of a significant antagonism of the 5-HT response by a submaximal dose of buspirone suggests that buspirone does not show antagonist properties at the dendritic level of pyramidal cells unlike the situation at the cell body layer (Andrade & Nicoll, 1987a; Colino & Halliwell, 1987; Sprouse & Aghajanian, 1988; Segal *et al.*, 1989). Further evidence that the action of buspirone was mediated through 5-HT receptors was shown by the antagonistic effects of the non-selective receptor antagonist methysergide and the putative 5-HT_{1A} antagonist spiroxatrine (Nelson & Taylor, 1986). No antagonism of this response was produced by ketanserin, a relatively selective 5-HT_2 receptor antagonist. Although methysergide has been shown to behave as a 5-HT_{1A} agonist/partial agonist (Andrade & Nicoll, 1987b) and Yakel *et al.* (1988) have shown that it can produce outward currents similar to 5-HT in the hippocampus, no change in the baseline e.p.s.p. was observed in the present study. Spiroxatrine has also been shown in biochemical studies to behave like an agonist at 5-HT_{1A} sites in the hippocampus *in vitro* (Nelson *et al.*, 1987; Herrick-Davies & Titeler, 1988). However the present findings indicate that spiroxatrine does not mimic 5-HT when given i.h. or i.p. *in vivo*. Indeed it was found to antagonize the effect of 5-HT when applied locally in the hippocampus. Since buspirone and spiroxatrine have been shown to have some affinity for the dopamine D_2 receptor type (Costall & Naylor, 1978; Garattini *et al.*, 1982) and buspirone and its metabolite 1(2-pyrimidinyl)-piperazine (1-PP) have some affinity for the α_2-adrenoceptor binding site in rat cortex (Rimele *et al.*, 1987), the possibility of the involvement of these receptors in their effects was investigated using trifluoperazine and idazoxan. These compounds were without inhibitory effect when applied alone and also did not antagonize the inhibitory response to local injection of buspirone. These findings strengthen the case that the effects of buspirone on the e.p.s.p. are indeed mediated by the 5-hydroxytryptaminergic system.

The receptor at which 5-HT and buspirone are acting on in the present study appears to conform to the requirements of Bradley *et al.* (1986) for classification as '5-HT_1-like' in that (a) methysergide was an effective antagonist, (b) ketanserin was inactive and (c) 5-CT mimicked the inhibitory effect of 5-HT. Although there are no generally agreed criteria for subdividing these receptors in relation to radioligand binding sites, there are strong parallels with the 5-HT_{1A} site. The relatively selective 5-HT_{1A} ligands, ipsapirone, gepirone and BMY 7378 all behaved like agonists. Previous electrophysiological studies in the rat hippocampus have shown that these close structural analogues of buspirone have mixed agonist/antagonist actions at putative 5-HT_{1A} receptors (Basse-Tomusk & Rebec, 1986; Rowan & Anwyl, 1986; Andrade & Nicoll, 1987a; Martin & Mason, 1987; Blier & de Montigny, 1987; Chaput & de Montigny, 1988; Sprouse & Aghajanian, 1988; Segal *et al.*, 1989). 8-OH-DPAT is usually considered to be an agonist at these sites (Beck *et al.*, 1985; Sprouse & Aghajanian, 1986; 1988; Van der Maelen *et al.*, 1987; Blier & De Montigny, 1987; Wu *et al.*, 1988) whereas in the present study it was found only to block the 5-HT-induced response. However the action of 8-OH-DPAT as a pure agonist in the hippocampus has been questioned. Colino & Halliwell (1986, 1987), Andrade & Nicoll (1987a,b) and Segal *et al.* (1989) reported that superfusion of 8-OH-DPAT onto submerged hippocampal slices can block the 5-HT-induced hyperpolarizing response. They suggested that 8-OH-DPAT had 5-HT_{1A} antagonist properties. The present results would seem to be most compatible with their data, although in their experiments buspirone, ipsapirone and gepirone also had marked antagonist properties. It is not clear why the apparent order of agonist activity of these compounds varies depending on the method of examining 5-HT receptors mediating inhibition of hippocampal cells. It is possible that the receptors are heterogeneous or that they are in different states in different subregions of the hippocampus (Yocca *et al.*, 1989). A study of a wider dose-range might help to clarify the situation. Since a number of compounds used are relatively selective for the 5-HT_{1A} site it seems unlikely that putative 1B, 1C or 1D receptors are involved (Hoyer, 1988). Although RU 24969 is a 5-HT_{1B} agonist (Tricklebank *et al.*, 1986) and is known to inhibit 5-HT release in the rat hippocampus by activation of the 5-hydroxytryptaminergic nerve terminal 5-HT_{1B} autoreceptor (Sharp *et al.*, 1989), it is approximately equipotent at hippocampal 5-HT_{1A} sites (Tricklebank *et al.*, 1986; Peroutka, 1986; Hamon *et al.*, 1986; Hoyer, 1988) and has been reported to act as an agonist at putative 5-HT_{1A} receptors mediating hypothermia in restrained rats when given s.c. at the dose used in the present study (Tricklebank *et al.*, 1986). It will be necessary to investigate a wider range of receptor selective drugs in order to clarify the situation completely with regard to receptor classification.

The effects of buspirone, ipsapirone and 8-OH-DPAT described here do not appear to be related to those described by Peroutka *et al.* (1987) on electrically evoked responses in the hippocampus *in vitro*, which were not apparently dependent on an interaction with 5-HT receptors. Mauk *et al.* (1988) and Hiner *et al.* (1988) have suggested that relatively high concentrations of these compounds have a presynaptic inhibitory effect on the excitability of afferent nerves in the stratum radiatum layer. Recent investigations in our laboratory (Rowan *et al.*, 1989) showed much higher doses and stimulation frequencies than those used in the present study were required to produce such effects with buspirone *in vivo*.

Overall, the present study showed that the 5-HT_1 ligands buspirone, ipsapirone, BMY 7378, gepirone and 5-CT, after direct intrahippocampal application, mimicked the inhibitory effect of 5-HT on excitatory synaptic transmission in the stratum radiatum and were thus probably acting as 5-HT_1 agonists at the doses tested. Local injection of 8-OH-DPAT and spiroxatrine into this region blocked the 5-HT effect. These compounds were therefore probably acting as 5-HT_1 antagonists. The fact that in the case of buspirone, BMY 7378

and spiroxatrine similar effects were observed under the present conditions after systemic administration indicates that in the alert rat, direct actions in the hippocampus appear to predominate over potentially opposing indirect ones (e.g. via alterations in raphe neuronal firing rates, e.g. Trulson & Trulson, 1986). This does not of course rule out a possible contribution from active metabolites when the drugs are given systemically (O'Connor *et al.*, 1989). The present data lend strong support for the functional importance of 5-HT_1 sites in the dendritic regions of hippocampal pyramidal cells *in vivo*.

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Reduction of NMDA receptors with dithiothreitol increases [^{3}H]-MK-801 binding and NMDA-induced Ca^{2+} fluxes

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1 We have investigated the modulation of N-methyl-D-aspartate (NMDA) receptor activation by the sulphydryl redox reagents dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB).

2 Increases in [^{3}H]-MK-801 binding produced by glutamate, glycine and spermidine were enhanced by DTT (2 mM) and diminished by DTNB (0.5 mM).

3 The inhibition of [^{3}H]-MK-801 binding by CGS 19755 and 7-chlorokynurenate was not altered by 2 mM DTT. However, the potency of the competitive polyamine antagonist, arcaine, was decreased by DTT.

4 NMDA-induced Ca^{2+} fluxes into primary cultures of rat forebrain neurones were enhanced by DTT in a DTNB-reversible fashion. In addition to augmenting the magnitude of NMDA-induced increase in intracellular free Ca^{2+}, 10 mM DTT also prolonged the duration of the Ca^{2+} signal. However, DTT had no effect on the increase in Ca^{2+} produced by depolarizing neurones with 50 mM KCl.

5 These studies show that the reduction of disulphide bonds on the NMDA receptor complex by DTT increases activation. The precise site of these groups remains unclear but they are unlikely to form an integral part of the glutamate, glycine or polyamine binding domains. The enhancement of the activation of the NMDA receptor by DTT is associated with increased Ca^{2+} fluxes. The possible pathophysiological consequences of receptor reduction are discussed.

Introduction

Activation of the N-methyl-D-aspartate (NMDA)-preferring subtype of glutamate receptor is subject to modulation by a variety of endogenous substances, including glycine, Mg^{2+}, Zn^{2+} (see Collingridge & Lester, 1989 for review) and polyamines (Ransom & Stec, 1988; Reynolds & Miller, 1989a; Williams *et al.*, 1989). In most cases the physiological consequence of the actions of these modulators is not entirely clear due to the difficulty in manipulating modulator levels *in vivo*. However, the action of substances that modulate the NMDA receptor have been the subject of great interest because of the likely therapeutic utility of NMDA receptor-directed drugs in neurological disorders including stroke, neurodegenerative diseases and epilepsy.

It was recently demonstrated that dithiothreitol (DTT) could enhance NMDA responses electrophysiologically (Aizenman *et al.*, 1989). As the actions of DTT could be fully reversed by 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), which oxidizes sulphydryl groups, it appeared that the actions of DTT were due to a chemical modification of one or more disulphide bonds on the NMDA receptor. Other studies have generally confirmed that the action of DTT on the NMDA receptor promotes activation (Lazarewicz *et al.*, 1989; Levy *et al.*, 1990; Sacaan & Johnson, unpublished observations). Furthermore, DTT enhances synaptic activity in areas of the brain where NMDA receptors are thought to be involved, including the hippocampus (Tolliver & Pellmar, 1987; 1988; Tauck & Ashbeck, 1990). However, the precise mechanism of action of DTT remains unclear.

In this study we have further investigated the actions of DTT on NMDA receptors. We have employed [^{3}H]-MK-801 to label the NMDA receptor-channel complex. This ligand can be used to monitor drug action at all of the known modulator sites on the NMDA receptor and, thus, it represents a powerful tool for probing the actions of DTT (Reynolds & Miller, 1988; 1989a; Ransom & Stec, 1988). In addition, we have studied the actions of DTT and DTNB on changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) produced by NMDA. This is a particularly important component to consider because the pathophysiological actions of NMDA appear to be Ca^{2+}-dependent (Choi, 1987).

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Methods

Radioreceptor binding assays

[^{3}H]-MK-801 binding to well washed membranes prepared from frozen rat brains (Pel-Freez, Rogers AR) was performed as previously described (Reynolds & Miller, 1989a). Assays consisted of 1 ml 10 mM HEPES/NaOH, pH 7.4 which contained 50–100 μg protein, 0.5 nM [^{3}H]-MK-801 and drugs as appropriate. Non-specific binding was determined by using 30 μM MK-801. Following a 2 h incubation at room temperature (21–25°C) assays were terminated by vacuum filtration over glass fibre filters (Schleicher and Schuell, Keene NH). Radioactivity was determined by liquid scintillation counting with an efficiency of approximately 50%. Basal [^{3}H]-MK-801 binding under these conditions ranged from 60–127 c.p.m. Estimates for EC_{50} values, as well as basal and maximum levels of binding for compounds which increased [^{3}H]-MK-801 binding were obtained using a simplex curve fitting routine which employed the following equation:

$$E = E_{max} \cdot D/(D + EC_{50}) + B$$

where E is the increase in binding for a given drug concentration, D, with a maximal response E_{max} and basal (unstimulated) binding of B. Spermidine, as previously noted (Reynolds & Miller, 1989a), produces a biphasic curve (see Figure 1). Only data points on the ascending phase of the dose-response curve were analysed for EC_{50} estimation. Dose-response curves performed in the presence of DTT or DTNB were compared to control curves by a repeated measures analysis of variance (ANOVA) test using both redox state and concentration as within subject factors. *Post hoc* comparison of means was performed by Tukey's HSD test with P values of less than 0.05 being considered significantly different.

Intracellular Ca^{2+} assays

$[Ca^{2+}]_i$ was monitored in primary cultures obtained from embryonic rat (E17) forebrain as previously described (Reynolds & Miller, 1989b). Cells were plated onto poly-D-lysine-coated glass cover slips in Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v foetal bovine serum, 100 u ml^{-1} penicillin and 5 $\mu g\,ml^{-1}$ streptomycin. After 24 h the medium was replaced by DMEM supplemented with antibiotics and 10% v/v horse serum, and the cover slips inverted. Cells were kept in a humidified incubator with 95% air, 5% CO_2 until use, which was usually 12–21 days. At the time of use, cover slips were turned over and rinsed in HEPES-buffered salt solution (HBSS) of the following composition (mM): NaCl 137, KCl 5, $MgSO_4$ 0.9, $NaHCO_3$ 3, NaH_2PO_4 0.6, K_2HPO_4 0.4, glucose 5.6 and HEPES 20, pH adjusted to 7.4 with NaOH. Cells were then incubated in HBSS containing 5 mg ml^{-1} bovine serum albumin and 5–7 μM fura-2 AM (Molecular Probes, Eugene OR, U.S.A.) for approximately 1 h at 37°C. Cover slips were then rinsed for 10 min in HBSS before use.

Recordings of fura-2 fluorescence were made as follows. Light from a 200 W mercury-xenon lamp (Oriel Corporation, Stratford CT, U.S.A.) was filtered by 340 and 380 nm narrow band-pass filters (Omega Optical, Brattleboro VT, U.S.A.). The filters were mounted in a wheel driven by computer-controlled stepping motor (Ludl Electronic Products, Hawthorne NY, U.S.A.) that moved filters rapidly (approx. 100 ms) to alternate between illumination at 340 and 380 nm wavelengths. The recording chamber (Biophysica Inc. Baltimore MD, U.S.A.) was mounted on the stage of a Nikon Diaphot fluorescence microscope. Fluorescence from single neurones was obtained by using a rectangular diaphragm to limit the visual field of the video port of the microscope. Levels of fluorescence were detected and quantified by a Thorn EMI bialkali photomultiplier and a C10 photon counting system (Thorn EMI Electron Tubes Inc, Fairfield NJ, U.S.A.). The photon counting system was interfaced with a 30386-based IBM compatible computer by custom designed data acquisition software. Values for $[Ca^{2+}]_i$ were estimated by comparing ratios obtained from single neurones with known values on a standard curve constructed with a range of EGTA-buffered Ca^{2+} concentrations. Values for $[Ca^{2+}]_i$ were calculated from the equation:

$$[Ca^{2+}]_i = K \cdot (\text{Ratio} - \text{Rmin})/(\text{Rmax} - \text{Ratio})$$

where K is a constant for the affinity of fura-2 for Ca^{2+} (Grynkiewicz *et al.*, 1985). The values for K, Rmin and Rmax were obtained from standard curves.

To monitor the effects of DTT and DTNB on NMDA-induced changes in $[Ca^{2+}]_i$ single neurones were first tested without addition of DTT or DTNB. DTNB (0.5 mM) was then added for 60 s and subsequently washed out before the addition of agonist. This condition gave the response to the agonist with a consistently oxidized state of the receptor. For each additional response the 60 s DTNB application was followed by 120 s incubation with the appropriate concentration of DTT. This agent was also washed out before the addition of agonist. Agonists were applied by perfusion, and application continued until the $[Ca^{2+}]_i$ change had reached a clear maximum. Reaching a maximum response rarely took more than 40 s. Following each addition of agonist, levels of $[Ca^{2+}]_i$ were allowed to return to baseline before additional drug treatments were performed. Baseline levels of $[Ca^{2+}]_i$ were usually stable for the duration of the experiment, which was usually less than 90 min. In experiments with 50 mM KCl, osmolarity was adjusted by substituting an appropriate amount of NaCl with KCl.

Drugs

[^{3}H]-MK-801 (15 Ci $mmol^{-1}$) was purchased from Du Pont/NEN (Boston MA, U.S.A.). Unlabelled MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) was a generous gift of Dr Richard Ransom (Merck, Sharp and Dohme, West Point PA, U.S.A.). CGS 19755 (1-(*cis*-carboxypiperidine-4yl)methyl-1-phosphonic acid) and 7-chlorokynurenate were generous gifts from Dr Paul Ornstein (Eli Lilly, Indianapolis, IN, U.S.A.). All other drugs and chemicals were from commercial sources.

Results

Effects of DTT and DTNB on [^{3}H]-MK-801 binding

Several studies have demonstrated that [^{3}H]-MK-801 binding to well-washed rat brain membranes is sensitive to the state of activation of the NMDA receptor (Reynolds *et al.*, 1987; Foster & Wong, 1987). We employed the [^{3}H]-MK-801 binding assay to attempt to establish the mechanism of action of DTT. Preliminary experiments showed that DTT produced a small increase (10–20%) in [^{3}H]-MK-801 binding to well-washed membranes at concentrations between 20 μM and 6 mM, while concentrations of this reducing agent above 8 mM decreased binding. To investigate the possible mechanisms underlying this action of DTT, we performed dose-response curves to the positive modulators of [^{3}H]-MK-801 binding, namely glutamate, glycine and spermidine (Figure 1). We employed 2 mM DTT as the test concentration to avoid the inhibition of binding seen at higher levels. DTT increased [^{3}H]-MK-801 binding at each concentration of glutamate tested. In contrast, the oxidizing agent DTNB generally decreased [^{3}H]-MK-801 binding. A similar pattern was observed when dose-response curves to glycine were performed (Figure 1b). DTT had rather modest effects on the increase in [^{3}H]-MK-801 binding produced by spermidine (Figure 1c). The ascending phase of the curve was shifted to the left slightly, but there was no difference in the maximum response. Interestingly, higher concentrations of spermidine more effectively decreased [^{3}H]-MK-801 binding in the presence of DTT. In the presence of DTNB, spermidine increased [^{3}H]-MK-801 binding less effectively when compared to controls.

The binding parameters for the agonists shown in Figure 1 are summarized in Table 1. DTT and DTNB did not alter the EC_{50} of the agonists tested. The fold increase in binding shown in Table 1 is expressed as the agonist-induced increase in binding over basal levels in the presence of DTT and DTNB as appropriate. From these analyses it was apparent that the principle action of DTT was to raise the baseline level of binding rather than to modify the glutamate- or glycine-induced increase in [^{3}H]-MK-801 binding. In contrast, DTNB tended to decrease basal binding. However, when binding was expressed as a proportion of control (i.e. no DTT or DTNB), as is shown in Figure 1, the actions of DTT and DTNB were more apparent.

Table 1 Effects of dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) on ligand binding to N-methyl-D-aspartate (NMDA) receptors

	EC_{50} (μM)	*Fold increase*[a]
Glutamate		
Control	0.484 ± 0.15	3.17 ± 0.2
+ DTT 2 mM	0.364 ± 0.07	2.73 ± 0.1
+ DTNB 0.5 mM	0.456 ± 0.09	2.75 ± 0.3
Glycine		
Control	0.372 ± 0.04	3.04 ± 0.6
+ DTT 2 mM	0.359 ± 0.05	3.11 ± 0.3
+ DTNB 0.5 mM	0.442 ± 0.11	2.44 ± 0.03
Spermidine		
Control	8.12 ± 0.47	13.5 ± 3.6
+ DTT 2 mM	6.78 ± 0.59	12.1 ± 3.5
+ DTNB 0.5 mM	9.47 ± 0.39	10.4 ± 2.7

Results are the mean ± s.e.mean of 4–5 curves performed in duplicate as described in Methods.
[a] Fold increase in [^{3}H]-MK-801 binding above basal levels.

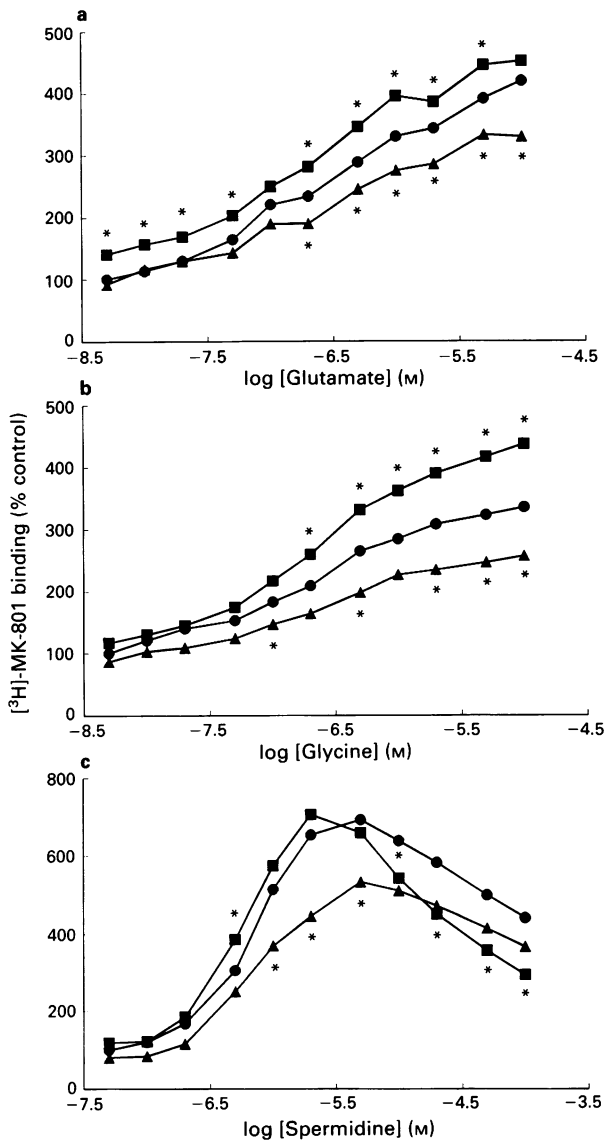

Figure 1 Effects of dithiothreitol (DTT) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) on the modulation of [^{3}H]-MK-801 binding by glutamate, glycine and spermidine. Concentration-response curves were performed using (a) glutamate, (b) glycine and (c) spermidine in the absence of redox reagents (●), or in the presence of 2 mM DTT (■) or 0.5 mM DTNB (▲). Curves with glutamate were obtained in the presence of 0.03 μM glycine, while curves with glycine were obtained in the presence of 0.1 μM glutamate. Curves with spermidine were obtained in the presence of 0.1 μM glutamate and 0.03 μM glycine. Standard error bars were omitted for clarity and usually represented less than 15% of the values shown. Results represent the mean of 4–5 experiments preformed in duplicate. Data points on curves performed in the presence of DTT and DTNB that are significantly different ($P < 0.05$) from corresponding values on the control curve are indicated by an asterisk.

We also examined the effects of DTT on the inhibition of [^{3}H]-MK-801 binding by competitive antagonists of glutamate, glycine and spermidine, namely CGS 19755, 7-chlorokynurenate and arcaine, respectively (Table 2). DTT had rather modest effects on the binding of these antagonists. However, a small but significant decrease in the affinity of the spermidine antagonist, arcaine, was found.

Effects of DTT and DTNB on NMDA-induced $[Ca^{2+}]_i$ changes

NMDA and glycine applied together to rat forebrain neurones in culture produced a rapid rise in $[Ca^{2+}]_i$. We tested 9 cells with up to 4 DTT concentrations per cell. Preliminary

Table 2 Effects of dithiothreitol (DTT) on antagonist binding to N-methyl-D-aspartate (NMDA) receptors

	IC_{50} (μM)
CGS 19755	
Control	4.20 ± 0.51
+ DTT 2 mM	2.99 ± 0.32
7-Chlorokynurenate	
Control	4.74 ± 0.88
+ DTT 2 mM	5.91 ± 1.30
Arcaine	
Control	0.79 ± 0.28
+ DTT 2 mM	4.58 ± 0.32**

Results are the mean ± s.e.mean of 4–6 curves performed in duplicate as described in Methods.
** Significantly different from control ($P < 0.01$, Student's t test).

experiments demonstrated that DTT (2 mM) could enhance responses to NMDA and glycine. To avoid differences in DTT effects arising from variations in the basal redox state of the neurones (Aizenman *et al.*, 1989) we used the following protocol: NMDA (30 μM) and glycine (1 μM) were applied to each cell at the beginning of the experiment, and then following a 60 s incubation with 0.5 mM DTNB, which was washed out before the agonists were re-applied. Following DTNB treatment responses to NMDA and glycine were reduced to 88.8 ± 5.1% of control. DTNB was then re-applied and washed out before each DTT treatment in order to return each cell to an oxidized state. Recordings from a typical cell are shown in Figure 2a. DTT increased NMDA and glycine-induced $[Ca^{2+}]_i$ changes between 1 and 10 mM. This effect could be completely reversed by the addition of DTNB. The concentration-dependence of the action of DTT on peak $[Ca^{2+}]_i$ responses to NMDA and glycine are shown in Figure 2b.

Interestingly, 10 mM DTT always increased the time taken to recover from the NMDA and glycine stimulus. Thus, while $[Ca^{2+}]_i$ normally returned to basal levels rapidly when the agonists were washed out, $[Ca^{2+}]_i$ remained elevated for as long as 20 min following treatment with 10 mM DTT. Although the actual recovery time varied from cell to cell a prolongation was seen in all cells tested with 10 mM DTT ($n = 8$). This phenomenon was seen less frequently with 3 mM DTT and never seen at all with 1 mM DTT. In order to determine whether this represented an action of DTT on Ca^{2+} homeostasis rather than a specific effect on NMDA receptors, we depolarized cells with 50 mM KCl to activate voltage-sensitive Ca^{2+} channels. As shown in Figure 3, this procedure produced rapid and reversible $[Ca^{2+}]_i$ changes that were similar in magnitude to those produced by NMDA with glycine. However, while the $[Ca^{2+}]_i$ signal produced by NMDA was increased by 10 mM DTT, KCl-induced increases in $[Ca^{2+}]_i$ were not altered. This demonstrated that DTT apparently did not alter Ca^{2+} homeostatic processes in the cell, but modified responses that were associated with NMDA receptor function.

Discussion

These studies have confirmed and extended the observation that activation of the NMDA receptor can be modulated by redox reagents (Aizenman *et al.*, 1989). One goal of this investigation was to establish the mechanism by which DTT produced its effects. We employed the [^{3}H]-MK-801 binding assay to monitor the effects of DTT on the actions of glutamate, glycine and spermidine. We observed a general increase in [^{3}H]-MK-801 binding which was consistent with the increase in NMDA receptor function associated with DTT. However, we did not find any specific alterations in the effects of the modulators tested. As DTT increased [^{3}H]-MK-801 binding even in the presence of maximal levels of glutamate

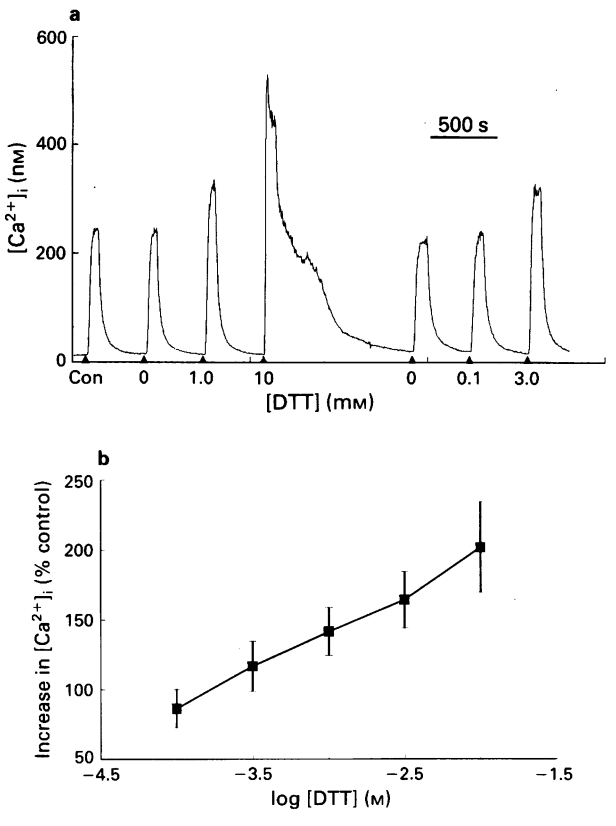

Figure 2 Effects of dithiothreitol (DTT) on N-methyl-D-aspartate (NMDA) and glycine-induced $[Ca^{2+}]_i$ changes in rat single cultured forebrain neurones. (a) $[Ca^{2+}]_i$ recording from a single neurone. NMDA (30 μM) and glycine (1 μM) were added at times indicated by triangles. Agonists were applied for 40 s and then removed by perfusion. With the exception of the first response (marked 'Con') cells were treated for 60 s with 0.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) followed by 120 s incubation with the concentration of DTT indicated, which was then washed out before agonist application. DTNB treatment alone reduced responses to 89 ± 5% of control levels. Where the DTT concentration is zero, cells were pretreated with DTNB only before NMDA and glycine application, thus indicating that DTNB completely reversed the actions of DTT. Following agonist application cells were perfused continuously until $[Ca^{2+}]_i$ levels returned to baseline. (b) Concentration-response relationship for effect of DTT on responses to NMDA and glycine. The data represent the peak NMDA and glycine-induced increase of $[Ca^{2+}]_i$ above baseline $[Ca^{2+}]_i$ levels in DTT-treated cells and are expressed as a percentage of control (untreated) responses. Each data point represents the mean of 4–8 cells and vertical lines show s.e.mean. Up to six different DTT concentrations were tested per cell. Responses to NMDA and glycine following DTNB but not DTT treatment near the end of each cell were 94 ± 10% of control responses, suggesting that responses were relatively stable even over the 60–90 min duration of these experiments.

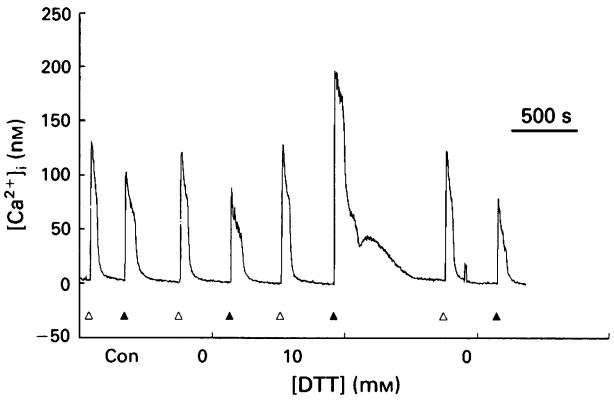

Figure 3 Effects of dithiothreitol (DTT) on $[Ca^{2+}]_i$ responses produced by N-methyl-D-aspartate (NMDA) and glycine and by depolarization with KCl in single cultured rat forebrain neurones. The trace represents a $[Ca^{2+}]_i$ recording from a single neurone. NMDA (30 μM) and glycine (1 μM) together, or KCl (50 mM) were added at times indicated by closed and open triangles, respectively. Agonists were applied for 40 s and then removed by perfusion. With the exception of the first response (marked 'Con') cells were treated for 60 s with 0.5 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) followed by 120 s incubation with the concentration of DTT indicated, which was then washed out before agonist application. Where the DTT concentration is zero, cells were pretreated with DTNB only before NMDA and glycine application, thus indicating that DTNB completely reversed the actions of DTT. Following agonist application cells were perfused continuously until $[Ca^{2+}]_i$ levels returned to baseline. This trace is representative of four separate cells tested under these conditions.

and glycine it is unlikely that DTT acts simply by increasing the affinity of these ligands. Consistent with this, we did not observe an increase in affinity of glutamate and glycine. Moreover, DTT did not increase the degree to which glutamate and glycine enhanced [^{3}H]-MK-801 binding. Finally, DTT had little effect on the actions of the competitive glutamate and glycine antagonists CGS 19755 and 7-chlorokynurenate. Together these findings suggest that the redox site that is the target of the action of DTT and DTNB is probably not located within the binding site for glutamate or glycine.

The actions of polyamines are a little more complex. It has been proposed that polyamines are positive modulators of the NMDA receptor based on their effects on [^{3}H]-MK-801 or [^{3}H]-TCP binding (Ransom & Stec, 1988; Reynolds & Miller, 1989a; Williams *et al.*, 1989). DTT did not increase the magnitude of the stimulant actions of spermidine, and the concentration-response curve was only slightly shifted to the left. However, DTT did enhance the inhibitory actions of spermidine which are observed at higher concentrations of this polyamine. We have previously found that the decrease in [^{3}H]-MK-801 binding produced by high concentrations of polyamines is more pronounced when the receptor is fully activated (Reynolds & Miller, 1989a). Thus, the interaction between spermidine and DTT is consistent with a more activated state of the receptor. Conversely, DTNB reduced the overall action of spermidine. In addition, we observed that the actions of the competitive polyamine antagonist, arcaine (Reynolds, 1990), were less potent in the presence of DTT. The significance of these findings remains obscure as a physiological effect of the polyamines has not yet been demonstrated. In the absence of a profound modification of the actions of spermidine it seems unlikely that DTT or DTNB modifies the polyamine binding site directly either. As polyamines are synthesized intracellularly and there is little evidence to suggest that they are secreted, it seems reasonable to suggest that the polyamine site may be located on the intracellular face of the receptor. The physiological actions of DTT that we have observed are probably restricted to the external face of the receptor, since this reducing agent is not lipid permeable. In addition, it is unlikely that the effects of DTT are mediated by a second messenger as effects on NMDA receptors have been observed electrophysiologically in excised outside-out patches of membrane (Wright & Nowak, 1990). Thus, it is unlikely that the actions of DTT are mediated by a modification of the polyamine site.

Perhaps the most interesting findings of this study relate to the effects of DTT on NMDA-induced Ca^{2+} influx. Many of the physiological and pathophysiological consequences of activating NMDA receptors, including neuronal cell death, are directly related to Ca^{2+} and presumably increased $[Ca^{2+}]_i$ (MacDermott *et al.*, 1986; Choi, 1987). In this study we have demonstrated that the increases in ligand binding to the NMDA receptor are accompanied by increases in NMDA-induced changes in $[Ca^{2+}]_i$. These actions of DTT may be considered specific chemical changes to NMDA receptors in that (i) DTT does not have to be present during NMDA exposure to produce an enhanced response, (ii) all the effects of DTT can be reversed by DTNB, and (iii) DTT does

not alter changes in $[Ca^{2+}]_i$ produced by KCl depolarization. In contrast to results obtained with the [^{3}H]-MK-801 binding assay, we did not observe a decrease in NMDA-induced changes in $[Ca^{2+}]_i$ at higher concentrations of DTT. However, unlike the [^{3}H]-MK-801 binding assay, DTT was washed out before the addition of agonists, whereas DTT was present continuously during the incubation of the binding assay. The inhibition observed in the [^{3}H]-MK-801 assay probably reflects a low affinity inhibitory action of DTT that is unrelated to modification of sulphydryl groups.

An intriguing action of higher concentrations of DTT is illustrated by Figure 2a. Following treatment with 10 mM DTT the duration of the NMDA-induced $[Ca^{2+}]_i$ change was drastically increased. The time taken for the recovery from the NMDA application, which lasted 40 s, was greatly in excess of the time necessary to exchange the bath volume completely with rinse solution many times. We initially suspected that the prolonged Ca^{2+} signal was a consequence of delayed extrusion of free Ca^{2+} from the cytoplasm. However, as demonstrated in Figure 3, a response to a potassium depolarization of similar magnitude to the NMDA response was not altered by DTT. This observation apparently excludes the possibility that altered Ca^{2+} homeostasis can account for the difference. It is possible that NMDA and KCl increase $[Ca^{2+}]_i$ in different compartments within the cell, which would allow differential modulation of Ca^{2+} homeostasis by DTT. It is also interesting to note that the concentration-dependence of the two actions of DTT may be different. Thus, DTT increases the peak $[Ca^{2+}]_i$ response at lower concentrations than are required to prolong the $[Ca^{2+}]_i$ signal. This may indicate that modification of two or more distinct disulphide bonds are involved in these processes. Nevertheless, the physiological consequences of this modification remain to be determined.

Several studies have shown that a certain minimum time of trauma is required in cultured neurones in order to produce delayed cell death. If the NMDA receptor can be reduced *in vivo* by physiological agents, it is possible that this would result in a sensitizing effect to marginal traumatic events. Alternatively, regional variations in constitutive levels of reduction or oxidation could underlie the selective vulnerability that has been observed in many neurotoxicity studies (Pulsinelli, 1985; Wieloch, 1985).

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CI-977, a novel and selective agonist for the κ-opioid receptor

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1 CI-977 is a new, nonpeptide κ-opioid compound that has been synthesized and its pharmacological properties determined in a series of *in vitro* and *in vivo* rodent models.

2 In radioligand binding studies, with guinea-pig forebrain homogenates, CI-977 bound with high affinity to [^{3}H]-U69593-labelled κ-sites (K_i = 0.11 nM) but with low affinity to [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO) labelled μ-sites (K_i = 99 nM) and [^{3}H]-[D-Pen^{2,5}]enkephalin (DPDPE) labelled δ-sites (K_i = 1.04 μM). CI-977 also bound with negligible affinity to [^{3}H]-(+)-3-(1-propyl-3-piperidinyl)phenol (3-PPP) labelled σ-sites (K_i = 1.9 μM) and [^{3}H]-1-(1-[2-thienyl]cyclohexyl)piperidine (TCP) labelled PCP sites (K_i > 10 μM).

3 CI-977 produced a potent inhibition of the electrically-evoked contractions of the guinea-pig ileum and rabbit vas deferens with IC_{50} values of 0.087 nM and 3.3 nM, respectively. The pK_B values for the opioid antagonists naloxone (7.6) and norbinaltorphimine (10.5) supported the κ nature of the CI-977-mediated effects in the smooth muscle assays.

4 CI-977 was a potent antinociceptive agent against a mechanical noxious stimulus in rats following intravenous, intramuscular, subcutaneous and oral administration. CI-977 was also effective against mechanical and chemical noxious stimuli in the mouse but ineffective against a thermal stimulus. The antinociceptive effects produced by CI-977 were completely reversed by naloxone (1 mg kg^{-1}, s.c.).

5 At doses close to those required to produce antinociception, CI-977 also caused a naloxone-reversible diuresis and inhibition of locomotor activity.

6 The *in vitro* and *in vivo* pharmacological profile of CI-977 demonstrates that it is a potent and selective agonist at the κ-opioid receptor.

Introduction

The κ-opioid receptor can be clearly distinguished from either the μ or δ sub-types by various *in vitro* biochemical and pharmacological models (Chang, 1984; Paterson *et al.*, 1984). In addition, with the main exception of antinociception, activation of the κ-receptor *in vivo* has been associated with a distinct behavioural profile. Thus, κ-agonists have been shown to cause sedation and diuresis at doses close to those producing antinociception (Leighton *et al.*, 1987; Dykstra *et al.*, 1987) but, more importantly, would also appear not to cause emesis, constipation, respiratory depression or to have a dependence liability; effects particularly associated with occupation of the μ-receptor (Martin, 1984).

These characteristics have been established principally through the use of compounds from the recent arylacetamide series such as U50488, U69593 and PD117302, which have demonstrated suitable selectivity for the κ-receptor (Von Voigtlander *et al.*, 1983; Lahti *et al.*, 1985; Clark *et al.*, 1988).

Previously, it had been difficult to ascribe a definitive pattern of behaviour to a specific action at the κ-receptor. This was mainly a consequence of the poor selectivity of the prototype benzomorphan ligands, such as ketocyclazocine, which were used in the original classification of the receptor (Martin *et al.*, 1976). These compounds have been shown to interact with comparable potency at μ- and δ-opioid, (Kosterlitz *et al.*, 1981; Gillan & Kosterlitz, 1982) as well as non-opioid (Zukin & Zukin, 1981; Su, 1982; Tam, 1983) types of receptor.

Nevertheless, despite this lack of selectivity, benzomorphans such as ethylketocyclazocine and bremazocine have maintained a higher affinity and/or potency at the κ-receptor than many of the arylacetamides (Lahti *et al.*, 1985; Leighton *et al.*, 1987; Clark *et al.*, 1988).

In the present study we describe the pharmacological characteristics of CI-977, a novel compound from the arylacetamide series that is under clinical evaluation at present. CI-977 exhibited the highest affinity yet described for an arylacetamide at the κ-receptor and, in addition, had the highest potency and efficacy in a series of *in vitro* and *in vivo* functional models.

A preliminary account of the results obtained in this study has been communicated to the British Pharmacological Society (Hunter *et al.*, 1990).

Methods

Radioligand binding assays

Guinea-pig forebrain homogenates were prepared as previously described (Smith *et al.*, 1989; Meecham *et al.*, 1989). Briefly, membrane homogenates were resuspended in 50 mM Tris-HCl (5 mM Tris-HCl for the [^{3}H]-TCP assay), to a final concentration of 10 mg ml^{-1} wet weight of original tissue. Membrane aliquots (400 μl) were added to a final incubation assay volume of 500 μl consisting of a single concentration of radioligand and at least six increasing concentrations of unlabelled ligand. The concentrations of the opioid radioligands were approximately equal to the respective equilibrium dissociation constants (K_D) determined by saturation analysis in a separate series of experiments (Meecham *et al.*, 1989). The radioligands used (K_D in parentheses) were the κ-selective [^{3}H]-U69593 (1.0 nM), the μ-selective [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO; 0.4 nM) and the δ-selective [^{3}H]-[D-Pen^{2,5}] enkephalin (DPDPE; 2.0 nM). The non-specific binding was determined as that remaining in the presence of 1 μM naloxone. [^{3}H]-(+)-3-(1-propyl-3-piperidinyl)- phenol (3-PPP; 1 nM) and [^{3}H]-1-(1[2-thienyl]cyclohexyl)- piperidine (TCP; 1 nM) were used to label the σ and phencyclidine binding sites, respectively. Non-specific binding was defined by 5 μM pentazocine ([^{3}H]-(+)-3-PPP) and 5 μM phencyclidine ([^{3}H]-TCP). The K_D values for [^{3}H]-(+)-3-PPP (30 nM) and [^{3}H]-TCP (7 nM) were determined in a preliminary series of experiments. All radioligands were incubated at 25°C for 80 min, with the exception of [^{3}H]-TCP (30 min), following which the assay was terminated by rapid filtration through Whatman GF/B glass fibre filter strips on a Brandel M-48 cell harvester. The filters were then washed with

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3 × 4 ml ice-cold 50 nM Tris-HCl and the radioactivity determined by liquid scintillation spectrometry.

Analysis of binding data

The competition binding data were analysed by ALLFIT (DeLean *et al.*, 1978) and the K_i values calculated from the Cheng-Prusoff (1973) equation: $K_i = IC_{50}/[1 + L/K_D]$, where the IC_{50} represents the concentration which inhibited 50% of the radioligand specific binding and L and K_D represent the assay concentration and equilibrium dissociation constant for the radioligand respectively.

In vitro *smooth muscle preparations*

The guinea-pig ileum and rabbit vas deferens bioassay preparations were set up as described by previous studies from this laboratory (Clark *et al.*, 1988; Meecham *et al.*, 1989).

Control log dose-response curves and those in the presence of antagonist were analysed by ALLFIT (DeLean *et al.*, 1978) and dose-ratios calculated at the IC_{50} level. The pK_B (negative log of the equilibrium dissociation constant) values for either the non-selective opioid antagonist, naloxone, or the more κ-selective antagonist norbinaltorphimine (nor-BNI) (Portoghese *et al.*, 1987; Birch *et al.*, 1987) were then determined by the method initially defined by Arunlakshana & Schild (1959).

In vivo *antinociception models*

The methods used to evaluate antinociceptive activity in tests employing different types of noxious stimuli, i.e. mechanical, chemical and thermal, were performed as previously described (Leighton *et al.*, 1987). The effects of test compounds on nociceptive pressure (mechanical) thresholds were determined in either the rat (70–100 g, Wistar males, Interfauna, Huntingdon, U.K.) paw pressure or mouse (20–25 g, CFLP males, Interfauna) tailclip models. The effect of test compounds on chemically- and thermally-induced nociceptive thresholds were determined by use of the mouse acetylcholine-induced abdominal constriction and hotplate models, respectively (Leighton *et al.*, 1987).

Route of administration

For all compounds under investigation in the present study, animals were dosed by either the intravenous (i.v.), subcutaneous (s.c.) or oral (p.o.) route of administration in a manner previously described (Leighton *et al.*, 1987).

In addition, CI-977 was also assessed for antinociceptive activity in the rat paw pressure test following intramuscular dosing. Animals were dosed by this route 5 min before testing with a dose volume of 0.01 ml per 100 g injected into the large muscle group at the back of the thigh.

To determine the time course of antinociceptive activity rats were dosed with CI-977 either intramuscularly or orally and the nociceptive response to a mechanical (paw pressure) stimulus determined at one of the following time intervals: 5, 10, 15, 30, 45, 60, 120, 180, 240, 300 or 360 min after dosing. Of the rats used none was tested more than once.

Additional in vivo *tests*

Locomotor activity Male mice (CFLP, 35–50 g, Interfauna, Huntingdon) were group housed under conditions of constant temperature (21 ± 1°C) and humidity on a fixed 12 h light 12 h dark cycle with food and water available *ad libitum*. Locomotor activity was measured by an automated activity monitoring system consisting of twelve clear polycarbonate cages each bi-sected by a single infrared beam (Marshall *et al.*, 1987). Test compounds were administered subcutaneously in a dose volume of 1 ml per 100 g body weight and immediately after dosing the animals were placed individually in the cages of the activity monitoring system. Locomotor activity was measured for 1 h following the placement of the animals in the activity monitor. All experiments were timed to start at the beginning of the dark period of the light/dark cycle. A group size of nine animals was used for all treatments.

Urine output The methodology for measuring the effect of compounds on urine output in rats was as described in previous studies from this laboratory (Leighton *et al.*, 1987). Briefly, male, normally hydrated Wistar rats (250–350 g) were injected subcutaneously with a test substance and then placed individually in metabolism cages. Urine output was then determined over a subsequent 6 h period. Group sizes of 6 animals were used throughout.

Materials

The following drugs were obtained from external sources: ethylketocyclazocine (Sterling-Winthrop), bremazocine (Sandoz), MR2034 ((−)-α-(1**R**,5**R**,9**R**)-5,9-di-methyl-2-(L-tetrahydrofurfuryl)-2′-hydroxy-6,7-benzomorphan, Boehringer-Ingelheim), DAMGO (Cambridge Research Biochemicals), DPDPE (Bachem), naloxone hydrochloride (Sigma) and U69593 ((5α, 7α, 8β)-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5) dec-8-yl]benzeneacetamide, Upjohn Co.).

U50488 (*trans*-(±)-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulphonate; spiradoline (5α,7α,8β)-(±)-3,4-dichloro-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide mesylate); PD117302 ((±)-*trans*-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[b]thiophene-4-acetamide monohydrochloride) and CI-977 ((5**R**)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide monohydrochloride) were all synthesized in the Department of Medicinal Chemistry, Parke-Davis Research Unit, Cambridge, U.K.

The following radioligands were used: [^{3}H]-U69593 (40–60 Ci mmol^{-1}), [^{3}H]-(+)-3-PPP (80–110 Ci mmol^{-1}), and [^{3}H]-TCP (40–60 Ci mmol^{-1}) were all purchased from New England Nuclear. [^{3}H]-DAMGO (40–60 Ci mmol^{-1}) and [^{3}H]-DPDPE (25–50 Ci mmol^{-1}) were both obtained from Amersham International.

Results

Radioligand binding studies

CI-977 produced a concentration-dependent inhibition of specific [^{3}H]-U69593 (κ) binding with a K_i value of 0.11 nM (Table 1). CI-977 also inhibited specific [^{3}H]-DAMGO (μ) and [^{3}H]-DPDPE (δ) binding with K_i values of 99.6 nM and 1036 nM, respectively (Table 1). The μ/κ and δ/κ ratios were 905 and 9420, respectively.

In comparison ethylketocyclazocine (EKC, 0.21 nM), bremazocine (0.12 nM) and MR2034 (0.09 nM) had κ affinities comparable to CI-977, but were considerably less selective with μ/κ ratios between 3.4 and 4.9 and δ/κ ratios between 9.2 and 42 (Table 1). In contrast, U50488 (0.69 nM), U69593 (0.67 nM), PD117302 (0.5 nM) and, to a lesser extent spiradoline (0.35 nM), had lower affinities for the κ-sites, and only U69593 (3672) had a higher μ/κ ratio, compared with CI-977 (Table 1). However, the arylacetamides in general had substantially higher κ selectivity than the benzomorphans with μ/κ and δ/κ ratios increasing from 125 (spiradoline) and 9418 (CI-977), respectively.

CI-977 was found to inhibit specific [^{3}H]-(+)-3-PPP binding to σ sites with a K_i value of 1.9 μM but had no detectable affinity (>10 μM) for the [^{3}H]-TCP-labelled phencyclidine (PCP) binding sites (Table 2). U50488 (1.04 μM), spiradoline (1.06 μM), U69593 (>10 μM) and PD117302 (1.2 μM) all had low affinities similar to CI-977 for the σ-site while bremazocine (496 nM), MR2034 (287 nM) and EKC (495 nM) were found to have higher affinities for this site (Table 2). At the PCP site, bremazocine (156 nM), MR2034

Table 1 Inhibition constants (K_i) obtained for CI-977 and standard opioid ligands at κ, μ and δ binding sites in guinea-pig forebrain membranes

Ligand	κ [^3H]-*U69593* K_i values (nM)	μ [^3H]-*DAMGO* K_i values (nM)	μ/κ	δ [^3H]-*DPDPE* K_i values (nM)	δ/κ
CI-977	0.11 ± 0.02 (6)	99.6 ± 4.3 (9)	905	1036 ± 77 (7)	9418
PD117302	0.50 ± 0.05 (6)	399 ± 90 (5)	798	7200 ± 920 (4)	14400
U50488	0.69 ± 0.04 (4)	435 ± 95 (2)	630	9200 ± 1400 (2)	13333
U69593	0.67 ± 0.04 (7)	2460 ± 157 (5)	3672	9900 ± 810 (4)	14776
Spiradoline	0.35 ± 0.06 (5)	43.7 ± 3.9 (6)	125	4530 ± 300 (6)	12943
Bremazocine	0.12 ± 0.006 (3)	0.46 ± 0.06 (3)	3.8	1.1 ± 0.1 (3)	9.2
MR2034	0.09 ± 0.02 (3)	0.31 ± 0.06 (5)	3.4	3.8 ± 0.2 (4)	42
EKC	0.21 ± 0.03 (5)	1.02 ± 0.25 (4)	4.9	7.0 ± 1.6 (4)	33
DAMGO	164 ± 16 (3)	0.55 ± 0.05 (3)	—	177 ± 14 (3)	—
DPDPE	>10000 (4)	790 ± 84 (4)	—	1.4 ± 0.2 (4)	—

Each value represents the mean ± s.e.mean for the number of experiments shown in parentheses, with each experiment performed in triplicate. EKC = ethylketocyclazocine.

(1.5 μM) and EKC (7.2 μM) had low, but demonstrable affinities while all arylacetamide ligands had no detectable affinity up to a concentration of 10 μM (Table 2).

In vitro *smooth muscle preparations*

CI-977 produced a concentration-dependent inhibition of the contractile responses to nerve stimulation of the guinea-pig ileum (GPI) and rabbit vas deferens (LVD) (Figure 1), with a full suppression of the nerve response at the highest doses tested. The IC_{50} values were 0.087 nM and 3.3 nM in the GPI and LVD respectively (Table 3).

The CI-977 responses were antagonized in a competitive manner by the relatively non-selective opioid antagonist naloxone and the κ-selective antagonist norbinaltorphimine (norBNI). The pK_B values (mean ± s.e. mean) following Schild plot analysis were 7.6 ± 0.1 (GPI) and 7.5 ± 0.1 (LVD) for naloxone and 10.5 ± 0.2 for nor-BNI in the ileum. Slope values in each case were not significantly different from unity ($P > 0.1$; Mann-Whitney U-Test).

The arylacetamide ligands, U50488, U69593, PD117302 and spiradoline, all produced responses qualitatively similar to CI-977 in both tissues but with IC_{50} values 10 to 40 fold lower in the GPI and 10 to 70 fold lower in the LVD (Figure 1).

In comparison, the benzomorphans EKC (IC_{50} = 0.26 nM), bremazocine (IC_{50} = 0.04 nM) and MR2034 (IC_{50} = 0.92 nM) all produced a full, concentration-dependent inhibition of contractions in the GPI while, in the LVD this was only achieved by EKC (Figure 1) with an IC_{50} of 22 nM (Table 3). The maximum response to MR2034 was only a partial inhibition of the nerve response with a range of 23–85%. The concentration of MR2034 that produced a response equivalent to 50% of the maximum effect of the drug was 18.4 ± 5.9 nM (n = 7). However, bremazocine produced a full inhibition in 7/19 tissues (IC_{50} = 10.1 nM; Table 3) but only a partial inhibition (Figure 1) in 12/19 tissues with a range of 25%–64%. In such tissues the concentration that produced a response equivalent to 50% of the maximum effect of the drug was 9.2 ± 2.1 nM.

Table 2 Inhibition constants (K_i) obtained for CI-977 and standard κ opioid ligands at σ and phencyclidine binding sites in guinea-pig forebrain membranes

Ligand	σ [^3H]-(+)-*3-PPP* K_i values (nM)	*Phencyclidine* [^3H]-*TCP* K_i values (nM)
CI-977	1916 ± 215 (5)	>10000 (3)
PD117302	1173 ± 236 (5)	>10000 (3)
U50488	1044 ± 146 (5)	>10000 (3)
U69593	>10000 (3)	>10000 (3)
Spiradoline	1056 ± 70 (5)	>10000 (3)
Bremazocine	496 ± 40 (4)	156 ± 12 (5)
MR2034	287 ± 42 (4)	1475 ± 56 (2)
EKC	495 ± 88 (5)	7180 ± 1090 (3)

Data represent the means ± s.e.mean for the number of experiments shown in parentheses.

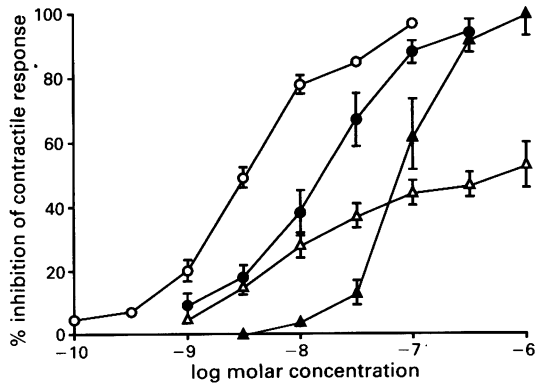

Figure 1 Concentration-dependent inhibitions of the electrically-induced contractions of the rabbit vas deferens by CI-977 (○), U62066 (●), U69593 (▲) and bremazocine (△). Each symbol represents the mean, and vertical lines the s.e.mean, of at least six independent preparations.

Antinociception in the rat

CI-977 caused a dose-dependent elevation in the paw pressure (mechanical) nociceptive threshold with MPE_{50} (dose producing a response that was 50% of the maximum possible effect

Table 3 A comparison of IC_{50} (nM) values obtained for CI-977 and reference κ-opioid ligands in the guinea-pig ileum (GPI) and rabbit vas deferens (LVD)

Compound	*GPI* (IC_{50}, nM)	*LVD* (IC_{50}, nM)
CI-977	0.087 ± 0.007 (31)	3.3 ± 0.5 (19)
PD117302	1.01 ± 0.14 (16)	58.0 ± 10.0 (11)
U50488	1.64 ± 0.09 (5)	222 ± 74 (4)
EKC	0.26 ± 0.10 (5)	22.4 ± 4.0 (4)
Spiradoline	0.88 ± 0.1 (13)	28.7 ± 4.4 (12)
U69593	3.4 ± 0.9 (7)	89.8 ± 12.1 (6)
Bremazocine	0.042 ± 0.016 (5)	10.1 ± 3.3 (7)[a]
MR2034	0.92 ± 0.07 (7)	—[b]

Each value represents the mean ± s.e.mean for the number of experiments shown in parentheses.

[a] Bremazocine produced a full inhibition of the nerve response in seven experiments. In a further twelve experiments it produced only a partial inhibition (range 22–64%) of the nerve response (see text).

[b] — MR2034 produced only a partial inhibition of the nerve response (range 23–85%) in all experiments (n = 7) (see text).

of the test) values of 0.02 and 1.8 mg kg^{-1} following intravenous (i.v.) and oral (p.o.) administration, respectively. (Figure 2a; Table 4).

The MPE_{50} values (95% confidence limits in parentheses) for CI-977 following intramuscular (i.m.) and subcutaneous (s.c.) administration were 0.07 (0.03–0.1) and 0.08 (0.04–0.1) mg kg^{-1}, respectively.

Following oral administration the antinociceptive effect for CI-977 remained significantly ($P < 0.05$, Dunnett's t test) above control levels for three hours at an MPE_{50} dose (2 mg kg^{-1}, p.o.) and for five hours at a dose (20 mg kg^{-1}, p.o.) ten fold higher than the MPE_{50} dose (Figure 3a). In comparison, at slightly below the MPE_{50} dose, CI-977 (0.03 mg kg^{-1}, i.m.) had a duration of action lasting 30 min which was prolonged with increasing dose to approximately two hours at four times (0.3 mg kg^{-1}, i.m.) the MPE_{50} dose (Figure 3b).

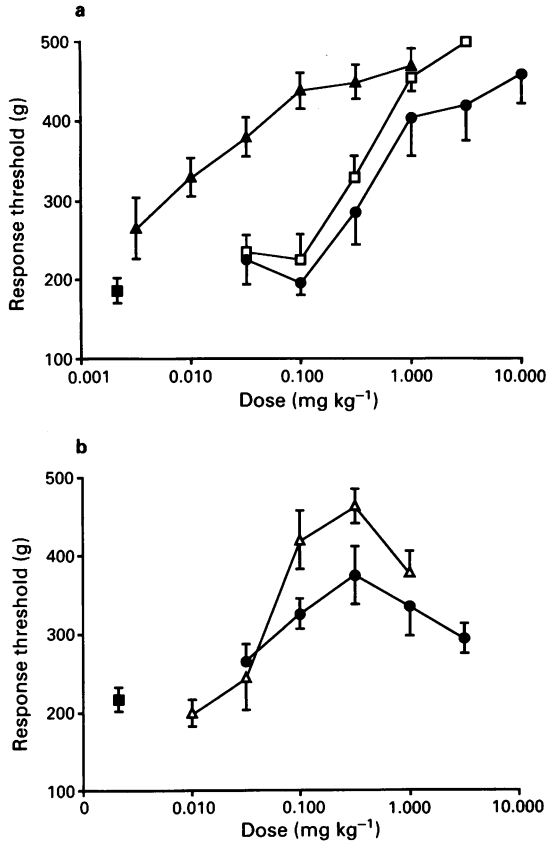

Figure 2 Dose-response curves for (a) CI-977 (▲), spiradoline (□) and U69593 (●) and (b) MR2034 (△) and bremazocine (●) in the rat paw pressure test. All compounds were given by the intravenous route of administration 5 min before testing. Each data point represents the mean, and vertical lines the s.e.mean, for groups of at least six animals. (■) Represents controls.

Table 4 Comparison of the antinociceptive effect of CI-977 with other κ-agonists in the rat paw pressure test

	MPE_{50} (mg kg^{-1}) i.v.	p.o.
CI-977	0.02 (0.008–0.043)	1.8 (1.5–2.4)
PD117302	1.41 (0.90–2.20)	25.3 (14.6–43.9)
Spiradoline	0.38 (0.16–0.87)	48.5 (29.9–78.7)
U69593	0.67 (0.32–1.28)	18.4 (4.8–71.0)
U50488	1.96 (1.67–2.29)	9.6 (4.6–19.8)
Bremazocine	—[a]	>30
EKC	0.09 (0.02–0.37)	>30
MR2034	0.07 (0.04–0.13)	>30

Each value represents the mean with 95% confidence limits in parentheses for groups of at least six animals.
[a] — The maximum antinociceptive response to bremazocine was lower (60%) than the maximum possible effect in this test. The dose of bremazocine that produced a response equivalent to 50% of the maximum effect of the drug was 0.096 mg kg^{-1} (see text).

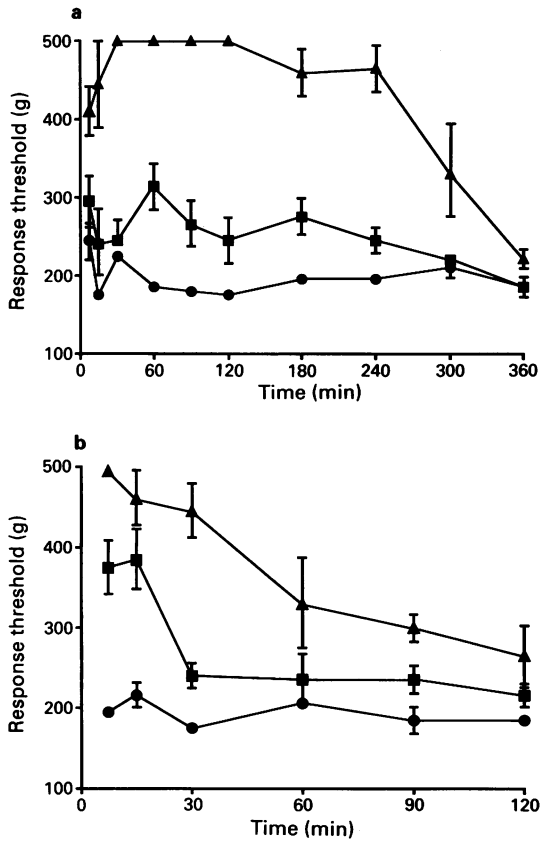

Figure 3 Duration of action of CI-977 in the rat paw pressure test following (a) oral administration, (●) control, (■) 2 mg kg^{-1} CI-977, (▲) 20 mg kg^{-1} CI-977 and (b) intramuscular administration, (●) control, (■) 0.03 mg kg^{-1} CI-977, (▲) 0.3 mg kg^{-1} CI-977. Group sizes of six were used throughout with no animal being tested more than once. Data are shown as mean and vertical lines indicate s.e.mean.

Spiradoline (Figure 2a), U69593 (Figure 2a), PD117302 and U50488, following i.v. and oral administration, all produced a dose-dependent increase in the nociceptive threshold qualitatively similar to CI-977, with maximum antinociception at the highest doses tested. However, the MPE_{50} values for these compounds (Table 4) were between 20 (spiradoline) and 100 (U50488) fold less potent (i.v.) and between 10 (U69593) and 26 (spiradoline) fold less potent (p.o.) than CI-977.

Following i.v. administration, MR2304 (Figure 2b) and EKC also produced maximum antinociception in the paw pressure test but were 3 to 5 fold less potent than CI-977. In contrast, the antinociception produced by bremazocine (i.v.) reached a plateau that was approximately 60% of the maximum possible effect attainable in this model (Figure 2b). The dose (mean with 95% confidence limits) of bremazocine that produced a response equivalent to 50% of the maximum effect of the drug was 0.096 (0.04–0.21) mg kg^{-1}. Following oral administration, none of the benzomorphans produced antinociception at doses up to 30 mg kg^{-1} (Table 4).

The opioid nature of the antinociceptive effects of all agents was confirmed by complete reversibility with naloxone (15 min pretreatment with 1 mg kg^{-1}, s.c.).

Antinociception in the mouse

CI-977 produced a dose-dependent increase in the nociceptive threshold in the acetylcholine-induced abdominal constriction (chemical) and tailclip (mechanical) nociception models with MPE_{50} values of 5 and 30 μg kg^{-1} (s.c.) respectively. In con-

trast it was ineffective up to 10 mg kg^{-1} (s.c.) in the mouse hotplate (thermal) test (Table 5).

MR2034 and U69593 were effective against both chemical and mechanical noxious stimuli but were 16 and 20 fold less potent, respectively, than CI-977 in the abdominal constriction test and 3 and 47 fold less potent, respectively, in the tailclip model. Bremazocine also caused a maximum increase in the nociceptive threshold in the abdominal constriction and tailclip tests but, in contrast to MR2034 and U69593, was equipotent with CI-977 in both models. All three compounds were inactive in the hotplate test (Table 5).

Spiradoline was effective against all three noxious stimuli including the thermal stimulus (MPE_{50} 13 mg kg^{-1}, s.c.). In the abdominal constriction and tailclip tests spiradoline was 20 and 40 fold less active, respectively, than CI-977 (Table 5).

Locomotor activity

All compounds tested produced a dose-dependent suppression of spontaneous locomotor activity (sedation) in mice with MPE_{50} values (95% confidence limits in parentheses) of 0.09 (0.03–0.23), 0.7 (0.2–3.6), 2.6 (0.6–10.2) and 6.8 (2.0–22.6) mg kg^{-1} (s.c.) for CI-977, spiradoline, PD117302 and U50488, respectively.

However, U69593 produced a biphasic effect consisting of a short lasting (30–40 min) period of hypoactivity (MPE_{50} = 2.4 (0.2–28.5) mg kg^{-1} s.c.) followed by rebound hyperactivity. All other compounds suppressed activity for the entire 60 min test period. The MPE_{50} values for bremazocine, EKC and MR2034 were 0.08 (0.04–0.15), 0.7 (0.3–1.7) and 1.5 (0.9–2.5) mg kg^{-1} (s.c.), respectively.

Diuretic effects

CI-977, spiradoline and U69593 all produced a potent diuresis in rats with MPE_{50} values (95% confidence limits in parentheses) of 0.16 (0.11–0.23), 0.65 (0.29–1.45) and 1.1 (0.4–2.6) mg kg^{-1} (s.c.), respectively. The diuresis observed with these agents was of short duration with the rate of production being maximal during the first 2 h measurement interval. The maximum volume of urine produced by each agent was 15.5 ± 1.2, 14.8 ± 1.1 and 14.1 ± 1.0 ml per 6 h for CI-977, spiradoline and U69593, respectively. Bremazocine (0.03–0.3 mg kg^{-1}, s.c.) and MR2034 (0.01–1.0 mg kg^{-1}, s.c.) also produced a diuretic response but with a maximum output of urine that was significantly below that of CI-977. The maximum volume of urine produced by bremazocine and MR2034 was 11.8 ± 0.7 and 8.2 ± 0.4 ml per 6 h, respectively. Since both benzomorphans appeared to be acting like partial agonists in this test an MPE_{50} value was not determined.

Discussion

In the present study it has been demonstrated that the novel arylacetamide CI-977 is a potent and selective agonist for the κ-opioid receptor.

In the *in vitro* opioid ligand binding and smooth muscle assays, CI-977 exhibited superior affinity and agonist potency, respectively, at the κ-receptor over the established arylacetamides. Moreover, the activity of CI-977 in each assay was either comparable to, or indeed, in the rabbit vas deferens, higher than the most potent benzomorphan ligand. Of the compounds tested only U69593 had a higher μ/κ selectivity than CI-977.

CI-977 would also appear to be more potent and of comparable selectivity to the more recently described ICI 197067 and its congeners (Costello *et al.*, 1988). The pK_B values for the non-selective opioid antagonist naloxone (Hutchinson *et al.*, 1975) and the κ-selective norbinaltorphimine (Portoghese *et al.*, 1987; Birch *et al.*, 1987) against CI-977 in the smooth muscle assays confirmed that the agonist effects of the compound were mediated through the κ-receptor.

In the rabbit vas deferens, a tissue with a lower κ-receptor reserve than the ileum (Miller *et al.*, 1986), there was, in most cases, an increase in the potency of CI-977 relative to the other agents, which would be consistent with it having a higher relative efficacy (Kenakin, 1984). This was further exemplified by comparing the ratio of agonist potency to receptor affinity which, as an empirical measure of receptor occupancy (Kenakin, 1984), was found to be between 3 and 10 fold lower for CI-977 relative to those agents observed to be full agonists in the rabbit vas deferens. However, the difference in efficacy was particularly apparent with bremazocine and MR2034 both of which failed to inhibit completely the contractions of the vas deferens at maximally-effective concentrations, indicative of partial agonism (Miller *et al.*, 1986; Verlinde & De Ranter, 1988). In contrast, in approximately 40% of vasa investigated, a sufficient κ-receptor reserve appeared to be present for bremazocine to act as a full agonist, such variability confirming previous observations (Verlinde & De Ranter, 1988).

In the *in vivo* rodent nociception models, following systemic administration, against either a mechanical or chemical noxious stimulus, the relative potencies for antinociception followed a similar pattern to those obtained in the *in vitro* assays. CI-977 was clearly the most potent antinociceptive compound of the arylacetamide series in the rat paw pressure and mouse tailclip and writhing models. Moreover, CI-977 again had a higher potency than the benzomorphans, with the exception of bremazocine which was equipotent with CI-977 in both the mouse tests. However, in the paw pressure test the

Table 5 A comparison between the effects of CI-977 and other κ-agonists against different types of noxious stimuli in the mouse

	Tailclip	*Abdominal constriction* MPE_{50} (mg kg^{-1})	*Hotplate*
CI-977	0.03 (0.008–0.12)	0.005 (0.003–0.009)	>30
PD117302[a]	2.2	0.8	>100
Spiradoline	0.81 (0.21–3.2)	0.3 (0.08–1.2)	13.1 (9.2–18.7)
U69593	1.4 (0.63–3.3)	0.6 (0.03–0.11)	>30
U50488[a]	6.4	1.0	>100
Bremazocine	0.03 (0.005–0.138)	0.006 (0.002–0.016)	>30
MR2034	0.08 (0.01–0.54)	0.08 (0.0003–17.9)	>30
EKC[a]	NT	0.2	>30

Each value represents the mean, with 95% confidence limits in parentheses where appropriate, for groups of at least six animals.
NT = not tested.
[a] Mean MPE_{50} values taken from Leighton *et al.* (1987).

potency and efficacy of bremazocine was considerably lower, as shown by the low ceiling to its effect when compared with CI-977.

The additional ineffectiveness of CI-977 against a thermal noxious stimulus of the intensity applied in the mouse hotplate (55°C) was consistent with its profile as a κ-receptor agonist (Tyers, 1980; Leighton *et al.*, 1987).

Interestingly, although most of the arylacetamides and benzomorphans shared a similar lack of antinociceptive potency in this model, spiradoline was found to be active. However, rather than being an indication of increased efficacy at the κ-receptor, the activity of spiradoline is more likely to be mediated through the μ-receptor. Previous studies have demonstrated that the (+)-enantiomers of racemic benzeneacetamides have agonist potency and selectivity for the μ-receptor (Von Voigtlander & Lewis, 1988; Meecham *et al.*, 1989).

CI-977 would also appear to have a clear superiority in potency when compared to morphine particularly when this involves mechanical and chemical noxious stimuli. However, morphine, as would be expected of a μ-agonist, maintains its potency against the type of thermal stimulus intensity used in the hotplate test (Leighton *et* al., 1987).

The pronounced sedation and diuresis produced by CI-977 at doses close to the antinociceptive dose was again consistent with the expected profile for a κ-receptor agonist (Leander, 1983; Ukai & Kameyama 1985; Leighton *et al.*, 1987; Dykstra *et al.*, 1987; Peters *et al.*, 1987). Furthermore, CI-977 does not cause inhibition of gastrointestinal motility, a dependence liability or respiratory depression (unpublished observations), effects normally associated with actions at the μ-receptor (Martin, 1984). The rank order of potency for the sedative and diuretic properties was similar to that obtained for antinociception in the rat paw pressure test. Moreover, the partial agonist properties of both bremazocine and MR2034 were again demonstrated by the low ceiling nature of the volume of urine produced by each agent relative to CI-977.

It has been suggested that σ, as well as PCP, sites might play some role in the mediation of the dysphoric (or psychotomimetic) behaviour associated with some of the previous, non-selective benzomorphan κ-agonists (Martin *et al.*, 1976; Steinfels *et al.*, 1988; Tam *et al.*, 1988). In this respect the negligible affinity of CI-977 at either site could be considered advantageous and might, therefore, improve the therapeutic utility of this compound.

However, additional studies have suggested that κ-receptors themselves may be involved in the mediation of dysphoria (Pfeiffer *et al.*, 1986; Shearman & Stenfors, 1986; Shippenberg *et al.*, 1988). Consistent with this notion spiradoline has been shown to be clinically dysphoric (Peters & Gaylor, 1989). However, although spiradoline exhibited acceptable κ-selectivity in the *in vitro* tests, the selectivity of the compound *in vivo* remains questionable (Von Voigtlander & Lewis, 1988; Peters & Gaylor, 1989), perhaps due to its relative lack of potency in producing an antinociceptive response. It should, therefore, prove interesting to observe the clinical effects of a compound like CI-977 that maintains its selectivity for the κ-receptor in animal behavioural models.

In conclusion, CI-977 was the only arylacetamide to have both an affinity and potency at the κ-receptor that is comparable to the most potent benzomorphan ligands. In addition, it would appear that CI-977 may also have a higher relative efficacy. It remains to be observed whether these apparent advantages can be translated into a clinical analgesic efficacy that will be comparable to the currently-available μ-agents such as morphine.

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Volatile anaesthetics inhibit a cyclic AMP-dependent sodium-potassium current in cultured sensory neurones of bullfrog

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1 Cultured dorsal root ganglion cells of the bullfrog were voltage-clamped in the whole-cell configuration.

2 An adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent cationic inward rectifier (I_H) was inhibited by bath application of enflurane (0.2–0.8 mM) and halothane (0.2–0.5 mM), which thereby induced an outward current at the resting potential, and a membrane hyperpolarization in unclamped cells.

3 The main effect of enflurane (0.5 mM) was to displace the steady-state I_H activation curve to a hyperpolarizing direction by about 10 mV, as well as to reduce the maximum H-conductance to about 20%.

4 Forskolin (1–10 μM), which enhances I_H by producing a depolarizing shift in the I_H activation curve and increasing the maximum H-conductance, recruited I_H even when the current had already been eliminated by enflurane (1 mM).

Introduction

It has been proposed that general anaesthetics may affect metabolisms of intracellular cyclic nucleotides (Triner *et al.*, 1980; Vulliemoz *et al.*, 1986) and that adenosine 3':5'-cyclic monophosphate (cyclic AMP) regulates the resting membrane potential of vertebrate nerve cells by changing the voltage-dependence of I_H (Bobker & Williams, 1989; Pape & McCormick, 1989; Tokimasa & Akasu, 1990a). Therefore, the effects of enflurane and halothane were tested on I_H of bullfrog primary afferent neurones which is also regulated by cyclic AMP-dependent protein kinase (Tokimasa & Akasu, 1990b).

Methods

Small (200–250 g) bullfrogs (*Rana catesbeiana*) were used. The whole-cell I_H was recorded from A-cells at 22–24°C as described previously (Tokimasa & Akasu, 1990b): the cells were superfused with a nominally calcium-free Ringer solution containing tetrodotoxin (3 μM, Sankyo), 4-aminopyridine (1 mM, Sigma) and the chloride salt of sodium (80 mM), potassium (5 mM), magnesium (10 mM), cobalt (2 mM), barium (2 mM) and tetraethylammonium (20 mM) (pH adjusted with 4 mM-HEPES, 1 mM-Tris and HCl to 7.2). A pipette solution contained Na_2ATP (5 mM, Sigma) and EGTA (1 mM, Sigma) (Tokimasa & Akasu, 1990a,b). The concentration of enflurane (Dinabbott) and halothane (Takeda) in the recording chamber was determined by gas chromatography (see Fujiwara *et al.*, 1988). Statistics are expressed as mean ± s.e.mean for the cells tested.

Results

I_H was activated during step hyperpolarizations (10–70 mV, 1–10 s) from a holding potential less negative than −60 mV or the threshold of I_H activation. Cyclic AMP-dependence of the properties of I_H was essentially the same as described previously (Tokimasa & Akasu, 1990a,b). Bath application of enflurane (0.2–0.8 mM) decreased the amplitude of I_H in all twenty cells tested (Figure 1a–c) without significantly changing the reversal potential of I_H; −27.1 ± 2.1 mV in control and −25.5 ± 2.2 mV in the presence of 0.2 mM enflurane ($n = 3$). The main effects of enflurane (0.5 mM) on I_H were as follows. First, enflurane caused an outward current at the resting potential (e.g. 165 pA at −75 mV in Figure 1a) by reducing a tonic inward flow of I_H, which thereby induced a membrane hyperpolarization (4–6 mV) in unclamped cells ($n = 3$, not shown). Second, the maximum H-conductance (12–21 nS at −120 mV) was reduced to 21 ± 2% ($n = 6$). Finally, the steady-state I_H activation curve now centred at −102.3 ± 3.1 mV as opposed to −91.6 ± 2.1 mV in control ($n = 5$). Consistent with this, I_H activated more slowly but deactivated more rapidly in the presence of enflurane than in the control ($n = 20$). In seven cells, the voltage-dependence of the time constant for the final exponential phase (see Tokimasa & Akasu, 1990a) of the activation (e-fold per 22.1 ± 2.3 mV) and deactivation (e-fold per 15.5 ± 1.4 mV) was not significantly changed by enflurane. In all three cells, essentially the same results as with enflurane were obtained with halothane (0.2–0.5 mM, not shown).

Enflurane (0.5–0.8 mM) depressed I_H even when the current had already been enhanced by forskolin (10 μM, Sigma) in all three cells tested. Enflurane now produced an outward (hyperpolarizing) shift of the holding current even at the holding potential of −50 mV ($n = 2$). These observations are compatible with the forskolin-induced displacement in the threshold potential for I_H from −60 to about −45 mV (Tokimasa & Akasu, 1990a,b). Forskolin (10 μM) recruited I_H even when the current had already been depressed by 0.5 mM enflurane ($n = 3$, Figure 1d) or eliminated by 1 mM enflurane ($n = 1$, not shown). However, comparable I_H recruitment by forskolin was never encountered when I_H was eliminated by 2 mM caesium ($n = 3$).

Discussion

The results presented here have demonstrated that enflurane and halothane at concentrations similar to those used clinically (0.2–1.2 mM, see Fujiwara *et al.*, 1988) inhibit I_H, an action opposite to that of forskolin but quite similar to that of protein kinase inhibitors such as H-8 (Tokimasa & Akasu, 1990a,b). Therefore, it can be speculated that primary target sites for the general anaesthetics could be related to direct actions of the anaesthetics on adenylate cyclase, phosphodiesterase or cyclic AMP-dependent protein kinase. The interaction between forskolin and enflurane on I_H (Figure 1d) may

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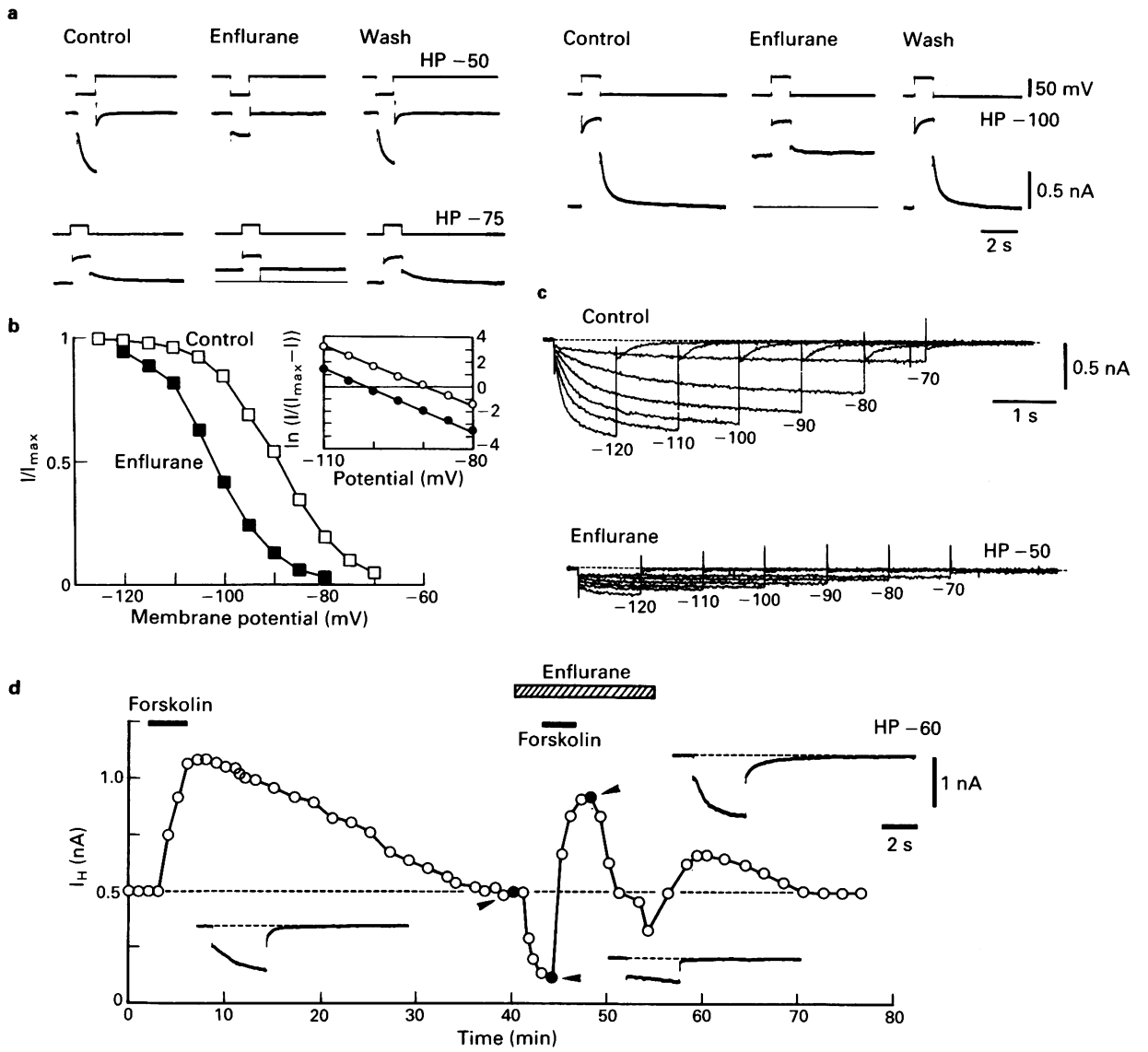

Figure 1 I_H inhibition by enflurane (0.5 mM). Results in (a)–(c) were obtained from a single cell. The resting potential was −75 mV when examined in the normal Ringer solution. (a) I_H was activated either briefly (1 s) during step hyperpolarizations from the holding potential (HP) of −50 to −100 mV (upper left panel) or continuously by setting the HP at −75 mV (lower left panel) and −100 mV (right panel). I_H was then deactivated for 1 s during step depolarizations to −50 mV either from −75 or −100 mV. Each column within the panels represents I_H before (left), during (middle) and after washout (right) of enflurane. Enflurane caused 170 pA outward shift of the holding current at HP −75 mV (700 pA at −100 mV). (b) The steady-state I_H activation curve in control (□, ○) and in the presence of enflurane (■, ●). I/I_{max} denotes the fractional I_H at given potentials (Tokimasa & Akasu, 1990a). Inset represents a semi-log plot of $I/(I - I_{max})$ showing that a hyperpolarizing shift in the half-activation voltage of the activation curve from −89 mV to −102 mV. (c) Sample recordings plotted in (b). Commanding levels are indicated beside each current trace. (d) Results were obtained from another cell. Ordinate scale denotes the amplitude of the activating I_H at −95 mV (HP −60 mV) during the course of experiments in which 10 μM forskolin was added to the superfusate for a period indicated by a solid bar and then to the superfusate containing enflurane (a hatched bar denotes the bath application of enflurane). Insets show the actual current traces at the time indicated by filled circles and arrows.

strengthen our working hypothesis. Alternatively, enflurane and halothane might change the conformation of some sites within the channel molecule at which the phosphorylation occurs by acting, for example, on the lipid surrounding the channel (Bean *et al.*, 1981; Haydon & Urban, 1986). Further experiments are in progress to answer these questions.

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Carbamylcholine- and 5-hydroxytryptamine-induced contraction in rat isolated airways: inhibition by calcitonin gene-related peptide

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1 The effects of rat and human α-calcitonin gene-related peptide (α-CGRP) were investigated in isolated smooth muscle preparations obtained from three levels of the rat respiratory tract.

2 Neither peptide (10^{-10}–10^{-6} M) had any effect on resting tension or on carbamylcholine (10^{-6} M)-induced tone of trachea or main bronchus. In contrast, CGRP sometimes reduced spontaneous or carbamylcholine-induced tone of lung parenchymal strips.

3 CGRP produced a significant rightward shift of the log concentration-response curves to carbamylcholine and 5-hydroxytryptamine (5-HT) in the main bronchus. A rightward shift was also seen in trachea and parenchymal strips but this did not achieve the level of significance. The maximal response to 5-HT was reduced in the main bronchus and lung parenchyma whereas the maximal contraction to carbamylcholine was decreased in parenchymal strip only.

4 In all three airway preparations, CGRP caused concentration-dependent inhibition of responses elicited by challenges with 10^{-7} M carbamylcholine or 5×10^{-7} M 5-HT. The inhibitory effect of the peptide was inversely related to the size of the airways: the smaller the calibre, the greater the inhibition.

5 The inhibitory action of CGRP was not modified by pretreatment with tetrodotoxin (10^{-6} M), propranolol (10^{-6} M) or indomethacin (10^{-6} M).

6 The results strongly suggest that (a) CGRP has a nonspecific inhibitory action on airway smooth muscle cells, (b) CGRP may act as a potent inhibitor of responses elicited by bronchoconstrictor substances and (c) its inhibitory activity may be most powerfully expressed in peripheral regions of the respiratory tract.

Introduction

Calcitonin gene-related peptide (CGRP) is a mammalian peptide containing 37 amino acid residues with a disulphide bridge between Cys_2 and Cys_7. It is generated by tissue-specific RNA processing of the primary transcript of the calcitonin gene (Amara *et al.*, 1982) and is expressed in thyroid parafollicular cells as well as in nuclei and nerve fibres of the central and peripheral nervous systems (Rosenfeld *et al.*, 1983). In both rat and human, this peptide is known to occur in two forms, designated α- and β-CGRP, which differ in their primary sequence by one and three amino acids respectively (Breimer *et al.*, 1988).

Using radioimmunoassay and immunocytochemical techniques, we have previously demonstrated that high concentrations of α-CGRP are found in the rat lung where the peptide exhibits a unique and widespread distribution, being the first peptide to be localized both in peripheral nerves and endocrine cells (Cadieux *et al.*, 1986a). CGRP-immunoreactive nerve fibres were found to be mostly sensory in nature. Their cell bodies lie in the nodose, jugular and dorsal root ganglia and axodendritic processes are carried in the vagus and the sympathetic nerves to form peripheral branches in the lung (Springall *et al.*, 1987). The physiological significance of CGRP in rat lung remains unclear, but the close association of CGRP-positive nerves with smooth muscle of the tracheobronchial tree suggests that this peptide may be involved in regulating bronchomotor tone. Recently, studies have established the presence of specific binding sites for CGRP in areas in which immunoreactivity was localized (Carstairs, 1987). These findings support the hypothesis that CGRP may participate in bronchomotor regulation by acting as a neurotransmitter or neuromodulator substance.

The present experiments were carried out to determine whether CGRP had any direct effect on the smooth muscle of rat isolated airways. The ability of CGRP to inhibit contraction induced by carbamylcholine and 5-HT was also investigated. A preliminary account of some of this work was presented at the APS/ASPET Joint Meeting, 1988. (Cadieux *et al.*, 1988).

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Methods

Tissue preparation

Male Sprague Dawley rats weighing 200–225 g were killed by exsanguination following intraperitoneal injection of pentobarbitone sodium (50 mg kg^{-1}). The trachea and the lungs were quickly removed and placed in cold Krebs solution. The trachea was separated at its junction with the main bronchi, and tracheal chains were prepared from the intrathoracic part of the trachea according to the method of Castillo & De Beer (1947). Both right and left main bronchi were dissected out and cut helically as described by Sirois *et al.* (1987). Finally, parenchymal strips (approximately 15 mm × 3 mm × 3 mm) were cut from the periphery of either right or left lower lobes of the lungs (Lulich *et al.*, 1976). Each preparation was mounted in a 5 ml jacketted organ bath containing Krebs solution gassed with 95% O_2, 5% CO_2 and maintained at 37°C. Contractions were measured isometrically with a Grass FT03 force displacement transducer and recorded on a Grass polygraph (Model 7D) as tension changes (g). All tissues were subjected to an initial loading tension of 1 g and allowed to equilibrate for 60 min (with changes of bath medium every 15 min) before CGRP and other agents were tested.

Functional studies

The effect of CGRP on the three different tissue preparations, initially was examined by testing cumulatively-applied CGRP (10^{-10}–10^{-6} M) against the resting tension and against tension induced by carbamylcholine (10^{-6} M).

To test for inhibitory activity of CGRP in isolated airways, cumulative concentration-response (C/R) curves to carbamylcholine (10^{-8}–10^{-3} M) or 5-HT (10^{-8}–10^{-4} M) were constructed in the presence of CGRP (10^{-7} M), added to the organ bath 2 min before constructing the C/R curve. Control experiments were performed in parallel on isolated airways where only the peptide vehicle (Krebs solution) was added.

Finally, the inhibitory action of CGRP was further characterized by studying its effect on contractions induced by single concentration of the two bronchoconstrictor agents. Contractions were elicited by carbamylcholine (10^{-7} M) or 5-HT (5×10^{-7} M) at 15 min intervals with a drug contact time just sufficient to record the peak response. CGRP (10^{-10}–10^{-6} M) was then added to the organ bath and 2 min later a further spasmogen challenge occurred. The contraction produced in the presence of the peptide was then compared with the mean of two control responses to carbamylcholine or 5-HT observed prior to CGRP administration. A further concentration of CGRP was tested only when responses to the spasmogen showed no further recovery. Concentrations of the peptide were tested in a random order in each experiment. In this latter series of experiments, the inhibitory effect of CGRP was also examined in the presence of tetrodotoxin (10^{-6} M), propranolol (10^{-6} M) or indomethacin (10^{-6} M).

Calculations

On all isolated airway preparations, C/R curves were plotted as molar concentration of drug versus contractile response expressed as g of tension. EC_{50} values were calculated by linear regression analysis of all points between 20 and 80% of maximal response and expressed as pD_2. pD_2 values were calculated from each individual C/R curve and the mean ± s.e.mean calculated from these data. The inhibitory effect of CGRP was assessed by comparing pD_2 values in the absence and the presence of CGRP. Results are presented as means ± s.e.mean. Throughout, mean values were compared by use of Student's t test for paired or unpaired data as appropriate. A probability level of $P < 0.05$ was considered statistically significant.

Drugs and solutions

The Krebs solution had the following composition (mM): NaCl 118.1, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11.1. The following drugs were used: synthetic rat and human α-CGRP (IAF Biochem), carbamylcholine chloride, histamine dihydrochloride, indomethacin, propranolol hydrochloride, 5-hydroxytryptamine creatinine sulphate (5-HT), atropine sulphate and tetrodotoxin (TTX) (each Sigma). Methysergide bimaleate was a generous gift from Sandoz Ltd (Basel, Switzerland). All drugs were freshly diluted in the stock Krebs solution with the exception of the peptides. The latter agents, in lyophilized form, were dissolved in distilled water at an initial concentration of 1 mg ml^{-1} and kept frozen at −20°C. The required working solutions were made by dilution in the stock Krebs solution immediately before the experiments. All concentrations referred to are final bath concentrations. The glassware (including the organ baths) were siliconised (Prosil-28) in order to minimize non specific adsorption.

Results

Effect of CGRP on spontaneous and carbamylcholine-induced tone

Figure 1(a,b) shows that the resting tension of rat main bronchus was not affected by either rat or human α-CGRP (10^{-10}–10^{-6} M). Neither peptide suppressed contraction induced by 10^{-6} M carbamylcholine in this preparation. Similar results were obtained with rat trachea. However, in about 40% of the lung parenchymal strips, CGRP caused a significant reduction of spontaneous tone (Figure 1d). When these same preparations were pre-contracted with carbamylcholine 10^{-6} M, CGRP caused concentration-dependent relaxation (Figure 1e). CGRP-induced relaxation was not reduced by tetrodotoxin (10^{-6} M) propranolol (10^{-6} M) or indomethacin (10^{-6} M).

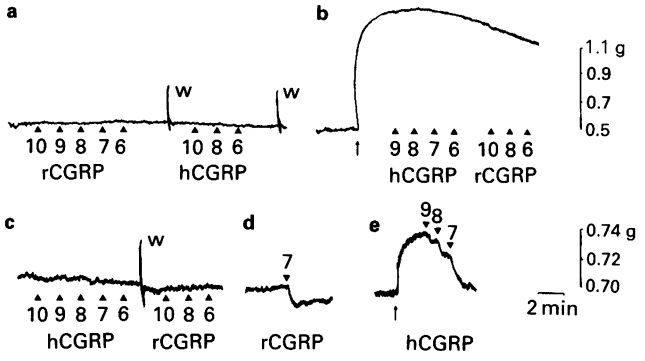

Figure 1 Original recordings showing the effects of increasing concentrations of rat and human α calcitonin gene-related peptide (α-CGRP) on the resting tension (a,c,d) and on carbamylcholine-induced tone (b,e) of rat isolated airways. Upper panel: main bronchus. Lower panel: parenchymal strips. Cumulative additions of CGRP (▲) at concentrations from 10^{-10} to 10^{-6} M did not exert any contractile or relaxant effect in main bronchus. Note however, that some parenchymal strips relax when challenged with CGRP (d,e) while others fail to respond (c). Ordinate scale: changes of tension in g. Abscissa scale: time in min. W: washouts. Arrows: carbamylcholine (10^{-6} M). r-CGRP: rat αCGRP. h-CGRP: human αCGRP.

Effect of CGRP on concentration-response curves of carbamylcholine and 5-HT

The addition of 5-HT (10^{-8}–10^{-4} M) or carbamylcholine (10^{-8}–10^{-3} M) caused, in all three preparations, rapidly-developing and concentration-dependent contraction. 5-HT and carbamylcholine were strongly antagonized by methysergide (10^{-6} M) or atropine (5×10^{-6} M) respectively. Both carbamylcholine and 5-HT were more potent on the trachea and main bronchus than on lung parenchymal strips (Table 1). Histamine had no effect on the resting tension of the same preparations.

Preincubation of the airways with 10^{-7} M of rat α-CGRP resulted in a significant shift to the right of the carbamylcholine C/R curve in the main bronchus but not in trachea and parenchymal strips (Figure 2, Table 2). In the trachea and main bronchus the maximal responses to carbamylcholine were not affected by the presence of CGRP whereas in lung parenchymal strips the presence of the peptide caused a 31.4% reduction in the maximal contraction. Although the rightward shift for carbamylcholine was not sig-

Table 1 pD_2 values and developed maximal contraction (m.c.) for carbamylcholine and 5-hydroxytryptamine (5-HT) in tissues isolated from rat lung

		Carbamylcholine	*5-HT*
Trachea	pD_2	6.71 ± 0.03	6.15 ± 0.03
	m.c. (g)	0.36 ± 0.03	0.175 ± 0.015
		(6)	(14)
Main bronchus	pD_2	6.49 ± 0.03	5.89 ± 0.02
	m.c. (g)	1.23 ± 0.1	0.37 ± 0.03
		(6)	(17)
Parenchyma	pD_2	5.34 ± 0.09	5.29 ± 0.06
	m.c. (g)	0.102 ± 0.006	0.133 ± 0.009
		(10)	(7)

The pD_2 values are expressed as mean ± s.e.mean. Figures in parentheses indicate the number of tissues.

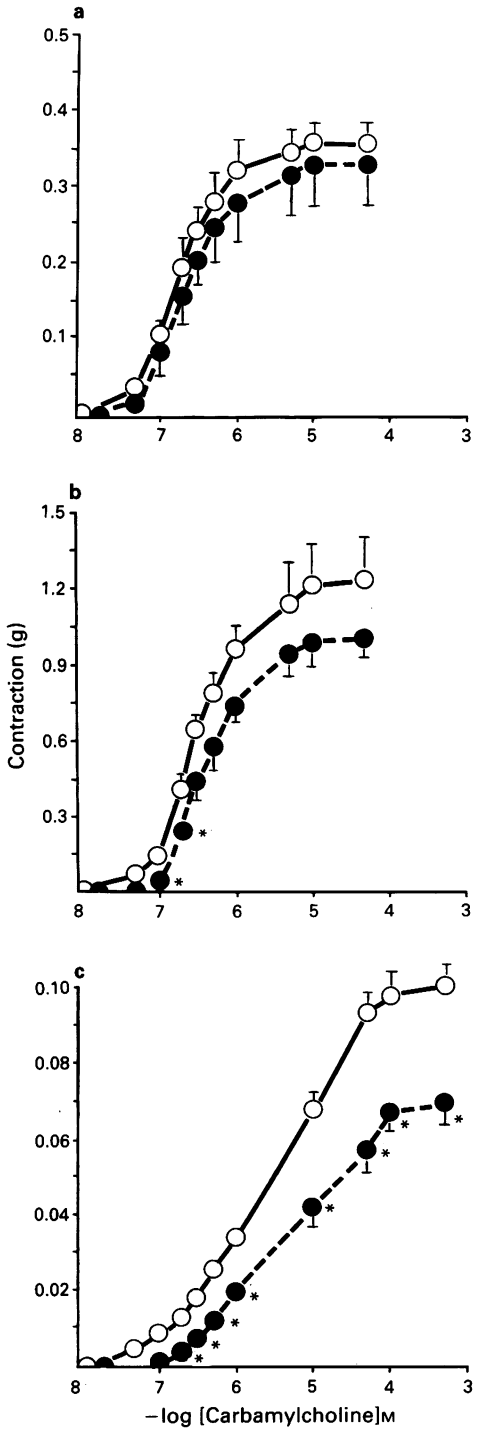

Figure 2 Cumulative concentration-response curves for carbamylcholine in the absence (○) and in the presence (●) of calcitonin gene-related peptide (CGRP, 10^{-7} M): (a) trachea, (b) main bronchus and (c) parenchymal strips. The peptide was added to the organ bath 2 min prior to the administration of the cholinoceptor agonist. Values are expressed as g tension. Each point and bar represents the mean and s.e.mean of data from 4 to 12 tissues. Asterisks: significantly different from controls.

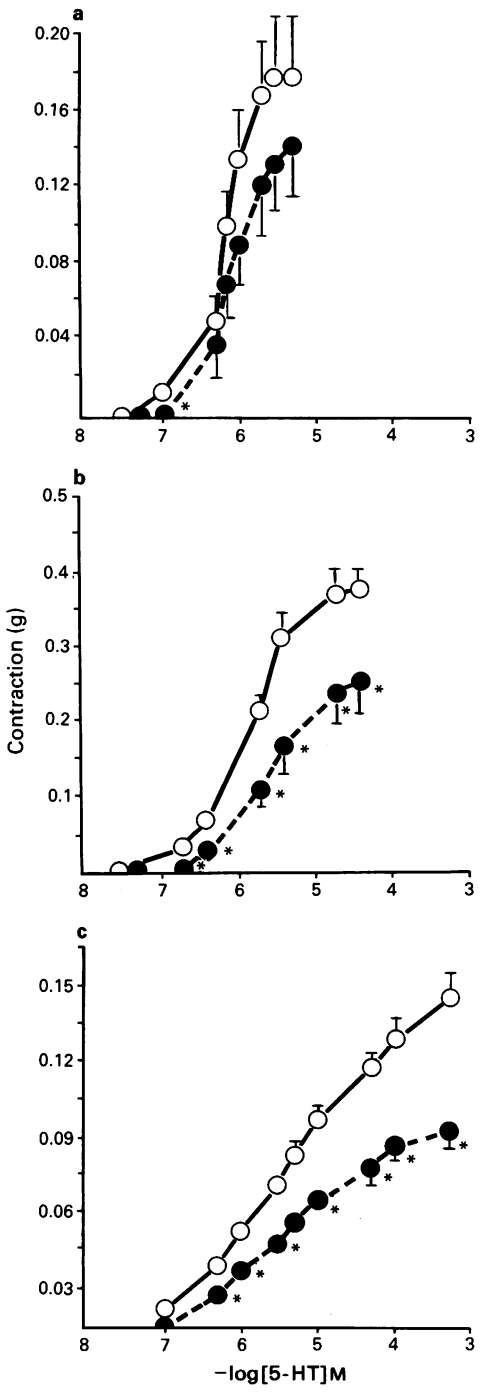

Figure 3 Cumulative concentration-response curves for 5-hydroxytryptamine (5-HT) in the absence (○) and in the presence (●) of calcitonin gene-related peptide (CGRP, 10^{-7} M): (a) trachea, (b) main bronchus and (c) parenchymal strips. The peptide was added to the organ bath 2 min prior to the administration of 5-HT. Values are expressed as g tension. Each point and bar represents the mean and s.e.mean of data from 8 to 16 tissues. Asterisks: significantly different from controls.

nificant in parenchymal strips, responses of both low and high concentrations of the cholinoceptor agonist were diminished in the presence of CGRP. This differs from the main bronchus where only contractile responses in the lower third of the C/R curve were decreased by pretreatment with the peptide.

5-HT-induced contractions were similarly affected by CGRP (Figure 3, Table 2) except that CGRP caused a significant reduction (30.8%) in the maximal response to 5-HT in the main bronchus. In parenchyma, the decrease in the maximal contraction following the administration of CGRP, averaged 42.1%.

Effect of various concentrations of CGRP on responses to single concentrations of carbamylcholine and 5-HT

The results presented above suggest that the modulatory effect of CGRP on the contractions induced by the two spasmogens may differ with respect to target tissue (distal versus proximal airways). An increase in the carbamylcholine concentration overcame the inhibitory effect of CGRP in the main bronchus

Table 2 pD_2 values for carbamylcholine 5-hydroxytryptamine (5-HT) and rat airways in the absence and presence of calcitonin gene-related peptide (CGRP) 10^{-7} M

Spasmogen	*Target tissue*	*Control* pD_2	*CGRP* pD_2	*Significance level*
Carbamylcholine	Trachea	6.71 ± 0.03 (6)	6.68 ± 0.06 (4)	NS
	Main bronchus	6.49 ± 0.03 (6)	6.38 ± 0.03 (4)	$P < 0.05$
	Parenchyma	5.34 ± 0.09 (10)	5.23 ± 0.07 (12)	NS
5-HT	Trachea	6.15 ± 0.03 (12)	6.02 ± 0.06 (11)	NS
	Main bronchus	5.89 ± 0.02 (16)	5.72 ± 0.08 (8)	$P < 0.05$
	Parenchyma	5.29 ± 0.06 (7)	5.39 ± 0.08 (8)	NS

Each value is the mean ± s.e.mean. Figures in parentheses indicate the number of tissues. NS: not significant.

but not in lung parenchymal strips. Similarly an increase in the concentration of 5-HT overcame CGRP-induced inhibition in the trachea but not in the main bronchus or parenchymal strip. This issue was further investigated by comparing the inhibitory effect of increasing concentrations of CGRP on carbamylcholine- and 5-HT-induced contractions in all three airway preparations.

The spasmogen challenges (10^{-7} M for carbamylcholine; 5×10^{-7} M for 5-HT), induced contractions varying between 5 and 20% of maximal responses depending on the airway preparation used. As illustrated in Figure 4, preincubation of rat bronchus with rat α-CGRP (10^{-10} M) caused a slight reduction of carbamylcholine-induced contraction but did not alter either the amplitude or the shape of the contractile response to added 5-HT. In contrast, pretreatment with higher concentrations of the peptide significantly decreased the contractions induced by the two bronchoconstrictor agents (Figures 4 and 5). The effect of CGRP did not show tachyphylaxis and the inhibitory action was rapidly reversible at lower doses of the peptide. In contrast, the inhibitory effect of high doses of CGRP ($> 10^{-8}$ M) tended to persist for 30 to 45 min despite repeated washings. Generally, in each preparation from one animal, the same dose of CGRP added repeatedly during the experiment, produced similar inhibition of the contraction.

The inhibitory effects of increasing concentrations of CGRP on responses to 10^{-7} M carbamylcholine and 5×10^{-7} M 5-HT are summarized in Figures 5a and 5b. As shown in Figure 5a, rat α-CGRP added 2 min before carbamylcholine inhibited contractions of the airways in a concentration-dependent manner. The inhibitory effect of the peptide was more marked in the lower segments of the tracheobronchial tree than in the trachea and, at a concentration of 10^{-6} M, reached an inhibitory effect of 63.3 ± 5.9% ($P < 0.05$) in the parenchymal strips, 32.5 ± 3.2% ($P < 0.05$) in the main bronchus and 7.3 ± 3.3% ($P > 0.05$) in the trachea. Similar results were obtained with 5-HT as the spasmogen. Pretreatment with 10^{-6} M CGRP, 2 min before the administration of 5×10^{-7} M 5-HT decreased the agonist-induced contractions by 42.6 ± 3.8% in lung parenchyma ($P < 0.05$), 31.3 ± 3.8% in main bronchus ($P < 0.05$) and 13.4 ± 0.65% in trachea ($P > 0.05$) of the controls, respectively (Figure 5b).

Human α-CGRP was equipotent with rat α-CGRP and exhibited similar inhibitory activity on contractions induced by carbamylcholine and 5-HT in all segments of the respiratory tract (Figures 5a and b). Finally, the inhibitory effect of rat α-CGRP on airways contractility was unchanged after

Table 3 Effect of tetrodotoxin, indomethacin and propranolol on the inhibitory action of calcitonin gene-related peptide (CGRP) on carbamylcholine- and 5-hydroxytryptamine (5-HT)-induced contractions in rat isolated parenchymal strips

	% inhibition of spasm induced by CGRP (10^{-7} M)	
	Carbachol (10^{-7} M)	*5-HT* (5×10^{-7} M)
Control	51.2 ± 3.1 (5)	39.9 ± 6.2 (3)
Tetrodotoxin (10^{-6} M)	49.5 ± 2.6 (3)	37.4 ± 2.5 (3)
Indomethacin (10^{-6} M)	51.7 ± 8.4 (5)	45.1 ± 7.7 (4)
Propranolol (10^{-6} M)	48.6 ± 6.7 (4)	40.7 ± 2.1 (3)

Each value is the mean ± s.e.mean. Figures in parentheses indicate the number of tissues.

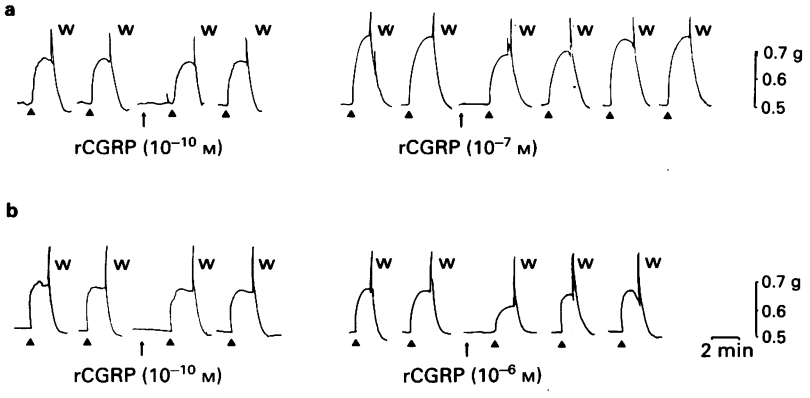

Figure 4 Recordings of the inhibitory effect of rat α-calcitonin gene-related peptide (α-CGRP) on contractions evoked by 10^{-7} M carbamylcholine (a) and by 5×10^{-7} M 5-hydroxytryptamine (b) in rat isolated main bronchus. Contractions were elicited by the two agonists at 15 min intervals in the absence or in the presence of various concentrations of CGRP. Note that the inhibitory action of CGRP tended to persist longer when high concentration (10^{-7} or 10^{-6} M) was added to the organ bath. In each panel, the two recordings were obtained from the same tissue. Ordinate scale: changes of tension in g. Abscissa scale: time in min. W: washouts.

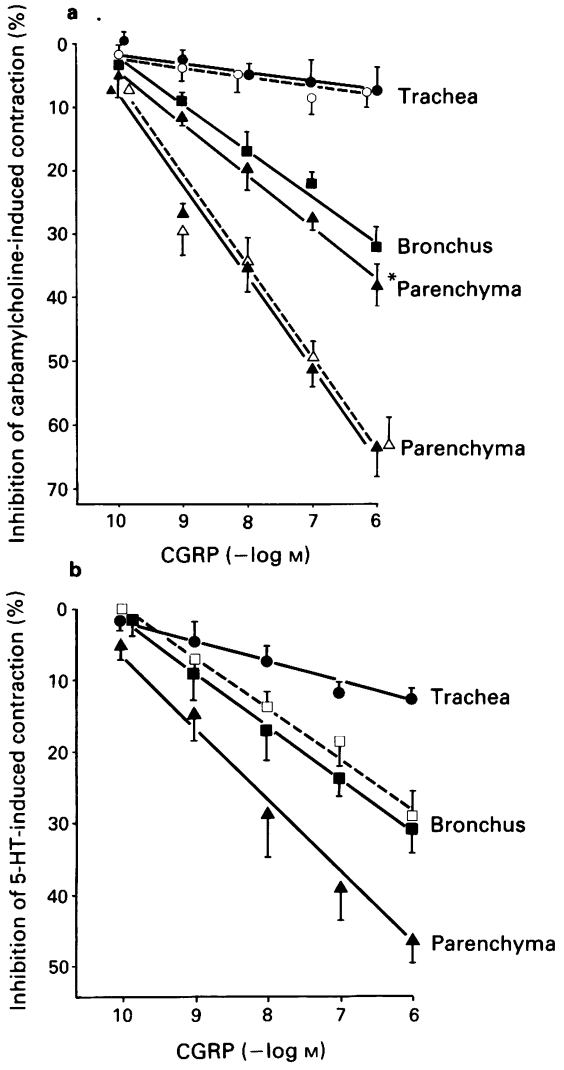

Figure 5 Log concentration-response curves for calcitonin gene-related peptide (CGRP) in inhibiting contractile responses to (a) carbamylcholine (10^{-7} M) and (b) 5-hydroxytryptamine (5-HT, 5×10^{-7} M) in rat isolated airways. CGRP was added in a manner similar to that described in the legend to Figure 4. Ordinate scale: degree of inhibition produced by CGRP expressed as percentage of contraction developed by the agonist alone. Abscissa scale: concentration of CGRP. Each point and bar represents the mean and s.e.mean of data from 3 to 10 tissues. *Contractile responses were elicited by 10^{-6} M carbamylcholine. Solid line and symbols: rat α-CGRP, broken line with open symbols: human α-CGRP.

20 min pretreatment with tetrodotoxin (10^{-6} M), propranolol (10^{-6} M) or indomethacin (10^{-6} M) (Table 3).

Discussion

The results obtained in this study revealed that rat and human α CGRP exert no direct contractile effect in rat isolated trachea and main bronchus. However, CGRP sometimes decreased spontaneous or carbamylcholine-induced tone of lung parenchymal strips. In agreement with Lulich & Paterson (1980) and Vornanen & Tirri (1981), all of these three classical airway smooth muscle preparations were insensitive to histamine but contracted in a concentration-dependent manner to carbamylcholine and 5-HT, both agonists being more potent in the trachea and main bronchus than in lung parenchymal strips. The apparent lack of responsiveness of the rat proximal airways to CGRP also corroborates previous reports that CGRP does not affect the resting tension of rat trachea *in vitro* (Hamel & Ford-Hutchinson, 1988) nor guinea-pig tracheal and bronchial smooth muscle *in vitro* (Martling *et al.*, 1988) and *in vivo* (Lundberg *et al.*, 1985).

In contrast to the findings of Hamel & Ford-Hutchinson (1988), our study showed that about 40% of the lung parenchymal strip preparations relaxed in response to CGRP. A likely explanation for this action of the peptide could be that different lung strips from the same or from different rats contained different proportions of vascular and airways smooth muscle (Lulich *et al.*, 1976; Goldie *et al.*, 1982; Bertram *et al.*, 1983). Furthermore, it has been reported that vascular smooth muscle may contribute significantly in responses of this preparation to agonists which stimulate airways smooth muscle (Mirbahar & Eyre, 1980; Evans & Adler, 1981). Since CGRP is a potent vasodilator agent in several species including man (Brain *et al.*, 1985; Gennari & Fischer, 1985) and causes relaxation of isolated pulmonary arteries (McCormack *et al.*, 1988; Martling *et al.*, 1988) and since CGRP does not affect the resting tension of isolated trachea and hilus bronchi of rodents (Lundberg *et al.*, 1985; Martling *et al.*, 1988; Hamel & Ford-Hutchinson, 1988; present study) we suggest that the relaxation induced by CGRP in some parenchymal preparations was due to the presence of a relatively higher proportion of vascular than airways smooth muscle cells. This relaxation was not blocked by tetrodotoxin, propranolol or indomethacin which is consistent with the finding that CGRP relaxes blood vessels by acting via its own receptors located on the vascular smooth muscle cells (Brain *et al.*, 1985). These parenchymal strips were thus considered not to reflect the activity of the smooth muscle of the fine peripheral airways and all parenchymal strips displaying a relaxation in response to 10^{-7} M CGRP were eliminated and not used in further experiments.

The present study provides strong evidence that CGRP is not totally devoid of biological effect on rat airways smooth muscle. Concentration-response curves to carbamylcholine and 5-HT were displaced to the right by CGRP in the main bronchus. Corresponding curves in the trachea and parenchymal strip were altered in shape. In addition, the CGRP-induced inhibition of contractile responses to the two spasmogens was inversely related to the size of the airways; the smaller the calibre the greater the inhibition. CGRP-induced reduction of responses to carbamylcholine and 5-HT standards (10^{-7} and 5×10^{-7} M respectively) was consistent with its downward displacement effect on the C/R curves of the agonists. Its inhibitory action was concentration-dependent and was most powerfully expressed on parenchymal strips and least powerfully expressed on trachea. These results are consistent with a neurotransmitter role for CGRP in regulating airway smooth muscle tone.

The finding that the magnitude of the inhibitory effect of CGRP on carbamylcholine- or 5-HT-induced contraction was greatest in parenchymal strips and smallest in trachea corresponds with the reported pattern of distribution of CGRP binding sites in rodent lungs. CGRP receptor sites are abundant in intrapulmonary bronchi of all sizes including the alveolar ducts but are sparsely distributed in smooth muscle of large airways (Carstairs 1987; Mak & Barnes, 1988). CGRP may therefore be of functional importance mainly in the periphery of the respiratory tract; hence providing confirmation of the heterogeneity of smooth muscle responsiveness through the lung (Burns & Doe, 1978; Lulich & Paterson, 1980; Vornanen & Tirri, 1981). The inhibitory action of CGRP was expressed against both carbamylcholine- and 5-HT-induced contraction and was not mediated by the release of endogeneous prostaglandins since indomethacin, an inhibitor of prostaglandin synthesis, had no effect. Moreover, the activity of CGRP was not modified by tetrodotoxin (10^{-6} M) or propranolol (10^{-6} M) which indicates that the action of CGRP is not related to the discharge of neuronal action potentials or to β-adrenoceptor activation consequent to noradrenaline

release. These results suggest instead that CGRP exerts a direct, non-specific inhibitory action on the smooth muscle cells. Furthermore, the low threshold dose required to induce an effect as well as the long duration of action of CGRP (especially noted by the slow recovery of the tissue following the administration of high doses of the peptide) suggest a high affinity of CGRP for its receptor. This would be in keeping with the demonstration of receptors with high affinity for CGRP in airway smooth muscle of mammalian lung (Mak & Barnes, 1988).

There is some suggestion that rat and human α-CGRP may differ slightly in biological activity (Marshall *et al.*, 1986) but the structure-activity relationships have not yet been examined in detail. According to our observations both peptides are equipotent in inducing inhibition of agonist-induced contractions in rat isolated airways. This is consistent with previous findings in the cardiovascular system where both rat and human α-CGRP behave similarly (Brain *et al.*, 1986; Tippins *et al.*, 1986). This suggest that the substitutions in position 1, 3, 25 and 35 in the amino acid sequence between rat and human α-CGRP (Rosenfeld *et al.*, 1983; Breimer *et al.*, 1988) have little or no effect on the biological activity of the peptide at least in some organs and/or tissues.

Evidence is accumulating that CGRP may be an important neurotransmitter in the respiratory system. In animals and man CGRP is present in sensory nerves which are associated either with vascular and non vascular smooth muscle of the lung (Rosenfeld *et al.*, 1983; Lundberg *et al.*, 1985; Uddman *et al.*, 1985; Cadieux *et al.*, 1986a; Martling *et al.*, 1988). CGRP is released from the lung upon stimulation with capsaicin (Martling *et al.*, 1988) and following cigarette smoke exposure (Cadieux *et al.*, 1986b). There has been much debate about the possible role of CGRP in the respiratory system but agreement exists between authors that a common effect of the peptide is to regulate blood flow (Joos *et al.*, 1986; Lundberg & Saria, 1987; Laitinen *et al.*, 1987; Uddman & Sundler, 1987). Hence, CGRP relaxes isolated pulmonary arteries obtained from both rodents (McCormack *et al.*, 1988) and human lungs (Martling *et al.*, 1988) and potentiates tachykinin-induced vascular permeability in dog (Laitinen *et al.*, 1987) and guinea-pig trachea (Lundberg *et al.*, 1985; Haass & Skofitsch, 1985). The involvement of CGRP in the regulation of the airway smooth muscle currently remains unclear. In human isolated airway smooth muscle there is evidence for (Palmer *et al.*, 1987) and against (Martling *et al.*, 1988) a bronchoconstrictor action of CGRP. Similarly, Lundberg *et al.*, (1985) and Martling *et al.*, (1988) showed that CGRP does not exert any contractile or relaxant effect in guinea-pig trachea and hilus bronchi, whereas Hamel & Ford-Hutchinson (1988) claimed that CGRP is one of the most potent contractile agents on guinea-pig trachea yet described. Results of the present study suggest that CGRP has an anti-bronchoconstrictor action in rat isolated airways, and is consistent with the report of Gatto *et al.* (1989) that CGRP potently inhibits the bronchoconstrictor effects of substance P and bombesin in the guinea-pig *in vivo*.

In summary, our findings indicate that CGRP exerts an inhibitory effect against bronchoconstrictor agents in rat isolated airways. This inhibitory effect is greatest in the periphery of the tracheobronchial tree and represents a direct effect of CGRP on the membrane of the smooth muscle cells. These observations support the hypothesis that CGRP may play a role in regulating airway tone.

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Contractile and relaxant responses of the canine isolated spinal artery to vasoactive substances

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1 Effects of vasoactive substances were investigated in the canine isolated spinal branch of the intercostal artery (SBICA).

2 Addition of angiotensin II (AII), vasopressin, noradrenaline (NA), adrenaline, 5-hydroxytryptamine (5-HT), and dopamine each produced concentration-dependent contraction in the SBICA, whereas prostaglandin $F_{2\alpha}$, histamine, and tyramine caused only slight contraction. The decreasing order of the potency of contractile agents was AII $\gg$ vasopressin = NA > 5-HT > adrenaline $\gg$ dopamine.

3 Although the pD_2 value for phenylephrine (5.31 ± 0.36) was smaller than that for NA (6.48 ± 0.13), there was no significant difference in E_{max} value between these two agonists in the SBICA. On the other hand, xylazine produced only a slight contraction, the pD_2 value being 3.59 ± 0.08. Phentolamine (10^{-8}–10^{-6} M) and prazosin (10^{-8}–10^{-6} M) competitively inhibited the NA-induced contraction, while yohimbine (10^{-8}–10^{-6} M) did not.

4 Acetylcholine (ACh), sodium nitroprusside (SNP), ATP, ADP, and adenosine caused concentration-dependent relaxations in SBICA following contraction with NA. On the other hand, isoprenaline up to 10^{-4} M did not produce any relaxation. The decreasing order of potency of the relaxant agents was ACh > SNP $\gg$ ATP = ADP = adenosine.

5 The ACh-induced relaxation was competitively inhibited by atropine and was abolished by mechanical removal of the endothelium. Aspirin (5×10^{-5} M) did not affect the relaxant response to ACh, while oxyhaemoglobin (10^{-5} M) and methylene blue (10^{-5} M) produced significant attenuation.

6 These results suggest that NA produces contraction of the isolated canine SBICA which is mainly mediated via α_1-adrenoceptors and that ACh causes a relaxation of the SBICA due to release of endothelium-derived relaxing factor (EDRF) from the endothelial cells.

Introduction

The neural and humoral regulation of regional blood flow in the central nervous system is of both physiological and clinical interest. Many studies, both *in vivo* and *in vitro*, have been carried out to evaluate the mode of regulation of cerebral circulation (see Edvinsson & MacKenzie, 1977; Heistad & Kontos, 1983 for review). On the other hand, studies on the regulation of spinal cord blood flow have been limited. Blood flow in the spinal cord has been measured by means of *in vivo* techniques including hydrogen clearance (Korbine *et al.*, 1974), ^{133}Xe clearance (Smith *et al.*, 1969; Griffiths, 1973), the microsphere method (Marcus *et al.*, 1977), and [^{14}C]-iodoantipyrine autoradiography (Sandler & Tator, 1976). Furthermore, new methods such as Laser-Doppler flowmetry (Sakamoto *et al.*, 1988) and the [^{14}C]-butanol 'indicator-fractionation' technique (Lindsberg *et al.*, 1989) have been validated for quantitating spinal blood flow *in vivo*. These studies have suggested that neural, chemical, metabolic, and autoregulatory factors may play important roles in the regulation of spinal cord blood flow. In contrast to the *in vivo* studies, there are few *in vitro* studies that have examined directly the effects of vasoactive substances on isolated blood vessels which supply blood to the spinal cord.

The spinal cord receives its blood supply from arteries which are branches of either the vertebral artery, intercostal artery, lumbar artery, or iliolumbar artery. The present study was undertaken to compare the effects of various vasoactive substances on the isolated spinal branch of the intercostal artery (SBICA) of the dog, and to investigate pharmacologically the mechanisms of smooth muscle contraction and relaxation induced by noradrenaline and acetylcholine, respectively.

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Methods

Mongrel dogs of either sex, weighing 6–13 kg, were anaesthetized with sodium pentobarbitone (25 mg kg^{-1}, i.v.) and killed by bleeding. Spinal branches of the 10th, 11th, and 12th intercostal arteries (0.6–0.8 mm outer diameter) were quickly dissected out. After the arteries were cleaned of fat and connective tissue, they were cut into cylindrical segments (2 mm long) under a dissecting microscope. Two tungsten wires were run through the lumen of the arterial segment. The preparations were suspended in 10 ml organ baths which were perfused with Krebs solution at a constant rate of 4 ml min^{-1}. The composition of the solution (mM) was as follows: NaCl 120.0, KCl 5.9, $NaHCO_3$ 25.0, NaH_2PO_4 1.2; $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 5.5. The solution was maintained at 36.0 ± 0.5°C and aerated with a gas mixture of 95% O_2 and 5% CO_2 to give a pH of 7.3–7.4. The upper end of the preparation was connected to a force-displacement transducer (Shinko Tsushin UL-10), and the lower end was fixed to the bottom of the bath. The resting tension was adjusted to 1.0 g, which was optimal for obtaining maximum contractile response to 80 mM KCl solution.

Experimental protocol

All arterial segments were allowed to equilibrate for 90 min. After the equilibration period, cumulative concentration-response curves for vasoconstrictor agents except angiotensin II (AII) and vasopressin were obtained by stepwise increase in concentration of the drug by a factor of 10, as soon as a steady response to the previous administration had been achieved. Concentration-response curves for AII and vasopressin were obtained by the addition of single concentration of the peptides every 30–120 min, long enough to avoid tachyphylaxis. At the beginning and end of each experiment, the

contractile response to 80 mM KCl solution was obtained. The extent of contraction induced by an agonist was expressed as a percentage of the KCl-induced contraction in each preparation. When α-adrenoceptor antagonists were tested, the drugs had been dissolved in oxygenated Krebs solution and perfused at least 30 min before the concentration-response curves for α-adrenoceptor agonists were determined. Controls were always run with no antagonist to observe time-dependent changes in the sensitivity of the preparations to the agonists.

When responses to vasorelaxant agents were observed, the preparations were contracted by 5×10^{-6}–10^{-5} M noradrenaline (NA), which resulted in 40–60% of the 80 mM KCl-induced maximum contraction in each preparation. After the contraction reached a plateau, cumulative concentration-response curves for the relaxants were obtained in the same way as for constrictor agents. The extent of relaxation was expressed as a percentage of maximum relaxation induced by 10^{-5} M sodium nitroprusside. In some experiments, the effects of atropine, aspirin, methylene blue, and oxyhaemoglobin on the relaxant responses to acetylcholine (ACh) were investigated. These inhibitors had been perfused at least 30 min before ACh was added. Oxyhaemoglobin was prepared by the technique described by Martin *et al.* (1985). The responses to ACh were also examined in preparations from which the endothelial cells had been removed by mechanically rubbing the intimal surface with cotton sticks. In four rubbed canine SBICA preparations, the absence of endothelial cells was confirmed histologically by the silver staining procedure (Poole *et al.*, 1958).

Drugs

The following drugs were used: acetylcholine chloride (Daiichi Seiyaku), atropine sulphate (Tanabe), ATP disodium (Wako), isoprenaline hydrochloride (Nikken Kagaku), methylene blue (Merck), phentolamine mesylate (Ciba-Geigy), prazosin hydrochloride (Pfizer), prostaglandin $F_{2\alpha}$ (Ono Yakuhin), sodium nitroprusside (Merck), and xylazine hydrochloride (Bayer). (−)-Adrenaline bitartrate, angiotensin II, aspirin, dopamine hydrochloride, haemoglobin, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, (−)-noradrenaline bitartrate, tyramine hydrochloride, vasopressin, and yohimbine hydrochloride were obtained from Sigma Chemical Co. Adenosine, ADP disodium, and phenylephrine hydrochloride were purchased from Kowa Co. The concentrations of drugs are expressed in log molar terms.

Statistics

Experimental values shown in the text, figures, and tables are mean ± standard error of the mean (s.e.mean). The negative logarithm of ED_{50}, the concentration of agonists causing one half of the maximum contraction, is expressed as the pD_2 value. Statistical analyses were performed by use of Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's test. The difference in means were considered significant when $P < 0.05$.

Results

Contractile responses of isolated spinal arteries

Administration of angiotensin II (AII), vasopressin, noradrenaline (NA), 5-hydroxytryptamine (5-HT), adrenaline, and dopamine produced concentration-dependent contractions in spinal branches of intercostal arteries (SBICA) of the dog (Figure 1). The order of potency of the contractile agents was AII ≫ vasopressin = NA > 5-HT > adrenaline ≫ dopamine. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), histamine, and tyramine caused only very small contractions in these preparations (Figure 1).

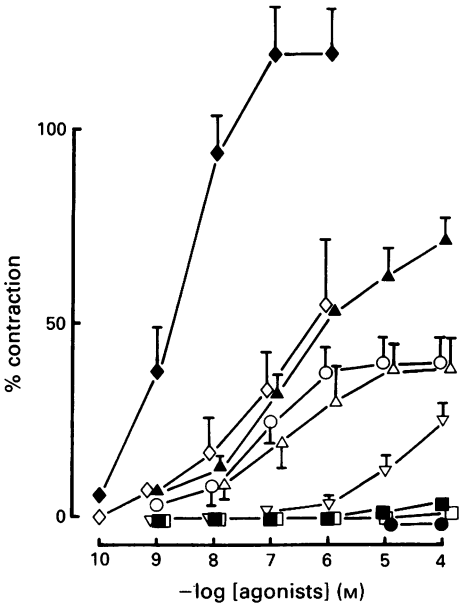

Figure 1 The contractile responses of canine isolated spinal branches of intercostal arteries to angiotensin II (◆), vasopressin (◇), noradrenaline (▲), 5-hydroxytryptamine (○), adrenaline (△), dopamine (▽), prostaglandin $F_{2\alpha}$ (■), histamine (□), and tyramine (●). On the ordinate scale, contractions induced by 80 mM KCl solution were taken as 100% in each experiment. All points represent the mean of 5 observations, and the vertical lines indicate the standard error of the mean.

Figure 2 demonstrates concentration-response curves of α-adrenoceptor agonists in five preparations. Phenylephrine (α_1-agonist) produced a concentration-dependent contraction. Although the pD_2 value for phenylephrine was smaller ($P < 0.05$) than that for NA, there was no significant difference in the E_{max} values of these two agonists (Table 1). On the other hand, xylazine (α_2-agonist) produced only a small contraction (Figure 2). The pD_2 and E_{max} values for xylazine were significantly smaller ($P < 0.01$) than those for NA (Table 1).

Phentolamine (10^{-8}–10^{-6} M) and prazosin (10^{-8}–10^{-6} M) inhibited the responses to NA in a concentration-dependent manner (Figure 3a,b). On the other hand, yohimbine in the concentration range 10^{-8} M–10^{-6} M did not affect the contrac-

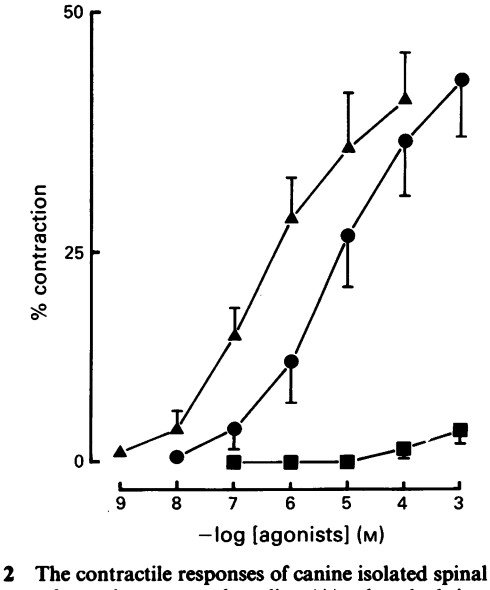

Figure 2 The contractile responses of canine isolated spinal branches of intercostal arteries to noradrenaline (▲), phenylephrine (●), and xylazine (■). The ordinate scale is the same as in Figure 1. All points represent the mean of 5 observations, and the vertical lines indicate s.e.mean.

Table 1 pD_2 and E_{max} values for various α-adrenoceptor agonists in the canine spinal branch of the intercostal artery

Agonist	n	pD_2	E_{max}[a] (%)
Noradrenaline	5	6.48 ± 0.13	43.4 ± 5.6
Phenylephrine	5	5.31 ± 0.36*	45.7 ± 6.9[NS]
Xylazine	5	3.59 ± 0.08**	3.7 ± 1.6**

[a] Maximum contraction produced by α-agonists as a percentage of KCl response.
* Significantly different from the value for noradrenaline, $P < 0.05$, ** $P < 0.01$.
NS: not significant.
The values are mean ± s.e.mean, and n is the number of observations.

tile response to NA (Figure 3c). Table 2 shows the slope and pA_2 values for these antagonisms obtained by the Arunlakshana-Schild plot (1959). The slope values for phentolamine and prazosin did not differ significantly from unity.

Relaxant responses of isolated spinal arteries

Acetylcholine (ACh), sodium nitroprusside (SNP), ATP, ADP, and adenosine caused concentration-dependent relaxations in the SBICA precontracted by 5×10^{-6}–10^{-5} M NA (Figure 4). ACh was the most potent vasorelaxant among these substances. The order of potency of relaxant agents was ACh > SNP ≫ ATP = ADP = adenosine. Addition of isoprenaline ranging from 10^{-8} M to 10^{-4} M did not produce any relaxation in these preparations (Figure 4).

Atropine (10^{-8}–10^{-6} M) caused a parallel shift to the right of the concentration-response curve to ACh. Schild plot

Table 2 Slope and pA_2 values of α-adrenoceptor antagonists against noradrenaline in canine spinal branch of intercostal artery

Antagonist	n	*Slope*	pA_2
Phentolamine	5	1.07 ± 0.12[NS]	8.79 ± 0.39
Prazosin	5	1.07 ± 0.14[NS]	10.20 ± 0.47
Yohimbine	5	No effect	No effect

[NS] The value does not significantly differ from unity.
The values are mean ± s.e.mean, and n is the number of observations.

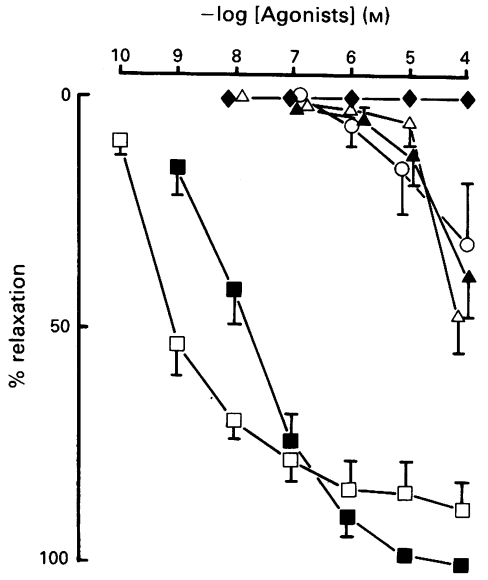

Figure 4 The relaxant responses to acetylcholine (□), sodium nitroprusside (■), ATP (△), ADP (○), adenosine (▲), and isoprenaline (◆) in canine isolated spinal branches of intercostal arteries contracted by 5×10^{-6}–10^{-5} M noradrenaline. On the ordinate scale, relaxations induced by 10^{-5} M sodium nitroprusside were taken as 100% in each experiment. All points represent the mean of 5 observations, and the vertical lines indicate s.e.mean.

analysis showed that the slope was 1.11 ± 0.12, which did not differ from unity and the pA_2 value was 10.6 ± 0.54 ($n = 6$).

As shown in Figure 5b, addition of ACh (10^{-11}–10^{-3} M) did not cause relaxation in preparations without endothelium, but SNP 10^{-5} M did. Pretreatment with 5×10^{-5} M aspirin did not affect ACh-induced relaxation (Figure 5c). On the other hand, the effect of ACh was significantly attenuated by pretreatment with 10^{-5} M methylene blue (Figure 5d). Pretreatment with oxyhaemoglobin 10^{-5} M increased the threshold concentration and decreased the maximum relaxation produced by ACh. These results are summarized in Figure 6.

Discussion

Contractile responses of isolated spinal arteries

The present results revealed that vasoactive substances such as AII, vasopressin, NA, 5-HT, adrenaline and dopamine

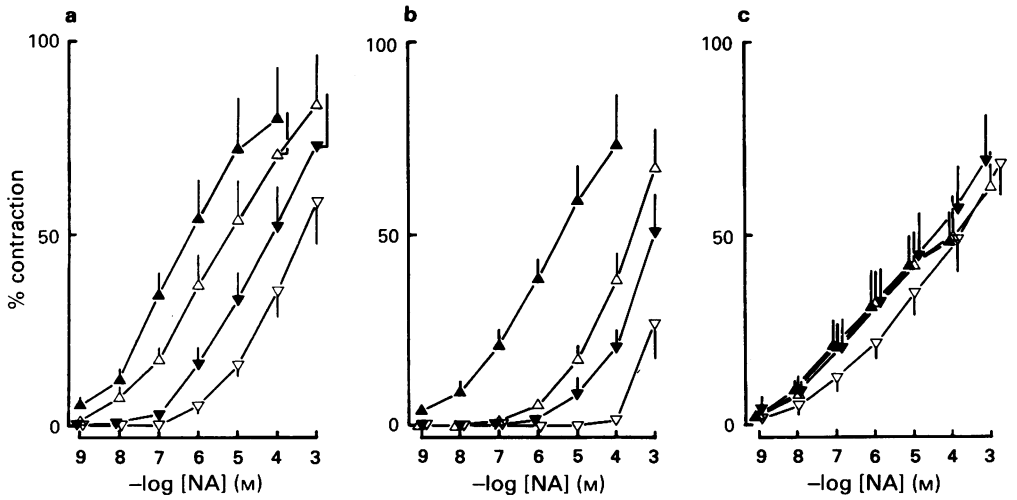

Figure 3 The effects of various α-adrenoceptor antagonists on contractile respones to noradrenaline in canine isolated spinal branches of intercostal arteries; (a) control (▲), 10^{-8} M (△), 10^{-7} M (▼), 10^{-6} M (▽), phentolamine ($n = 7$); (b) control (▲), 10^{-8} M (△), 10^{-7} M (▼), 10^{-6} M (▽), prazosin ($n = 6$); (c) control (▲), 10^{-8} M (△), 10^{-7} M (▼), 10^{-6} M (▽), yohimbine ($n = 6$). The ordinate scale is the same as in Figure 1. The vertical lines indicate the standard error of the mean.

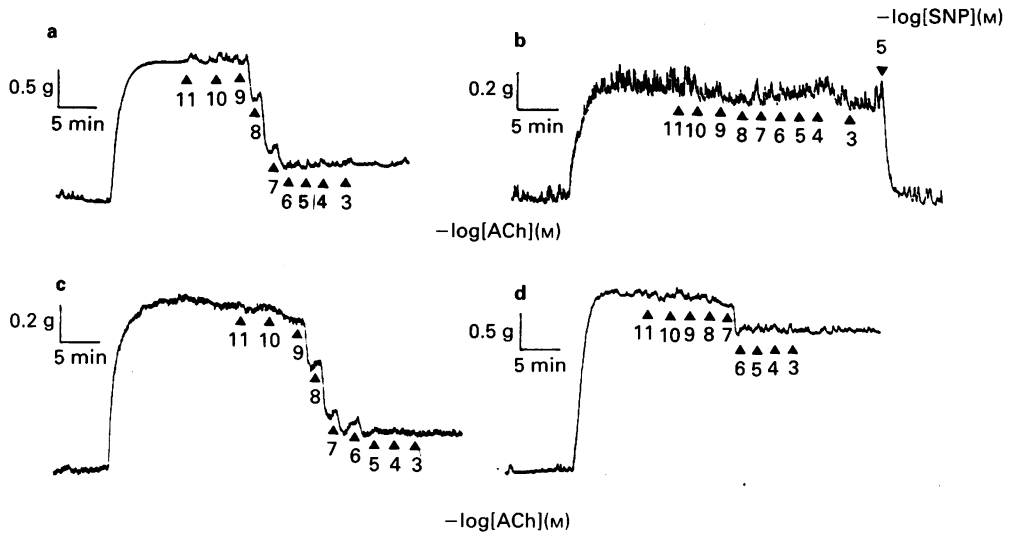

Figure 5 Typical responses of isolated canine spinal branches of intercostal arteries to acetylcholine; (a) control; (b) responses to acetylcholine (ACh) and sodium nitroprusside (SNP) in the preparation from which endothelial cells were removed; (c) pretreatment with 5×10^{-5} M aspirin; (d) pretreatment with 10^{-5} M methylene blue. All concentrations in this figure are given as negative logs.

caused a concentration-dependent contraction in the canine isolated spinal branch of the intercostal artery (SBICA). Among these substances, AII was the most potent vasoconstrictor (Figure 1). In general, AII is regarded as a potent vasoconstrictor agent in isolated small arterial preperations. However, there is a regional difference in the response of isolated arteries to AII, and it produces only a slight contraction in canine cerebral arteries (Toda & Miyazaki, 1978). In canine isolated cerebral arteries, AII also causes a relaxation in preparations contracted by $PGF_{2\alpha}$ (Toda & Miyazaki, 1981). This relaxation seems to be mediated mainly by the release of prostacyclin from the endothelial cells. In contrast to the cerebral arteries, pretreatment with 5×10^{-5} M aspirin produced no effect on the AII-induced contraction in the SBICA. Thus, there may be a difference in AII-induced release of endogenous vasodilator prostaglandins between cerebral and spinal arteries.

$PGF_{2\alpha}$ produces a marked contraction in isolated cerebral arteries of various species (White & Hagen, 1982). However, $PGF_{2\alpha}$ up to 10^{-4} M elicited only a small contraction of the SBICA in the present experiments. Thus, the response of the SBICA to $PGF_{2\alpha}$ seems to be markedly different from that of cerebral arteries.

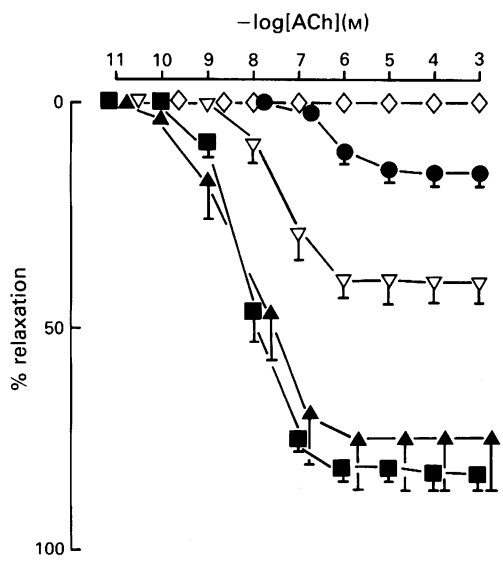

Figure 6 The effects of aspirin 5×10^{-5} M (▲, $n = 7$), methylene blue 10^{-5} M (▽, $n = 12$), oxyhaemoglobin 10^{-5} M (●, $n = 7$) and removal of endothelium (◇, $n = 6$) on relaxant responses to acetylcholine in isolated canine spinal branches of intercostal arteries. The control concentration-response curve to acetylcholine is represented by (■, $n = 5$). The vertical lines indicate s.e.mean.

Electron microscopic (Iwayama *et al.*, 1970; Nielsen *et al.*, 1971; Sato & Suzuki, 1975) and histochemical studies (Nielsen & Owman, 1967; Hernández-Pérez & Stone, 1974; Kawai & Ohhashi, 1986) demonstrated a dense adrenergic innervation in cerebral arteries of various animals. However, electrical stimulation of the sympathetic nerves produces only slight or no contraction of cerebral arteries (Raper *et al.*, 1972; Toda & Fujita, 1973). Furthermore, contraction of cerebral arteries, including the internal carotid artery, induced by exogenous NA is considerably smaller than that produced by other agonists such as 5-HT and $PGF_{2\alpha}$ (Bohr *et al.*, 1961; Toda & Fujita, 1973; Kawai *et al.*, 1984). Thus, the physiological significance of sympathetic nerves in the regulation of cerebral circulation is not clear. In the present study, the addition of tyramine produced no contraction in SBICA (Figure 1). This does not imply, however, that sympathetic nerves do not play an important role in the regulation of the spinal circulation. Morphological studies demonstrated the existence of sympathetic innervation of the smaller arteries supplying the spinal cord (Ohgushi, 1968; Marcus *et al.*, 1977; McNicolas *et al.*, 1980).

The pharmacological characteristics of the α-adrenoceptors in SBICA were investigated with selective agonists and antagonists. The results showed that NA (α_1- and α_2-agonist) and phenylephrine (α_1-agonist) caused almost the same maximum contraction, although the pD_2 value for phenylephrine was smaller than that for NA (Table 1). On the other hand, xylazine (α_2-agonist) produced only a small contraction (Figure 2, Table 1). These results suggest that NA and phenylephrine contract the SBICA mainly through activation of α_1-adrenoceptors on the smooth muscle cells. This view is also supported by the present results that phentolamine (α_1- and α_2-antagonist) and prazosin (α_1-antagonist) competitively inhibited the NA-induced contraction but yohimbine (α_2-antagonist) did not (Figure 3). Previous work has suggested that the α-adrenoceptor subtype in smooth muscle cells of canine cerebral arteries was α_2 in the middle cerebral and basilar arteries (Sakakibara *et al.*, 1982; Skärby *et al.*, 1983; Toda, 1983), and both α_1 and α_2 in the internal carotid arteries (Kawai *et al.*, 1988). On the other hand, NA-induced contractions are mediated by α_1-adrenoceptors in canine extracranial arteries of various tissues (Sakakibara *et al.*, 1982; Toda, 1983). Our findings suggest that the α-adrenoceptor subtype in the SBICA is similar to that in the extracranial arteries rather than in the cerebral arteries of dogs.

Relaxant responses of isolated spinal arteries

The addition of ACh, SNP, ATP, ADP, and adenosine produced concentration-dependent relaxations in SBICA which had been contracted by NA. On the other hand, isoprenaline up to 10^{-4} M did not cause any relaxation in the preparations. Stimulation of β-adrenoceptors produces cerebral vasodilatation *in vivo* (Edvinsson & MacKenzie, 1977). In addition, β-adrenoceptor agonists induce a relaxation of isolated cerebral vessels when the preparations have intrinsic tone. These findings suggest that β-adrenoceptors exist in cerebral arteries, contrasting with the present results in the SBICA.

Among the vasodilators used, ACh was the most potent on the SBICA. This relaxation seems to be mediated via muscarinic receptors, since it was competitively inhibited by atropine. Relaxation induced by ACh, but not that induced by SNP, was abolished by mechanical removal of the endothelium. It is well known that endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and prostacyclin (Moncada *et al.*, 1977) are released from vascular endothelial cells and cause vascular smooth muscle cells to relax. In the present experiments, treatment with aspirin, an inhibitor of cyclo-oxygenase, did not affect the ACh-induced relaxation, suggesting that prostacyclin did not play an important role in the ACh-induced relaxation. On the other hand, oxyhaemoglobin, an inhibitor of EDRF, and methylene blue, an inhibitor of guanylate cyclase, markedly suppressed the ACh-induced relaxation in the spinal arteries. All these findings strongly suggest that the ACh-induced relaxation in the SBICA is mainly mediated through release of EDRF. A previous report suggests that ACh-induced endothelium-dependent relaxation is relatively small in canine cerebral arteries (Tsukahara *et al.*, 1986). There may be a regional difference in endothelial function or sensitivity of vascular smooth muscles to EDRF between spinal and cerebral arteries.

The present experimental results suggest that the pharmacological characteristics of SBICA are quite different from those of the isolated canine cerebral arteries reported in previous studies. In the canine SBICA, the addition of NA produces considerable contraction which is mainly mediated via α_1-adrenoceptors. On the other hand, ACh causes a marked relaxation of the SBICA due to the release of EDRF from the endothelial cells.

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Effects of pre-contraction with endothelin-1 on α_2-adrenoceptor- and (endothelium-dependent) neuropeptide Y-mediated contractions in the isolated vascular bed of the rat tail

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1 The pressor effects to bolus doses of the α_2-adrenoceptor agonist UK-14,304 were studied in the isolated vascular bed of the perfused rat tail before and after increasing the perfusion pressure with infusions of endothelin-1. Those of neuropeptide Y were studied before and after pre-constriction with endothelin-1 or 5-hydroxytryptamine. The pressor effects of neuropeptide Y were studied before and after functional disruption of the endothelium with the detergent CHAPS.

2 Endothelin-1 and the α_1-adrenoceptor agonist phenylephrine induced dose-dependent vasoconstriction, endothelin-1 being some 10^4 times more potent than phenylephrine [log dose (mol) of the ED_{50} for endothelin-1 and phenylephrine: -11.8 ± 0.2 ($n = 7$), -8.2 ± 0.2 ($n = 5$) respectively].

3 Under control conditions, at basal perfusion pressures, UK-14,304 and neuropeptide Y were virtually inactive as vasoconstrictors. Following a sustained increase in perfusion pressure by infusions of endothelin-1 (2.5–10 nM at 0.8 ml min^{-1}), however, both UK-14,304 and neuropeptide Y induced dose-dependent pressor responses and both were some 10^2 times more potent than phenylephrine [log dose (mol) of the ED_{50} for UK-14304 and neuropeptide Y: -10 ± 0.5 ($n = 6$), -10.3 ± 0.4 ($n = 6$) respectively]. Responses to neuropeptide Y also were uncovered when vascular tone was increased with 5-hydroxytryptamine (5–20 nM) [log dose (mol) of the ED_{50} for neuropeptide Y: -10.2 ± 0.2 ($n = 6$)].

4 Pre-constriction-induced pressor responses to UK-14,304 were inhibited by 1 μM rauwolscine whilst those to neuropeptide Y were inhibited by disruption of the endothelium. Removal of the endothelium had no significant effect on the pressor responses to 4 pmol or 8 pmol endothelin-1 and had no effect on the increase in perfusion pressure induced by the endothelin-1 infusions but did decrease the time-course of pressor responses to bolus injections of endothelin-1. Endothelial disruption had no significant effect on the vasoconstriction induced by all but one of the doses of phenylephrine administered [log dose (mol) of the ED_{50} for phenylephrine after CHAPS: -8.6 ± 0.2 ($n = 5$)], indicating that the responsiveness of the vascular smooth muscle was not destroyed by CHAPS. This treatment did, however, slow the onset and prolong the time course of the phenylephrine-induced responses.

5 These results indicate that, in the isolated vascular bed of the rat tail, pressor responses to both α_2-adrenoceptor- and neuropeptide Y receptor-activation are uncovered by agonist-induced preconstriction including that to endothelin-1. Neuropeptide Y-induced vasoconstriction was endothelium-dependent.

Introduction

The pithed rat demonstrates the existence of post-junctional α_1- and α_2-adrenoceptors in the vasculature, both of which contribute to systemic pressor responses (Drew & Whiting, 1979; Docherty *et al.*, 1979; Docherty & McGrath, 1980). Until recently, however, there were few *in vitro* examples of arterial vessels or resistance beds that possess functional populations of postjunctional α_2-adrenoceptors sensitive to the selective α_2-adrenoceptor antagonists yohimbine, rauwolscine and idazoxan and resistant to the selective α_1-adrenoceptor antagonist prazosin. However, in the isolated vascular bed of the rat tail, rauwolscine-sensitive and prazosin-resistant α_2-adrenoceptors were uncovered by increasing the vascular tone with arginine vasopressin (Templeton *et al.*, 1989). The ability to uncover postjunctional α_2-adrenoceptor-mediated responses is not restricted to arginine vasopressin i.e. 'tone' induced by 5-hydroxytryptamine also uncovers responses to UK-14,304 in the isolated perfused rat tail (Templeton, 1988); prostaglandin $F_{2\alpha}$, phenylephrine, acetylcholine, 5-hydroxytryptamine and histamine in the canine portal vein uncover responses to the selective α_2-adrenoceptor agonist B-HT 920 (Furuta, 1988); non-pressor doses of angiotensin II enhance responses to the selective α_2-adrenoceptor agonist UK-14,304 in the rabbit saphenous artery (Dunn *et al.*, 1989) and in the canine plantaris artery, responses to B-HT 920 are uncovered in the presence of the calcium channel activator Bay K 8644 (Sulpizio & Hieble, 1987).

Neuropeptide Y is co-stored with noradrenaline in the large dense cored vesicles of sympathetic nerve endings (Ekblad *et al.*, 1984) and they are co-released from the sympathetic varicosities upon nerve stimulation (Lundberg *et al.*, 1984). Previous *in vitro* studies have shown that, like α_2-adrenoceptor agonists, neuropeptide Y is not a potent vasoconstrictor in its own right (Edvinsson *et al.*, 1987), although low levels of neuropeptide Y can potentiate the vasoconstriction induced by noradrenaline, sympathetic nerve stimulation and other vasoconstrictor agents (Edvinsson *et al.*, 1984; 1987). Furthermore, in cat cerebral vessels and the pig spleen vasculature, it has been shown that neuropeptide Y and α_2-adrenoceptor agonists share the ability to inhibit adenylate cyclase through a pertussis-toxin-sensitive mechanism and to decrease intracellular levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Fredholm *et al.*, 1985; Lundberg *et al.*, 1988). We have therefore now investigated and compared the influence of agonist-induced tone on the vascular responses to neuropeptide Y and an α_2-adrenoceptor agonist.

Endothelin-1 is a potent vasoconstrictor in several isolated veins and arteries (Yanagasawa *et al.*, 1988; D'Orleans-Juste *et al.*, 1988) and can facilitate pressor effects of noradrenaline in rabbit ear artery (Wong-Dusting *et al.*, 1989) but little is known about its interaction with peripheral postjunctional α_2-adrenoceptor activity.

We have studied the interactions between endothelin-1, neuropeptide Y and the α_2-adrenoceptor agonist UK-14,304

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in the isolated vascular bed of the rat tail. Vasoconstrictor responses to UK-14,304 were studied in the presence of endothelin-1 induced tone and those to neuropeptide Y were studied in the presence of tone induced by both endothelin-1 and 5-hydroxytryptamine. Previous studies have suggested that neuropeptide Y-induced vasoconstriction is endothelium-dependent (Daly & Hieble, 1987; Hieble *et al.*, 1989) and this was investigated here by disruption of the vascular endothelium of the rat tail vasculature with the detergent CHAPS.

Methods

Male Wistar rats (280–320 g) were killed by stunning followed by cervical dislocation. The ventral surface of the tail was shaved and the skin reflected for 3–5 cm around the proximal end of the tail. The tail artery was exposed with blunt forceps and cleaned of fat and connective tissue and cannulated with a polyethylene cannula. The tail was amputated by cutting through an intervertebral disc and placed on an elevated plastic platform allowing the arterial cannula to be connected to a perfusion circuit. The tail vasculature was perfused at a rate of 0.8 ml min^{-1} with a modified physiological salt solution the temperature of which, at the point of entry into the tail artery, was monitored with a digital thermometer (Digitron Instrumentation Ltd) and maintained at 37°C ± 0.4°C ($n = 12$). The temperature of the perfusate leaving the tail was also monitored and was found to be 26.5°C ± 0.3°C ($n = 12$). After the start of perfusion, blood filled drops were observed from the cut end of the tail, indicating a successful cannulation. The perfusion pressure was allowed to stabilize for 45 min before the start of any experimental procedure.

Measurement of agonist-mediated responses at basal and raised perfusion pressure

Dose-response curves to endothelin-1, neuropeptide Y, phenylephrine and UK-14,304 were constructed by bolus injection of drugs into the perfusing solution via an injection port close to the point of cannulation. The injectate volume was constant at 0.01 μl and doses are expressed as quantity (nmol etc.). Drugs added to the perfusate reservoir are expressed as concentration (nM etc.). In all experiments, the perfusion pressure was allowed to return to baseline before the administration of a subsequent dose of agonist. In those preparations in which endothelin-1 dose-response curves were constructed, the number of doses it was possible to administer in each was variable due to the long recovery period required for the higher doses. The actual doses administered to each tail were therefore variable and the '*n*' values for each dose were correspondingly variable in these experiments (i.e. see Figure 5a).

After establishing control responses to neuropeptide Y and UK-14,304, endothelin-1 (2.5–10 nM) was included in the perfusate until the perfusion pressure was approximately doubled. After a stable perfusion pressure had been maintained over a 10 min period, dose-response curves for the two agonists were repeated. In other experiments, a dose-response curve to neuropeptide Y was determined before and after doubling the vascular perfusion pressure with 5-hydroxytryptamine (5–20 nM). The concentrations of endothelin-1 and 5-hydroxytryptamine used were variable as the dose required to double the vascular perfusion pressure varied between preparations. In those preparations in which UK-14,304 was studied, after establishing the second dose-response curve, 1 μM rauwolscine was added to the perfusate (still containing endothelin-1) and the dose-response curve for UK-14,304 repeated for a third time.

Effect of endothelium removal on neuropeptide Y-, endothelin-1- and phenylephrine-induced responses

In those preparations in which responses to neuropeptide Y were studied in the presence of endothelin-1, after establishing the second dose-response curve, destruction of the endothelium was achieved by perfusion of the vascular bed with 0.3% (w/v) solution of the detergent CHAPS in distilled water at 2 ml min^{-1} for 90 s. The infusion of endothelin-1 was then immediately resumed. After a 30 min re-equilibration period, the dose-response curve for neuropeptide Y (still in the presence of endothelin-1) was repeated for a third time. Removal of the endothelium was confirmed both histologically and pharmacologically. Histologically, the presence or absence of the endothelium was determined by use of cacodylate buffered fixative (Sabatini *et al.*, 1963) on artery samples which were cut on a cryostat. Pharmacologically, in four preconstricted preparations, the effect of CHAPS on acetylcholine-induced vasorelaxation was determined as it is known that removal of the vascular endothelium abolishes endothelium-dependent vasorelaxation to acetylcholine.

In order to establish if treatment with CHAPS affected the responsiveness of the vascular smooth muscle and if the pressor effect of endothelin-1 was affected by such treatment, in those preparations in which dose-response curves for phenylephrine and endothelin-1 had been established, the responsiveness of the vasculature to these agonists was determined after CHAPS treatment.

Drugs and solutions

The composition of the physiological salt solution was (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11 and Na_2EDTA 0.023. Ficoll (2%, molecular weight approximately 70,000) was included to prevent water retention. The following compounds were used: rauwolscine HCl (Roth); UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer); phenylephrine HCl (Sigma); 5-hydroxytryptamine creatinine sulphate, (BDH biochemicals); acetylcholine chloride (Sigma); CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1- propanesulphonate, Sigma); neuropeptide Y (I.C.I. Pharmaceuticals Division, Cheshire) and endothelin-1 (human, Scientific Marketing Associates).

Statistical analysis

All results are given as the mean ± s.e.mean. The ED_{50}s for the agonists were calculated for each individual preparation (by BBC microcomputer graphical interpolation) and meaned within groups. For all experiments, differences between means were compared by paired Student's *t* test. For those experiments in which the *n* for the first dose-response-curve was greater than that for the subsequent one to be compared, a paired Student's *t* test was applied only to those experiments in which both procedures were conducted, and it is these results which are given below.

Results

Pressor responses to UK-14,304 in the presence and absence of endothelin-1-induced tone, and the effect of rauwolscine

Figure 1 shows the vasoconstrictor responses to UK-14,304 at basal perfusion pressure and in the presence of endothelin-1-induced tone (in the absence and presence of 1 μM rauwolscine). At a basal perfusion pressure of 76 ± 8 mmHg ($n = 6$), UK-14,304 produced a maximum response of only 6 mmHg. During an infusion of endothelin-1 (2.5–10 nM), sufficient to double the perfusion pressure of each individual preparation (162 ± 18 mmHg; $n = 6$), UK-14,304 elicited dose-dependent increases in perfusion pressure. The maximum response was approximately 90 mmHg and the log dose (mol) of the ED_{50} for UK-14,304 was −10 ± 0.5 ($n = 6$). Following an injection of UK-14,304, perfusion pressure returned to baseline after 5–20 min depending on the dose administered. The highest dose of UK-14,304 induced a pressor response

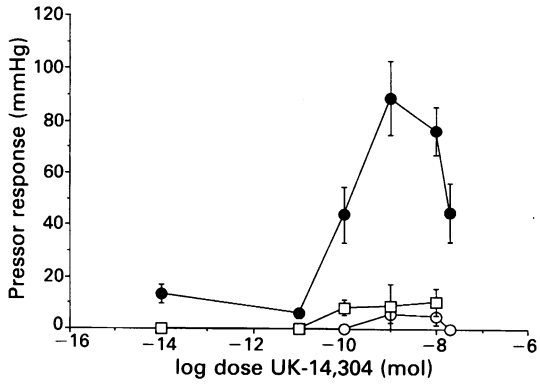

Figure 1 Pressor responses to UK-14,304 in the isolated perfused vascular bed of the rat tail: (○) control responses ($n = 6$); (●) responses in preparations in which the vascular tone has been raised with 2.5–10 nM endothelin-1 [0.8 ml min^{-1}] ($n = 6$) and (□) responses in preparations in which vascular tone has been raised with endothelin-1 and 1 μM rauwolscine included in the perfusate ($n = 4$). The points show the mean and the vertical lines show s.e.mean.

which was significantly less than the maximum response attained ($P < 0.05$). Figure 1 also shows that the responses induced by UK-14,304 were virtually abolished by including 1 μM rauwolscine in the perfusion fluid. Rauwolscine itself did not affect the increase in vascular tone induced by endothelin-1, the perfusion pressure after rauwolscine being 162 ± 18 mmHg ($n = 4$).

Pressor responses to neuropeptide Y in the presence and absence of endothelin-1- or 5-hydroxytryptamine-induced tone and the effect of endothelium removal

Figure 2a shows the vasoconstrictor responses to neuropeptide Y at basal perfusion pressure, after vascular tone was doubled with endothelin-1 and after removal of the endothelium with CHAPS (in the presence of endothelin-1). At a basal perfusion pressure of 72 ± 7 mmHg ($n = 6$), neuropeptide Y failed to elicit a response greater than 14 ± 6 mmHg. After vascular tone had been increased to 157 ± 14 mmHg ($n = 6$), neuropeptide Y induced a dose-dependent increase in perfusion pressure which returned to baseline in 10–40 min depending on the dose administered. The maximum response elicited was 110 mmHg and the log dose (mol) of the ED_{50} for neuropeptide Y was −10.3 ± 0.4 ($n = 6$). In 3 experiments it was also confirmed that neuropeptide Y-induced responses were not affected by 1 μM rauwolscine. Figure 2a shows that removal of the endothelium with CHAPS (still in the presence of endothelin-1) abolished all responses to neuropeptide Y. CHAPS treatment did not significantly affect the increase in vascular tone induced by the endothelin-1 infusion, the perfusion pressure after treatment with CHAPS being 129 ± 16 mmHg ($n = 4$). In 3 experiments it was also confirmed that CHAPS did not affect the ability of the rat tail vasculature to respond to UK-14,304.

Figure 2b shows the vascular responses to neuropeptide Y before ($n = 6$) and after increasing the vascular tone with 5-hydroxytryptamine [5–20 nM] ($n = 6$). At a basal tone of 69 ± 7 mmHg, neuropeptide Y induced a pressor response of 54 ± 11 mmHg only at the highest dose used. After vascular tone was increased with 5-hydroxytryptamine to 148 ± 18 mmHg, neuropeptide Y elicited dose-dependent pressor responses, attaining a maximum response of 210 mmHg with a log dose (mol) of the ED_{50} of −10.2 ± 0.2. From Figure 2b it can be seen that the dose-response curve for neuropeptide Y in the presence of 5-hydroxytryptamine was still rising at the highest dose of neuropeptide Y used (the highest available) and it is possible that even greater pressor responses could have been attained at higher doses. It should also be noted that the ED_{50} for neuropeptide Y may, therefore, not have been calculated from a true maximum and may be higher in value.

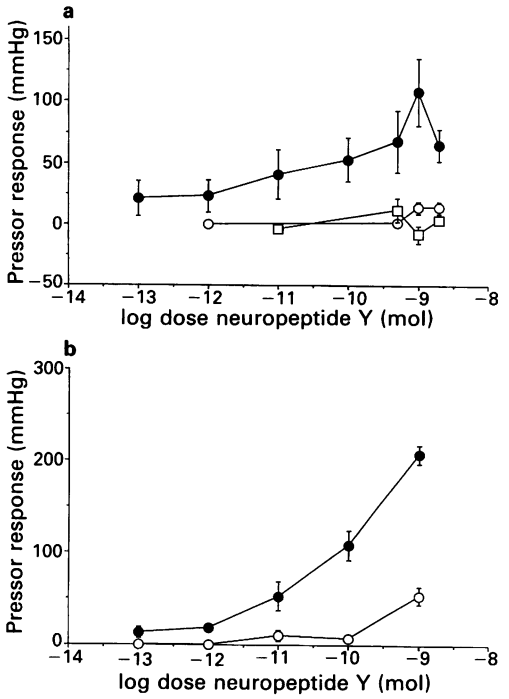

Figure 2 Pressor responses to neuropeptide Y in the isolated perfused vascular bed of the rat tail. (a) Preconstriction with endothelin-1: (○) control responses ($n = 6$); (●) responses in preparations in which the vascular tone has been raised with 2.5–10 nM endothelin-1 (0.8 ml min^{-1}) ($n = 6$) and (□) responses obtained in preparations in which the endothelium had been destroyed with CHAPS (in the continued presence of endothelin-1) ($n = 4$). (b) Preconstriction with 5-hydroxytryptamine: (○) control responses ($n = 6$) and (●) responses in preparations in which the vascular tone has been raised with 5–20 nM 5-hydroxytryptamine ($n = 6$). All points show the mean and the vertical lines show s.e.mean.

In 2 preparations in which vascular perfusion pressure was increased from mean values of 60 mmHg to 200 mmHg with 5 nM 5-hydroxytryptamine, acetylcholine (5.5 nmol) induced a vasorelaxation of 80 mmHg. This was abolished after perfusion of the preparation with CHAPS. Likewise, in 2 preparations in which tone was increased with 1 μM phenylephrine (from means of 60 mmHg to 150 mmHg) acetylcholine (5.5 nmol) induced an initial pressor response followed by a vasorelaxation (Figure 3a). After CHAPS treatment the pressor response remained whilst the vasorelaxation was abolished (Figure 3b). This indicates that the endothelium was successfully removed with CHAPS but the ability of the smooth

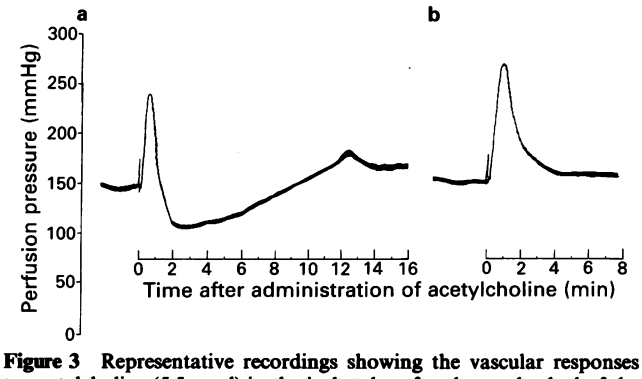

Figure 3 Representative recordings showing the vascular responses to acetylcholine (5.5 nmol) in the isolated perfused vascular bed of the rat tail preconstricted with 1 μM phenylephrine. (a) Control response. (b) Response after disruption of the vascular endothelium with CHAPS.

muscle to contract was not affected. Removal of the endothelium with CHAPS was also confirmed histologically.

Pressor responses to phenylephrine and endothelin-1 and the effect of endothelium removal

Figure 4a shows the vasoconstrictor responses to the selective α_1-adrenoceptor agonist phenylephrine before ($n = 5$) and after perfusion of the tail vascular bed with CHAPS ($n = 5$). Before CHAPS infusion, the basal perfusion pressure was 69 ± 6 mmHg and phenylephrine produced a maximum response of 210 mmHg and its log dose (mol) of the ED_{50} was -8.2 ± 0.2. Following phenylephrine administration, the perfusion pressure recovered after 1–15 min. Treatment with CHAPS did increase the perfusion pressure in some preparations (e.g. see Figure 4b) but overall, had no statistically significant effect on the basal perfusion pressure of the CHAPS-treated group of preparations (93 ± 11 mmHg), and no significant effect on the ability of the vascular bed to respond to phenylephrine (log dose (mol) of the ED_{50} for phenylephrine in the presence of CHAPS was -8.6 ± 0.2) except at the 2 nmol dose. At this one dose, removal of the endothelium with CHAPS significantly decreased the phenylephrine-induced vasoconstriction. Removal of the endothelium slowed the onset and prolonged the time course of the phenylephrine response as depicted in Figure 4b.

Endothelin-1 induced dose-dependent vasoconstriction from a basal perfusion pressure of 73 ± 5 mmHg ($n = 5$–9). Its maximum pressor response was 110 mmHg and the log dose (mol) of its ED_{50} was -11.8 ± 0.2 ($n = 7$) [Figure 5a]. Perfusion pressure returned to baseline some 5–60 min after endothelin-1 administration, depending on the dose administered. Removal of the endothelium with CHAPS had no significant effect on the vascular responses to bolus injections of endothelin-1. With 4 pmol endothelin-1 the response before CHAPS was 51 ± 9 mmHg ($n = 9$) and after CHAPS was 40 ± 8 mmHg ($n = 5$). With 8 pmol endothelin-1 the response before CHAPS was 70 ± 13 mmHg ($n = 7$) and after CHAPS was 127 ± 27 mmHg ($n = 4$). In 2 out of 4 of these preparations, the perfusion pressure was increased by CHAPS treatment and, in all 4, the time course of the endothelin-1 response was altered by removal of the endothelium, being slower in onset and shorter in duration (Figure 5b).

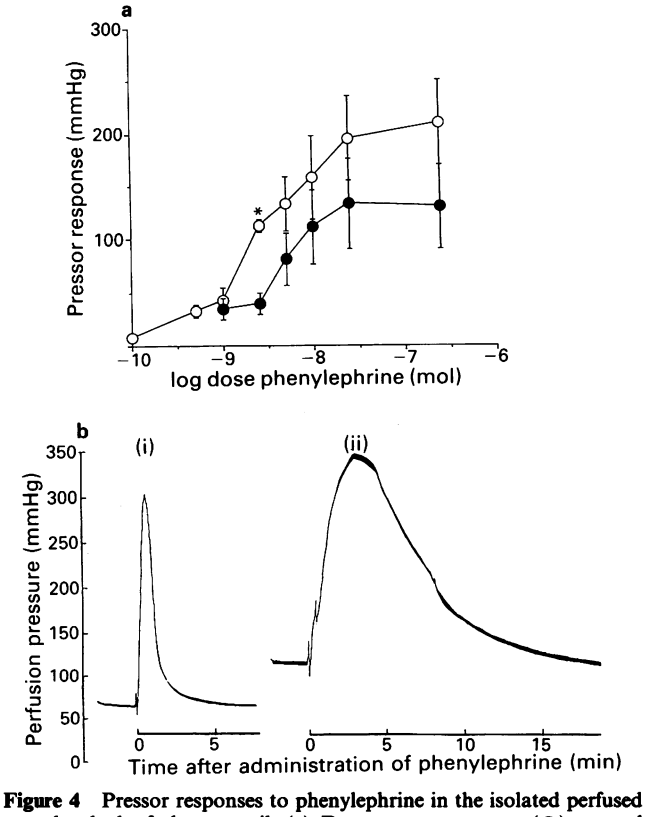

Figure 4 Pressor responses to phenylephrine in the isolated perfused vascular bed of the rat tail. (a) Dose-response curve: (○) control responses ($n = 5$) and (●) the responses obtained in preparations in which the endothelium had been destroyed with CHAPS ($n = 5$). The points show the mean and vertical lines show s.e.mean. * $P < 0.05$ compared with CHAPS-treated group (paired Student's t test). (b) Representative recordings showing the time-course of phenylephrine (10 pmol) induced responses in the isolated perfused vascular bed of the rat tail: (i) before removal of the endothelium; (ii) after endothelium removal with CHAPS.

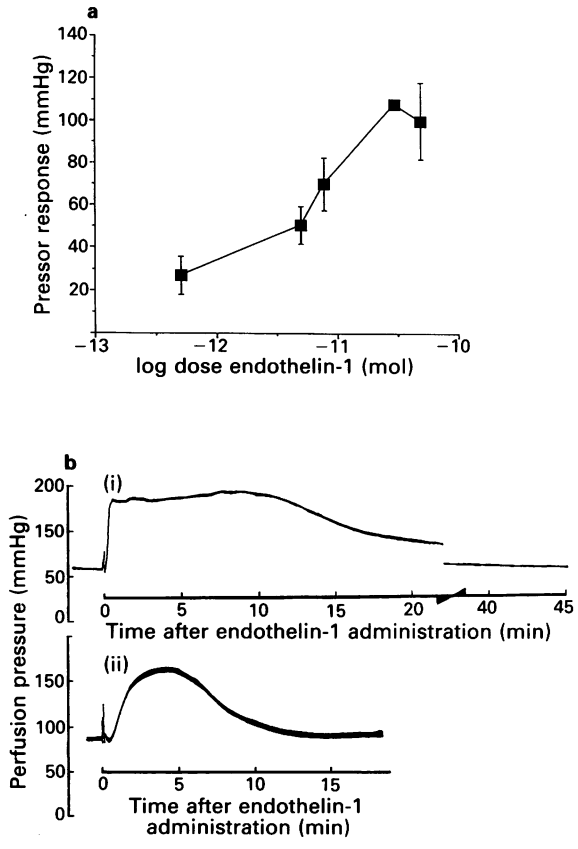

Figure 5 Pressor responses to endothelin-1 in the isolated perfused vascular bed of the rat tail. (a) Dose-response curve ($n = 5$–9). The points show the mean and the vertical lines show s.e.mean. (b) Representative recordings showing the time-course of endothelin-1 (8 pmol)-induced responses in the isolated perfused vascular bed of the rat tail: (i) before removal of the endothelium; (ii) after endothelium removal with CHAPS.

Discussion

The influence of vascular tone on α_2-adrenoceptor- and neuropeptide Y-receptor-mediated vasoconstriction

Under the control experimental conditions used here the temperature of the perfusate was decreased by 10.5°C on passage through the tail indicating that the vasculature was markedly vasodilated. This confirms the importance of the rat tail vasculature in thermoregulation (Rand *et al.*, 1965; Raman *et al.*, 1983). This study shows that, in the isolated perfused vascular bed of the rat tail, at basal vascular tone, the α_2-adrenoceptor agonist UK-14,304 is virtually inactive at inducing pressor responses. Induction of vascular tone with endothelin-1 uncovered a functional population of α_2-adrenoceptors indicated by the ability of the selective α_2-adrenoceptor agonist UK-14,304 to elicit dose-dependent vasoconstrictions which were sensitive to a selective concentration of the α_2-adrenoceptor antagonist rauwolscine. A previous study using a similar preparation has shown that

arginine vasopressin-induced tone will also uncover rauwolscine-sensitive, prazosin-resistant α_2-adrenoceptor-mediated responses and has no effect on vascular responses to phenylephrine (Templeton *et al.*, 1989).

Some previous studies using segments of the isolated tail artery of the rat and selective α_1-adrenoceptor and α_2-adrenoceptor antagonists have indicated postjunctional α_2-adrenoceptor involvement in vasoconstriction induced by nerve stimulation or catecholamines (Medgett & Langer, 1984; 1986; de Moraes *et al.*, 1988). Raising the vascular tone with endothelin-1 not only uncovered responses to UK-14,304 but showed it to be some 10^2 times more potent than the α_1-adrenoceptor agonist phenylephrine (as indicated by its ED_{50} value) though producing maximum responses some 50% of those elicited by phenylephrine. The responses to UK-14,304 were also of longer duration than those to phenylephrine. The highest dose of UK-14,304 induced a pressor response that was significantly less than the maximum response. The reason for this is unclear, though there is some evidence to suggest that in both un-constricted and pre-constricted rat isolated tail arteries, high doses of α_2-adrenoceptor agonists may induce release of an endothelium-dependent relaxing factor which counteracts their direct contractile activity (Matsuda *et al.*, 1985).

At basal vascular tone, neuropeptide Y was virtually inactive at inducing pressor responses. However, in those vascular beds pre-constricted with endothelin-1, neuropeptide Y elicited dose-dependent vasoconstrictions. Like UK-14,304, neuropeptide Y induced a maximum response only 50% that of phenylephrine, was 10^2 times more potent than phenylephrine as indicated by its ED_{50} value and its responses were of a longer duration than those of phenylephrine. Pre-constriction with 5-hydroxytryptamine instead of endothelin-1 had a somewhat different effect on responses induced by neuropeptide Y. Although its ED_{50} value was not changed, in the presence of 5-hydroxytryptamine, neuropeptide Y elicited responses twice the magnitude of those elicited in the presence of endothelin. The results indicate that, in the perfused vascular bed of the rat tail, the magnitude of the response to neuropeptide Y depends upon the pre-constricting substance. Furuta (1988) also demonstrated that, in canine isolated portal vein, the α_2-adrenoceptor agonist B-HT 920 failed to elicit responses at basal vascular tone but could induce concentration-dependent responses, the maxima of which depended both on the pre-contraction levels and on the pre-contracting substance.

Under conditions of increased sympathetic discharge such as occurs in hypertension, haemorrhagic shock and hypoxia, co-release of neuropeptide Y may contribute to the observed increases in regional resistance. That neuropeptide Y overflow is increased under conditions of increased sympathetic discharge is indicated by the observation that circulating neuropeptide Y levels increase following haemorrhage in rats (Morris *et al.*, 1987) and exercise in man (Morris *et al.*, 1986). There is increasing evidence that, in man, circulating levels of endothelin-1 are raised in conditions in which regional vasoconstriction is increased such as in acute renal failure (Tomita *et al.*, 1989) and essential hypertension (Saito *et al.*, 1990). Recalculating the data in these studies shows that the circulating concentration of endothelin-1 is increased to approximately 10 pmol–1 nmol in such patients. This range of endothelin-1 concentration has been shown to be sufficient to induce contraction of human umbilical vessels *in vitro* (Haegerstrand *et al.*, 1989). Thus, combinations of increased sympathetic tone, increased circulating endothelin-1 and increased circulating neuropeptide Y levels may promote increased expression of prolonged α_2-adrenoceptor- and neuropeptide Y-mediated regional vasoconstriction in such disease states.

Acetylcholine-induced vasorelaxations in the perfused rat tail are inhibited by both haemoglobin (an inhibitor of endothelium-dependent relaxing factor, Griffith *et al.*, 1984) and atropine (Templeton, 1988). Perfusion of blood vessels with the detergent CHAPS has been shown to remove the vascular endothelium without any significant effect upon the underlying vascular smooth muscle (Tesfamariam *et al.*, 1984; Hiley *et al.*, 1987). In the tail bed, the vasoconstrictor responses to phenylephrine, endothelin-1 and acetylcholine were not significantly affected, whilst the vasorelaxation induced by acetylcholine was abolished by CHAPS. This proof of selective endothelium removal was supported by histological evidence of endothelial damage. The simplest explanation of these results is that, in the rat perfused tail, perfusion of CHAPS disrupts the vascular endothelium without affecting the underlying smooth muscle.

The neuropeptide Y-induced contractions in the presence of agonist-induced tone were not observed after endothelium removal. This is consistent with previous studies showing that neuropeptide Y-induced potentiation of adrenoceptor-mediated contractions in the rabbit ear artery and canine saphenous vein are endothelium-dependent (Daly & Hieble, 1987; Hieble *et al.*, 1989) but contrary to other studies showing that the direct vasoconstrictor effect of neuropeptide Y on small human skeletal muscle arteries and pig splenic arteries is not endothelium-dependent (Pernow, 1989). This indicates that there may be species or tissue differences in the endothelium-dependency of neuropeptide Y-induced responses. In the rat tail vasculature, it is possible that neuropeptide Y requires the presence of an endogenous endothelium-dependent constricting factor, such as endothelin, to elicit its vasoconstrictor response. The continuous infusion of endothelin-1 did not, however, permit neuropeptide Y-induced responses in the absence of endothelium. This could be because endothelin-1 is not the endogenous endothelium-dependent constricting factor involved in the neuropeptide Y response or that it is crucial for such a factor to be released endogenously from the endothelium to exert its permissive effect on the vascular smooth muscle. The actual endothelial mechanism of the neuropeptide Y response is currently under investigation.

It was of interest to investigate whether or not neuropeptide Y pre-constriction uncovers α_2-adrenoceptor-induced responses. Whilst vascular tone could not be elevated and sustained by neuropeptide Y in the isolated perfused vascular bed of the rat tail, in 2 experiments, infusions of neuropeptide Y (100–200 pmol min^{-1}) did not uncover UK-14,304-induced responses (data not shown). Aubert *et al.* (1988) found that neuropeptide Y potentiated pressor responses to noradrenaline but not to B-HT 933 in the conscious rat. However, Hieble *et al.* (1989) demonstrated that neuropeptide Y potentiated the α_2-adrenoceptor-mediated contraction in canine saphenous vein. This indicates that there may be species or tissue differences in this effect of neuropeptide Y. The potentiation of α_2-adrenoceptor- and neuropeptide Y-mediated responses by preconstriction is probably due to a generalized change such as an increase in $[Ca^{2+}]_i$ regardless of the effector pathway leading to such an increase. It follows from this that responses to both α_2-adrenoceptor agonists and neuropeptide Y would be extremely sensitive to changes in Ca^{2+} availability. Indeed, in the rat isolated tail artery, α_2-adrenoceptor agonist-induced responses are more sensitive than are α_1-adrenoceptor agonist-induced responses to the Ca^{2+} channel antagonists nifedipine and nicardipine, to decreased extracellular Ca^{2+} and to the Ca^{2+} entry promoter Bay K 8644 (Su *et al.*, 1986; Abe *et al.*, 1987). Neuropeptide Y-induced vasoconstriction in human skeletal muscle and renal arteries is sensitive to nifedipine (Pernow, 1988) and in the pithed rat, neuropeptide Y-induced potentiation of α-adrenoceptor activation is also Ca^{2+}-dependent (Dahlöf *et al.*, 1985).

The effect of endothelium removal on pressor responses induced by endothelin-1 and phenylephrine

Removal of the endothelium with CHAPS did not significantly affect the maximum responses to phenylephrine or its ED_{50} and significantly reduced the response at only one indi-

vidual dose. However, there is no positive evidence for endothelium-dependency of the phenylephrine-induced response. The effect of phenylephrine seems to be directly on the vascular smooth muscle and the ability of the vascular smooth muscle to contract is certainly not greatly affected by treatment with CHAPS. In some preparations, removal of the endothelium did increase the basal perfusion pressure. This could indicate a degree of tonic endothelium-derived relaxing factor release from the rat tail vasculature although, as this effect was not seen in all CHAPS-treated preparations, it may simply indicate a less specific 'damage'. More consistently, however, the time-course of the phenylephrine-induced response was markedly prolonged upon removal of the endothelium, usually by a factor of three and the onset of the response was slowed. Garland (1989) reported that, in the rabbit basilar artery, removal of the endothelium inhibited the production of phenylephrine-induced action potentials and that this was associated with a slower development in tension. A similar effect could explain the present increased time-course of the response to phenylephrine after CHAPS. Alternatively, in the rat tail vasculature under normal circumstances, the responses to phenylephrine may be buffered by release of an endothelium-dependent relaxing factor and/or facilitated by an endothelium-dependent constricting factor. This is such a clear effect that it deserves further study.

The most potent agonist used in this study was endothelin-1, being 10^4 times more potent than phenylephrine and 10^2 times more potent than UK-14,304 and neuropeptide Y (even in the presence of vascular tone). This is consistent with its very high potency in most vascular preparations on which it has been tested (e.g. Yanagasawa *et al.*, 1988; D'Orleans-Juste *et al.*, 1988). Removal of the endothelium did not significantly affect the responses to bolus injections of endothelin-1, although there was an increase in endothelin-1 infusion-induced perfusion pressure in some preparations following CHAPS treatment which may be due to the ability of prolonged infusions of endothelin-1 to induce release of endothelium-dependent relaxing factor from the intact endothelium (Warner *et al.*, 1989). As with phenylephrine, however, the evidence suggests that the vasoconstrictor effect of endothelin-1 is via receptors located on the vascular smooth muscle. The time course of the endothelin-1 induced response was reduced by removal of the endothelium and this was associated with slower development of the pressor response. Again, this may indicate that, in the endothelium-intact rat tail vasculature some endothelium-dependent factor may contribute to the development and maintenance of endothelin-1-induced responses.

In conclusion, in the rat isolated tail vasculature, the α_2-adrenoceptor agonist UK-14,304 did not induce vasoconstriction until the vascular tone of the preparation was increased with endothelin-1. Likewise, neuropeptide Y did not cause vasoconstriction until the vascular bed was preconstricted with either endothelin-1 or 5-hydroxytryptamine and the maximum response to neuropeptide Y was higher in the presence of 5-hydroxytryptamine. The neuropeptide Y-induced pressor responses were endothelium-dependent. It is suggested that a preconstriction-induced increase in $[Ca^{2+}]_i$ is necessary for responses to these two agonists. The prolonged, and potent responses to α_2-adrenoceptor agonists and neuropeptide Y, uncovered in the presence of increased vascular tone, may contribute to regional vasoconstriction observed in pathological conditions in which sympathetic nerve activity is increased.

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Evidence for the presynaptic action of 5-hydroxytryptamine and the involvement of purinergic innervation in the rabbit lower urinary tract

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1 The effects of 5-hydroxytryptamine (5-HT) were studied *in vitro* on bladder and urethral muscle strips from the rabbit. 5-HT produced dose-dependent contraction in the detrusor and urethra.

2 The 5-HT-induced contraction could be dose-dependently inhibited by the 5-HT_3 antagonists MDL 72222, ICS 205-930 and BRL 43694. No effect of ketanserin, methysergide or metitepine was observed on the contractile response to 5-HT.

3 Atropine and α,β-methylene ATP both partially blocked the contractile response to 5-HT. Together they caused more inhibition than either alone.

4 Atropine and α,β-methylene ATP also inhibited the contractile response to electrical field stimulation. The 5-HT_3 antagonist MDL 72222 had no effect on the contraction to field stimulation.

5 The atropine- and α,β-methylene ATP-resistant components of 5-HT-induced contraction were not affected by the 5-HT_1 antagonists metitepine, the 5-HT_2 antagonists ketanserin and methysergide or the 5-HT_3 antagonists MDL 72222, ICS 205-930 and BRL 43694.

6 Tetrodotoxin, hexamethonium, phentolamine and prazosin had no effect on the contractile response to 5-HT.

7 These results suggest that in the rabbit lower urinary tract (i) there are 5-HT_3 receptors, (ii) the contractile response to 5-HT is mediated by presynaptic stimulation, (iii) there is non-adrenergic, non-cholinergic excitatory neurotransmission.

Introduction

5-Hydroxytryptamine (5-HT) was discovered a little over 40 years ago by Rapport and colleagues (1948) and has been found to increase the activity of various visceral structures. In 1957, Gaddum & Picarelli showed that two distinct 5-HT effects were observed in the smooth muscle of guinea-pig ileum. One was associated with a direct contraction of smooth muscle (D-response), the other appeared to mediate depolarization of the cholinergic neurones (M-response). Scientific interest in 5-HT has increased dramatically over the past few years. There has been a recent upsurge of interest in a classification of 5-HT receptors to replace the M- and D-receptors proposed in 1957 by Gaddum & Picarelli. There is now strong evidence that 5-HT receptors can be divided into three types; 5-HT_1, 5-HT_2, and 5-HT_3, with further subdivisions of the 5-HT_1 type, and possibly of the other types as well (Bradley *et al.*, 1986).

Although it has been recognized for a long time that the mammalian urinary bladder contracts in response to 5-HT (Gyermek, 1961; 1962; Ambache & Zar, 1970; Taira, 1972; Saum & de Groat, 1973), the mechanism of action of 5-HT and the identification of the receptors in the lower urinary tract of various species is still unclear. It is known that different 5-HT receptors are present in the lower urinary tract of various species (Saxena *et al.*, 1985; Klarskov & Hørby-Petersen, 1986; Holt *et al.*, 1986).

The atropine-resistant component of the contraction evoked by parasympathetic nerve stimulation in the bladders of several species has been known for many years (Langley & Anderson, 1895; Henderson & Roepke, 1934; Ambache, 1955). This has been interpreted by most workers to be due to the presence of non-adrenergic, non-cholinergic excitatory nerves (Ambache & Zar, 1970; Burnstock *et al.*, 1972; Downie & Dean, 1977), and there is now considerable evidence that adenosine 5'-triphosphate (ATP) is an excitatory transmitter in the urinary bladder of small mammals. The ATP analogue, α,β-methylene ATP, which is resistant to hydrolysis has been shown to activate and then desensitize P_2-purinoceptors. It not only abolishes ATP-induced contraction but also the atropine-resistant response to excitatory nerve stimulation (Kasakov & Burnstock, 1983; Fujii, 1988; Brading & Mostwin, 1989).

The purpose of the present study was (1) to investigate the mechanism and the nature of the response to 5-HT in the rabbit lower urinary tract, (2) to test whether non-adrenergic, non-cholinergic transmission is involved in the 5-HT-induced contraction, and (3) to identify the 5-HT receptor in the rabbit lower urinary tract by application of several 5-HT antagonists.

Methods

Preparation of specimens

Rabbit bladder and urethra were obtained from New Zealand White rabbits of either sex, weighing from 600 g to 2500 g. These were stunned by a blow to the neck and exsanguinated. The specimens were placed in oxygenated Krebs solution. A longitudinal cut was made from the anterior wall of the urethra up through the bladder neck to the bladder dome. The mucosa was then dissected free from the bladder and urethral muscles. Strips were cut from the anterior wall, and the posterior wall of the bladders from both sexes. The urethral strips were made in either longitudinal or transverse direction. An operating microscope was used to ensure that there was good longitudinal alignment of the muscle bundles within a strip. All strips of lower urinary tract smooth muscle measured approximately 8 mm × 1 mm × 1 mm unstretched.

Tension recording and stimulation

Fine silk ligatures were tied to each end of the strip which was then mounted between platinum ring electrodes 1 cm apart in a specially constructed Perspex organ bath. The organ bath had a capacity of 0.2 ml and was continuously perfused with warmed (35–37°C) Krebs solution at a flow rate of 1 ml min^{-1}

(Brading & Sibley, 1983). Initially, the strips were allowed to equilibrate for at least one hour, after a resting tension of 1 g had been applied. Tension was measured isometrically with Pioden UF1 transducers and recorded on a Watanabe multichannel pen recorder after amplification.

Activation of intrinsic nerves was achieved by electrical field stimulation by pulses with the following parameters: 50 V, 0.05 ms width, 5 s trains at varying frequency. Successive trains of stimuli were given at least 5 min after the previous contraction had returned to baseline. After each drug-induced response recovery periods of 10–30 min were allowed before further drug application. Drugs and solutions were applied by dipping the ends of the feeder tubes for the perfusion system into the appropriate solutions. This allowed accurately timed exposures of the tissues to different solutions, and by following the bubbles introduced when the solution was changed the instant of tissue contact was recorded.

At the commencement of each experiment, the contractile response of the strips to a 2 min application of 126 mM KCl was obtained and subsequent responses were recorded as a percentage of the control response. This dose of KCl produced a near maximal contraction.

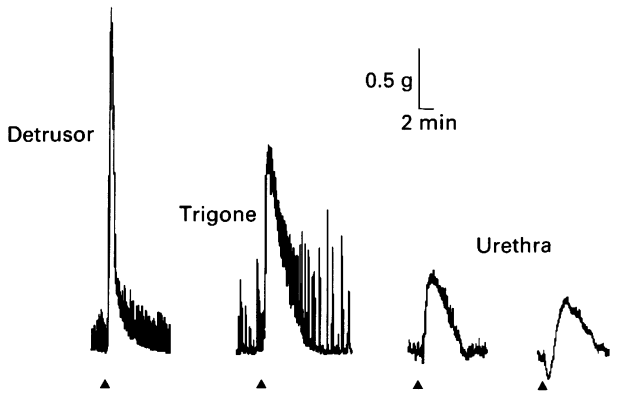

Figure 1 Contractile response to 5-hydroxytryptamine (5-HT) 10^{-3} M applied for 30 s (▲) to the rabbit lower urinary tract smooth muscles. A larger and more rapid contraction was present in the detrusor than in the trigone and urethral preparations. The biphasic response of some urethral preparations is shown as well as the more common monophasic response.

Drugs

The following drugs were used: 5-hydroxytryptamine creatinine sulphate complex (5-HT), adenosine 5′-triphosphate (ATP), tetrodotoxin (TTX), α,β-methyleneadenosine 5′-triphosphate (α,β-methylene ATP), hexamethonium bromide, prazosin hydrochloride (all these drugs were obtained from Sigma); atropine sulphate (B.D.H.); methysergide bimaleate and ICS 205-930 (3α-tropanyl-1H-indole-3-carboxylic acid ester, Sandoz); phentolamine mesylate (Ciba); BRL 43694 (Granisetron, [endo]N-(9-methyl-9-azabicyclo-[3,3,1]-non-3-yl)-1-methyl-1H-indazole-3-carboxamide hydrochloride, Beecham); ketanserin tartrate (Janssen), MDL 72222 (1αH, 3α,5αH-tropan-3-yl-3,5-dichlorobenzoate, Merrell Dow); metitepine monomethanesulphonate (Roche). Drugs were, where possible dissolved in distilled water to make a concentrated stock solution, these were refrigerated until needed. MDL 72222 was made up as a stock solution of 10^{-3} M with 75% ethanol, prazosin was dissolved in DMA (1:9, NN-dimethylacetamide, B.D.H.) as a stock solution of 10^{-3} M, and diluted appropriately with Krebs solution; 5-hydroxytryptamine, ATP and α,β-methylene ATP, were kept frozen. Drug concentrations presented are the final bath values. The vehicle was checked to have no effect. The Krebs solution used had the following composition (mM): NaCl 120.0, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, NaH_2PO_4 1.0 and glucose 11.5. All solutions were equilibrated with 97% O_2, 3% CO_2, pH 7.4 at 35–37°C. High K^+ solution (126 mM) was prepared by replacing NaCl with an equimolar amount of KCl in normal Krebs solution.

Statistical analysis

Student's *t* test and analysis of variance were used to compare differences in responses between the control and experimental curves. A probability level of $P < 0.05$ was accepted as significant. When appropriate, results are presented as means ± s.e.mean. pA_2 values were calculated for each concentration of antagonist from a Schild plot according to: $pA_2 = -\log\{[\text{antagonist}]/(DR - 1)\}$ (Mackay, 1978).

All points of each graph are means of at least 6 muscle strips taken from 3 different rabbits.

Results

Effect of 5-HT and 5-HT antagonists

A 30 s application of 5-HT produced dose-dependent contractions in the rabbit bladder (10^{-8} to 10^{-3} M) and in the urethra (10^{-6} to 10^{-3} M). A larger and more rapid contraction was seen in the detrusor than in the trigone and urethra (Figures 1 and 2). Biphasic responses which consisted of a small relaxation followed by contraction were evoked by 5-HT in some rabbit urethra strips. When high concentrations ($\geq 10^{-5}$ M) or long superfusion periods (≥30 s) were used, the responses to 5-HT were easily desensitized ($n = 9$). Thus, only one dose of the higher concentrations of 5-HT was applied for 30 s in any one experiment. There was no difference in the response to 5-HT between the anterior and posterior detrusor, or between the sexes.

Putative 5-HT antagonists were tested. Ketanserin (10^{-7} to 10^{-6} M), methysergide (10^{-8} to 10^{-7} M) and metitepine (10^{-8} to 10^{-7} M) had no effect on the contractile response to 5-HT. However, BRL 43694 (5×10^{-11} to 2×10^{-10} M), ICS 205–930 (5×10^{-12} to 1×10^{-10} M) and MDL 72222 (5×10^{-10} to 5×10^{-9} M) after 30 min exposure all competitively antagonized the 5-HT-induced contraction in the rabbit bladder strips (Figure 3). The pA_2 values were: for MDL 72222 9.3 ± 0.04 ($n = 9$), for BRL 43694 10.5 ± 0.01 ($n = 6$) and for ICS 205-930 12.5 ± 0.36 ($n = 8$). The Schild plot slopes were: for MDL 72222 1.52 ± 0.12, for BRL 43694 2.19 ± 0.03 and for ICS 205-930 0.68 ± 0.12. These values, although empirically useful, should be treated with some caution in view of the difference of the slope from 1.

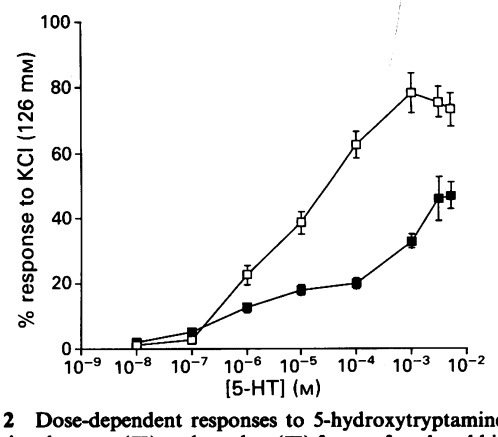

Figure 2 Dose-dependent responses to 5-hydroxytryptamine (5-HT) in anterior detrusor (□) and urethra (■) from a female rabbit. Points represent means of 15–20 experiments of detrusor and 9–12 experiments of urethra; vertical lines show s.e.mean. Desensitization of the contractile response to 5-HT with higher concentrations ($\geq 10^{-3}$ M) can be seen for the detrusor experiments.

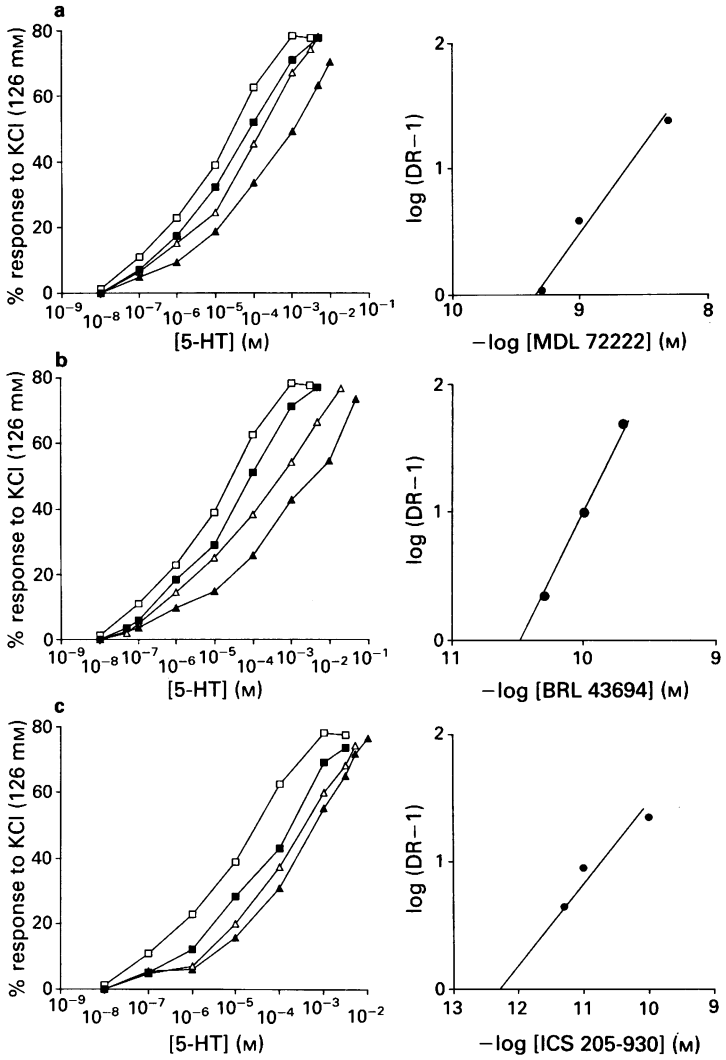

Figure 3 Effect of the 5-hydroxytryptamine (5-HT) antagonists MDL 72222, BRL 43694 and ICS 205-930 on rabbit detrusor preparations. (a) MDL 72222 5×10^{-10} M (■), 10^{-9} M (△) and 5×10^{-9} M (▲), control (□). The pA_2 value from the Schild plot was 9.3 ± 0.04. (b) BRL 43694 5×10^{-11} M (■), 10^{-10} M (△) and 2×10^{-10} M (▲). The pA_2 value was 10.5 ± 0.01 ($n = 6$). (c) ICS 205-930 5×10^{-12} M (■), 10^{-11} M (△) and 10^{-10} M (▲), its pA_2 value was 12.5 ± 0.36 ($n = 8$). Each point represents the mean of at least 9 experiments from 6 animals.

Atropine (10^{-7} M) and α,β-methylene ATP (10^{-6} M) both partially inhibited the 5-HT-induced contractions. When applied together, they produced more inhibition of the contractile response to 5-HT than either alone (Figure 4). There was a small atropine and α,β-methylene ATP-resistant component of the 5-HT-induced contraction in the bladder strips, which was not abolished by either the 5-HT_1 antagonist metitepine (10^{-7} M), the 5-HT_2 antagonists ketanserin (10^{-7} M) and methysergide (10^{-7} M), or the 5-HT_3 antagonists MDL 72222 (10^{-7} M), ICS 205-930 (10^{-6} M) and BRL 43694 (10^{-7} M).

TTX (1.6×10^{-6} M), hexamethonium (10^{-6} M), phentolamine (10^{-6} M) and prazosin (10^{-6} M) did not affect the 5-HT-induced contraction of the rabbit bladder.

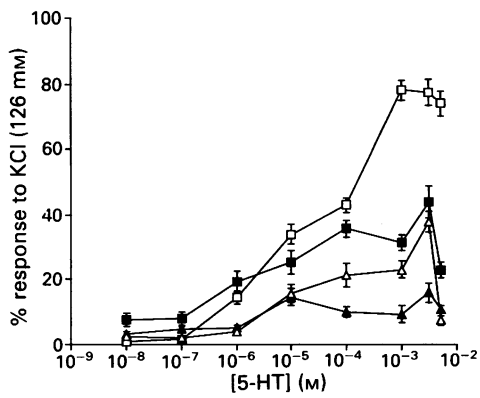

Figure 4 Effect of atropine and α,β-methylene ATP on the contractile response to 5-hydroxytryptamine (5-HT) in anterior detrusor of male rabbits. A combination of atropine 10^{-6} M and α,β-methylene ATP 10^{-6} M (▲, $n = 12$) caused greater inhibition than either atropine (■, 10^{-6} M, $n = 12$) or α,β-methylene (△, 10^{-6} M, $n = 12$) alone. Vertical lines show s.e.mean; $P \leq 0.001$ compared to control (□) for each group at concentrations of 10^{-4} M, 10^{-3} M, 3×10^{-3} M and 5×10^{-3} M and for α,β-methylene ATP or combination group at 10^{-6} M and 10^{-5} M. The effect of the combination of atropine and α,β-methylene ATP was significantly different from that of atropine alone at 10^{-5} M to 5×10^{-3} M ($P \leq 0.01$) and from that of α,β-methylene ATP alone at 10^{-4} M to 3×10^{-3} M ($P \leq 0.01$).

Electrical field stimulation

Nerve-mediated responses of the rabbit bladder were studied by use of electrical impulses at frequencies of 1, 5, 10, 20, 30, 40, and 50 Hz to stimulate the intramural nerves selectively. The contractile response increased at frequencies up to 30 Hz, and then reached a plateau. There was no difference in the response to field stimulation between male and female rabbit bladder. Abolition of the responses by TTX (1.6×10^{-6} M) was complete at all frequencies. A 20 min pretreatment with atropine caused dose-dependent (10^{-8} to 10^{-6} M) inhibition of

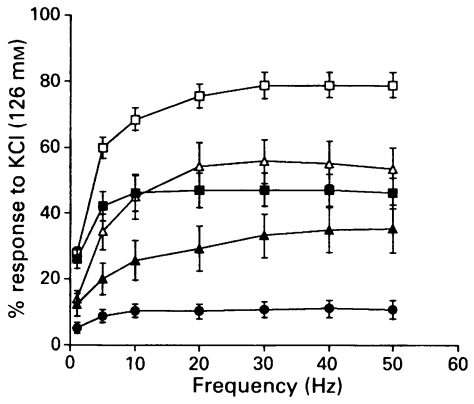

Figure 5 Effects of atropine and α,β-methylene ATP on the contractile response to electrical field stimulation in the posterior detrusor of male rabbits. Results show that a combination of 10^{-7} M atropine and 10^{-6} M α,β-methylene ATP (●, $n = 9$) caused significantly ($P \leq 0.05$) greater inhibition than either atropine (■, 10^{-7} M, $n = 9$) or α,β-methylene ATP (△, 10^{-6} M, $n = 12$; ▲, 10^{-5} M, $n = 10$) alone at 5–50 Hz. Atropine significantly decrease responses ($P \leq 0.05$) from 5–50 Hz, and both concentrations of α,β-methylene ATP significantly decreased ($P < 0.05$) responses from 1–50 Hz when compared to control responses. Vertical lines show s.e.mean (□) Control responses.

the response to field stimulation in the rabbit bladder preparations, with a maximum inhibition at high frequencies of 40% (Figure 5). The residual response was termed the atropine-resistant component. α,β-Methylene ATP caused a dose-dependent inhibition at all frequencies; at 10^{-6} M, about 35% inhibition was seen. Together, atropine (10^{-7} M) and α,β-methylene ATP (10^{-6} M) produced a greater inhibition of the contractile response to field stimulation than either alone (Figure 5). However, the 5-HT_3 antagonist MDL 72222 (10^{-7} M) showed no effect on the contraction to field stimulation of the strips.

Discussion

The contractile response of the bladder and urethra to 5-HT are complicated, and may involve more than one type of receptor (Cohen, 1989). The responses could be caused either by direct effects on the smooth muscle cells, or indirectly via effects on the autonomic innervation of these organs. Both actions have been implicated in the effects of 5-HT on cat bladder (Saxena *et al.*, 1985), but the majority of the work in this field implicates indirect actions as being of major importance. The ability of 5-HT to stimulate ganglion cells in other tissues, for instance in the enteric nervous system, is well recognized (Gaddum & Picarelli, 1957; Drakontides & Gershon, 1968; Costa & Furness, 1979; Jin *et al.*, 1989), and there is evidence that 5-HT may actually be one of the neurotransmitters to ganglion cells (Richardson *et al.*, 1985; Bradley *et al.*, 1986). Since urinary tract smooth muscles may receive excitatory innervation from both parasympathetic and sympathetic pathways (for review, see Kuru, 1965), it has been of interest to determine which of these autonomic pathways was involved in the response to 5-HT. Experimental evidence strongly implicates effects via the parasympathetic pathway in all species studied (dog: Gyermek, 1962; cat: Saum & de Groat, 1973; frog: Hirai & Koketsu, 1980; rat: Aas, 1983), the indoleamine thought to be activating ganglion cells which result in the release of acetylcholine onto the smooth muscle cells. The results described in this paper also suggest the involvement of the parasympathetic pathway in the effects of 5-HT on the rabbit urinary tract smooth muscles, since atropine dose-dependently inhibited the responses. However, there was a clear atropine-resistant component of the 5-HT response in this species.

An atropine-resistant component of the parasympathetic innervation has long been recognised in most species (for review, see Brading, 1987), and ATP has been implicated as a second excitatory transmitter (Burnstock *et al.*, 1972; Fujii, 1988; Brading & Mostwin, 1989). The ATP analogue α,β-methylene ATP, which has been shown to activate and desensitize P_2-purinoceptors, not only abolishes ATP-induced contraction but also the atropine-resistant responses to excitatory nerve stimulation (Kasakov & Burnstock, 1983; Fujii, 1988). It was therefore of interest to investigate the atropine-resistant component of the 5-HT response in the rabbit bladder and to compare it to the atropine-resistant response to excitatory nerve stimulation. The present studies revealed that after desensitization of the P_2-purinoceptors with α,β-methylene ATP, the atropine-resistant component of the 5-HT response was inhibited, and that a combination of atropine and desensitization of the P_2-purinoceptors abolished all but a small component of the 5-HT response. A very similar pattern of blockade was seen with transmural stimulation of excitatory nerves. In addition, the 5-HT responses were not blocked by phentolamine, prazosin or hexamethonium. This strongly suggests that 5-HT is acting to release the excitatory transmitters from the intrinsic nerves in the rabbit and that an adrenergic mechanism is not involved.

TTX, which is known to block Na^+ channels in the nerve axons, and which completely blocks the excitatory effect of transmural nerve stimulation in the rabbit, had no effect on the 5-HT-induced contraction in the present study. It has also been demonstrated that TTX did not affect the 5-HT-induced responses in rabbit nodose ganglia (Higashi & Nishi, 1982), although it abolished responses to 5-HT in the mouse duodenum (Drakontides & Gershon, 1968) and in the guinea-pig intestine (Costa & Furness, 1979). A possible explanation is that 5-HT may act on receptors on the nerve terminal to induce membrane depolarization and transmitter release. Shuster *et al.* (1985) demonstrated that 5-HT can close cyclic AMP-sensitive K^+ channels in *Aplysia* neurones, which would lead to depolarization, and Higashi & Nishi (1982) showed that 5-HT can open Na^+ channels in the nodose ganglion cells of the rabbit. It has also been suggested that excitation of 5-HT receptors on nerve terminals may release acetylcholine in rat bronchi (Aas, 1983) and rabbit heart (Fozard, 1984b), and Holt *et al.* (1986) showed that 5-HT could enhance the responses to excitatory (cholinergic) innervation presynaptically in the mouse bladder.

The classes of 5-HT receptors involved in physiological responses have been extensively studied by means of radioligand binding studies and classical pharmacological organ-bath techniques. At least three classes of receptor have been demonstrated (Bradley *et al.*, 1986), one of which, the 5-HT_3-receptor, has clearly been identified in the peripheral nervous system (Fozard, 1984a; Richardson & Engel, 1986; Fake *et al.*, 1987; Sanger, 1987). In the present study, the 5-HT_3-receptor antagonists ICS 205-930, BRL 43695 and MDL 72222 were all effective at blocking the excitatory responses of the bladder, whilst the 5-HT_1- and 5-HT_2-antagonists were ineffective. Thus in the rabbit 5-HT_3-receptors mediate the release of ATP and acetylcholine from the nerve terminals. The relative potency of the three 5-HT_3-receptor antagonists obtained in the present study (ICS 205-930 > BRL 43694 > MDL 72222) is the same as seen by Fozard (1989) on the rabbit vagus, although in that preparation the pA_2 values were 10.2, 9.9 and 7.9, whereas for the rabbit bladder the antagonists were more potent with pA_2 values of 12.5, 10.5 and 9.3 respectively. The affinity of binding sites for 5-HT_3-receptor antagonists in rat cortical membranes showed a slightly different order, with K_i values (nM) of 0.4 for ICS 205-930, 0.26 for BRL 43694 and 5.3 for MDL 72222 (Nelson & Thomas, 1989). [^{3}H]-ICS 205-930 also labels 5-HT_3-like binding sites in neuroblastoma cells (Hoyer & Neijt, 1987).

Although in the rabbit bladder the majority of the contractile response to 5-HT was abolished by the 5-HT_3-receptor antagonists, there was still a residual contractile response to 5-HT, which was not blocked by the 5-HT_1, 5-HT_2 or 5-HT_3

receptor antagonists. This suggests that there is another 5-HT-receptor which has a small effect either directly on the smooth muscle, or indirectly through an effect on other nerve endings. The fact that there was also a residual response to transmural nerve stimulation in the presence of atropine and desensitization of the P_2-purinoceptors, which was blocked by TTX, suggests that in this species a minor component of excitation involving a third transmitter may exist.

In summary, the involvement of the release of acetylcholine and a non-adrenergic, non-cholinergic neurotransmitter in the 5-HT-induced contraction is strongly suggested by the experiments. The results also identify the receptor involved in the rabbit lower urinary tract as the 5-HT_3 receptor.

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General anaesthetics and field currents in unclamped, unmyelinated axons of rat olfactory cortex

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1 The effects of seven general anaesthetics and one local anaesthetic having a wide range of physical and chemical properties were studied on nerve terminal Na- and K-mediated currents in slices of olfactory cortex. These currents were measured from the groups of fine unmyelinated axons traversing the surface of the olfactory cortex and which give off synapses *en passant*. The amplitude of the K-current was visualized by depolarizing the axons via an electrode polarization.

2 The anaesthetics tested were ketamine (0.1–2 mM), pentobarbitone (0.1–5 mM), urethane (5–200 mM), halothane (0.5–5 mM), ether (10–200 mM), alphaxalone (0.001–0.05 mM), diisopropylphenol (0.05–0.5 mM) and lignocaine (0.01–0.5 mM). All had depressant effects on the axonal Na-current (at the higher concentrations tested) and on the K-current (at slightly lower concentrations). The apparent lower potency on the Na-current was considered to be due to a masking of effect as a consequence of the reduction in the K-mediated membrane rectification rather than any real difference in the susceptibilities of the Na and K-currents.

3 Some of the depressant effect of pentobarbitone and alphaxalone was γ-aminobutyric acid (GABA)-mediated as indicated by the reduced potency in the presence of bicuculline. The actions of ketamine and halothane were unaffected by bicuculline.

4 For some anaesthetics these axonal depressant effects might contribute to general anaesthesia, while for other substances the relatively high concentrations necessary would suggest that this mode of action does not produce effective anaesthesia *in vivo*.

Introduction

A wide variety of preparations and tissues have been used to study the mechanisms of general anaesthetic action. A substantial body of evidence indicates that many anaesthetics enhance central inhibitory synaptic transmission at low concentrations (Nicoll, 1972; 1975; Nicoll *et al.*, 1975; Barker & Ransom, 1978; Pickles & Simmonds, 1975; Scholfield, 1978; 1980; Harrison & Simmonds, 1984). On the other hand, some authors have examined the effects of these substances on excitatory synaptic transmission of various types and have drawn other conclusions: anaesthetics act at synapses either to interfere with the action of the transmitter substance with its receptor (Barker & Grainer, 1973; Adams, 1976; Richards & Smaje, 1976) or they have a presynaptic depressant action (Larrabee & Posternak, 1952; Weakly, 1969; Richards, 1972; Goldring & Blaustein, 1982). In previous studies, pentobarbitone was found to have a depressant effect on conduction along the lateral olfactory tract (LOT) (Pickles & Simmonds, 1976; Scholfield & Steel, 1985). However, pentobarbitone was an effective depressant only at concentrations which in plasma are considered lethal in whole animals. The possibility arises that the unmyelinated axons which make *en passant* synaptic connections are more anaesthetic-sensitive than the myelinated axons of the LOT from which they derive. This might explain the apparent greater anaesthetic susceptibility of synaptic transmission. We have therefore studied the actions of some general anaesthetics on these fine presynaptic axons using an extracellular recording method to distinguish the Na- and K-currents (Scholfield, 1987). The present experiments show that a variety of general anaesthetics non-selectively block both the Na- and K-currents, albeit at concentrations which are generally considered as somewhat higher than those existing in body fluids during anaesthesia.

Methods

Rats of either sex weighing 150–200 g were decapitated and the brains removed. Slices of olfactory cortex were cut to have a thickness of 100–200 μm and were maintained in solution bubbled with 95% O_2:5% CO_2 for at least 60 min before use. Each slice was bisected parallel with the axons radiating from the LOT and then, immediately before recording, another vertical cut was made perpendicular to the axonal orientation (Scholfield, 1988). The slice was placed on a nylon mesh suspended within a recording bath of effective volume 0.5 ml. A glass electrode having an angled tip and a fire-polished orifice 40 μm in diameter was offered up to the freshly cut face of the slice. Gentle suction was applied to draw in Krebs solution followed by a 50 μm length of the tissue containing the unmyelinated axons (Scholfield, 1988). The electrode was connected to a current recording amplifier of input resistance 10 kΩ and capacitively coupled to subsequent stages (time constant 3 s). The current amplifier also contained the facility for applying various polarizations to the recording electrode between +200 and −200 mV. These polarizations were applied as square wave pulses of 600 ms duration beginning 400 ms before application of the stimulus. This is a long period relative to the latency of the response and was necessary because the onset of the polarization caused a large and slow capacitive current. With a 400 ms prepolarization, the recording baseline had settled down to an acceptable level. The axons were stimulated by a pair of 100 μm diameter lacquered tungsten wires placed close to the area where the axons leave the LOT. Stimuli of 20–40 V, 0.2 ms duration were delivered at 0.1 Hz. Statistical comparisons were made by the two-tailed *t* test, paired or unpaired as appropriate. Results were expressed as a mean ± standard error of mean.

Solutions

The normal Krebs solution had the following composition (mM): Na 144, K 6.1, Ca 2.5, Mg 1.3, Cl 132, HCO_3 25 and (+)-glucose 11. Experiments were conducted at 23–25°C and anaesthetics were dissolved directly into the solution perfusing the recording bath. In the case of the volatile anaesthetics (halothane and ether), particular precautions were exercised to avoid loss of the substance through evaporation and partitioning into the silicon rubber tubing connecting the reservoir to the recording bath. All the long lengths of rubber tubing were replaced by glass or stainless steel. The substances

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were dissolved, by gentle agitation, directly into solution freshly equilibrated with 95% O_2:5% CO_2 and with the air space over the solution replaced by 95% O_2:5% CO_2. No further gassing of the solution was performed and the solution made only a single passage through the recording chamber at 5 ml min^{-1}: the normal practice is to recirculate solutions from a gassed reservoir once the previous solution has been flushed out. Increasing the bath perfusion rate to 10 ml min^{-1} produced no further effect of the anaesthetic. If ether solution was recirculated after an effect had equilibrated, the preparation recovered at the same rate as it did when the ether was washed out with normal solution. The dissolving of alphaxalone was aided by an ultrasonic cleaning bath. All anaesthetics were applied for 15 min periods in sequentially increasing concentrations. Some experiments were also conducted in the presence of 10 μM bicuculline which was added 15 min before the anaesthetic. A stock solution of bicuculline in HCl (10 mM) was diluted into the bathing solution immediately before use.

Results

Figure 1 shows typical recordings obtained from the olfactory cortex with a current amplifier. Without electrode polarization, application of a single electrical stimulus to the axons derived from the LOT resulted in a positive current transient (of 19.3 ± 1.3 nA in 56 slices) followed by a small negative current (of 2.8 ± 0.4 nA). Stimulation during the application of a +100 mV electrode polarization caused the negative current to become greatly enlarged (to 13.6 ± 1.0 nA) and the positive current reduced a little (to 15.9 ± 1.4 nA).

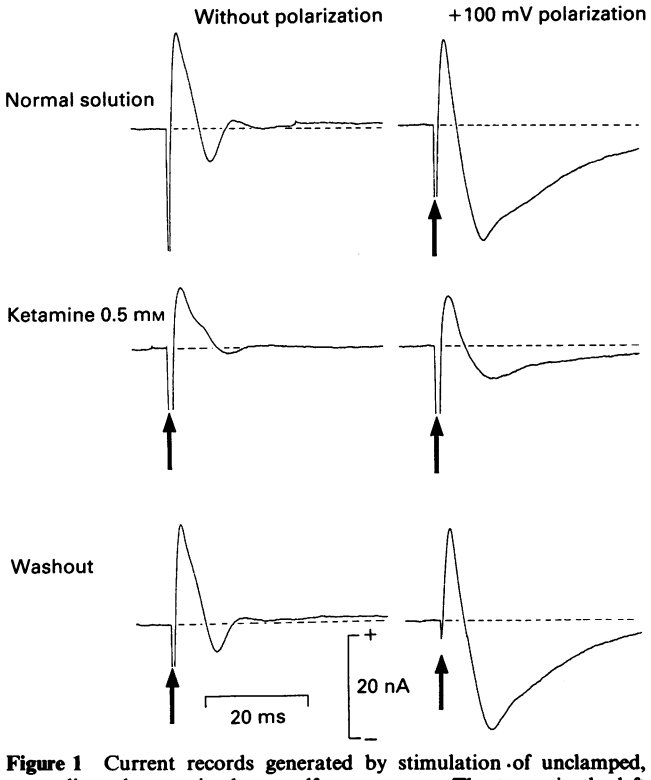

Figure 1 Current records generated by stimulation of unclamped, unmyelinated axons in the rat olfactory cortex. The traces in the left column were obtained by using a recording electrode without any polarization and those on the right during application of a +100 mV polarization. In the upper pair of traces the preparation was in normal solution and then the preparation was bathed in ketamine (0.5 mM) for 15 min (middle traces). In the lower traces, the ketamine had been washed out for 20 min. The dashed line shows the extrapolated recording base-line. These are single responses recorded with 5 kHz filtering and were replayed from a computer on to a digital plotter. The arrows show the point of stimulus application and stimulus artefact. The latter tend to be large and variable because of the closeness of the stimulating electrodes to the recording electrode.

In previous experiments, the negative current was shown to have a pharmacological profile compatible with a K-mediated event and the earlier short positive current a Na-event (Scholfield, 1988; 1989). By this recording technique the K-current depends upon the generation of the propagated Na-current. Thus any synchronous effect on both the Na- and K-currents might be the result of a selective depression of either the Na channels or both Na and K channels. Therefore concentration-effect curves were constructed to agents known to block specifically Na- and K-currents (tetrodotoxin (TTX), and 3,4-diaminopyridine (3,4-DAP) respectively) to provide a base to compare the effects of other drugs which may have specific actions on one or other current.

TTX was added for 15 min periods at progressively increasing concentrations between 10 and 100 nM and the initial positive current was depressed in a concentration related manner (Figure 2a). The after negative current was also depressed but

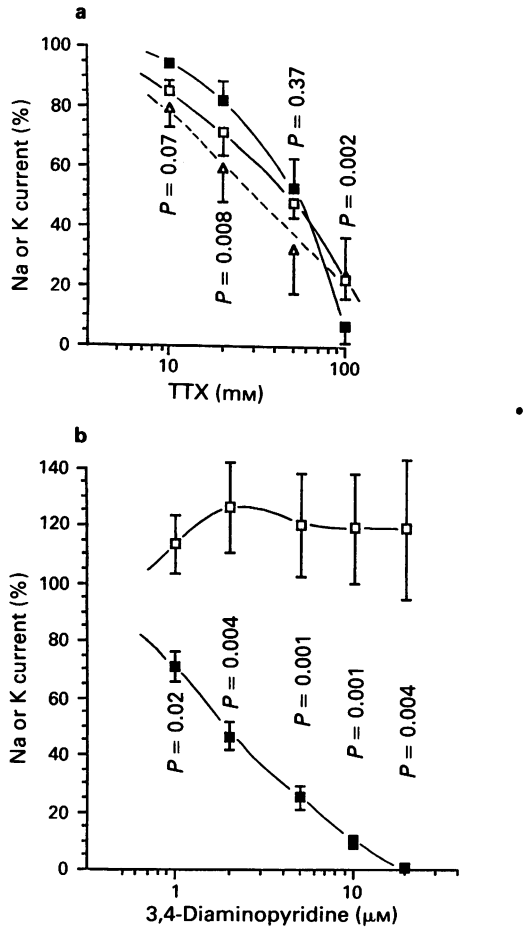

Figure 2 Concentration-effect curves for tetrodotoxin (TTX) and 3,4-diaminopyridine (3,4-DAP) on the field currents generated in unmyelinated axons of rat olfactory cortex. (a) In 6 slices, TTX was added in four sequential 15 min applications of increasing concentration in normal solution. The negative after-current is plotted as (■) and the initial positive current as (□). The *P* values aligned at each concentration denote the level of the significance between these two currents. The continuous lines were interpolated by 'eye'. In another set of 6 slices, TTX was added in the same concentrations in the presence of 100 μM 3,4-DAP and 5 mM TEA (△). There was no significant difference between the initial positive current in normal solution and 3,4-DAP/TEA. (b) A similar curve for 3,4-DAP in the presence of 5 mM TEA and 100 μM Cd with another 6 slices. The Na current is plotted as (□) and the K current as (■). The *P* value for the elevation of the Na current at various concentrations was 0.07 to 0.2. Ordinates: Maximum amplitude of the appropriate current as a percentage of that before addition of the first concentration. Abscissae: concentration of TTX (a) or 3,4-DAP (b) in the bathing solution. When appropriate, the s.e.means at each point are shown in one direction to reduce cluttering. The absence of a s.e.mean indicates it is smaller than the point.

at the lower concentrations, consistently less than the positive current, while at higher concentrations, the negative after-current was relatively more depressed (Figure 2a). To exclude secondary effects of the K-current on the Na-current, TTX was also tested in the presence of 3,4-DAP and TEA (Figure 2a).

A concentration-effect curve to K-channel blockade was also constructed. In this preparation, several pharmacologically distinct K-conductances are present (Scholfield, 1989). Of these, the aminopyridine-sensitive current is principally responsible for repolarizing the axonal membrane after the action potential. Therefore a concentration-effect curve to 3,4-DAP in the presence of 5 mM TEA and 100 mM Cd (to block slower and Ca-activated K-currents) was constructed. The negative after-current was depressed while the initial positive current was enhanced (Figure 2b). Since the aminopyridines are regarded as specific for the 'A' type K-conductance at the concentrations used in the present experiments, then the elevation of the Na-current was an indirect consequence of this blockade of the K-current. In the absence of TEA and Cd, the elevation is more consistent (Scholfield, 1988).

Since the recording electrode does not voltage clamp the axons, a large part of these responses were currents resulting from changing potentials across the axonal membrane capacitance (Scholfield, 1989). Therefore this procedure should be seen solely as a way of distinguishing the two pharmacologically characterised currents rather than a study of their voltage dependence and kinetics.

Effect of ketamine

Figure 1 shows a depression produced by ketamine on the currents recorded by this technique. The onset of the depressant effect occurred when the ketamine reached the slice and was complete within 15 min of continuous application (Figure 3). On washing out the ketamine both the Na and K-currents recovered over a 30 min period. In a further 8 slices, ketamine was applied in a serially increasing range of concentrations between 0.1 and 2 mM. The depressant effect of ketamine was concentration-dependent (Figure 4a) and ketamine appeared to have a slightly more selective effect on the K-current (Figures 1, 3 and 4a). In another 6 slices, ketamine was found to be significantly more effective in the presence of 3,4-DAP and TEA (Figure 4a). In the presence of bicuculline (10 μM) the depressant action of ketamine was indistinguishable from that without bicuculline.

Halothane

Ten preparations were used to examine the effects of halothane over a concentration-range of 0.5 to 5 mM. Halothane showed depressant effects in a concentration-related manner between 1 and 5 mM (Figure 4b). The K-current appeared a little more sensitive to the action of halothane than the Na-current. Halothane was retested in a further 5 slices at one concentration (3 mM) alone. Then after washing out and recovery, halothane was reapplied in the presence of 10 μM bicuculline. In 3 mM halothane, the amplitude of the Na-current was 69 $\pm$ 7% of that in normal solution and 64 $\pm$ 8% in bicuculline and the respective amplitudes for the K-current were 78 $\pm$ 6 and 84 $\pm$ 10%. The responses in bicuculline were not significantly different by paired *t*-test. In another 10 slices, halothane had a significantly more potent depressive effect on the Na-current in the presence of 3,4-DAP and TEA than in normal solution (Figure 4b).

Ether

In six experiments, ether was tested at concentrations between 1 and 200 mM. It had little effect up to 20 mM but at concentrations higher than this, both the Na and K-currents were depressed in a concentration-dependent manner (Figure 4c). On washing out ether, the response fully recovered within 3 min. This contrasts with all the other agents tested where full recovery required 15–40 min of washing out.

Diisopropylphenol

This substance was tested in the concentration range 0.05–0.5 mM and again the K-current was more depressed than the Na-current at each concentration (Figure 4d). In the presence of 3,4-DAP and TEA, diisopropylphenol was more effective on the Na-current (Figure 4b).

Lignocaine

For comparison, a local anaesthetic was also tested and this depressed the Na-current and also, more potently, the K-current (Figure 4e) with a time-course and manner similar to the depression produced by general anaesthetics. Lignocaine was more effective on the Na-current in the presence of 3,4-DAP and TEA (Figure 4d).

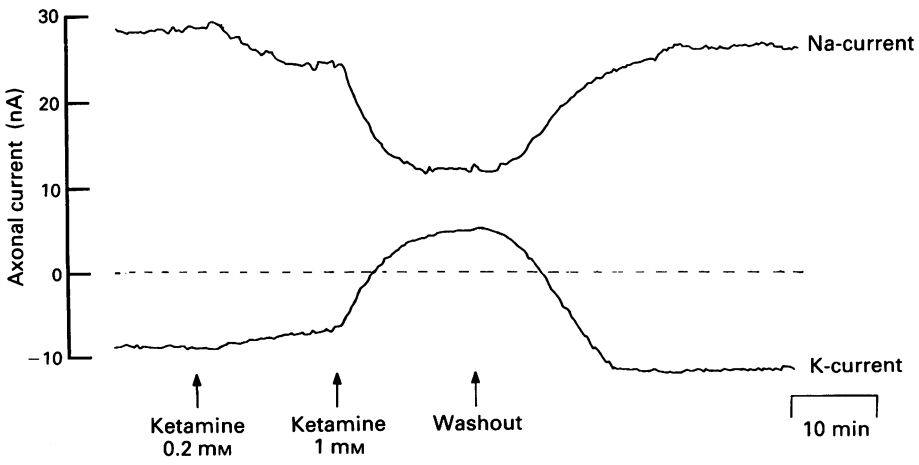

Figure 3 Continuous time-courses for the maximum amplitude of the Na-current (upper trace) and the K-current (lower trace). The preparation was stimulated at 0.05 Hz and the current waveforms stored by computer. The peak height of the positive current for each waveform was measured by computer and plotted out on an X-Y plotter. The lower trace ('K-current') was obtained by measuring the amplitude of the current at a constant latency (15 ms after the stimulus). During the experiment, 0.2 mM ketamine and then 1 mM ketamine were added to the solution superfusing the slice at the points arrowed followed by their washout (washout). Records were obtained throughout this experiment during an electrode polarization of +100 mV. In the presence of 1 mM ketamine, the K-current decreased and was replaced by a positive tail.

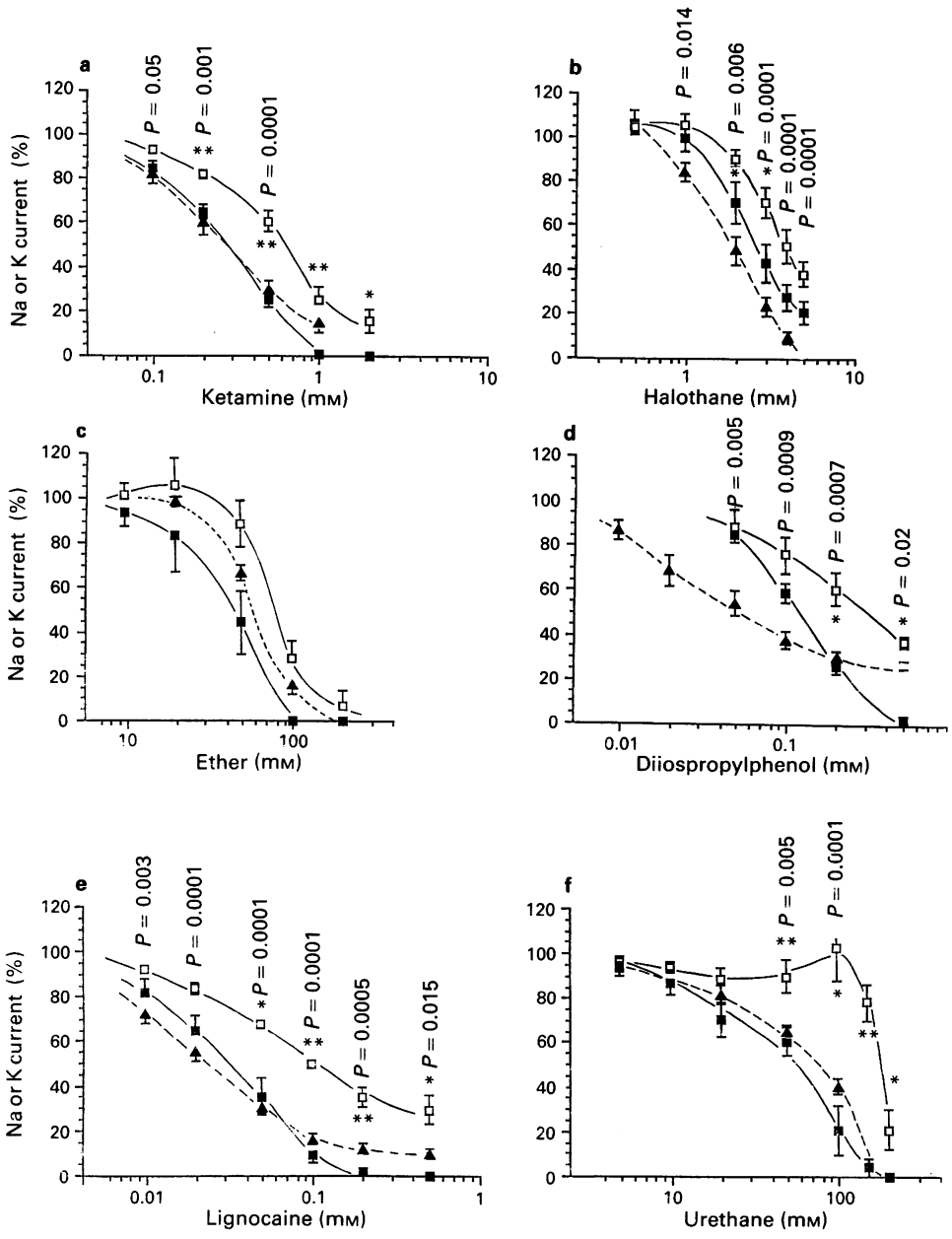

Figure 4 Dose-response curves for the maximum amplitude of the Na-current (unpolarized electrode) and maximum K-current (+100 mV polarization) obtained by sequential increases in concentration of ketamine (a), halothane (b), ether (c), diisopropylphenol (d), lignocaine (e) and urethane (f): (□) Na-current; (■) K-current (continuous lines); (▲) Na-current in the presence of 100 μM 3,4-diaminopyridine (3,4-DAP) and 5 mM tetraethylammonium (TEA). The *P* values given at each point are for the differences between the Na-current in normal solution and in the presence of 3,4-DAP and TEA. The asterisks denote the level of significance between the effects on the Na- and K-currents under the same conditions: * $P < 0.02$ and ** $P < 0.002$. Ordinate scales: The amplitude of the respective current is expressed as a percentage of that before application of anaesthetic. For some points the s.e.mean bar is shown in one direction only to reduce cluttering. Points not showing bars have errors smaller than the symbol. The lines are drawn 'by eye'.

Urethane

Six slices were used to study the effect of 5–200 mM urethane. At all concentrations above 10 mM there was a depression of the K-current while the Na-current was only depressed at 150 and 200 mM (Figure 4f). In the preceding experiments, the anaesthetics were also more effective on the K-current but this was particularly marked with urethane (Figure 4f): in some experiments, the Na-current was a little elevated in 100 mM urethane. This might have resulted from a selective depressant action on the K-current such as is observed with K-channel blockers (Scholfield, 1987). Therefore 10 slices were pre-equilibrated with 100 μM 3,4-diaminopyridine and 5 mM tetraethylammonium to block axonal K-currents (Scholfield & Steel, 1988). Under these conditions, urethane had a more potent effect on the Na-current than when the K-current was intact (Figure 4f). The implication of this result is that the depressant action of urethane on the Na-current in normal solution is masked by a blockade of the K-current.

Pentobarbitone

This substance was tested at 0.1 to 5 mM in 15 slices. Little effect was observed at the lowest concentration tested (0.1 mM), but between 0.1 and 2 mM, both the Na- and K-currents were progressively depressed with increasing concentration (Figure 5a). Pentobarbitone was also tested in the presence of bicuculline where its action was noticably reduced (Figure 5a, 5 slices) such that little depression of the Na-current occurred until the concentration reached 1 mM. The depressant action of pentobarbitone on the K-current was less influenced by bicuculline (Figure 5a). The effect of 3,4-DAP

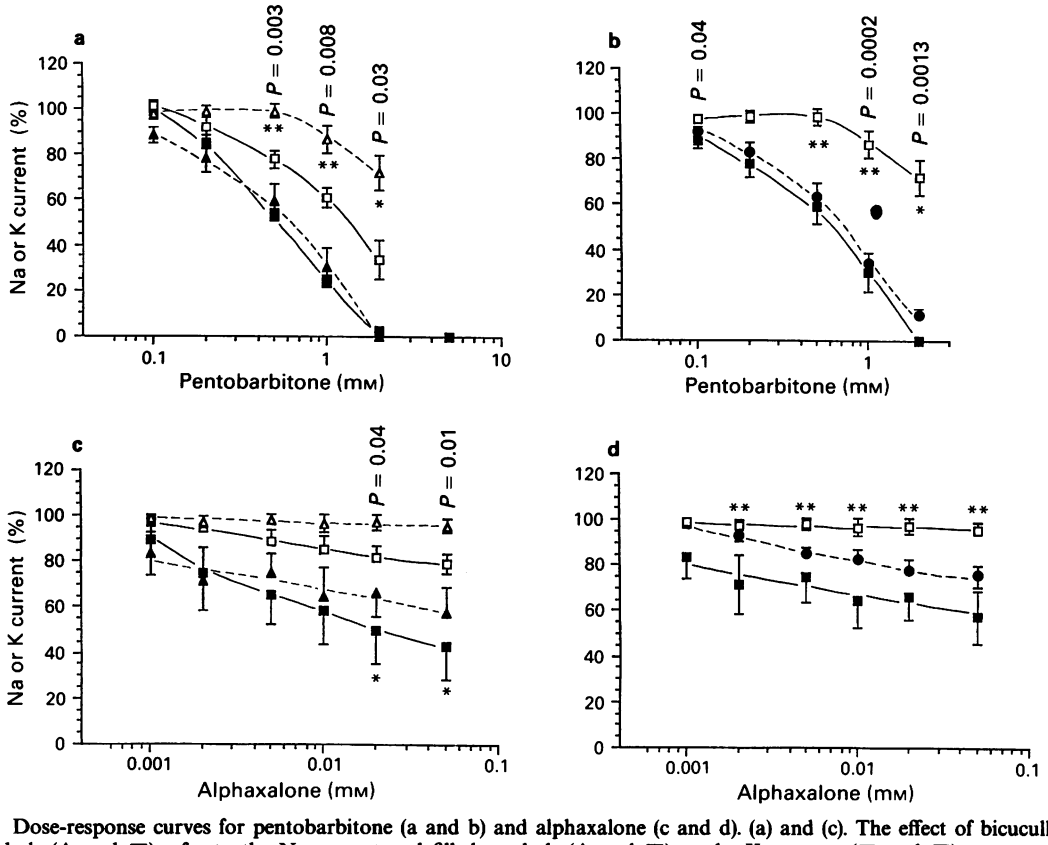

Figure 5 Dose-response curves for pentobarbitone (a and b) and alphaxalone (c and d). (a) and (c). The effect of bicuculline. The open symbols (△ and □) refer to the Na-current and filled symbols (▲ and ■) to the K-current; (□ and ■) connected by the continuous line are for the Na- and K-currents in normal solution and (△ and ▲) and dashed lines refer to the currents obtained in the presence of 10 μM bicuculline. The P values given at each point are for the differences between the Na-current in normal solution and in the presence of 10 μM bicuculline. (b) and (d) The effect of K channel blockade. The Na-current is represented by (□) and the K-current by (■); (●) Na-current in the presence of 100 μM 3,4-diaminopyridine (3,4-DAP) and 5 mM tetraethylammonium (TEA). Bicuculline (10 μM) was present throughout all the experiments. The P values given at each point are for the differences between the Na-current in normal solution and 3,4-DAP. The asterisks refer to the differences in the percentage reduction between the Na- and K-currents: * $P < 0.02$; ** $P < 0.002$.

and TEA on the action of pentobarbitone was also tested in the presence of bicuculline in a further 12 slices (Figure 5b): the Na-current showed a sensitivity to pentobarbitone similar to that of the K-current in normal solution.

Alphaxalone

In six experiments, alphaxalone was tested at concentrations between 1 and 50 μM. The Na-current was weakly depressed and the K-current a little more depressed by alphaxalone (Figure 5c). In another 6 slices, the alphaxalone was tested in the presence of bicuculline (10 μM). Under these conditions the depressant effect of alphaxalone on the Na-current was not observed and the effect on the K-current was weaker (Figure 5c). Alphaxalone was also tested in the presence of bicuculline, 3,4-DAP and TEA. In this solution, alphaxalone showed a slight depression but the effect was not significant.

Discussion

Seven general anaesthetics having a wide range of physical and chemical properties have been tested on the Na- and K-currents generated in unmyelinated axons of the rat olfactory cortex. All the substances showed some evidence of depressant effects on axonal currents. Do these effects have any relevance to the cellular mechanism of general anaesthesia *in vivo*?

Since the depressant action of the general anaesthetics was shared by low concentrations of lignocaine, a substance not regarded as a general anaesthetic, it would seem unlikely that the depression of axonal function by these substances could entirely account for the cellular actions of general anaesthetics during anaesthesia *in vivo*. Several cellular mechanisms of action of general anaesthetics in mammals have been proposed. Among them are: (i) enhanced inhibition; (ii) depression of transmitter release or action and (iii) depression of axonal conduction.

(i) In the olfactory cortex, pentobarbitone, alphaxalone, and to a lesser extent halothane and diisopropylphenol, enhance γ-aminobutyric acid (GABA)-mediated inhibition at concentrations lower than those used in the present study (Pickles & Simmonds, 1978; Scholfield, 1978; 1980; Harrison & Simmonds, 1984; Cottrell *et al.*, 1987; Collins, 1988). In the case of pentobarbitone and alphaxalone, the enhancement of inhibition occurs at relatively low concentrations. The effect is seen at the anaesthetic concentrations seen *in vivo* (Chenoweth *et al.*, 1962; Sear & Prys-Robert, 1979) and this is considered by several authors to be their principal mechanism of action. Furthermore, some of the depressant effect on axonal function can be accounted for by an action via GABA or axonal Cl-channels. In contrast, halothane and ketamine showed no such bicuculline-sensitivity although they have a weak potentiating effect on inhibition (Scholfield, 1980). In this present study, thin slices were used to avoid postsynaptic cells. Such slices would also exclude inhibitory neurones (Scholfield, 1978; Halliwell, 1982) which might give rise to GABA-mediated feedback inhibition on the presynaptic axons (Schmidt, 1963; Nicoll, 1975; Pickles & Simmonds, 1976; Cain & Simmonds, 1982). With larger slices or *in vivo*, the effect of alphaxalone and pentobarbitone may be more substantial, particularly with high stimulus rates.

(ii) Ether, halothane and diisopropylphenol are more potent depressors at some excitatory synapses (Richards, 1973;

Richards & Smaje, 1976; Collins, 1988) than they were depressors of axonal function in the present study. Thus the action at the excitatory synapse may make a major contribution to the action of these substances as general anaesthetics *in vivo*.

(iii) Urethane and ketamine had a similar potency in depressing excitatory transmission (Scholfield, 1980) and depressing axonal currents in the present study. Since excitatory transmission is dependent on axonal function, the possibility exists that a major cellular effect of these substances is via this axonal action. Notwithstanding this, enhancement of inhibition (Nicoll, 1972; Scholfield, 1980) or NMDA glutamate receptor antagonism (Johnson & Lodge, 1984; Harrison & Simmonds, 1985) are more likely to make major contributions to the *in vivo* actions.

Thus the depression of axonal currents should not be considered as being responsible for the cellular action of the general anaesthetics studied here. However, the effect may contribute in part or in whole to the lethal actions of these substances.

In a previous study (Scholfield & Harvey, 1975) lignocaine was found to be a slightly more potent depressor of the excitatory postsynaptic potential (e.p.s.p.) (ED_{50} = 0.18 mM) than of the action potential of the myelinated LOT (ED_{50} = 0.26 mM) in the olfactory cortex. These concentrations compare to an ED_{50} of 0.10 mM for the unmyelinated axons (Figure 4). This difference between potency values for the myelinated and unmyelinated axons is substantial and indicates that the LOT axons can be a poor indicator of presynaptic susceptibilities.

Judging from the effect of TTX in normal solution (without K-channel blockade), any substance which selectively blocks Na-channels will have a slightly more preferential effect on the Na-current than the K-current. In all the experiments performed in the present study, the Na-current was consistently less sensitive than the K-current. Therefore, it seems unlikely that these substances had any selective effect on the Na channels in this preparation as is the case with most anaesthetics on squid axon (Haydon & Urban, 1986). This contrasts with the preferential effect of urethane on the invertebrate Na-current (Gierasimov & Janiszewski, 1967; Pichon, 1979).

The K-currents which normally aid the recovery of the membrane from the action potential appeared to be more sensitive to the depressant action of all these substances. This might be expected for two reasons: (i) with the present methodology, the generation of the K-current is dependent upon the prior activation of the Na-current and therefore any selective effect on the Na-current would be overlooked; (ii) as the K-current is depressed, there will be some enhancement of the Na-current as is the case for substances such as the aminopyridines which selectively block the 'A' type current (Scholfield & Steel, 1988; Scholfield, 1988). With 100 mM urethane there was a suggestion of augmentation of the Na-current for this reason. When the K-current was blocked, the Na-current had a similar sensitivity to the K-current. Thus, at the level of the independent channel currents, urethane at least shows little selectivity for Na or for K.

In conclusion, a wide range of anaesthetics have non-selective depressant effects on both the Na- and K-currents but with regard to the concentrations required, it would seem that most of the substances do not make a major contribution to anaesthesia *in vivo* via this mechanism.

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Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells

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1 The effects of suramin on an adenosine 5'-triphosphate (ATP)- activated inward current were investigated in PC12 phaeochromocytoma cells with whole-cell voltage-clamp techniques.

2 Suramin (30 to 300 μM) inhibited the ATP-activated current in a dose-dependent manner. The inhibitory effects were reversible and competitive.

3 Suramin also suppressed the current activated by adenosine 5'-O-(3-thiotriphosphate) but did not affect the current activated by nicotine. Suramin did not affect the suppression of a K current induced by methacholine.

4 The results suggest that suramin antagonizes the ATP-receptor-operated membrane current reversibly and selectively.

Introduction

Evidence has been provided to suggest that adenosine 5'-triphosphate (ATP) is released with noradrenaline or acetylcholine from nerve terminals in smooth muscle and neuronal cells (Burnstock & Kennedy, 1985, Bean & Fiel, 1989). Recent studies revealed that excitatory responses mediated by P_2-purinoceptors are triggered by ATP-activated cationic inward currents in smooth muscle cells (Benham *et al.*, 1987; Benham & Tsien, 1987; Nakazawa & Matsuki, 1987; Friel, 1988; Bean & Fiel, 1989) and non-muscle cells (Krishtal *et al.*, 1988; Inoue *et al.*, 1989). It was reported that suramin, a trypanocide drug, antagonized an excitatory P_2-purinoceptor response, namely, smooth muscle contraction, in mouse vas deferens (Dunn & Blakeley, 1988). This finding suggested the possibility that suramin is a reversible blocker of P_2-purinoceptors. However, there have been no reports concerning effects of suramin on ATP-activated currents.

In the present study, we have examined the effects of suramin on the ATP-activated current in PC12 phaeochromocytoma cells. We also examined whether suramin affected other receptor-operated membrane currents.

Methods

PC12 cells were cultured as previously described by Inoue & Kenimer (1988). Briefly, PC12 cells (1×10^6; passage 50–60) were plated in collagen-coated polystyrene dishes and cultured for 2 to 3 days in Dulbecco's modified Eagle's medium containing 5% foetal bovine serum, 5% heat-inactivated horse serum, 2 mM L-glutamine and 50 μg ml^{-1} gentamicin sulphate at 37°C, with an atmosphere of 90% air: 10% CO_2.

Membrane currents were measured with whole-cell patch-clamp techniques (Hamill *et al.*, 1981) under conditions described elsewhere (Nakazawa *et al.*, 1989). The cells were superfused with an extracellular solution containing (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, glucose 11.1 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10.0 (adjusted with NaOH to pH 7.4). Patch pipettes were filled with an intracellular solution containing (mM): CsCl 150, HEPES 10.0 and glycoletherdiamine- N,N,N',N'-tetracetic acid (EGTA) 5.0. Membrane current measurements were made at room temperature (about 22°C). In calculating the amplitude of currents activated by depolarizing voltage-steps, a linear component of leak current, estimated from a hyperpolarizing step from −60 mV to −100 mV, was subtracted.

Drugs used were ATP (adenosine 5'-triphosphate disodium salt, Sigma), suramin monosodium salt (Bayer), ATPγS (adenosine 5'-O-(3-thiotriphosphate) tetralithium salt, Boehringer Mannheim), nicotine (Sigma) and methacholine (acetyl-β-methylcholine chloride, Sigma). Drugs were dissolved in extracellular solution and applied to the cells by superfusion.

Results

ATP activated a rapid inward current, which reached a maximum within 1 to 2 s and inactivated with a time constant of about 5 s, in PC12 cells (Inoue *et al.*, 1989). Figure 1a shows the blocking effect of suramin on the ATP-activated current. Suramin (30 μM) partially blocked an inward current activated by 100 μM ATP. The effect was reversible and the current comparable to the control response was obtained after washout of suramin. Suramin blocked the ATP-activated current in a dose-dependent manner between 30 and 300 μM (Figure 1b). Figure 1 shows the dose–response curves for the ATP-activated current in the presence and absence of 30 μM suramin. The dose–response curve was shifted to the right in a parallel manner with suramin. Maximal responses obtained with ATP (up to 3 mM) were not affected by 30 μM suramin (control: 1016 ± 120 pA at −60 mV (means ± s.e.mean, $n = 5$); suramin: 1143 ± 175 pA at −60 mV ($n = 5$)).

ATPγS activated a current similar to that produced by ATP but the response was somewhat more sustained (Nakazawa *et al.*, 1990). Suramin also suppressed the current activated by 300 μM ATPγS (Figure 2a). The current was decreased upon superfusion with suramin (100 μM) and reversed by returning to the solution containing ATPγS alone. Suramin (100 μM) inhibited the ATPγS (300 μM)-activated current by about 80% in 5 cells tested.

We examined the selectivity of the antagonistic effect of suramin. First, we studied the effects on a nicotinic receptor-operated inward current (Bormann & Matthaei, 1983; Nakazawa *et al.*, 1989). Nicotine activates an inward current much smaller than that activated by ATP in PC12 cells (Inoue *et al.*, 1989). As shown in Figure 2b, suramin (300 μM) did not affect

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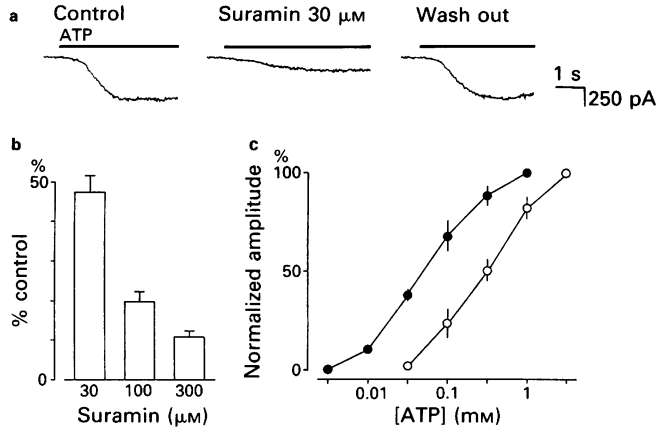

Figure 1 Effect of suramin on ATP-activated inward current in PC12 cells. Holding potential was −60 mV. (a) Current activated by 100 μM ATP at 5 min intervals in the absence (left and right) and presence (middle) of 30 μM suramin. Suramin was added 2 min before ATP application. (b) Dose-dependence of the inhibitory effect of suramin on the ATP-activated current. Peak amplitude of the current activated by 100 μM ATP in the presence of suramin was normalized to that in the absence of suramin. Each column represents the mean from 4 to 6 cells tested. Vertical bars show s.e.mean. (c) Log concentration curves for peak amplitudes of the current activated by ATP in the absence (●) and presence (○) of suramin (30 μM). All responses were normalized with respect to the maximal response in each cell. Each point represents the mean from 4 to 6 cells tested. Vertical bars show s.e.mean where it exceeded the size of the symbol.

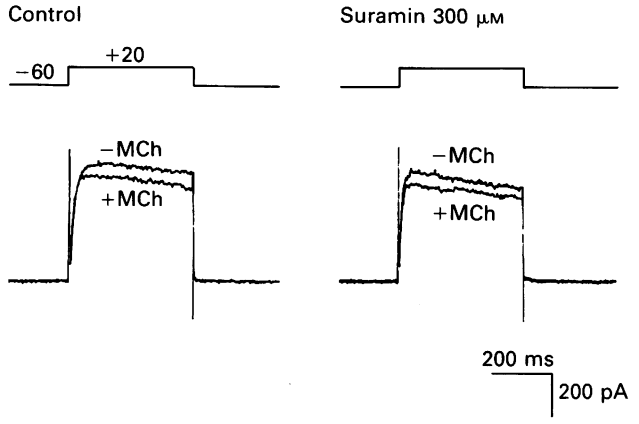

Figure 3 Lack of effect of suramin on the suppression by methacholine of a K current. The K current was activated by a 400 ms voltage-step to +20 mV from a holding potential of −60 mV every 5 s. The cell was first exposed to 300 μM methacholine and rinsed with drug-free solution for 5 min. The cell was next superfused with 300 μM suramin for 1 min and then exposed to 300 μM methacholine (suramin was continuously present). The current traces just before (−MCh) and 30 s after (+MCh) the exposure to methacholine in the absence (left) or presence of 300 μM suramin (right) were superimposed.

the current activated by 10 μM nicotine (93.0 ± 4.3% of control, $n = 5$). Next, we studied the effects of suramin on a muscarinic receptor-mediated response in PC12 cells, namely, suppression of a voltage-gated K current (Nakazawa *et al.*, 1989). In preliminary experiments, suramin (300 μM) itself slightly decreased the K current (92.4 ± 3.5% of control, $n = 5$). Suramin did not affect the suppression of the K current induced by 300 μM methacholine, a selective agonist for muscarinic receptors. The K current which remained after the application of 300 μM methacholine was 76.5 ± 3.7% of control in the presence of 300 μM suramin ($n = 6$; the current in the presence of suramin was taken as control) whereas the value was 83.6 ± 2.2% of control in the absence of suramin ($n = 6$). The values were not significantly different (Student's t test, $P > 0.05$).

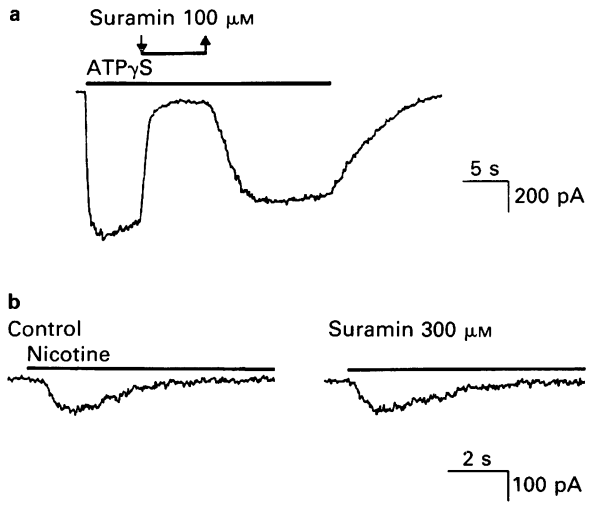

Figure 2 Effects of suramin on inward currents activated by adenosine 5'-O-(3-thiotriphosphate) (ATPγS) (a) and nicotine (b). Holding potential was −60 mV. (a) Current activated by 300 μM ATPγS. Suramin (100 μM) was applied during the ATPγS-activated current. The current was suppressed during the application of suramin and recovered after washout. (b) Current activated by 10 μM nicotine in the absence (left) and presence (right) of 300 μM suramin. Note that suramin did not affect the nicotine-activated current but depressed markedly the response to ATPγS.

Discussion

It was reported that suramin antagonizes both excitatory and inhibitory P_2-purinoceptor responses in smooth muscle tissues (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989). In the present study, suramin reversibly and competitively antagonized the ATP-activated inward current in PC12 cells. This finding for the first time showed that suramin is also an antagonist for purinoceptors in non-muscle cells. Suramin antagonized the current activated by ATPγS but did not affect the changes in membrane currents induced by nicotine and methacholine.

ATP exerts excitatory effects by activation of receptor-operated channels in smooth muscle cells (Benham *et al.*, 1987; Benham & Tsien, 1987; Nakazawa & Matsuki, 1987; Friel, 1988) and neuronal cells (Krishtal *et al.*, 1988; Bean & Friel, 1989). The receptor-operated channels in smooth muscle and neurones appear to have many properties in common (Bean & Friel, 1989). We recently characterized the ATP-activated current in PC12 cells (Nakazawa *et al.*, 1990): the ionic pathway for the current has very similar properties including kinetics, voltage-dependency, ion-selectivity, and agonist-selectivity to those reported for the ATP-activated channels in other excitable cells (Bean & Fiel, 1989). Therefore, it is probable that the blocking of the ATP-activated current is a common mechanism underlying antagonism by suramin of excitatory effects of ATP both in muscle and non-muscle cells. Competitive antagonism by suramin of the ATP-activated current in the present study suggests that suramin may compete with ATP for the receptor which seems directly coupled to the channels (Benham *et al.*, 1987; Bean & Friel, 1989).

In conclusion, we have shown that suramin reversibly and selectively antagonizes the ATP-activated current in PC12 cells. The blockade of receptor-operated channels may be a common method of action of suramin on excitatory responses mediated by P_2-purinoceptors.

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Haemodynamic alterations in anaesthetized and acutely intoxicated newborn piglets

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1 We investigated the haemodynamic alterations in 2-day old anaesthetized piglets after acute ethanol intoxication using the microsphere technique.

2 After ethanol infusion, mean arterial blood pressure (MABP) decreased by 6%, cardiac output (CO) decreased by 26%, heart rate (HR) increased by 20% and systemic vascular resistance (SVR) increased by 36%.

3 Arterial perfusion to the kidneys, gastrointestinal (GI) organs and carcass decreased by 39%, 34% and 26%, respectively.

4 In piglets pretreated with 4-methylpyrazole (4-MP), an alcohol dehydrogenase inhibitor, the decrease in MABP and increase in HR were not observed after ethanol infusion, but the reduction in CO and increase in SVR were maintained.

5 Arterial blood flow to the GI organs and carcass, but not the kidneys, remained at significantly reduced levels.

6 These observations indicate that ethanol can adversely affect CO and arterial perfusion to GI and musculoskeletal structures while metabolites of ethanol, such as acetaldehyde, affect MABP and HR. Therefore, the clinical effects observed after acute ethanol intoxication in neonates may vary with their rate of ethanol metabolism.

Introduction

Although the adverse effects of chronic ethanol exposure on the foetus (Jones *et al.*, 1974; Hanson *et al.*, 1976) are well characterized, the effects of acute ethanol intoxication on the newborn are still unclear. Previous studies in foetal and newborn lambs, as far as mean arterial blood pressure (MABP) and heart rate (HR) are concerned, have produced conflicting results (Mann *et al.*, 1975; Ayromlooi *et al.*, 1979; Cook *et al.*, 1981; Cumming *et al.*, 1985). Previously, we have found that newborn piglets have rates of ethanol metabolism close to those reported for human neonates ($2.0 \pm 0.2\,mmol\,l^{-1}\,h^{-1}$), and that acutely intoxicated piglets develop significant hypotension and tachycardia which can be reversed by blocking ethanol metabolism with 4-methylpyrazole (4-MP) (Chandler *et al.*, 1989). The present series of experiments was undertaken to determine cardiac output (CO), systemic vascular resistance (SVR) and regional blood flow distribution in acutely intoxicated newborn piglets and to assess the effects of blocking ethanol metabolism with 4-MP.

Methods

Surgical preparation

Healthy farm-bred two-day old Camborough piglets with a mean weight of 1.34 ± 0.06 kg were the subjects of this study. All incisions were infiltrated with 1% lignocaine hydrochloride. Anaesthesia was induced with 1% halothane and tracheostomies were performed. After tracheostomies, piglets were paralyzed with $0.3\,mg\,kg^{-1}$ (+)-tubocurarine, halothane was discontinued and anaesthesia was maintained with 70% nitrous oxide in oxygen. Tidal volume and ventilation rate were adjusted via a Harvard Apparatus respirator to maintain a $PaCO_2$ range of 35 to 45 mmHg. Polyethylene catheters (PE-60) were placed in the femoral artery to monitor MABP and to collect blood ethanol, arterial blood gas and microsphere reference samples and in the femoral vein for ethanol or 5% dextrose in water infusion. Additional catheters (PE-100) were placed into the left ventricle via the left carotid artery for the injection of microspheres and into the left jugular vein for central venous pressure (CVP) measurements. Placement of the catheter in the left ventricle was performed by following the ventricular pressure tracing. The location of all catheters was verified at autopsy. Body temperature was maintained constant with a servo-controlled overhead heater at 38°C. All piglets were allowed to rest for 2 h after surgical procedures and before experiments were started to insure stability after the surgical stress and to allow for recovery from the single dose of (+)-tubocurarine.

Series 1 experiments

The aim of this study was to examine the haemodynamic effects of acute ethanol intoxication. Piglets were randomly assigned into one of two groups. Six piglets (E) received ethanol, $1.4\,g\,kg^{-1}$, over 30 min while 6 piglets (C) received an equivalent volume of dextrose solution. Blood flow to the various regions was assessed by the microsphere technique before the ethanol or dextrose infusion and 90 min after the end of the infusion. MABP and HR were monitored continuously and arterial blood gases, haematocrit and CVP measurements were carried out before each microsphere measurement. Arterial blood samples (0.5 ml) were taken immediately prior to the last microsphere measurement to determine plasma ethanol concentrations.

Series 2 experiments

The aim of this study was to determine the effects of blocking ethanol metabolism with 4-MP on ethanol-induced haemodynamic changes. Piglets were randomly assigned into one of two groups. Four piglets (CMP) received 4-MP, $50\,mg\,kg^{-1}$, intravenously over a 15 min infusion interval prior to dextrose infusion while six piglets (EMP) received 4-MP prior to ethanol infusion. Blood flow to the various regions was assessed by the microsphere technique before the 4-MP infusion, 15 min after the end of the 4-MP infusion and 90 min after the

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end of the dextrose or ethanol infusion. MABP and HR were monitored continuously while arterial blood gases, haematocrit and CVP determinations were made prior to each microsphere measurement. Arterial blood samples (0.5 ml) were taken at 5, 30 and 90 min after the end of the dextrose or ethanol infusion to determine plasma ethanol concentrations.

Microsphere technique

Microspheres, $15 \pm 5\,\mu m$ diameter, were labelled with ^{85}Sr, ^{141}Ce or ^{51}Cr (3M Company, St. Paul, Minnesota, U.S.A.). Approximately 1×10^6 microspheres were suspended in 1 ml of 10% dextran and 0.02% Tween 80 and injected into the left ventricle. The catheter was then flushed with 2 ml of 0.9% saline. A reference sample of blood from the abdominal aorta was collected continuously with a pump for 2 min beginning 10 s prior to the microsphere injection at a constant withdrawal rate of $0.97\,ml\,min^{-1}$. At the end of the experiment, each anaesthetized animal was killed by an injection of saturated KCl into the left ventricle.

The brain was separated from the spinal cord at the level of the first cervical vertebra and divided into left and right hemispheres. The brain was then dissected into regions representing the cerebellum, cortex, basal ganglia and brainstem. All sections were weighed and placed in counting vials to heights of 1 cm.

The heart, stomach and right and left kidneys were removed, weighed and placed in counting vials. The small and large bowel, liver and the remaining parts of the piglet (carcass) were weighed separately and individually incinerated at 300°C for 72 h. After obtaining the dry weight of the tissues, the organs were ground into a fine powder with a mortar and pestle and aliquots of the resulting ash were placed in counting vials to a height of 1 cm.

Radioactivity from all regions and reference samples was measured by an automatic gamma counting system (1185 Series, Nuclear-Chicago). Blood flow to the various regions was calculated according to the equation: organ flow = (c.p.m. organ/c.p.m. reference sample) × rate of withdrawal of reference sample. All blood and tissue samples contained at least 400 microspheres. Adequate mixing of the microspheres was confirmed by comparing blood flow to the left brainstem, basal ganglia, cortex, cerebellum and kidney with the respective blood flow to corresponding structures on the right side.

Ethanol analysis

Arterial blood samples were placed in tubes containing potassium oxalate and sodium fluoride, mixed and placed on ice. Within 2 h, the samples were centrifuged for 2 min in a Fisher microcentrifuge (model 235A) and the plasma separated and stored at −20°C.

Plasma ethanol concentrations were determined by gas-liquid chromatography on a Hewlett Packard model 402 instrument using a flame ionization detector and a Poropak Q/R column (Cumming *et al.*, 1984). Operating parameters included a column and injector port temperature of 150°C and a detector temperature of 250°C. Nitrogen was used as the carrier gas and propanol-1 (0.02% v/v) as the internal standard.

Data analysis

The calibration curve for ethanol quantitation was determined by the least squares linear regression analysis of the peak height ratio of ethanol:propranol-1 to assess the goodness of fit (Goldstein, 1964).

Analysis of variance for repeated measurements was used to detect statistical significance. Multiple group comparisons were made with Duncan's multiple range test with $P < 0.05$ indicating statistical significance. All values are expressed as means ± s.e.mean.

Results

Arterial blood gases, pH and haematocrit values for control (C) and ethanol-treated (E) piglets are shown in Table 1. Similar data for the control 4-methylpyrazole-treated (CMP) and 4-methylpyrazole and ethanol-infused (EMP) piglets are shown in Table 2. There was no significant difference in any of these parameters between C and E or CMP and EMP piglets. In addition, 4-MP pretreatment did not affect arterial blood gases, pH or haematocrit.

Baseline MABP, HR, CO and SVR values for the four groups of piglets are summarized in Table 3. Changes in these parameters 90 min after dextrose or ethanol infusion,

Table 1 Arterial blood gases, pH and haematocrit (HCT) changes in Series 1 piglets

Parameter	*Group*	*Pre-infusion*	*Post-infusion*
Po_2	C	107 ± 7	106 ± 6
(mmHg)	E	117 ± 6	114 ± 6
Pco_2	C	37 ± 1	38 ± 1
(mmHg)	E	37 ± 1	37 ± 1
pH	C	7.41 ± 0.03	7.40 ± 0.02
	E	7.42 ± 0.02	7.40 ± 0.03
HCT	C	29 ± 1	29 ± 1
	E	28 ± 1	29 ± 1

Values are means ± s.e.mean for 6 piglets. Control (C) piglets received dextrose while treated (E) piglets received ethanol. Pre-infusion before dextrose or ethanol infusion and post-infusion is 90 min after the end of dextrose or ethanol infusion.

Table 2 Arterial blood gases, pH and haematocrit (HCT) values in Series 2 piglets

Parameter	*Group*	*Pre 4-MP*	*Post 4-MP*	*Post-infusion*
Po_2	CMP	130 ± 9	135 ± 10	130 ± 12
(mmHg)	EMP	120 ± 10	124 ± 10	122 ± 9
Pco_2	CMP	38 ± 1	39 ± 1	37 ± 1
(mmHg)	EMP	37 ± 1	39 ± 1	37 ± 1
pH	CMP	7.34 ± 0.03	7.30 ± 0.04	7.33 ± 0.01
	EMP	7.34 ± 0.01	7.33 ± 0.01	7.33 ± 0.01
HCT	CMP	28 ± 1	28 ± 1	29 ± 1
	EMP	28 ± 1	28 ± 1	28 ± 1

Values are means ± s.e.mean. Four control (CMP) piglets received 4-methylpyrazole (4-MP) prior to 5% dextrose in water infusion while six treated (EMP) piglets received 4-MP prior to ethanol infusion. 'Pre 4-MP' is before 4-MP infusion, 'Post 4-MP' is after 4-MP infusion and 'Post-infusion' is 90 min after the end of dextrose or ethanol infusion.

Table 3 Baseline mean arterial blood pressure, heart rate, cardiac output and systemic vascular resistance values in Series 1 and 2 piglets

Group	*MABP*	*HR*	*CO*	*SVR*
C	86 ± 4	225 ± 10	20 ± 2	3.5 ± 0.3
E	95 ± 7	196 ± 6	27 ± 3	3.4 ± 0.4
CMP	71 ± 2	213 ± 9	24 ± 4	3.0 ± 0.3
EMP	81 ± 5	197 ± 14	25 ± 2	3.2 ± 0.3

Values are means ± s.e.mean. There are six animals per group, except for CMP piglets where only four animals were studied. Mean arterial blood pressure (MABP) is expressed as mmHg, heart rate (HR) as beats min^{-1}, cardiac output (CO) as ml $100\,g^{-1}\,min^{-1}$ and systemic vascular resistance (SVR) as mmHg ml^{-1} $100\,g^{-1}min^{-1}$. For other abbreviations, see text.

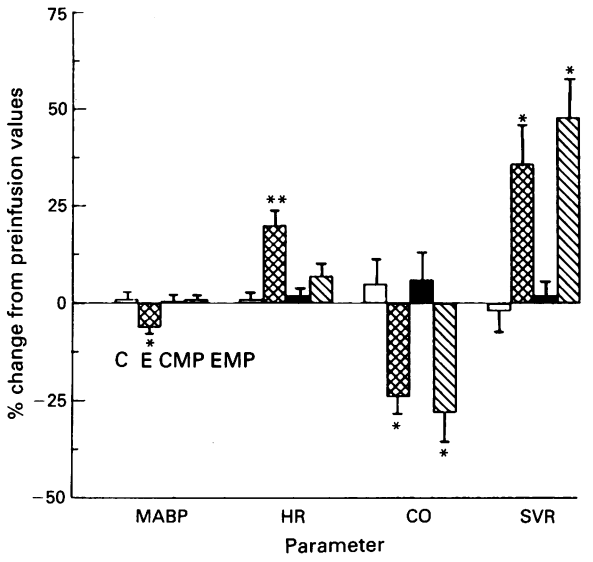

Figure 1 Changes in mean arterial blood pressure (MABP), heart rate (HR), cardiac output (CO) and systemic vascular resistance (SVR). Each column represents the value 90 min after dextrose or ethanol infusion as a percentage of its preinfusion value. Series 1 control piglets (C), open columns; Series 1 ethanol-treated piglets (E), cross hatched columns; Series 2 control 4-methylpyrazole-treated piglets (CMP) solid columns; Series 2 ethanol-infused 4-MP-treated piglets (EMP) hatched columns. Values are means ± s.e.mean. * $P < 0.05$ and ** $P < 0.01$ compared to preinfusion value. There are six animals per group, except for CMP piglets, where only four animals were studied.

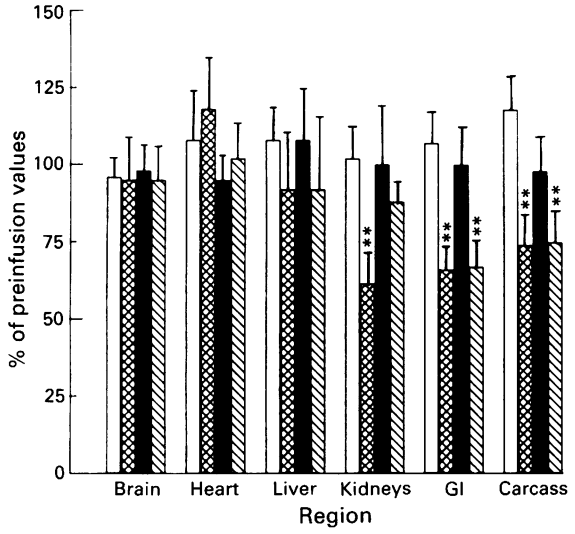

Figure 2 Changes in regional blood flow. Each column represents arterial perfusion 90 min after dextrose or ethanol infusion as a percentage of its preinfusion value. Series 1 C piglets, open columns; Series 1 E piglets, cross-hatched columns; Series 2 CMP piglets, solid columns; Series 2 EMP piglets, hatched columns. Values are means with s.e.mean shown by vertical bars. ** $P < 0.01$ compared to preinfusion value. There are six animals per group, except for CMP piglets, where only four animals were studied.

Table 5 Haemodynamic parameters in Series 2 piglets before (Pre 4-MP) and after (Post 4-MP) 4-methylpyrazole infusion

Parameter	*Group*	*Pre 4-MP*	*Post 4-MP*
MABP	CMP	72 ± 3	71 ± 2
	EMP	82 ± 5	81 ± 5
HR	CMP	208 ± 3	213 ± 9
	EMP	200 ± 12	197 ± 14
CO	CMP	23 ± 3	24 ± 4
	EMP	25 ± 2	25 ± 2
SVR	CMP	3.3 ± 0.3	3.0 ± 0.3
	EMP	3.3 ± 0.4	3.2 ± 0.3

Values are means ± s.e.mean. Four animals were studied in the CMP group and six in the EMP group. Mean arterial blood pressure (MABP) is expressed in mmHg, heart rate (HR) in beats min^{-1}, cardiac output (CO) in ml $100\,g^{-1}$ min^{-1} and systemic vascular resistance (SVR) in mmHg $100\,g^{-1}\,min^{-1}$. For other abbreviations, see text.

expressed as a percentage of the value immediately prior to dextrose or ethanol infusion, are illustrated in Figure 1. After the end of the ethanol infusion, a 6% decrease in MABP ($P < 0.05$) and 20% increase in HR ($P < 0.01$) were observed in E piglets while no change was seen in EMP piglets receiving 4-MP prior to ethanol infusion. In C and CMP piglets, MABP and HR values after dextrose infusion were not different from values obtained prior to dextrose infusion. There was a reduction in CO ($P < 0.05$) and an increase in SVR ($P < 0.05$) after ethanol infusion in E and EMP piglets. In C and CMP piglets, CO and SVR values after dextrose infusion were not different from values obtained prior to dextrose infusion.

Baseline CVP values were 0.8 ± 0.4, 0.8 ± 0.4, 0.8 ± 0.2 and 0.8 ± 0.2 cmH_2O in C, E, CMP and EMP piglets, respectively. After dextrose or ethanol infusion, the corresponding values were 0.7 ± 0.4, 0.6 ± 0.3, 0.6 ± 0.3 and 0.6 ± 0.3 cmH_2O.

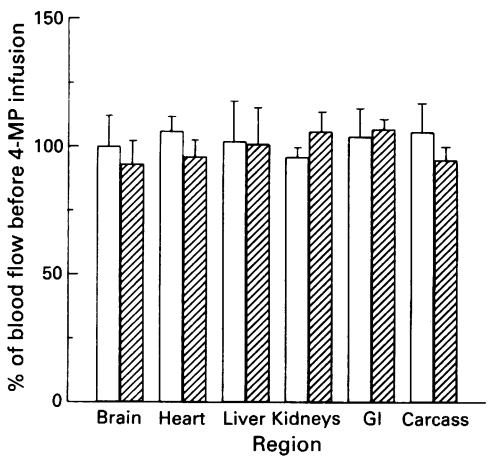

Figure 3 Changes in regional blood flow after 4-methylpyrazole (4-MP) infusion. Open columns, CMP piglets; hatched columns, EMP piglets. Values are means with s.e.mean shown by vertical bars. Four animals were studied in the CMP group and six in the EMP group.

Table 4 Baseline arterial blood flow (ml $100\,g^{-1}\,min^{-1}$) in Series 1 and 2 piglets

Group	*Brain*	*Heart*	*Liver*	*Kidneys*	*GI*	*Carcass*
C	53 ± 8	184 ± 36	53 ± 11	198 ± 25	62 ± 12	11 ± 1
E	59 ± 6	186 ± 33	77 ± 21	188 ± 18	93 ± 32	16 ± 2
CMP	76 ± 18	218 ± 31	61 ± 13	175 ± 25	61 ± 9	14 ± 3
EMP	68 ± 7	210 ± 35	44 ± 9	186 ± 17	73 ± 8	15 ± 1

Values are means ± s.e.mean. There are six animals per group, except for CMP piglets, where only four animals were studied. For abbreviations, see text.

Table 6 Regional cerebral blood flow (ml $100\,g^{-1}\,min^{-1}$) in Series 1 and 2 piglets before (Pre) and 90 min after (Post) the end of dextrose or ethanol infusion

Region	*Group*	*Pre*	*Post*
Brainstem	C	54 ± 8	62 ± 6
	E	60 ± 7	58 ± 3
	CMP	76 ± 22	80 ± 16
	EMP	76 ± 3	74 ± 8
Cerebellum	C	60 ± 4	58 ± 3
	E	64 ± 3	64 ± 9
	CMP	81 ± 19	80 ± 8
	EMP	67 ± 6	68 ± 11
Basal ganglia	C	79 ± 8	75 ± 7
	E	85 ± 9	86 ± 9
	CMP	117 ± 30	116 ± 21
	EMP	101 ± 7	98 ± 12
Cortex	C	53 ± 6	51 ± 7
	E	64 ± 5	56 ± 8
	CMP	76 ± 18	67 ± 9
	EMP	68 ± 8	68 ± 7

Values are means ± s.e.mean. There are six animals per group, except for CMP piglets, where only four animals were studied. In series 1, control (C) piglets received dextrose infusion and treated (E) piglets received ethanol infusion. In series 2, control (CMP) piglets received 4-methylpyrazole (4-MP) prior to the dextrose infusion, and treated piglets (EMP) received an ethanol infusion after pretreatment with 4-MP.

There was no significant difference in CVP between piglet groups or after dextrose or ethanol infusion.

Baseline regional blood flow data are presented in Table 4. Changes in these values 90 min after dextrose or ethanol infusion, expressed as a percentage of the value immediately prior to dextrose or ethanol infusion, are illustrated in Figure 2. Blood flow to all regions tested remained constant in C and CMP piglets. There were decreases in renal, GI and carcass blood flow ($P < 0.01$) after ethanol administration in E piglets while only GI and carcass blood flow reductions ($P < 0.01$) were observed in EMP piglets.

Plasma ethanol concentrations were higher in piglets receiving 4-MP prior to ethanol infusion compared to those receiving ethanol alone ($P < 0.05$). Ninety min after the end of the ethanol infusion, plasma ethanol concentration was $36.3 \pm 3.9\,mmol\,l^{-1}$ in EMP piglets and $31.1 \pm 2.2\,mmol\,l^{-1}$ in E piglets. Plasma ethanol concentration remained steady over the study duration in EMP piglets, showing that 4-MP effectively blocked ethanol metabolism for the duration of the study.

There were no changes in MABP, HR, CO or SVR following 4-MP infusion in both CMP and EMP piglets (Table 5). Similarly, regional blood flow was not altered in these piglets after 4-MP infusion (Figure 3).

Blood flow to the brainstem, cerebellum, basal ganglia and cortex are shown in Table 6. Blood flow to these brain regions remained constant in all four groups with the basal ganglia receiving the most perfusion within groups.

Discussion

The major findings of these studies are the reduced cardiac output (CO) and altered regional blood flow observed after acute ethanol intoxication in the newborn piglet and the prevention of hypotension and tachycardia with 4-MP treatment prior to ethanol administration.

In our study, CO significantly decreased 26% from baseline values 90 min after the end of the ethanol infusion. Normal CO depends on both venous return and myocardial contractility. The maintenance of CVP readings does not indicate any pooling of blood in the peripheral compartments. In addition, the constancy of haematocrit values suggests that there is no significant alteration of plasma volume due to any diuretic effect of ethanol. These data strongly imply that the reduction in CO is the direct result of impaired myocardial contractility by ethanol.

It is recognized that acute ethanol intoxication may depress cardiac performance in adult animals (Horwitz & Atkins, 1974; Stratton *et al.*, 1981) and man (Lang *et al.*, 1985; Kelbaek *et al.*, 1985). In foetal lambs, Kirkpatrick *et al.* (1976) observed a significant depression in the extent and velocity of left ventricular myocardial fibre shortening at blood ethanol concentrations of 23.9 and $45.7\,mmol\,l^{-1}$. These indices of cardiac contractility were depressed in the absence of changes in end diastolic diameter, left atrial pressure and systemic arterial pressure. In foetal lambs, Urfer *et al.* (1984) reported a significant difference in the pre-ejection period to ejection time ratio observed before ethanol infusion to that seen 15 and 30 min after ethanol infusion, at blood ethanol concentrations of about $19.6\,mmol\,l^{-1}$. Both studies concluded that myocardial function is compromised with acute ethanol intoxication. Our observation of a 26% decrease in CO after ethanol administration in the neonatal piglet is consistent with the finding in the foetal lamb.

The effects of ethanol on regional blood flow in newborn animals have not been reported previously. In our study, arterial blood flow to the brain, heart and liver was maintained while flow to the kidneys, GI organs and carcass was decreased. The reduction in blood flow to GI organs observed after ethanol administration to the neonate is in contrast to the increase in splanchnic blood flow observed in adult humans (Stein *et al.*, 1963) and animals (Cheung *et al.*, 1975; Shaw *et al.*, 1977; Friedman *et al.*, 1979; Friedman *et al.*, 1981; McKaigney *et al.*, 1986). The differences observed in regional blood flow after ethanol administration may be related to age, species and type of anaesthetic used.

As in our previous studies (Chandler *et al.*, 1989), MABP was significantly decreased and HR increased by 90 min after the end of the ethanol infusion. These haemodynamic alterations suggest an adverse cardiovascular effect of acute ethanol administration. Such adverse effects are consistent with previous reports of human neonatal depression (Peden *et al.*, 1973; Cook *et al.*, 1975) and neonatal death (Jung *et al.*, 1980) after ethanol intoxication.

Total and regional CBF were maintained after ethanol intoxication. These observations confirm our previous reports of the maintenance of CBF and CBF autoregulation in intoxicated newborn piglets (MacIntyre *et al.*, 1987). Maintenance of CBF has also been observed by Mann *et al.* (1975) in foetal lambs at blood ethanol concentrations up to $47.8\,mmol\,l^{-1}$.

4-MP successfully blocked ethanol metabolism in newborn piglets. Steady-state plasma ethanol concentrations were achieved over the duration of our study with a dose of $50\,mg\,kg^{-1}$ of 4-MP without any significant haemodynamic responses or changes in arterial blood gases. These findings are consistent with studies on 4-MP in rats by Hellstrom & Tottmar (1982).

In piglets given 4-MP before ethanol infusion, the decrease in CO seen after ethanol infusion alone was still observed. Therefore, ethanol itself, rather than its metabolites, may be the major cause of the reduction in CO observed with acute ethanol intoxication. However, the decrease in MABP seen after ethanol administration alone was not observed in those piglets pretreated with 4-MP. In adult rats, Hellstrom & Tottmar (1982) also observed that 4-MP given prior to ethanol administration prevented the decrease in MABP observed with ethanol treatment. This suggests that the reduction in MABP could be due to a metabolite such as acetaldehyde. This is consistent with other reports on the vasodilator effects of acetaldehyde (Altura *et al.*, 1978; Hellstrom & Tottmar, 1982). Inhibition of alcohol dehydrogenase activity with 4-MP and consequent blockade of acetaldehyde production would abolish the hypotension observed after ethanol treatment.

4-MP prevented the decrease in renal flow observed after ethanol treatment alone. However, the reduction in GI blood flow was not abolished by 4-MP pretreatment. The preser-

vation of renal blood flow may be a beneficial effect of maintaining arterial blood pressure at baseline level.

Previous studies in foetal lambs have shown transient tachycardia but no consistent blood pressure changes (Ayromlooi *et al.*, 1979; Cook *et al.*, 1981). Those results may be accounted for by the significantly lower ethanol metabolic rate in foetal and newborn lambs (Cumming *et al.*, 1985) compared to newborn humans (Wagner *et al.*, 1970; Fuchs & Fuchs, 1981) and piglets (Chandler *et al.*, 1989).

In conclusion, acute ethanol intoxication caused significant hypotension, tachycardia, decreased CO, elevated SVR and altered regional blood flows in neonatal piglets. 4-MP pretreatment prevented the reduction in MABP and the increase in HR but did not affect the CO decrease or SVR increase observed after ethanol alone. Ethanol is a cardiac depressant while its metabolites, such as acetaldehyde, probably play a major role in the hypotension and tachycardia observed after ethanol intoxication.

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Structure-activity studies on endothelin (16–21), the C-terminal hexapeptide of the endothelins, in the guinea-pig bronchus

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1 We describe the results of a structure-activity study in which the C-terminal hexapeptide of the endothelins, endothelin (16–21), is compared with shorter fragments; hexapeptides bearing amino-acid substitutions and the corresponding C-terminal fragments of sarafotoxins. The guinea-pig bronchus was used in this study since it is the most sensitive preparation for endothelin (16–21) thus far developed.

2 The biological results obtained with endothelin (16–21) and analogues demonstrate that the contractile activity of the C-terminal hexapeptide of endothelin on the guinea-pig bronchus depends on quite close structural requirements, strongly suggestive of a receptor interaction. The following features appear to be essential for the biological activity: (a) the C-terminal free carboxylic function; (b) the L-configuration of Trp-21; (c) the β-carboxylic function of Asp-18; (d) the presence of Leu-17 and (e) the imidazole moiety of His-16.

3 The hexapeptide corresponding to the C-terminal portion of sarafotoxin, sarafotoxin (16–21), was devoid of biological activity. This behaviour might be related to the proposed existence of more than one receptor for the endothelin/sarafotoxin family in the guinea-pig bronchus.

Introduction

Endothelins are a family of 21 amino acid peptides recently discovered in the human genome (Yanagisawa *et al.*, 1988a,b; Inoue *et al.*, 1989). Of the three identified structures, termed endothelin-1, endothelin-2 and endothelin-3, the first is identical to porcine endothelin, which was originally isolated as a product of cultured aortic endothelial cells (Yanagisawa *et al.*, 1988a), while endothelin-3 has the same sequence as the peptide originally identified on the rat genome (Yanagisawa *et al.*, 1988b). Another class of structurally related peptides are the sarafotoxins S6, isolated from the venom of the snake *Atractaspis engaddensis* (Kloog *et al.*, 1988; Ambar *et al.*, 1989; Gu *et al.*, 1989; Kloog & Sokolovsky, 1989). Common characteristics of both endothelins and sarafotoxins are the two conserved disulphide bridges and the C-terminal hydrophobic hexapeptide. This latter portion (His-Leu-Asp-Ile-Ile-Trp-OH) is shared by all of the three endothelins, while three out of the four known sarafotoxins share a C-terminal sequence bearing Gln in the place of Leu at position 17 and Val in the place of Ile at position 19; the latter quite conservative substitution being absent in the fourth sarafotoxin sarafotoxin S6d.

Peptides of the endothelin/sarafotoxin family are potent vasoactive agents in various isolated blood vessels as well as in intact animals (De Nucci *et al.*, 1988; Lippton *et al.*, 1988; Tomobe *et al.*, 1988; Wright & Fozard, 1988; Han *et al.*, 1989; D'Orleans-Juste *et al.*, 1989a,b; Walder *et al.*, 1989; Warner *et al.*, 1989; Rodman *et al.*, 1989). Endothelins and sarafotoxins are also potent contractile agents on certain non-vascular smooth muscles (Uchida *et al.*, 1988; Maggi *et al.*, 1989c,d). Specific endothelin receptors have been demonstrated in various peripheral preparations (Power *et al.*, 1989; Hoyer *et al.*, 1989) and the existence of multiple receptors for endothelins and sarafotoxins has been proposed (Kloog et al., 1989; Maggi *et al.*, 1989a; 1990).

We have previously shown (Maggi *et al.*, 1989a,b) that the C-terminal fragment endothelin (16–21) is inactive in the rat isolated aorta, while it is a full agonist in the guinea-pig isolated bronchus, two sensitive tissues in which both endothelins and sarafotoxin-S6b exert a powerful contractile effect. Based on these findings, as well as on a more extensive study of the effects of endothelins, sarafotoxin S6b and endothelin (16–21) on several *in vitro* preparations, we have proposed the existence of at least two distinct receptors for endothelins.

We describe here the results of a structure-activity study in which the hexapeptide endothelin (16–21) is compared with: (a) shorter fragments; (b) hexapeptides bearing amino acid substitutions and (c) the corresponding C-terminal fragments of sarafotoxins S6. The guinea-pig bronchus was selected as the bioassay for this study, being the most sensitive preparation to endothelin (16–21) thus far found (Maggi *et al.*, 1990).

Methods

Male albino guinea-pigs (220–300 g) were stunned and bled. The main bronchus was excised and placed in gassed (96% O_2 and 4% CO_2) Krebs solution, pH 7.4. Bronchial rings were prepared for isotonic recording (the load was 0.5 g) after the removal of the bronchial epithelium by gentle rubbing of the internal surface. The preparation was placed in a 5 ml organ bath containing gassed Krebs solution, at 37°C, plus 5 μM indomethacin and a mixture of peptidase inhibitors (1 μM thiorphan, 1 μM captopril, 1 μM bestatin), i.e. conditions selected to improve the contractile response to endothelins (Maggi *et al.*, 1988a,b; 1990). Cumulative concentration-response curves to the peptides were obtained, the next concentration being added when the effect of the preceding one had reached a steady state. The responses were expressed as percentages of the maximal response to KCl (80 mM, added to the bath). Only one peptide was tested in each preparation. The maximum concentration tested for each peptide was 100 μM. For those peptides which at 100 μM failed to produce a response >50% of that to KCl, 10 μM endothelin (16–21) was added to the bath to assess the maximal response to this peptide.

Statistical analysis

Each value is the mean ± s.e.mean from 6 experiments. Regression analysis of log-concentration/effect curves was performed by the least squares method, considering linear such curves between 20 and 80% of the maximum response. EC_{50} and confidence limits were calculated accordingly. Analysis of data was performed by a computer programme (Tallarida & Murray, 1981).

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Peptides

All the peptides used in this study were prepared by the solid-phase method; syntheses were carried out with a SP 640 Peptide Synthesizer (Labortec AG, Switzerland) using the Fmoc protection strategy. Purification was performed in a single step by reverse-phase preparative high performance liquid chromatography (h.p.l.c.) (Waters Delta Prep 3000 apparatus; Deltapack C_{18} 19 × 300 mm column, linear gradient of CH_3CN/0.1% trifluoroacetic acid in 0.1% trifluoroacetic acid). Amino acid analysis of final products was always satisfactory; h.p.l.c. purity was greater than 98%.

Results

As described previously (Maggi *et al.*, 1989a,b) endothelin (16–21) produced a concentration-dependent contraction of the guinea-pig isolated bronchus, 10 nM being the threshold concentration. The maximal response to endothelin (16–21) was about 80% of the response to KCl. Among the peptides tested only endothelin (17–21) and [Val^{19}]ET (16–21) gave maximal responses comparable to that of endothelin (16–21). For the other peptides shown in Figure 1 values are expressed as a percentage of the response to 10 μM endothelin (16–21). The pentapeptide endothelin (17–21) was a full agonist, its activity being less, by about 100 times, than that of the hexapeptide (Table 1 and Figure 1). By contrast, the tetrapeptide endothelin (18–21) was weakly active even at 0.1 mM. Similar results were obtained when modifying the C-terminal Trp-21, either by amidating the carboxylic moiety, as in endothelin (16–21)-NH_2, or introducing the D-isomer, as in [D-Trp^{21}] endothelin (16–21). Also the two analogues modified in the N-terminal region, e.g. [Asn^{18}] endothelin (16–21), in which Asp-18 was replaced by the corresponding amide to block the carboxylic function on the side chain, and [Lys^{16}]endothelin (16–21), in which His-16 was replaced by Lys in order to enhance the positive charge on the side chain, were very weak agonists (ED_{50} > 0.1 mM; Table 1). The C-terminal tail common to sarafotoxin S6a, b and c, sarafotoxin (16–21), was only weakly active even at 0.1 mM (Figure 1). In contrast, the C-terminal hexapeptide of sarafotoxin S6d had some activity at a threshold concentration of 10 μM. At 100 μM the response to sarafotoxin S6d (16–21) did not exceed 50% of maximal response to endothelin (16–21) and for this reason an ED_{50} was not calculated. [Val^{19}]endothelin (16–21) was a full agonist, being about 5.5 times less potent than endothelin (16–21) (Table 1).

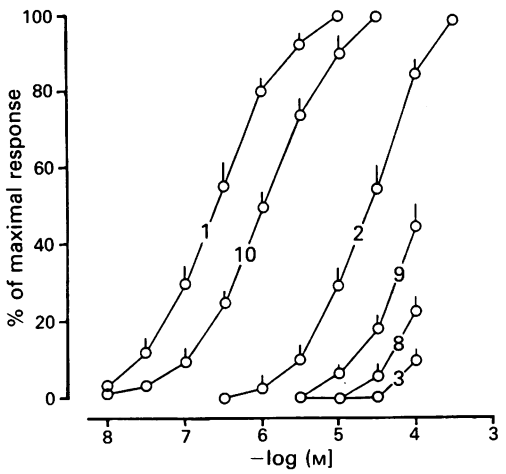

Figure 1 Concentration-dependent contraction of the guinea-pig isolated bronchus to endothelin (16–21) and some analogues. All values are mean of 6 experiments with s.e.mean shown by vertical lines: (1) Endothelin (16–21); (2) endothelin (17–21); (3) endothelin (18–21); (8) sarafotoxin (16–21); (9) sarafotoxin S6d (16–21); (10) [Val^{19}]endothelin (16–21). For peptides 1, 2 and 10 contractile values are expressed as a percentage of the contraction produced by endothelin (16–21) at 10 μM.

Discussion

The biological results obtained with endothelin (16–21) and its analogues demonstrate that the contractile activity of the C-terminal hexapeptide of the endothelins on the guinea-pig bronchus depends on quite strict structural requirements. In fact, the following features, at least, appear to be essential for the biological activity of endothelin (16–21): (a) the C-terminal free carboxylic function (Maggi *et al.*, 1989a); (b) the L-configuration of Trp-21; (c) the β-carboxylic function of Asp-18; (d) the presence of Leu-17 and (e) the imidazole moiety of His-16.

These close structural requirements are strongly suggestive of a receptor interaction. Moreover, some of the structural requirements described above are shared also by native endothelins. In fact, both the C-terminal amide of endothelin-1 (Kimura *et al.*, 1988) and [D-Trp^{21}]endothelin-1 (Nakajima *et al.*, 1989) have been shown to have very weak activity. In addition, the C-terminal moiety of endothelin molecules appears to be essential for biological activity; in fact, while the bicyclic N-terminal fragment endothelin (1–16) is devoid of biological activity (Kimura *et al.*, 1988), analogues characterized by the whole endothelin sequence, but with one or both the disulphide bridges disrupted, maintain some activity (Nakajima *et al.*, 1989; Randall *et al.*, 1989).

An interesting and unexpected finding of this study was the lack of activity of the hexapeptide corresponding to the C-terminal portion of sarafotoxins. Failure of this hexapeptide to induce a significant contractile activity is in sharp contrast with the action of sarafotoxin S6b, which is a full agonist showing high potency in the guinea-pig bronchus (Maggi *et al.*, 1990) and with the corresponding endothelin partial sequence, e.g. endothelin (16–21), which maintains full biological activity. This behaviour might be related to the proposed existence of more than one receptor for the endothelin/sarafotoxin family in the guinea-pig bronchus (Maggi *et al.*, 1990). Since sarafotoxin S6d (16–21) was a very weak agonist, while [Val^{19}]endothelin (16–21) was only slightly less active than endothelin (16–21), the crucial feature for

Table 1 Sequences and biological activity of analogues of endothelin (16–21) on guinea-pig isolated bronchus

No.	*Peptide*	*Sequence*	ED_{50} (μM)	95% *confidence limits*
1	Endothelin (16–21)	His-Leu-Asp-Ile-Ile-Trp-OH	0.228	(0.1–0.6)
2	Endothelin (17–21)	Leu-Asp-Ile-Ile-Trp-OH	25	(19–31)
3	Endothelin (18–21)	Asp-Ile-Ile-Trp-OH	>100	
4	Endothelin (16–21)-NH_2	His-Leu-Asp-Ile-Ile-Trp-NH_2	>100	
5	[D-Trp^{21}] endothelin (16–21)	His-Leu-Asp-Ile-Ile-Trp-OH	>100	
6	[Asn^{18}] endothelin (16–21)	His-Leu-Asn-Ile-Ile-Trp-OH	>100	
7	[Lys^{16}] endothelin (16–21)	Lys-Leu-Asp-Ile-Ile-Trp-OH	>100	
8	Sarafotoxin (16–21)	His-Gln-Asp-Val-Ile-Trp-OH	>100	
9	Sarafotoxin S6d (16–21)	His-Gln-Asp-Ile-Ile-Trp-OH	>100	
10	[Val^{19}] endothelin (16–21)	His-Leu-Asp-Val-Ile-Trp-OH	1.23	(1.10–1.35)

the differential activity between the C-terminal hexapeptides of endothelins and sarafotoxins is represented by the replacement of Leu-17 by Gln. Thus, considering the endothelin C-terminal tail as the minimal structural requirement sufficient for the activation of a putative ET_B receptor subtype (Maggi *et al.*, 1989a,b), an evolutionary differentiation of mammalian endothelin receptors might be postulated to account for ineffectiveness of the C-terminal hexapeptide of sarafotoxin. On the other hand we cannot exclude the possibility that the biological activity of endothelin (16–21) involves cross-talk between the endothelin fragment and receptors unrelated to those for endothelins, although the marked similarities between the structural requirements for the biological activity of the C-terminal hexapeptide and the entire endothelin molecule (see above) make this explanation less likely. Direct demonstration of competition between endothelin (16–21) and the entire endothelin molecule at common sites is now necessary to confirm or disprove the ET_A/ET_B receptor hypothesis.

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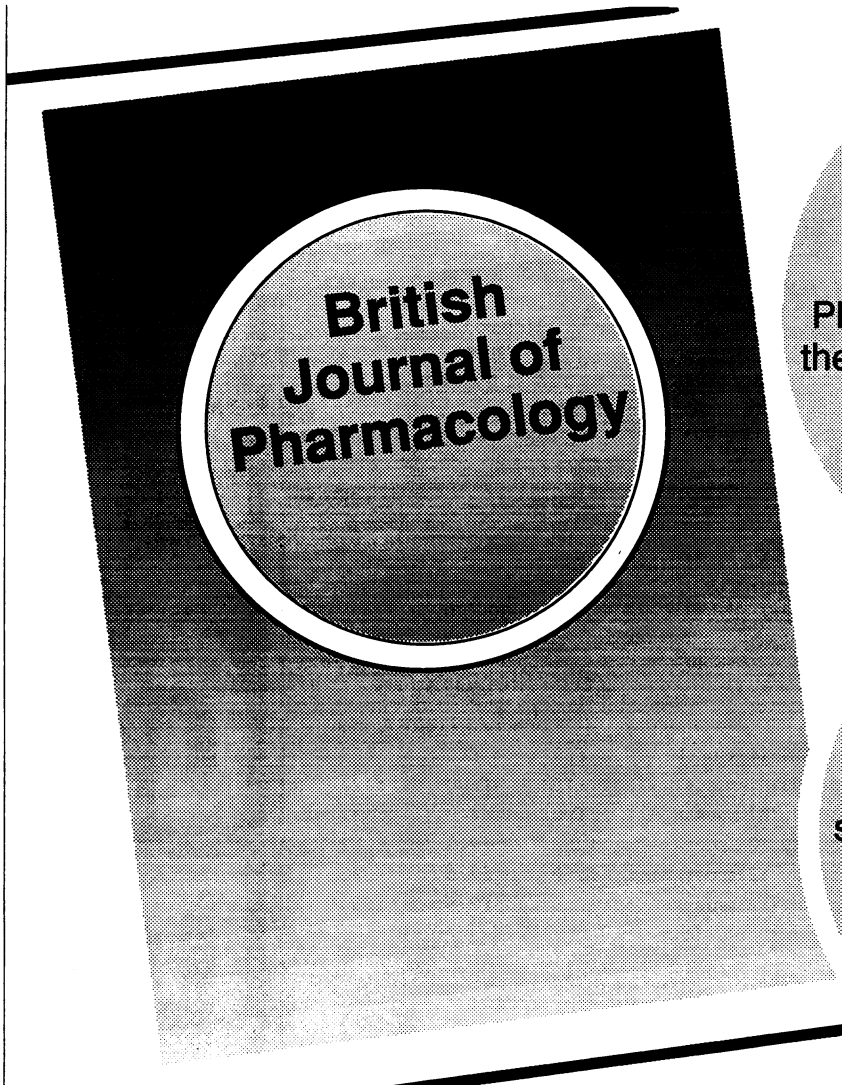

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