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Inability of endothelin to increase Ca^{2+} current in guinea-pig heart cells

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Effects of endothelin, a novel vasoconstrictor peptide derived from vascular endothelial cells, on cardiac contractility and membrane currents, were examined in guinea-pig cardiac preparations. Endothelin (3–1000 nM) produced a positive inotropic effect in papillary muscles in a concentration-dependent manner. In whole-cell voltage clamp recording, endothelin (250 nM) decreased the amplitude of Ca^{2+} current (I_{Ca}, 25.0 ± 6.6%) in ventricular myocytes. The endothelin-induced decrease in I_{Ca} was abolished by pretreatment with ryanodine (1 μM). These results suggest that endothelin does not activate cardiac sarcolemmal Ca^{2+} channels. The enhancement of the sarcoplasmic reticulum function may play an important role in the positive inotropic effect of endothelin.

Introduction Recently, a novel potent vasoconstrictor peptide derived from vascular endothelial cells, was discovered and termed endothelin (Yanagisawa *et al.*, 1988). In addition to its potent vasoconstrictor effect, Ishikawa *et al.* (1988) have shown that endothelin produces a positive inotropic effect in guinea-pig atria. They have also shown that the positive inotropic effect of endothelin was attenuated by a dihydropyridine Ca^{2+} antagonist, suggesting that the positive inotropic effect of endothelin may be mediated by an increase in transsarcolemmal Ca^{2+} influx. Therefore, we have evaluated directly the effects of endothelin on the Ca^{2+} current (I_{Ca}) of guinea-pig heart cells using the whole-cell voltage clamp technique.

Methods To measure cardiac contractile tension, the right ventricular papillary muscles were carefully dissected from guinea-pig heart according to the methods described by Hattori *et al.* (1986). The muscles were mounted vertically in water-jacketed baths containing Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2 at 30°C. The composition of Krebs-Henseleit solution (pH 7.4) was (in mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10. Isometric tension of the muscles was measured with a force transducer and recorded on a pen recorder. The muscles were paced electrically at a frequency of 0.5 Hz. The resting tension applied to the preparations was adjusted to 0.5 g. The concentration-response curves for the positive inotropic effect of endothelin were determined in a cumulative manner by increasing its concentration in steps of 0.5 log units. All experiments were carried out in the presence of propranolol (1 μM) to eliminate a possible modulation of the response by endogenously released noradrenaline.

To measure membrane currents, guinea-pig single ventricular cells were enzymatically isolated by the method described by Taniguchi *et al.* (1981). Membrane currents were recorded in the whole-cell voltage clamp mode of the patch-clamp technique (Hamill *et al.*, 1981). The pipette solution contained (in mM): KOH 110, KCl 20, $MgCl_2$ 1, K_2-ATP 5, K_2-creatine phosphate 5, aspartic acid 90–100 and EGTA 10. The pH was adjusted to 7.4 with 5 mM HEPES. For single cell experiments, the composition of the external solution was (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES-NaOH buffer 5.0 and the pH was adjusted to 7.4. The temperature of the perfusate was maintained at 34–36°C.

Endothelin was kindly given by Prof. T. Masaki, and also obtained from Peptide Institute Inc. (Osaka, Japan). Endothelin was dissolved in 10 mM Na-phosphate buffer (pH 7.0) to produce a 0.1 mM stock solution. Ryanodine (Progressive Agri Systems Inc., Wind Gap, PA, U.S.A.) was dissolved in dimethyl sulphoxide to give a 10 mM stock solution.

Statistical analyses of endothelin-induced changes were performed by use of Student's *t* test. Significance was established when the probability value was less than 0.05. All data are presented as means ± standard errors (s.e.).

Results Endothelin produced a positive inotropic effect in electrically paced guinea-pig papillary muscles. The threshold concentration for endothelin was 3 nM and the concentration to produce the maximal effect was 1 μM. Endothelin at a concentration of 1 μM increased the contractile tension by 219 ± 55% of the control values ($n = 6$, $P < 0.01$).

In order to examine the effects of endothelin on I_{Ca}, we applied the whole-cell configuration of the patch-clamp technique to single ventricular cells of guinea-pigs. The cells were held at −37 mV to avoid the influence of the fast Na^+ current. The I_{Ca} was elicited by a 300 ms depolarizing test pulse to +3 mV from the holding potential at 0.1 Hz. After the external application of 250 nM endothelin, I_{Ca} gradually decreased and reached a steady level within 3–5 min. In 8 cells, endothelin decreased the amplitude of I_{Ca} (which was defined as the difference between the peak current and the current at the end of test pulse) by 25.0 ± 6.6% of the control values ($P < 0.01$). Actual current traces and time course of the decrease in I_{Ca} of a representative experiment are shown in Figure 1b(i), (ii) and Figure 1a. The current-voltage relation of I_{Ca} obtained in the presence of endothelin showed that I_{Ca} decreased at every membrane potential tested from −27 mV to +63 mV (data not shown).

The endothelin-induced decrease in I_{Ca} was abolished by pretreatment with ryanodine, a specific inhibitor of sarcoplasmic reticular (SR) function (Sutko & Kenyon, 1983). In the presence of 1 μM ryanodine, I_{Ca} remained unchanged until 5 min after the application of 250 nM endothelin, as shown in Figure 1a and Figure 1b(iii and iv). In 6 cells pretreated with 1 μM ryanodine, 250 nM endothelin decreased the amplitude of I_{Ca} by only 2.9 ± 10.3% of control values (NS).

Discussion As previously demonstrated in guinea-pig left atria (Ishikawa *et al.*, 1988), endothelin increased contractile tension in guinea-pig papillary muscles in a concentration-dependent manner. However, we found that endothelin decreased I_{Ca} rather than increased it in guinea-pig single ventricular cells at a concentration of 250 nM at which the inotropic response to endothelin was submaximal in the guinea-pig

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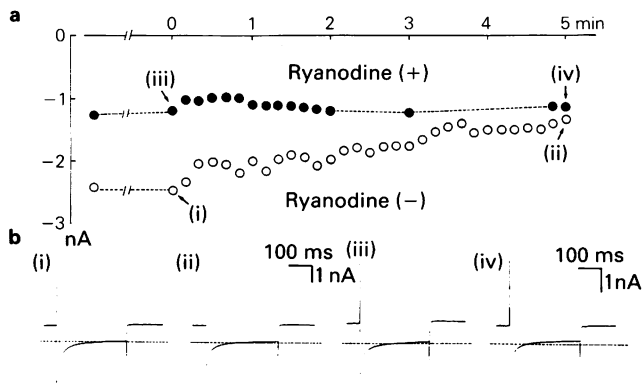

Figure 1 Effect of endothelin on I_{Ca} in the absence and presence of 1 μM ryanodine. (a) Time course change of an amplitude of I_{Ca} after 250 nM endothelin, which was given at time 0. The amplitude of I_{Ca} is defined as the difference between peak and steady-state current obtained by a 300 ms depolarizing test pulse to 3 mV from the holding potential of −37 mV. The test pulse was applied at 0.1 Hz. (○) No ryanodine present; (●) in presence of ryanodine 1 μM. (b) Representative examples of the actual current records at the time indicated in (a) (i–iv) are shown. The broken lines indicate zero current level.

papillary muscles. It is unlikely that the reduction in I_{Ca} produced by endothelin is due to its direct action on sarcolemmal Ca^{2+} channels because the peptide did not affect I_{Ca} in the presence of ryanodine.

It is not surprising to find that endothelin decreased rather than increased I_{Ca} in guinea-pig heart cells: if endothelin elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in some way other than activating Ca^{2+} channels, the elevation of $[Ca^{2+}]_i$ may produce a decrease in I_{Ca} (Ca^{2+}-inactivation) (Lee *et al.*, 1985). In the present study, we showed that ryanodine abolished the endothelin-induced decrease in I_{Ca}. This result suggests that endothelin may influence SR-function. Therefore, it is possible that a source of intracellular Ca^{2+} increased by endothelin is SR but not Ca^{2+}-influx through sarcolemmal Ca^{2+} channels. As cell membranes are not readily permeable to polypeptide compounds, the action of endothelin on SR seems to require some intracellular second messengers.

Yanagisawa *et al.* (1988) have speculated that endothelin may be an intrinsic Ca^{2+}-agonist which directly activates the L-type-Ca channels, the speculation being based on the observation that the dihydropyridine Ca^{2+} antagonist nicardipine, at a relative high concentration, slightly but significantly shifted the concentration-response curve for the positive inotropic effect of endothelin in guinea-pig left atria (Ishikawa *et al.*, 1988). On the other hand, Gu *et al.* (1989) recently reported that endothelin failed to bind to any calcium antagonist binding sites associated with L-type calcium channels in rat cardiac membrane fragments. The present observation that endothelin produced no direct effect on I_{Ca} in the presence of ryanodine in guinea-pig heart cells clearly indicates that endothelin can increase the cardiac contractile tension without activating Ca^{2+} channels.

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Role of endothelium and calcium channels in endothelin-induced contraction of human cerebral arteries

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Endothelin constricted human isolated cerebral arteries in a concentration-dependent manner. The maximal tension developed, as well as EC_{50} values were similar in arteries with and without endothelium. Removal of extracellular calcium or addition of the calcium antagonist nicardipine (10^{-6} M), attenuated but did not abolish responses to endothelin. These experiments show that the endothelin-induced contraction in human cerebral arteries is not linked to the presence of intact endothelial cells. The data also show that the contractile effects of endothelin cannot be explained solely by an action on voltage-dependent calcium channels.

Introduction Endothelin, a peptide secreted by endothelial cells in response to various stimuli, contracts vascular strips and produces a long-lasting increase in blood pressure (Yanagisawa *et al.*, 1988). With regard to the cerebral vessels, recent observations have shown that endothelin-1 contracts canine and feline basilar arteries when applied intracisternally but not when is given intraluminally (Shigeno *et al.*, 1989). It was concluded from these experiments that the endothelium forms a barrier which impedes the access of endothelin to the smooth muscle cells. The present experiments were designed to elucidate the effects of endothelin on human isolated cerebral arteries with special emphasis on endothelium-dependent responses as well as extracellular calcium requirements.

Methods Human cerebral arteries were obtained during autopsy of 12 patients (8 men and 4 women, aged 23–79 years) who had died 4 to 12 h before. The cause of death was variable: 4 were victims of car accidents, 5 died from myocardial infarction and 3 from cancer of the stomach. Cylindrical segments (4 mm in length and 0.7–1 mm in outside diameter) were cut from branches of the middle cerebral artery for isometric recording of tension. Each arterial segment was set up in a 4 ml bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 115, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1 and disodium EDTA 0.01, equilibrated with 95% O_2 and 5% CO_2 to give a pH of 7.3 to 7.4. Temperature was held at 37°C. To select resting tension for maximal force development, the arterial segments were exposed repeatedly to 60 mM KCl. Basal tension was increased gradually until contractions were maximal. The optimal resting tension was 1 g. The vessels were allowed to attain the steady level of tension during a 2 h accommodation period before testing. Calcium-free medium was prepared by substituting $CaCl_2$ with 1 mM EGTA. In some experiments the endothelium was removed mechanically by inserting a roughened stainless-steel wire into the lumen and gently rolling the vessel segment on a damp filter paper. This procedure removed more than 90% of the endothelial cells as observed from silver-stained whole-mount sections for light microscopy (Caplan & Schwartz, 1973). Functional integrity of the endothelium was confirmed by the presence of relaxation induced by acetylcholine (10^{-6} M) during contraction obtained with prostaglandin $F_{2\alpha}$ (3×10^{-6} M). The following drugs were used: endothelin-1-human (Scientific Marketing Associates); nicardipine (Ferrer International); acetylcholine chloride (Sigma) and prostaglandin $F_{2\alpha}$(Sigma).

Data are expressed as means ± s.e.mean and were analyzed by Student's *t* test. A probability value less than 0.05 was considered as significant.

Results Cumulative application of endothelin produced a sustained constrictor response which was concentration-dependent (Figure 1a). Maximal tensions developed were of similar size in arteries with and without endothelium: 2589 ± 434 and 2104 ± 323 mg ($P > 0.05$). Geometric mean EC_{50} values (95% confidence interval) were of comparable magnitude ($P > 0.05$) in arteries with and without endothelium 1.1×10^{-9} M (4.5×10^{-10}–2.4×10^{-9} M) and 6.9×10^{-10} M (3.5×10^{-10}–1.3×10^{-9} M). There was no significant difference ($P > 0.05$) in the contraction of arteries with and without endothelium to the addition of KCl 60 mM (2312 ± 151 versus 1972 ± 216 mg). Removal of extracellular calcium or addition of nicardipine (10^{-6} M) to the baths 10 min before the endothelin concentration-response curve was determined, reduced significantly ($P < 0.05$) the maximal response to endothelin to about 20 and 35%, respectively (Figure 1b).

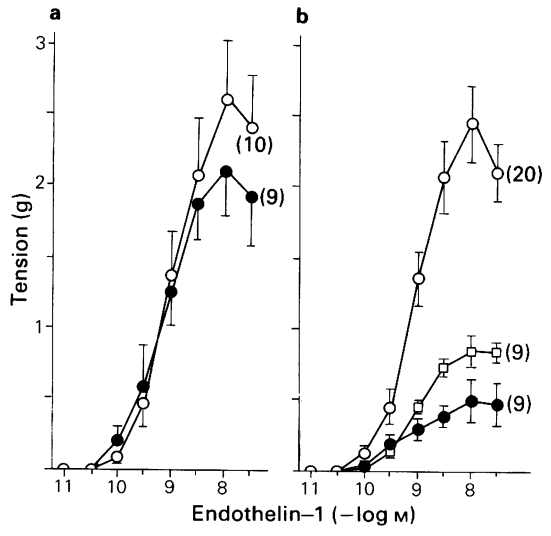

Figure 1 (a) Dose-response curves for endothelin-1 in human cerebral arteries with (○) and without (●) endothelium. (b) Dose-response curves for endothelin-1 in arteries incubated in normal medium (○), in calcium-free EGTA medium (●), and in normal medium containing nicardipine 10^{-6} M (□). Values are means with s.e.mean shown by vertical bars. Number of experiments are indicated within parentheses.

Discussion The present experiments demonstrate that endothelin, a vasoconstrictor peptide derived from endothelial

cells, is a potent agonist for cerebral smooth muscle contraction. The EC_{50} values are similar to those previously obtained for vasopressin (Lluch *et al.*, 1984) and lower than the values for 5-hydroxytryptamine, angiotensin II and noradrenaline (Conde *et al.*, 1981) in the same vascular preparations. The endothelin-induced contraction is not affected by the presence of endothelium indicating that vasodilator agents secreted by endothelial cells under basal, spontaneous conditions (Martin *et al.*, 1986) do not appear to counteract the local effects of endothelin. Therefore, it is likely that the contractile effects of endothelin are due to direct stimulation of vascular smooth muscle. This interpretation is consistent with the endothelium-independent contraction observed in human and rat cerebral arteries (Hardebo *et al.*, 1989). In contrast, in canine and feline cerebral arteries, the endothelium attenuates this contraction (Shigeno *et al.*, 1989; Edvinsson *et al.*, 1989).

The contraction of some vascular preparations induced by endothelin is completely inhibited in a calcium-free medium (Yanagisawa *et al.*, 1988). Furthermore, a previous report indicates that maximal endothelin-induced contractions in human isolated cerebral arteries are reduced to 15% in the presence of nimodipine 10^{-8} M (Hardebo *et al.*, 1989). The present experiments in human cerebral arteries indicate that the contraction produced by endothelin is, at least in part, independent of calcium influx since this peptide induced a slow tonic contraction in a calcium-free medium or after the addition of the calcium antagonist nicardipine 10^{-6} M. These results support previous experiments in rat aortic rings indicating that the contractile response to endothelin has two components, one independent of calcium influx and the other dependent on calcium influx (Auguet *et al.*, 1988). This latter component is only partially inhibited by calcium channel blockers, indicating that the action of endothelin may not be limited to opening of a calcium channel.

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Adverse effects on rat cardiac function *ex vivo* after repeated administration of the benzodiazepine partial inverse agonist, FG7142

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1 The Langendorff preparation was used to investigate functional changes in rat heart one week after the last of a course of repeated injections of the benzodiazepine inverse agonist, FG7142 (20 mg kg^{-1} i.p; three times weekly for five weeks).

2 Under these conditions, FG7142 caused a statistically significant reduction in both cardiac basal tension and the inotropic effect of noradrenaline at doses giving 50 and 100% of the maximum response.

3 Basal heart rate, basal coronary perfusion pressure and the effects of noradrenaline *ex vivo* on these parameters were all unaffected by repeated administration of FG7142.

4 FG7142 had no intrinsic effects on cardiac function when administered *in vitro*.

5 We discuss mechanisms which could underlie the effects of FG7142 on cardiac tension *ex vivo* and consider the possibility that this action may be related to the anxiogenic or proconvulsant actions of this drug.

Introduction

FG7142 (β-carboline-3-carboxylic acid methylamide) is a benzodiazepine partial inverse agonist with well-documented proconvulsant and anxiogenic effects (Dorow *et al.*, 1983; File *et al.*, 1985; but see: Thiebot *et al.*, 1988). When given repeatedly, this drug causes long-lasting changes in seizure threshold, culminating in the expression of kindled seizures (Little *et al.*, 1984). In recent experiments, we found that a single injection of this drug caused a marked increase in the number of β-adrenoceptors in rat cerebral cortex, a change which was attenuated by repeated injection of this drug (Stanford *et al.*, 1989).

There are several reasons for predicting that FG7142 would affect cardiac, as well as CNS, function. First, changes in central β-adrenoceptors, if a widespread effect, would influence the response of peripheral organs to catecholamines. Secondly, receptors for benzodiazepines have been found in the heart. Although the physiological role of these receptors is still unresolved (see, for example: Saano *et al.*, 1989), to our knowledge, the actions of benzodiazepine inverse agonists on these receptors have not been studied. Thirdly, an increased incidence of premature death attributed to cardiovascular disease has been found for patients suffering from anxiety (Coryell *et al.*, 1982; Black *et al.*, 1985); adverse changes in cardiac function might then also follow repeated administration of an anxiogenic drug, such as FG7142. Finally, recent tests of stimulus discrimination suggest that FG7142 acts as a chemical stressor (Leidenheimer & Schechter, 1988); stressful stimuli are know to induce cardiomyopathy in a range of species (Raab *et al.*, 1964; Johansson *et al.*, 1974; Corley *et al.*, 1977).

In the present study, peripheral effects of repeated administration of FG7142 were investigated in rats showing signs of long-lasting effects of drug treatment (expression of kindled seizures). The Langendorff preparation was used to study the effects of FG7142 on cardiac function *ex vivo* and *in vitro*. We demonstrated that while this drug has no acute intrinsic effects on cardiac function, prolonged administration of FG7142 caused a reduction in basal tension and the inotropic response to noradrenaline *ex vivo*. A preliminary account of these results has been presented (Stanford *et al.*, 1988).

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Methods

Animals and drug procedure

Male Sprague-Dawley rats, 150–170 g at the start of the experiment, were given an i.p. injection of FG7142 (20 mg kg^{-1}) three times weekly for five weeks. Controls received vehicle injections only (0.5% Tween 80 in distilled water). We looked for induction of myoclonic jerks and clonic convulsions known to occur after repeated injection of FG7142 ('chemical kindling' Little *et al.*, 1984). FG7142 does not cause these signs in normal animals, but does so after repeated administration, an effect that lasts apparently for the life-time of the animal. Our experimental criterion to ensure that the dose regimen was appropriate for induction of long-term physiological changes (in the central nervous system, at least) was the production of a clonic convulsion on administration of 20 mg kg^{-1} FG7142. All animals were then left, drug and vehicle injection-free for a period of fifteen weeks to ensure that any changes in cardiac function resulting from repeated FG7142 administration represented long-lasting effects of this drug. A final, single injection of either vehicle or FG7142 (20 mg kg^{-1} i.p.) was then administered, as before, to test for persistent drug effects. At this stage, 2 animals failed to reach the experimental criterion for lasting physiological changes after FG7142 administration and were excluded from subsequent aspects of the study.

Effects of FG7142 on rat heart ex vivo

One week after the final injection, animals were killed by a blow to the head and cervical dislocation. Animal weights at the end of the experiment were (g): vehicle-injected, 681 ± 27; FG7142-injected, 708 ± 14. The hearts were rapidly excised and cardiac function was evaluated by use of the Langendorff preparation (Langendorff, 1895): hearts were perfused via the aorta with oxygenated Krebs solution at 37°C. A Watson-Marlow flow inducer was used to maintain the flow rate at 13 ml min^{-1}, a rate somewhat faster than is traditional for perfusion of the rat heart. This was because the animals were considerably older at the end of the experiment, and the hearts consequently larger, than those usually used in this preparation (see, for example: Dai, 1976). Changes in coronary perfusion pressure, due to vascular resistance, were

recorded with a Statham P23 ID pressure transducer. The force generated by isometric contractions of the heart (tension) were measured with a Grass FT.03 force displacement transducer attached to the apex of the left ventricle; basal tension was set at 2.0 g. Changes in heart rate were monitored with a tachograph (grass 7P4D) triggered by an input from the tension transducer. All parameters were recorded on a Grass polygraph.

We recorded basal coronary perfusion pressure, heart rate and tension and the changes in these parameters induced by bolus doses of noradrenaline. This was administered at 20 min intervals by injection into the tubing carrying the perfusion medium, just above the aorta. The following doses of noradrenaline were used (mol): 1×10^{-11}, 5×10^{-11}, 2×10^{-10}, 1×10^{-9}, 2×10^{-9}, 1×10^{-8}, 5×10^{-8}; these separate doses of noradrenaline were administered in a volume of 0.1 ml in increasing order of concentration.

Each experiment examined hearts from a vehicle- and FG7142-injected rat simultaneously. Two such experiments were conducted each day; this yoked design minimised methodological differences between experiments. On each experimental apparatus, control and FG7142-treated hearts were studied alternately, to eliminate any possible systematic differences in equipment sensitivity. Hearts from 7 vehicle-injected and 7 FG7142-injected animals were examined. All experiments and measurements were carried out blind.

Effects of FG7142 on rat heart in vitro

FG7142 was dissolved in 0.05 M HCl; this vehicle had no consistent effects in the Langendorff preparation when administered alone. With the same experimental procedure as that described above, direct effects of FG7142, over the dose range 10^{-12}–10^{-6} mol, were investigated *in vitro* in rat hearts from untreated animals.

Statistics

The data were analysed by reading values from calibrated polygraph traces. Readings were taken both immediately before (basal values) and, at one second intervals, for 5 s after injection of drug (noradrenaline or FG7142). By this time, all responses were past their peak value. The traces were decoded with respect to FG7142 or vehicle treatment *in vivo* only when all data had been analysed. Student's *t* test (unpaired samples) and two way ANOVA (Genstat) were used to assess the significance of any differences.

Drugs and solutions

The ionic composition of the Krebs solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4 \cdot 7H_2O$ 1.2 and glucose 11. The solution was aerated with 95% O_2: 5% CO_2. Noradrenaline bitartrate was obtained from Sigma. FG7142 was a gift from Beecham's Pharmaceutical Division.

Results

Effects of FG7142 on cardiac function ex vivo

Cardiac tension Although individual pairs of data at each concentration of noradrenaline were not significantly different, two-way ANOVA revealed an overall effect of FG7142 on cardiac basal tension: the tension generated by hearts from drug-pretreated animals was significantly less than that of the controls ($P = 0.03$) (Figure 1). There was also a significant reduction in the basal tension generated by all hearts during the course of each experiment ($P < 0.001$), but this was not influenced by drug treatment.

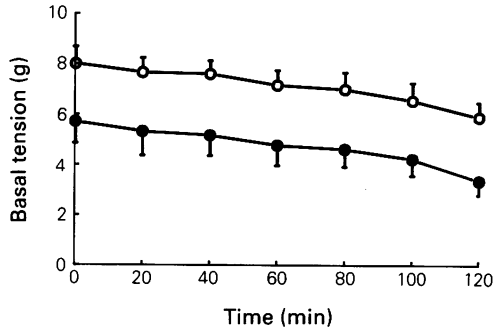

Figure 1 Effect of repeated administration of FG7142 on the basal tension generated by rat hearts. Control (○) and FG7142-pretreated (●) rats. Data show mean with vertical lines indicating s.e.mean. Sample number = 7 for both treatment groups.

The effects of FG7142 on inotropic responses to noradrenaline were assessed by subtraction of the basal tension (measured immediately before injection of noradrenaline) from

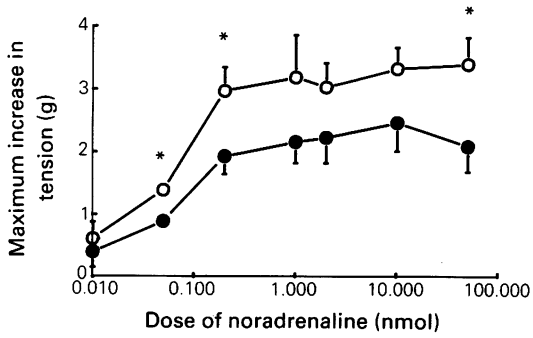

Figure 2 The increase in tension generated by hearts from control (○) and FG7142-pretreated (●) rats in response to noradrenaline. This was calculated by subtraction of basal tension from the peak tension generated on administration of noradrenaline. Data show mean with vertical lines indicating s.e.mean. * $P < 0.05$ (*t* test). Sample number = 7 for both treatment groups.

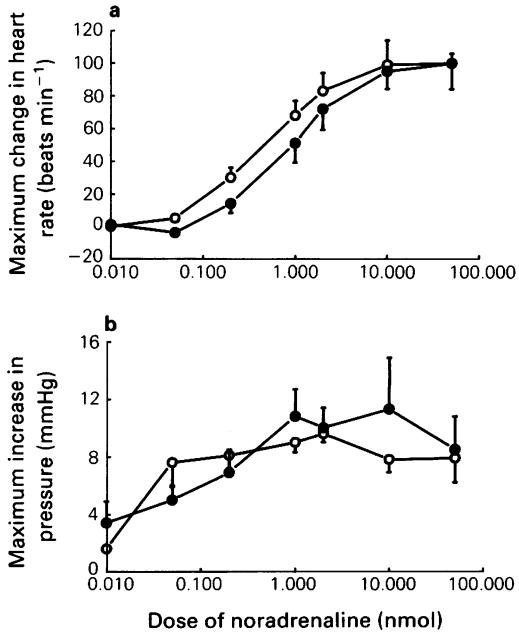

Figure 3 The increase in heart rate (a) and the increase in coronary perfusion pressure (b) induced by noradrenaline in hearts from control (○) and FG7142-pretreated (●) rats. Data show mean with vertical lines indicating s.e.mean. $n = 7$ for both treatment groups.

the maximum tension recorded after administration of noradrenaline. This measurement therefore reflects the change in tension due to noradrenaline. Figure 2 shows that in hearts from FG7142-pretreated animals there was a significant reduction in the change in cardiac tension at doses of noradrenaline giving approximately 50% of the maximal effect (5×10^{-11}–2×10^{-10} mol) and (5×10^{-8} mol) of the maximum effect, itself ($P < 0.05$, t test).

Heart rate Between the start and end of each experiment, basal heart rate ranged from 196 ± 4 to 199 ± 4 beats min^{-1} in control hearts and from 177 ± 10 to 193 ± 10 beats min^{-1} in hearts from FG7142-treated animals. There were no statistically significant time- or treatment-related changes. When the increase in heart rate induced by noradrenaline was calculated by subtraction of the rate immediately before injection of noradrenaline from the value recorded at the maximum chronotropic responses, there were no significant differences in response of hearts from FG7142- and vehicle-injected animals (Figure 3).

Coronary perfusion pressure Between the start and end of each experiment, basal coronary pressure ranged from (mmHg) 16.2 ± 1.0 to 26.6 ± 2.1 in control hearts and from 17.6 ± 2.4 to 25.8 ± 1.9 in hearts from FG7142-pretreated animals; FG7142 had no significant effects on basal coronary perfusion pressure. The increase in pressure caused by administration of noradrenaline was also unaffected by FG7142 (Figure 3).

Actions of FG7142 in vitro

Hearts from previously untreated animals showed no changes in cardiac tension, heart rate or coronary perfusion pressure when FG7142 was administered *in vitro* over the dose range: 1×10^{-12} to 1×10^{-6} mol (data not shown).

Discussion

The main finding from this study was that repeated administration to rats of the benzodiazepine partial inverse agonist, FG1742, caused a significant reduction in both basal tension and the inotropic effect of noradrenaline *ex vivo* at doses giving 50% and 100% of the maximum response. FG7142 did not affect basal heart rate or chronotropic responses to noradrenaline. Coronary perfusion pressure was similarly unaffected by this drug. These results suggest that FG7142 has a selective effect on cardiac contractility. Major questions arising from our findings concern the mechanisms underlying the effects of FG7142 on cardiac tension and whether or not they are related to the psychotropic or proconvulsant actions of this drug.

The effects of FG7142 could be mediated either centrally, or peripherally. Although results of studies are inconsistent, there is evidence that systemic administration of benzodiazepines induces a tachycardia through inhibition of vagal activity mediated by central γ-aminobutyric $acid_A$ ($GABA_A$)-receptors (Conahan & Vogel, 1986; Dimicco, 1987). Repeated administration of drugs like FG7142, which also influence $GABA_A$-receptor function, could therefore lead to adaptational or pathological changes in the heart as a result of actions within the CNS.

It is also possible that centrally-mediated effects of FG7142 on the heart would be linked to the anxiogenic or proconvulsant actions of this drug. FG7142 is anxiogenic in man (Dorow *et al.*, 1983) and modifies stress-related behaviour in animal models of anxiety (File *et al.*, 1985). Recent experiments on stimulus discrimination have shown that FG7142 may be regarded as a 'chemical stressor' (Leidenheimer & Schechter, 1988) and, although this compound does not seem to have any long-lasting effects on behavioural responses to mild stress such as novelty (Taylor *et al.*, 1988; Stanford *et al.*, 1989), it will substitute for the more severe stress of inescapable electroshock as the conditioning stress in development of learned helplessness, an animal model of depression (Drugan *et al.*, 1985).

There are many acounts of adverse effects of stress on the heart. These effects culminate in cardiomyopathy with fuschinophilia, myofibrillar degeneration and EEG changes (Johansson *et al.*, 1974; Corley *et al.*, 1975; 1977). It is well-known that acute stress activates sympathetic neurones innervating the heart and, in view of the cardiotoxic effects of noradrenaline (Ferrans *et al.*, 1969; Moss & Schenk, 1970), an increase in noradrenaline release could contribute to the cardiomyopathy caused by repeated stress. The β-carboline, β-carboline-3-carboxylic acid (β-CCE), a structural analogue of FG7142, has already been shown to cause a marked increase in plasma catecholamines in monkeys (Ninan *et al.*, 1982). The selective effects of FG7142 on cardiac tension could therefore be explained by a myofibrillar disorder associated with a stress-induced increase in noradrenaline.

Increased release of noradrenaline would be expected to attenuate both chronotropic and inotropic responses to noradrenaline through homologous desensitization of β-adrenoceptors. Although the inotropic response to noradrenaline was reduced by FG7142, chronotropic responses were unaffected. In contrast, prolonged infusion of catecholamines affects both chronotropic and inotropic responses to noradrenaline in rats (Chang *et al.*, 1982), while reduced chronotropic responses to noradrenaline in isolated right atria have been obtained after repeated inescapable footshock stress (Bassani & De Moraes, 1987) and acute restraint stress (Capaz & De Moraes, 1988). Our findings that the chronotropic response to noradrenaline was unchanged, while the inotropic response was reduced after repeated administration of FG7142, suggest that the effects of FG7142 differ from those of other forms of stress and that there is regional variation of the effects of this drug on cardiac function. Regional variation in intracardial changes in adrenoceptor binding or function have been noted previously with congestive heart failure in dogs (Fan *et al.*, 1987), after sympathectomy by 6-hydroxydopamine (6-OHDA) in chick atria (Williams *et al.*, 1987) and in rats with phaeochromocytoma (Rosenbaum *et al.*, 1987), but there is as yet no explanation for this variation.

A direct action on the heart is another possible mechanism underlying long-term effects of FG7142. When administered to the isolated, blood perfused atrium of the dog, the benzodiazepine, diazepam, induces a negative followed by a positive inotropic and chronotropic response (Akahane *et al.*, 1987); the negative effect is thought to involve a direct action on the heart. The mechanism underlying this response is unresolved but it may be highly relevant that benzodiazepine receptors have been found in peripheral tissues, including the heart (Le Fur *et al.*, 1983). As yet, there is no general consensus on the function of peripheral benzodiazepine receptors, but it is of particular interest that the density of these receptor sites is significantly reduced in the hearts of Maudsley Reactive rats, a strain specifically inbred for high levels of fearfulness (Drugan *et al.*, 1987). When tested *in vitro*, we found that FG7142 had no acute, intrinsic actions on the heart. Although this could suggest that peripheral benzodiazepine receptors are not involved in the actions of this drug, the possibility that changes develop after prolonged administration of FG7142 cannot be excluded.

Peripheral benzodiazepine receptors, which are also found in the brain, could link adverse effects of FG7142 on the heart to the proconvulsant actions of this compound. Little is known about functional changes in the periphery associated with seizure activity, but in both the CNS and heart a transient, post-ictal increase in the number of peripheral (Bassle *et al.*, 1987) and central benzodiazepine receptors (e.g. Syapin *et al.*, 1981) has been demonstrated. It has been suggested that

changes in peripheral benzodiazepine receptor function after repeated seizures are linked to increased intracellular Ca^{2+} and neuronal damage after repeated seizures (Basile *et al.*, 1987). It is equally possible that a similar process could adversely affect myocardial tissue.

The results of this study show that a drug noted for its anxiogenic and proconvulsant actions has marked effects on basal cardiac tension and inotropic responses to noradrenaline. These changes could be evoked by one or more of several different mechanisms, ranging from effects within the CNS to direct actions on the heart mediated by peripheral benzodiazepine receptors. Further investigation of these mechanisms underlying the effects of benzodiazepine receptor ligands on peripheral tissues could help explain why cardiac dysfunction is frequently associated with stress-related disorders.

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Structure-activity studies on bradykinin and related peptides: agonists

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1 Bradykinin, kallidin, T-kinin, [Hyp^3]-bradykinin and several analogues were prepared by solid-phase synthesis and purified by high performance liquid chromatography.

2 The various peptides were tested for their abilities to relax the dog carotid and renal arteries, or to contract the rabbit jugular vein and aorta, in order to measure their activities on BK_2 (the first three preparations) or BK_1 (the rabbit aorta) receptors. The dog renal artery without endothelium was also used as a BK_1 receptor system.

3 T-kinin was found to be less active than bradykinin, while the replacement of Pro^3 with Hyp favoured BK_2 receptor occupation. [Hyp^3,Tyr(Me)^8]-BK was found to be a selective BK_2 receptor agonist.

4 Amidation or methylation of the C-terminal carboxyl decreased activity, while extension of the N-terminal with Sar or D-Arg increased affinity and selectivity for BK_1 (Sar) and affinity for BK_2 (D-Arg) receptors. Acetylation of N-terminal amide brought affinity down to 10% or less.

5 Replacement of the peptide bonds Phe^8-Arg^9 to protect from kininase I and II, decreased affinities slightly, but was incompatible with additional changes at the N-terminal or in the peptide bond Gly^4-Phe^5.

6 Substitution of C-terminal Phe in desArg^9-BK (the BK_1 receptor stimulant) with D-Phe increased potency and selectivity for BK_1 receptors while protecting from carboxypeptidases. Sar[D-Phe^8]desArg^9-BK was found to be a potent and selective BK_1 receptor agonist.

Introduction

New kinins such as [Hyp^4]-kallidin and T-kinins have recently been discovered (Bedi *et al.*, 1983; Okamoto & Greenbaum, 1983a; Mindroiu *et al.*, 1988), but not yet fully characterized. Moreover, the need of selective kinins that act as pure agonists or antagonists on BK_1 or BK_2 receptors is strongly felt, especially after the reports that there may be new types of kinin receptors (Rifo *et al.*, 1987; Plevin & Owen, 1988) or that kinins may act indirectly through non-receptor mechanisms, such as the release of prostaglandins in the guinea-pig trachea (Rhaleb *et al.*, 1988) or that of histamine from rat peritoneal mast cells (Devillier *et al.*, 1985; 1988).

In the present study, some naturally occurring kinins and a few analogues of bradykinin, kallidin or desArg^9-bradykinin were prepared and tested in various pharmacological preparations in order to identify compounds that act as pure kinin-receptor agonists and are more selective than the naturally occurring kinins or their metabolites.

Methods

Pharmacological preparations

Experiments were performed on tissues taken from rabbits (Albino New-Zealand, 1.0–1.5 kg) and mongrel dogs (9–15 kg) of either sex. The rabbits were killed by stunning and exsanguination, while the dogs were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, intraperitoneally) and killed through section of the carotid arteries. Kinins and their analogues were tested on the rabbit jugular vein, the rabbit aorta, the dog carotid artery and the dog renal artery. In previous studies (Bérubé *et al.*, 1978; Gaudreau *et al.*, 1981; Regoli *et al.*, 1977) it was shown that several rabbit veins and the rabbit aorta respond to kinins with concentration-dependent contractions. On the other hand, the dog carotid artery, while contracted with noradrenaline (2.0×10^{-8} M), responds to kinins with relaxations, provided the endothelium is intact (D'Orléens-Juste *et al.*, 1985), while the dog renal artery responds to kinins with relaxations both when the endothelium is present or absent (Rhaleb *et al.*, 1989). The tissues were rapidly dissected away from the animals and prepared as strips, as described previously (Rhaleb *et al.*, 1989). They were then suspended in 10 ml organ baths containing warm (37°C), oxygenated (with 95% O_2 and 5% CO_2) Krebs solution of the same composition as that described by Rioux *et al.* (1973). The arterial vessels were stretched with an initial tension of 2 g and the isolated veins with 0.5 g. Changes of tension produced by the various agents were measured with Grass isometric transducers (FT 03C), calibrated as to 1 g = 30 mm. Contractions or relaxations were displayed on Grass Polygraph Model 7D. Before testing the drugs, the preparations were allowed to equilibrate for 90–120 min, during which time the tissues were repeatedly washed and tension adjusted.

Experimental protocols

Concentration-response curves were measured by cumulative (in the dog carotid and renal arteries and rabbit aorta) or consecutive (in the rabbit jugular vein) application of peptides, in order to obtain reliable estimations of the apparent affinities (which are expressed in terms of pD_2) and maximum responses. The contractile effects of kinins and analogues were measured on the rabbit jugular vein treated with captopril (1.1×10^{-6} M) in order to avoid the degradation of peptides by kininase II (Regoli *et al.*, 1986). The relaxant effects of kinins on dog carotid and renal arteries were evaluated in the presence of intact endothelium after contraction with noradrenaline (NA). Ethylenediamine-tetraacetic acid (EDTA) (5.9×10^{-5} M) and indomethacin (2.8×10^{-6} M) were added to the solution to prevent the oxidation of the catecholamines and block the synthesis of prostaglandins which may interfere with both the contractile effects of noradrenaline or the relaxing effects of the kinins. Effect of kinins on BK_1 receptors were evaluated on the rabbit aorta and the dog renal artery without endothelium contracted with NA and treated with EDTA as mentioned above. Removal of endothelium was assessed by the absence of response of the vessels to acetylcholine (2.6×10^{-6} M). The contractile effects of kinins on the rabbit aorta were measured after a period of incubation of 5 h,

in order to obtain measurable and stable responses (Regoli et al., 1986).

Peptides and other agents

The following peptides were used: bradykinin (BK), kallidin (KD: Lys-bradykinin), BK amide (BK-NH_2), BK methyl ester (BK-OMe), [Tyr(Me)^8]-BK, [Hyp^3]-BK, [Hyp^4]-KD, Ile-Ser-BK (T-kinin), [Hyp^3,Tyr(Me)^8]-BK, Sar-BK, D-Arg-BK, N-Ac-BK, [Aib^7]-BK, Sar[Aib^7]-BK, D-Arg[Aib^7]-BK, [Phe$^8\Psi$(CH_2-NH)Arg^9]-BK, Sar[Phe$^8\Psi$(CH_2-NH)Arg^9]-BK, [MePhe^5, Phe$^8\Psi$(CH_2 - NH)Arg^9] - BK, [Gly$^4\Psi$(CH_2 - NH) - Phe^5,Phe$^8\Psi$(CH_2-NH)Arg^9]-BK, [Phe$^9\Psi$(CH_2-NH)Arg^{10}]-KD, desArg^9-BK, desArg^{10}-KD, [D-Phe^8]-desArg^9-BK, Sar[D-Phe^8]-desArg^9-BK, Sar[Gly$^4\Psi$(CH_2-NH)Phe^5, D-Phe^8]-desArg^9-BK, [Hyp^3]-desArg^9-BK, [Hyp^4]-desArg^{10}-KD. All these peptides were prepared in our laboratory. The synthesis and purification of the peptides, except the pseudo-peptide analogues (containing the isosteric bond CH_2-NH), were performed with the procedure described by Drapeau & Regoli (1988). The synthesis of the pseudo-peptides was described briefly by Drapeau et al. (1988).

Concentrated solutions (1–5 mg ml^{-1}) of all agents were prepared in distilled water and stored at −20°C until used.

Other drugs used were noradrenaline (Sigma), indomethacin (Sigma) EDTA (Fisher) and ascorbic acid (Baker). Solutions of noradrenaline were prepared in 5% ascorbic acid in order to prevent the oxidation of the amines and solutions of indomethacin were prepared in 0.02 M Trizma base (Sigma).

Results are presented in terms of pD_2, (the apparent affinity of agonists, calculated from at least 8 concentration-response curves) and relative activity, calculated as a percentage of that of bradykinin for the BK_2 receptor system and of that of desArg^9-BK for the BK_1 receptor. pD_2 values evaluated in the rabbit jugular vein and the rabbit aorta were calculated from concentration-response curves in which the maximum effects were measured. pD_2 values measured on the dog carotid and renal artery are derived from concentration-response curves obtained in tissues previously contracted (to approximately 1 g) with noradrenaline and should therefore be considered as approximate.

Results

Pharmacological characterization of naturally occurring kinins and some analogues

The effects of kinins containing a hydroxyproline residue and those of T-kinin (Ile-Ser-BK), are shown in Table 1 in comparison with BK and KD. Replacement of Pro^3 by Hyp in bradykinin brings about an increase of activity in the rabbit jugular vein and the dog renal artery with endothelium (two sensitive BK_2 preparations), without change of activity on the rabbit aorta (the BK_1 receptor system).

Results obtained with [Hyp^4]-kallidin indicate that this peptide is more active than kallidin in the dog carotid and renal arteries and slightly less active than kallidin on the rabbit aorta.

T-kinin was found to be less active than BK in the three BK_2 receptor systems, as well as on the rabbit aorta (Table 1).

In an attempt to find BK_2 receptor agonists more potent and selective than BK, the Pro in position 3 of [Tyr(Me)^8]-BK was replaced with Hyp. As shown by the data of Table 1, [Hyp^3,Tyr(Me)^8]-BK is very potent on the BK_2 receptor systems (dog carotid and renal arteries and rabbit jugular vein) and is completely inactive on the rabbit aorta. This new compound is therefore the most active and selective BK_2 receptor agonist identified until now.

Analogues of bradykinin modified at the N or C-terminals or in position 7 (Table 2)

In a second series of experiments (Table 2), it was found that amidation of the C-terminal carboxyl is accompanied by drastic decrease of activity on the BK_2 (by a factor of 142 to 1000) and on the BK_1 (by a factor of 10) receptors. Methylation of the terminal carboxyl is better tolerated on the BK_2 than on the BK_1 receptor. Activity of BK-OMe is decreased by 69% to 89% on the BK_2 and by 94% on the BK_1 receptor.

Extension of the peptide chain at the N-terminal with Sar (sarcosine) is fairly well tolerated and it is associated with a small increase of affinity on the BK_1 receptor. Extension of the chain by a D-Arg is favourable both on the BK_2 and BK_1 receptor: D-Arg-BK is more active than BK in all three BK_2 receptor systems and is twice as active as BK on the BK_1. Acetylation of the N-terminal amine function in BK brings about a marked (more than 90%) decrease of activity on the BK_2 and BK_1 receptors.

Data from the literature (Vavrek & Stewart, 1985) indicated that the replacement of Pro^7 by aminoisobutyric acid (Aib) in BK increases affinity. This modification was made in three compounds and, as shown by the data of Table 3, it brought significant decreases of activities on the BK_2 and even more on the BK_1 receptor.

Pseudopeptide analogues of bradykinin

In order to prevent the degradation of bradykinin and kallidin at the C-terminal end, the peptide bond between Phe^8 and Arg^9 of BK or Phe^9 and Arg^{10} of KD were replaced by the pseudopeptide bond CH_2-NH. This modification was also made in the analogue Sar-BK in order to prevent the activity of aminopeptidases. An analogue with two pseudopeptide bonds, [Gly$^4\Psi$Phe^5,Phe$^8\Psi$Arg^9]-BK as well as [MePhe^5, Phe$^8\Psi$Arg^9]-BK were also prepared and tested. The purpose of these compounds was to protect the peptide from degradation by endopeptidases that may be active on the Gly^4-Phe^5 bond.

The results summarized, in Table 3, indicate that the two C-terminal pseudopeptide analogues of BK and KD show

Table 1 Activities of kinins, some natural homologues and some analogues on isolated vessels containing BK_1 or BK_2 receptors

	Dog				*Rabbit*			
	Carotid artery		*Renal artery*		*Jugular vein*		*Aorta*	
	pD_2	RA	pD_2	RA	pD_2	RA	pD_2	RA
Bradykinin (BK)	8.64	100	9.85	100	8.48	100	6.22	100
Kallidin (KD)	8.44	63	9.70	71	8.63	141	7.27	1122
[Hyp^3]-BK	8.62	98	10.14	195	8.88	254	6.17	89
[Hyp^4]-KD	8.73	123	10.10	178	8.48	100	7.22	1000
Ile-Ser-BK	7.77	13	8.83	10	8·18	50	5.97	56
[Tyr(Me)^8]-BK	8.64	100	9.89	110	8.59	129	Inact	
[Hyp^3,Tyr(Me)^8]-BK	9.07	270	10.22	234	8.56	123	Inact	

pD_2: The concentration of agonists that produce 50% of the maximum effect.
RA: Relative activity as a % of that of BK. Inact: inactive.

Table 2 Activities of kinins and their analogues on four isolated vessels

	Dog				*Rabbit*			
	Carotid artery		*Renal artery*		*Jugular vein*		*Aorta*	
	pD_2	RA	pD_2	RA	pD_2	RA	pD_2	RA
BK	8.64	100	9.85	100	8.48	100	6.22	100
BK-NH_2	6.49	0.7	6.87	0.1	5.51	0.1	5.22	10
BK-OMe	8.13	31	8.90	11	7.69	16	5.00	6
Sar-BK	8.15	32	9.78	85	8.41	85	6.39	148
D-Arg-BK	8.71	117	10.08	170	8.70	166	6.56	219
N-Ac-BK	7.68	11	8.64	6	7.31	7	5.39	1.3
[Aib^7]-BK	7.60	9	8.23	2	6.28	0.6		<0.1
Sar[Aib^7]-BK	6.96	2	7.47	0.4	6.84	2.3		<0.1
D-Arg[Aib^7]-BK	7.67	10.7	8.41	3.6	6.74	1.8		<0.1

pD_2 and RA same as in Table 1.

variable decrease in activities, between 35 and 70% on BK_2 receptor systems and are practically inactive on the rabbit aorta. Extension of the peptide chain at the N-terminal with a Sar appears to be unfavourable when associated with $Phe^8\Psi Arg^9$, since it is accompanied by a drastic decrease of activity on both BK_2 and BK_1 receptors. The same is true when the pseudopeptide bond is made simultaneously between positions 8–9 and 4–5, as well as when MePhe is used to replace Phe in bradykinin. This last compound is practically inactive on both BK_2 and BK_1 receptors.

Analogues of desArg^9-bradykinin

Some modifications were made in the sequences of $desArg^9$-BK and $desArg^{10}$-KD, which represent the most active and selective BK_1 receptor stimulants. The compounds were tested in five preparations, the same three BK_2 receptor systems used in the previous experiments and two BK_1 receptor systems, namely the rabbit aorta and the dog renal artery without endothelium. The results are summarized in Table 4. The replacement of the Pro residue in position 3 of $desArg^9$-BK and position 4 of $desArg^{10}$-KD with Hyp is associated with a decrease of activity on the rabbit aorta, while the activity on the dog renal artery without endothelium is increased. Indeed, this modification appears to be favourable for BK_2 as well as for the renal BK_1 receptor occupation. The other compounds analyzed in Table 4, were prepared to obtain BK_1 receptor stimulants which may be more stable than the natural compounds. Phe^8 of $desArg^9$-BK was therefore replaced by D-Phe. The analogue is a pure agonist, 3.4 times more active than $desArg^9$-BK on BK_1 receptors and inactive on BK_2 receptors. Affinity for BK_1 receptors is increased further when the peptide is extended at the N-terminal by the addition of a Sar. Indeed, Sar[D-Phe^8]$desArg^9$-BK is 11 times more active than $desArg^9$-BK on BK_1 and is inactive on BK_2 receptors. In order to make the compound even more stable, a pseudopeptide bond was placed between the Gly^4 and Phe^5, but the activity of the compound was found to be markedly diminished.

Table 3 Activities of kinin analogues, containing pseudopeptide ($CH_2 \cdot NH$) bonds, in four isolated vessels

	Dog				*Rabbit*			
	Carotid artery		*Renal artery*		*Jugular vein*		*Aorta*	
	pD_2	RA	pD_2	RA	pD_2	RA	pD_2	RA
BK	8.64	100	9.85	100	8.48	100	6.22	100
Ψ^{8-9}-BK	8.11	30	8.96	13	8.29	65	Inact	
Ψ^{9-10}-KD	8.29	45	9.51	46	8.23	56		<0.1
SarΨ^{8-9}-BK	7.51	7	9.09	17	7.56	12	Inact	
[$MePhe^5$]-Ψ^{8-9}-BK	Inact		6.30	0.03	Inact		Inact	
Ψ^{4-5},Ψ^{8-9}-BK	7.04	2.5	7.59	1	5.03	0.3		<0.1

pD_2 and RA same as in Table 1.
Ψ: pseudopeptide bond CH_2-NH.

Table 4 Activities of $desArg^9$-BK and some analogues on five preparations, two of which, the dog renal artery without endothelium (– Endo) and the rabbit aorta, are sensitive BK_1 receptor systems

	Dog carotid artery		*Dog renal artery*		*Rabbit jugular vein*		*Dog renal artery (– Endo)*		*Rabbit aorta*	
	pD_2	RA	pD_2	RA	pD_2	RA	pD_2	RA	pD_2	RA
$desArg^9$-BK	Inact		Inact			<0.1	8.60	100	7.29	100
$desArg^{10}$-KD	Inact		Inact			<0.1	8.60	100	8.60	2042
BK	8.64	100	9.85	100	8.48	100	8.25	45	6.22	9
[Hyp^3]$desArg^9$-BK	5.85	0.2		<0.1	5.37	0.08	9.11	324	6.52	17
[Hyp^4]$desArg^{10}$-KD	5.49	0.07		<0.1	—	<0.01	8.75	141	8.06	589
[D-Phe^8]$desArg^9$-BK	5.74	0.01		<0.1		<0.1	8.97	234	7.82	339
Sar[D-Phe^8]$desArg^9$-BK		<0.1		<0.1		<0.1	9.04	275	8.33	1096
Sar[Ψ^{4-5},D-Phe^8]$desArg^9$-BK		<0.1		<0.1		<0.1	8.93	216	6.71	26

pD_2 and RA same as in Table 1. Relative activity is expressed as % of that of BK in the dog carotid and renal arteries and rabbit jugular vein and as % of that of $desArg^9$BK in the dog renal artery without endothelium (– Endo) and rabbit aorta.

Discussion

With a view to improving agonist selectivity and potency, various modifications were made in the sequences of bradykinin, kallidin and the corresponding C-terminal desArg-peptides. Firstly, we evaluated the effect of replacing the second Pro residue from the N-terminals (Pro^3 of BK and Pro^4 of KD) with Hyp. This modification is favourable for BK_2 receptors and rather unfavourable for the BK_1. We therefore pushed this concept further and obtained the most potent and pure BK_2 receptor stimulant, namely [Hyp^3, $Tyr(Me)^8$]-BK which is 2–3 times as active as BK on the dog arteries and is completely inactive on BK_1 receptor systems. T-kinin was also evaluated and found to be less active than kinins, in accordance with previous findings by Okamoto & Greenbaum (1983b) and by Sugio & Greenbaum (1988).

Secondly, we replaced the C-terminal peptide bond Phe^8-Arg^9 with a pseudo peptide and a selective BK_2 receptor agonist, almost as active as BK on BK_2 but inactive on BK_1 receptors was obtained (Drapeau *et al.*, 1988). The pseudopeptide analogue of kallidin showed similar activity and no advantage with respect to the BK analogue. Extension of the chain from the N-terminal with Sar or modification of the peptide bond between Gly^4 or Phe^5 (either by a pseudopeptide or by MePhe), were accompanied by a great decrease of activity. It appears that the pseudopeptide modification cannot be easily combined with other changes, both at the N-terminal and or within the chain.

As to BK_1 receptors, the unfavourable effect of Hyp as a substitute for Pro with respect to BK_1 receptor activation was confirmed by the results obtained with [Hyp^3]-$desArg^9$-BK and [Hyp^4]-$desArg^{10}$-KD. The replacement of the C-terminal Phe by D-Phe, which should protect the peptide from degradation by carboxypeptidase, was found to be very good: the peptide ([D-Phe^8]-$desArg^9$-BK) showed a markedly increased affinity. Chain extension at the N-terminal by a Sar was tolerated since Sar[D-Phe^8]-$desArg^9$-BK was found to be 11 times more active than $desArg^9$-BK. Although this new compound is only half as active as $desArg^{10}$-KD, the presence of a Sar instead of a lysine at the N-terminal and that of D-Phe at the C-terminal should confer resistance against both carboxy and aminopeptidases; moreover, the new compound should be much less active as histamine releaser than $desArg^{10}$-KD (Devillier *et al.*, 1985). Sar[D-Phe^8]$desArg^9$-BK appears to be a potent BK_1 selective agonist without histamine releasing activity and somewhat more stable than $desArg^9$-BK.

An attempt to make the compound resistant to some endopeptidases by using a pseudopeptide bond between positions 4 and 5 was unsuccessful, since Sar[$Gly^4\Psi Phe^5$,D-Phe^8]-$desArg^9$-BK is very weak on BK_1 receptors, its relative affinity being only approximately 1/50 of that of Sar[D-Phe^8]-BK.

In regard to the three compounds containing AiB instead of Pro, we could not confirm the results of Vavrek & Stewart (1985), who showed that [AiB^7]BK is 4 times more active than BK on the guinea-pig ileum. In the three BK_2 receptor systems used in the present study, [AiB^7]BK and its homologues were found to be weak agonists. A few experiments performed on guinea-pig ileum longitudinal strip treated with atropine (Rhaleb *et al.*, unpublished) indicated that the AiB containing compounds (Table 2) were found to be as active as BK. Further studies are needed to explain this discrepancy.

Naturally occurring kinins appear to be nonselective since they act on BK_2 and BK_1 receptors. Selective BK_2 receptor stimulants have been obtained either by protecting the peptide from degradation by kininase I and II or by changes in positions 3 and 8, as in [Hyp^3,$Tyr(Me)^8$]-BK. Affinity and selectivity for BK_1 receptors were improved by replacing the C-terminal Phe of $desArg^9$-BK with a D-Phe, or by extending the peptide chain at the N-terminal with Sar. These modifications should increase the resistance of the peptides to degradation. Thus, selective agonists for BK_2 and BK_1 receptors, identified in the present experiments, should be useful for future studies on kinin receptors.

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Vasodilatation and inhibition of mediator release represent two distinct mechanisms for prostaglandin modulation of acute mast cell-dependent inflammation

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1 Intravital microscopy of the hamster cheek pouch was used to examine the influence of vasodilator prostanoids (prostaglandin E_2 (PGE_2), PGI_2), forskolin, and nitroprusside on the microvascular changes during acute inflammation induced by antigen or histamine. The results extend our previous finding that PGE_2 modulates allergic inflammation and histamine release in the cheek pouch model.

2 The microvascular actions of arachidonic acid and different cyclo-oxygenase products (PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$, and the thromboxane A_2 (TXA_2)-analogue U-44069) were first compared with respect to their effects on arteriolar tone. Of the prostaglandins, only PGE_2 and PGI_2 were potent vasodilators and markedly increased local blood flow. Nitroprusside and forskolin also caused vasodilatation and increased blood flow, but were somewhat less potent than PGE_2 and PGI_2.

3 Topically applied PGE_2 and PGI_2 in vasodilator concentrations suppressed the antigen-induced plasma leakage. On the other hand, although the antigen response was predominantly mediated by histamine, both prostaglandins enhanced the plasma leakage evoked by exogeneous histamine.

4 In contrast, the vasodilator nitroprusside, in a dose causing an increase in blood flow equal to that of PGE_2 and PGI_2, potentiated both the histamine-induced plasma leakage, as well as the plasma and leukocyte extravasation after antigen challenge, indicating that the anti-inflammatory actions of the prostaglandins were unrelated to their vasodilator properties *per se*.

5 Because forskolin, a specific activator of adenylate cyclase, mimicked the actions of PGE_2 and PGI_2, i.e. inhibition of the antigen-induced plasma extravasation and enhancement of the histamine response, it is possible that the observed antiallergic effects of the prostaglandins were related to accumulation of intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP).

6 Taken together, there appears to be a competition between pro- and anti-inflammatory effects of PGE_2 and PGI_2 in reactions involving release of endogeneous inflammatory mediators *in vivo*, i.e. enhancement of inflammatory mediator target action on one hand ('two mediator synergism'), and suppression of mediator release on the other. Moreover, the observations indicate that vasodilatation and inhibition of mediator release are two distinct actions of PGE_2 and PGI_2.

Introduction

Since the observation that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin formation (Vane, 1971; Smith & Willis, 1971; Ferreira *et al.*, 1971), considerable interest has focused on the involvement of prostaglandins and related compounds in inflammation. Prostaglandins of the E series (PGE_2, PGE_1) and prostacyclin (PGI_2) have generally been regarded as proinflammatory mediators because they are potent vasodilators and act synergistically with other inflammatory mediators (see Williams, 1983). However, there are also instances when these prostaglandins display actions which must be regarded as anti-inflammatory. For example, *in vitro* PGEs and PGI_2 may inhibit evoked release of inflammatory mediators from granulocytes (Lichtenstein & Bourne, 1971; Weissmann *et al.*, 1980; Camussi *et al.*, 1981; Ham *et al.*, 1983), mast cells (Loeffler *et al.*, 1971; Kaliner & Austen, 1974; Peachell *et al.*, 1988) and lung tissue (Walker, 1973; Hitchcock, 1978; Engineer *et al.*, 1978), with adenosine 3′:5′-cyclic monophosphate (cyclic AMP) suggested to act as the second messenger.

We have previously shown that intravital microscopy of the hamster cheek pouch can be used for studies of microvascular dynamics and *in vivo* histamine release during antigen-induced mast cell-dependent inflammation (Raud *et al.*, 1988; 1989a,b). In this model, topical administration of PGE_2 in vasodilator concentrations has been found to reduce significantly both histamine release and plasma extravasation evoked by antigen challenge (Raud *et al.*, 1988). On the other hand, the NSAIDs indomethacin and diclofenac enhance histamine release, plasma leakage, and leukocyte accumulation evoked by challenge with antigen or the mast cell secretagogue compound 48/80 in the check pouch (Raud *et al.*, 1988; 1989c). Moreover, PGE_2 effectively reverses the NSAID-induced potentiation of histamine release and inflammation (Raud *et al.*, 1988; 1989c). These observations support the view that endogenous vasodilator prostanoids may function as local regulators of mediator secretion in the hamster cheek pouch, and possibly also in other experimental models where NSAIDs have been shown to enhance inflammation (Higgs *et al.*, 1980; Hedqvist *et al.*, 1984; Lundberg & Gerdin, 1984). Nevertheless, it is evident that PGE_2 also exerts proinflammatory actions in the hamster cheek pouch, because plasma extravasation induced by challenge with exogenous inflammatory mediators is increased in the presence of topically applied PGE_2 in vasodilator concentrations. This potentiating effect of PGE_2 includes the plasma leakage from postcapillary venules produced by directly acting mediators such as histamine and leukotriene C_4, (LTC_4), as well as leukocyte-dependent plasma extravasation in response to LTB_4 (Raud *et al.*, 1988). Taken together, there appears to be a competition between pro- and anti-inflammatory effects of PGE_2 in reactions involving release of endogenous inflammatory mediators *in vivo*, i.e. enhancement of mediator target action on one hand, and suppression of mediator release on the other.

Prompted by the observations described above, the present study addresses two major questions: (1) Is the inhibition of the antigen response previously documented for PGE_2 a consequence of the vasodilatation *per se* or is it related to other factors such as cyclic AMP elevation? (2) Are there vasodilator cyclo-oxygenase products other than PGE_2 which may contribute to inhibition of antigen-induced inflammation in

the cheek pouch? Therefore, the microvascular actions in this particular model of the primary cyclo-oxygenase products PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2 (TXA_2, e.g. its analogue U-44069) were first compared with respect to their effects on arteriolar tone, and, as expected, only PGI_2 shared the vasodilator action of PGE_2. Thereafter, the relationship between vasodilatation (and increased blood flow) and effects on histamine or antigen-induced responses was investigated by comparing the influence of PGE_2 and PGI_2 with that of two structurally and pharmacodynamically different vasodilators, namely the cyclic AMP elevating agent forskolin (Seamon *et al.*, 1981) and nitroprusside which triggers cyclic GMP production (Rapoport & Murad, 1983).

The present observations support the view that vasodilator prostaglandins (i.e. PGE_2 and PGI_2) can inhibit mast cell-dependent inflammation by an action that is unrelated to vasodilatation and increased blood flow, but rather involves local suppression of mediator secretion, as previously documented for PGE_2 (Raud *et al.*, 1988). In addition, it is possible that the anti-inflammatory actions of these prostaglandins *in vivo* occur via induction of intracellular cyclic AMP accumulation.

Methods

Animals and immunization

Male Syrian hamsters (80–100 g) were immunized by i.p. injections of 10 μg ovalbumin in 0.2 ml saline, containing 10 mg $Al(OH)_3$. Four weeks later the animals were reinjected with 1 μg ovalbumin in 10 mg $Al(OH)_3$ i.p., and the experiments were done 7–10 days after the second injection.

Cheek pouch preparation and intravital microscopy

As described in detail elsewhere (Björk *et al.*, 1984), the left cheek pouch of anaesthetized (sodium pentobarbitone 6 mg $100\,g^{-1}$ body weight, i.p.) hamsters was everted and prepared for intravital microscopy under continuous superfusion ($7\,ml\,min^{-1}$) with a bicarbonate buffer maintaining physiological temperature, pH and gas tensions.

The exposed microvascular network in the cheek pouch was observed with a Leitz Orthoplan microscope equipped with × 10 oculars, a × 4 long-distance lens (for plasma extravasation), and × 25 or × 55 water immersion lenses (for vessel diameters and leukocyte emigration). The preparation was transilluminated with a halogen 12 V 100 W lamp or a mercury short arc 100 W lamp with proper filters for ordinary light and fluorescent light observations, respectively.

Plasma extravasation was quantified by use of FITC-dextran (25 mg $100\,g^{-1}$ of body weight was given i.v. as a $50\,mg\,ml^{-1}$ saline solution) as a tracer for plasma proteins and by counting the number of fluorescent leakage sites per cm^2 of cheek pouch area (Svensjö *et al.*, 1978). Total plasma leakage over a given time period was estimated by determining the area under the curve for the FITC-dextran leakage sites.

Vessel diameters and leukocyte actions were visualized on a TV monitor via a videocamera (Panasonic Wv-1550) and stored on videotape with a SONY VO-5630 videocasette recorder for subsequent measurement. Arteriolar diameters and blood flow were measured in vascular segments with an inner diameter of 13 ± 2.1 μm. Arteriolar erythrocyte velocity was measured on-line in single arterioles according to the dual-slit cross-correlation technique (Wayland & Johnson, 1967), and volume blood flow was calculated from the velocity data and the simultaneous values for arteriolar diameter measured off-line from the video recordings. In the experiments with antigen (ovalbumin) or LTB_4 challenge, leukocyte emigration around venules with an inner diameter of 30 ± 4.1 μm was quantitated from video recordings. The number of extravascular leukocytes was counted before and 40 min after challenge within a 190 × 140 μm frame around 7 venular segments (collectively designated 1 unit area).

Experimental design

After completion of the cheek pouch preparation, FITC-dextran was injected, and the responsiveness of the preparation to acetylcholine was assessed according to Duling (1973). The arterioles used for examination of changes in diameter and blood flow responded to acetylcholine (1 μM topically for 30 s) with prompt dilatation which lasted less than 2 min. No increase in microvascular permeability or signs of leukocyte activation were observed after acetylcholine. Ten min later the preparation was challenged topically (drugs added to superfusate to reach indicated concentrations around the cheek pouch) with ovalbumin, histamine, or LTB_4, either alone or in the presence of a given vasodilator (durations and concentrations given in text). Mepyramine was given topically throughout the experiments, starting 15 min before challenge. Substances dissolved in ethanol were diluted in the buffer solution to give a final concentration of ethanol below 0.05%, which in itself has no effect on the microvascular parameters studied.

Drugs and chemicals

Leukotriene B_4 (LTB_4) was kindly provided by Dr J. Rokach (Merck-Frosst, Pointe Claire, PQ, Canada), and PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ and U-44069 by Dr J. Pike (Upjohn, Kalamazoo, MI, U.S.A.). Arachidonic acid was from Nu-Chek Prep (Elysian, MN, U.S.A.). Stock solutions of arachidonic acid, LTB_4, PGE_2, PGD_2, $PGF_{2\alpha}$ and U-44069 were stored at −20°C in ethanol, and PGI_2 was made freshly for each experiment in a 10^{-2} M bicarbonate solution.

Acetylcholine chloride, fluorescein isothiocyanate-conjugated dextran (FITC-dextran, M_r 150,000), histamine dihydrochloride, pyrilamine maleate (mepyramine), and ovalbumin (grade III) were purchased from Sigma. Forskolin (stock solution in ethanol) was from Hoechst (Sweden), nitroprusside from Roche (Basel, Switzerland), and aluminium hydroxide ($Al(OH)_3$, dried gel, F2200) from Reheis (Kankakee, IL, U.S.A.).

Statistical analysis

Statistical evaluations were performed using the Mann-Whitney U-test. Data are expressed as mean values ± s.d.

Results

Effects of prostaglandins, forskolin, and nitroprusside on arteriolar tone and blood flow

Arachidonic acid, PGE_2 and PGI_2 caused dose-dependent arteriolar dilatation when added topically to the cheek pouch, whereas PGD_2 and $PGF_{2\alpha}$ failed to affect vessel diameters in concentrations up to 1 μM (Figure 1). The TXA_2 analogue U-44069 (10–100 nM), on the other hand, evoked arteriolar constriction as well as some platelet aggregation (not shown). The threshold concentrations for PGE_2 and PGI_2 were between 1 and 3 nM, and at 30 nM they caused ≈ 110% increase in arteriolar diameter and enhanced blood flow by 558% and 476%, respectively (Figure 2).

Forskolin and nitroprusside also caused dose-dependent arteriolar dilatation (Figure 1), and at a dose of 300 nM the arteriolar dilatation and increased blood flow induced by these two agents were of a magnitude similar to that caused by 30 nM PGE_2 or PGI_2 (Figure 1 and 2).

Microvascular responses to antigen challenge

As previously described (Raud *et al.*, 1988; 1989a), challenge of the hamster cheek pouch with antigen (ovalbumin,

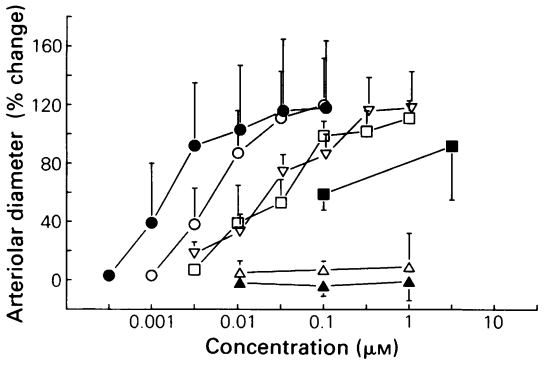

Figure 1 Changes of arteriolar diameters after topical challenge with prostaglandin E_2 (PGE_2, ○), PGI_2 (●), forskolin (▽), nitroprusside (□), PGD_2 (△), $PGF_{2\alpha}$ (▲), and arachidonic acid (■) in hamster cheek pouches. Data obtained by cumulatively increasing the dose of the vasodilators at 5 min intervals. The maximal change in vessel diameters relative to the control period was determined at the end of each 5 min challenge. Mean values with s.d. shown by vertical bars, *n* (number of animals) = 3–6 (one vessel per animal).

$10\,\mu g\,ml^{-1}$ topically for 5 min) evoked a brief vasospasm, followed by extensive leakage of plasma (i.e. FITC-dextran extravasation) (Figure 3a) and arteriolar dilatation (Figure 3b). Moreover, beginning 5–10 min after the start of challenge, there was a marked and long-lasting increase in the number of endothelium-adherent leukocytes, while few leukocytes emigrated to the extravascular interstitium during the 45 min observation time.

Effects of PGE_2 PGI_2, forskolin and nitroprusside on antigen-induced microvascular responses

When the 5 min antigen challenge was performed in the presence of PGE_2, PGI_2 or forskolin in equiactive vasodilator concentrations (topically for 15 min, starting 5 min before antigen), the plasma leakage responses were significantly reduced (Figure 3a and Figure 4). In these concentrations (30 nM of PGE_2 and PGI_2, and 300 nM of forskolin) the vasodilators *per se* caused virtually no increase in plasma extravasation (Figure 3a) and they appeared to be without effect on basal leukocyte-endothelium interactions.

The inhibitory activities of PGE_2, PGI_2 and forskolin on the antigen reaction seemed unrelated to vasodilatation, because nitroprusside (300 nM), which had very similar effects on arteriolar diameter, blood flow and basal microvascular parameters, enhanced the plasma leakage evoked by antigen (Figures 3 and 4). This despite the duration of nitroprusside-induced vasodilatation being shorter than that of PGE_2 or forskolin ($P < 0.05$ at 12.5 and 17.5 min) (Figure 3b).

Furthermore, while nitroprusside also strikingly potentiated the antigen-induced increase in leukocyte emigration, PGE_2, PGI_2, and forskolin were inactive in this respect (Figure 5).

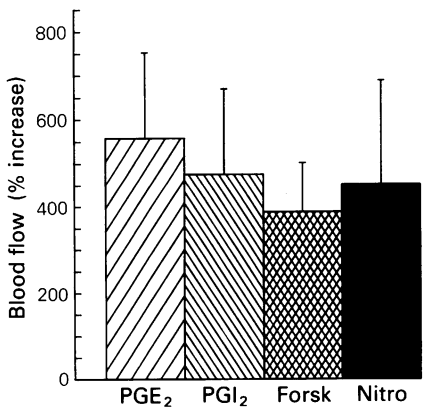

Figure 2 Effects of prostaglandin E_2 (PGE_2, 30 nM, $n = 6$), PGI_2 (30 nM, $n = 4$), forskolin (Forsk, 300 nM, $n = 4$), and nitroprusside (Nitro, 300 nM, $n = 4$) on arteriolar blood flow in hamster cheek pouches. Mean values with s.d. shown by vertical bars; *n* refers to number of animals (one vessel per animal).

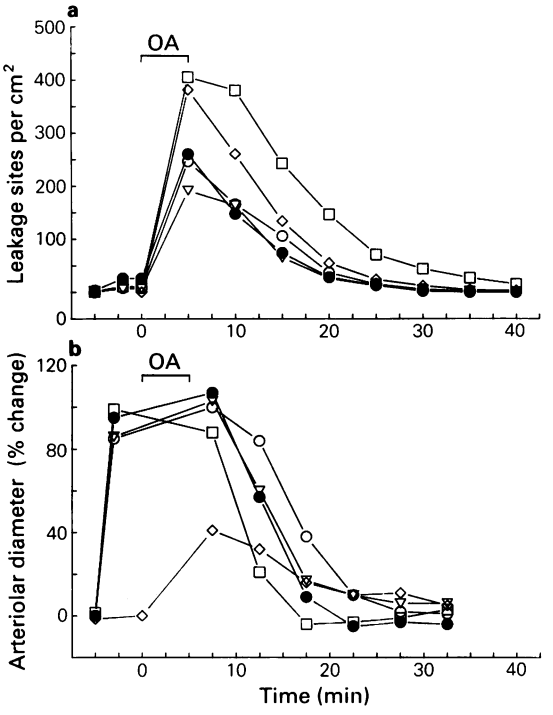

Figure 3 (a) Time course for plasma leakage (FITC-dextran leakage sites) in hamster cheek pouch after topical challenge with antigen (ovalbumin, OA) alone (control, $10\,\mu g\,ml^{-1}$ for 5 min, $n = 10$, ◇), and in the presence of prostaglandin E_2 (PGE_2, 30 nM, $n = 5$, ○), PGI_2 (30 nM, $n = 5$, ●), forskolin (300 nM, $n = 5$, ▽) or nitroprusside (300 nM, $n = 5$, □). (b) Changes in arteriolar diameters after antigen challenge (same experiments as in (a)). Vasodilators were given topically for 15 min, starting 5 min before antigen challenge. Mean values (error bars omitted for clarity); *n* refers to number of animals.

Effects of PGE_2, PGI_2, forskolin, and nitroprusside on histamine-induced microvascular responses

The antigen-induced plasma leakage was predominantly mediated by histamine, as indicated by experiments where

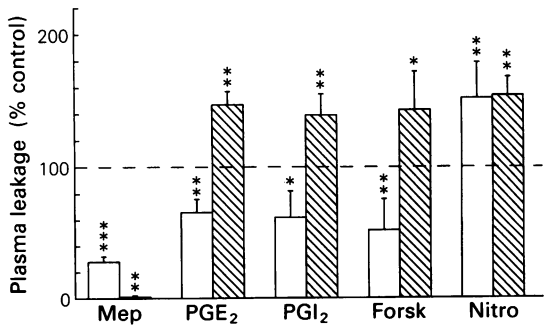

Figure 4 Total (0–40 min) leakage of plasma in hamster cheek pouch after topical challenge with antigen ($10\,\mu g\,ml^{-1}$, open columns) or histamine (10 μM, hatched columns) for 5 min. Effects (in % of control, dashed line, $n = 8$–10) of topically applied mepyramine (Mep, 1 μM, $n = 4$), prostaglandin E_2 (PGE_2, 30 nM, $n = 5$), PGI_2 (30 nM, $n = 5$), forskolin (Forsk, 300 nM, $n = 5$), and nitroprusside (Nitro, 300 nM, $n = 4$–5). Data with antigen ± vasodilators same as in Figure 3a. Mean values with s.d. shown by vertical bar * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ vs. control. *n* refers to number of animals.

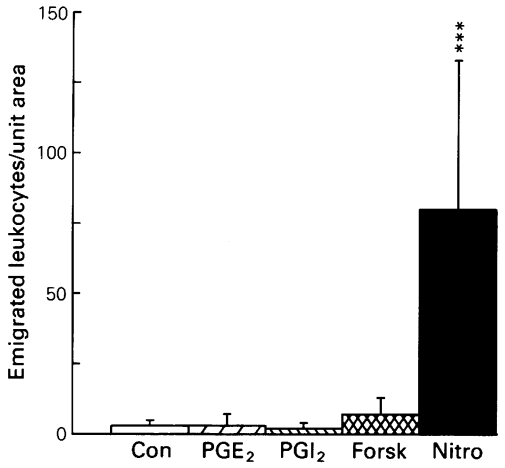

Figure 5 Increase in number of emigrated leukocytes in hamster cheek pouch 40 min after topical challenge with antigen alone (Con, 10 $\mu g ml^{-1}$ for 5 min, $n = 10$), and in the presence of prostaglandin E_2 (PGE_2, 30 nM, $n = 5$), PGI_2 (30 nM, $n = 5$), forskolin (Forsk, 300 nM, $n = 5$) or nitroprusside (Nitro, 300 nM, $n = 5$). Mean values with s.d. shown by vertical bars. *** = $P < 0.001$ vs. control. n refers to number of animals.

sensitized animals were pretreated with the H_1-receptor antagonist mepyramine (1 μM topically) (Figure 4). Therefore, it was of interest to examine whether PGE_2, PGI_2 and forskolin also could suppress histamine-induced plasma leakage. However, in contrast to the antigen response, these vasodilators enhanced the plasma leakage evoked by exogeneous histamine, and the same applied to nitroprusside (Figure 4). It should be noted in this context that the total (0–40 min) leakage of plasma after the dose of histamine used here (10 μM) was of similar magnitude to that induced by antigen (cf. Raud *et al.*, 1989a).

Effects of PGI_2 on LTB_4-induced microvascular responses

Recently, we showed that PGE_2 (30 nM topically) potentiates the leukocyte emigration and subsequent plasma leakage induced by the chemoattractant LTB_4 (Raud *et al.*, 1988). In the present study it was found that PGI_2 has a similar amplifying effect on LTB_4-induced inflammation in the cheek pouch. Thus, the total (0–40 min) plasma leakage response to LTB_4 (300 nM) in the presence of PGI_2 (30 nM, $n = 3$) was $192 \pm 61\%$ greater ($P < 0.05$) than that after LTB_4 alone ($n = 5$). In addition, the leukocyte emigration increased from 45 ± 37 emigrated leukocytes/unit area after LTB_4 alone (300 nM, $n = 5$), to 237 ± 112 in the presence of PGI_2 (30 nM, $n = 3$, $P < 0.05$).

Discussion

The present results confirm our previous observation that antigen-induced plasma extravasation in the hamster cheek pouch is suppressed by locally applied PGE_2 in a vasodilator concentration (Raud *et al.*, 1988). Because the antigen reaction is potentiated by pretreatment with indomethacin and appears to involve release of vasodilator prostaglandins (Raud *et al.*, 1988), it was of interest to examine if other vasodilator cyclo-oxygenase products exerted anti-inflammatory effects similar to those of PGE_2.

The initial characterization of the microvascular responses evoked by the major endogenous cyclo-oxygenase products revealed that only PGI_2 mimicked the vasodilator actions of PGE_2 in the cheek pouch, whereas PGD_2 or $PGF_{2\alpha}$ were inactive in this respect, and the TXA_2-analogue U-44069 caused vasoconstriction. Therefore, antigen challenge was performed in the presence of PGI_2 in a dose selected to cause vasodilatation equal to PGE_2. The results showed that PGI_2 and PGE_2 both reduced the antigen-induced plasma extravasation and to approximately the same extent.

Because PGE_2 or PGI_2 caused a close to maximal arteriolar dilatation and greatly increased local blood flow, it could be speculated that changes in microvascular haemodynamics were involved in the suppression of the antigen-induced plasma extravasation. However, when local blood flow in the cheek pouch was increased to the same degree with the potent vasodilator nitroprusside, the antigen-induced plasma leakage was instead potentiated. Moreover, PGE_2, PGI_2, and nitroprusside uniformly potentiated the plasma leakage evoked by challenge with exogenous histamine. The latter observation confirms our previous findings with PGE_2 (Raud *et al.*, 1988) and is in harmony with data reported for PGE_2 and PGI_2 by others (Williams & Morley, 1973; Komoriya *et al.*, 1978; Morley *et al.*, 1981, Rampart & Williams, 1986). Because histamine was a major mediator of the plasma extravasation evoked by antigen challenge (see Figure 4), it may be concluded that PGE_2 and PGI_2 did not inhibit the antigen response at the target site for histamine, i.e. the venules where the plasma extravasation occurs. Rather, the findings indicate that PGE_2 and PGI_2 inhibited the antigen response by suppression of histamine release. This is further supported by a previous study showing that PGE_2 indeed inhibits antigen-induced *in vivo* release of histamine in this particular model (Raud *et al.*, 1988). In addition, as indicated in the introduction, there are several *in vitro* studies documenting such a mode of action of PGE_2 and PGI_2. In this context, it is worth considering that the prostaglandins probably also suppressed antigen-induced release of chemotactic mediators. This interpretation is based on the following findings: (1) Prostaglandin E_2 and PGI_2 did not enhance the antigen-induced leukocyte activation, although they strikingly enhanced leukocyte emigration and subsequent plasma leakage evoked by LTB_4 (Raud *et al.*, 1988; this paper). (2) We have previously shown in the hamster cheek pouch that PGE_2 effectively inhibits the enhanced antigen-induced leukocyte emigration after pretreatment with indomethacin (Raud *et al.*, 1988). Because the potentiated leukocyte emigration is inhibited by 5-lipoxygenase inhibitors (Raud, 1989), it seems likely that this prostaglandin-sensitive response is mediated by LTB_4, or some other chemotactic 5-lipoxygenase product. (3) The leukocyte emigration after antigen challenge was markedly enhanced in the presence of nitroprusside-induced vasodilatation. Moreover, *in vitro* studies have shown that PGE_2 and PGI_2 can inhibit the release of chemotactic mediators from activated leukocytes (Camussi *et al.*, 1981; Ham *et al.*, 1983) and macrophages (Elliott *et al.*, 1989).

While the proinflammatory effects of vasodilator prostaglandins (i.e. PGEs and PGI_2) *in vivo* are generally attributed to increased microvascular blood flow and hydrostatic pressure (see Williams, 1983), it is suggested that cyclic AMP may act as the second messenger for the inhibition of inflammatory mediator release by these prostaglandins *in vitro* (Lichtenstein & Bourne, 1971; Kaliner & Austen, 1974; Weissmann *et al.*, 1980; Ham *et al.*, 1983; Peachell *et al.*, 1988). To gain some information as to whether the apparent link between cyclic AMP and suppression of mediator release by prostaglandins also exists *in vivo*, we added histamine or antigen to the cheek pouch in the presence of the diterpene derivative forskolin which is known to stimulate cyclic AMP production by a selective action on adenylate cyclase (Seamon *et al.*, 1981; Green & Clark, 1982). It was found that forskolin, in a dose causing equal increase in blood flow to PGE_2 and PGI_2, imitated the actions of the prostaglandins, i.e. the effect of antigen was inhibited while the histamine response was enhanced. Supported by previous observations that forskolin potentiates histamine-induced oedema formation (Sugio & Daly, 1983), and *in vitro* data that forskolin can inhibit anaphylactic release of histamine and leukotrienes from mast cells (Marone *et al.*, 1987; Peachell *et al.*, 1988), our results suggest that for-

skolin suppressed the antigen response by inhibiting mediator secretion. Although circumstantial, the forskolin data also indicate that the anti-inflammatory action of PGE_2 and PGI_2 may have been a consequence of cyclic AMP elevation. This notion gains further support by the failure of nitroprusside to suppress the antigen reaction, because nitroprusside stimulates the production of cyclic GMP rather than cyclic AMP (Rapoport & Murad, 1983).

Although most studies showing oedema potentiating effects of vasodilator prostaglandins *in vivo* have focused on microvascular target effects of exogenously applied directly acting inflammatory mediators, Williams & Morley (1973) and Williams *et al.* (1986) showed that locally applied PGE_1 also may enhance immunologically induced reactions in rabbit skin, dependent on endogenous inflammatory mediator release. This may seem in contrast to our findings; however, it could also illustrate that suppression of inflammatory mediator release by prostaglandins can be camouflaged by their proinflammatory vasodilator activities. It is possible that this interpretation is further reflected in a report by Morley *et al.* (1981), who showed in guinea-pig skin that local PGE_2 potentiated the plasma extravasation induced by various exogeneous mediators, including histamine and leukotrienes, but failed to affect immunologically triggered inflammation which was found to be dependent on endogenous release of histamine and leukotrienes. Accordingly, Komoriya *et al.* (1978) showed in rat skin, that PGE_1 and PGI_2 enhanced the increase in permeability evoked by histamine, but not that caused by adenosine 5'-triphosphate (ATP) which is known to cause mast cell degranulation (Kiernan, 1972) and histamine release (Diamant & Krüger, 1967). In analogy, Sugio & Daly (1983) reported that forskolin greatly enhanced histamine-induced plasma extravasation in rat skin, whereas corresponding skin reactions to 1 μg PGE_1 were reduced by forskolin. Interestingly, plasma extravasation in rat skin to such high doses of PGE_1 has been shown to involve histamine release (Crunkhorn & Willis, 1971).

These seemingly discordant observations regarding the influence of vasodilator prostaglandins on inflammatory responses involving release of endogenous mediators may be reconciled if results in different models are considered with respect to the degree of basal blood flow in each particular system. Thus, in tissues with low basal blood flow, PGE_2 and PGI_2 would be more prone to enhance inflammation as a result of mediator synergism. On the other hand, in a system with high blood flow, it is likely that inhibition of mediator release will be the most obvious action of these prostaglandins. Such an interpretation would explain the different actions of PGEs on immunologically evoked inflammation in the skin of guinea-pigs and rabbits, and in the hamster cheek pouch. Specifically, in the hamster cheek pouch, which is well perfused, the data with PGE_2 and PGI_2 strongly indicate that inhibition of mediator release predominates over mediator synergism. In contrast, in the rabbit skin, which is likely to have a relatively lower basal blood flow, the dominant effect of PGE_2 will be enhancement of inflammation due to vasodilator synergism with released mediators. That the hamster cheek pouch model and the rabbit skin model are different is for example illustrated by comparing the action of LTB_4 in the two systems. Thus, while LTB_4 itself effectively promotes plasma leakage in the hamster cheek pouch (Björk *et al.*, 1982), it can only induce oedema in the rabbit skin in the presence of a vasodilator such as PGE_2 (Bray *et al.*, 1981). It seems feasible that these differences to a large extent relate to variations in basal blood flow between the two models.

Taken together, the present observations indicate that vasodilatation and inhibition of mediator release are two distinct actions of PGE_2 and PGI_2 which modulate inflammatory reactions involving release of endogenous mediators. As discussed in detail above, the final outcome of these opposite actions of vasodilator prostanoids is likely to vary with different species, tissues and inflammatory reactions. Accordingly, such a dual prostaglandin action may contribute to the understanding of variable results in studies where NSAIDs or prostaglandins are used to modify different types of inflammation.

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Stereoselective inhibition of muscarinic receptor subtypes by the enantiomers of hexahydro-difenidol and acetylenic analogues

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1 The affinities of the (R)- and (S)-enantiomers of hexahydro-difenidol (*1*) and its acetylenic analogues hexbutinol (*2*), hexbutinol methiodide (*3*) and *p*-fluoro-hexbutinol (*4*) (stereochemical purity > 99.8%) for muscarinic receptors in rabbit vas deferens (M_1), guinea-pig atria (M_2) and guinea-pig ileum (M_3) were measured by dose-ratio experiments.

2 The (R)-enantiomers consistently showed higher affinities than the (S)-isomers. The stereoselectivity ratios [(R)/(S)] were greatest with the enantiomers of *1* (vas deferens: 550; ileum: 191; atria: 17) and least with those of the *p*-Fluoro-analogue *4* (vas deferens: 34; ileum: 8.5; atria: 1.7).

3 The enantiomeric potency ratios for compounds *1–4* were highest in rabbit vas deferens, intermediate in guinea-pig ileum and much less in guinea-pig atria. Thus, these ratios may serve as a predictor of muscarinic receptor subtype identity.

4 (S)-*p*-Fluoro-hexbutinol [(S)-*4*] showed a novel receptor selectivity profile with preference for M_3 receptors: $M_3 > M_2 \geqslant M_1$.

5 These results do not conform to Pfeiffer's rule that activity differences between enantiomers are greater with more potent compounds.

Introduction

A large body of evidence derived from both functional and radioligand binding studies suggests that there are at least three pharmacological muscarinic receptor subtypes [M_1, M_2 ($M_{2\alpha}$) and M_3 ($M_{2\beta}$)] (Eglen & Whiting, 1986; Mitchelson, 1988; Giraldo *et al.*, 1988; Waelbroeck *et al.*, 1988a; 1989; Lambrecht *et al.*, 1989d). This subclassification was recently confirmed by cloning, sequencing and expression of complementary DNA encoding five muscarinic receptors (m1–m5) (Kerlavage *et al.*, 1987; Peralta *et al.*, 1987; Akiba *et al.*, 1988; Brann *et al.*, 1988). The antagonist binding properties of m1–m3 and their patterns of expression in various tissues closely correspond to those of the M_1, M_2 and M_3 receptors (Peralta *et al.*, 1987; Akiba *et al.*, 1988; Maeda *et al.*, 1988; Buckley *et al.*, 1989). The selective muscarinic antagonists pirenzepine (Hammer *et al.*, 1980; Lambrecht *et al.*, 1988a; Waelbroeck *et al.*, 1988a; 1989), methoctramine (Melchiorre *et al.*, 1987; Wess *et al.*, 1988), AF-DX 116 (Giachetti *et al.*, 1986), hexahydro-(sila-)difenidol (Mutschler & Lambrecht, 1984; Lambrecht *et al.*, 1988a; 1989d; Waelbroeck *et al.*, 1988a; 1989; Akiba *et al.*, 1988; Buckley *et al.*, 1989) and *p*-fluoro-hexahydro-sila-difenidol (Lambrecht *et al.*, 1988a; 1989b,d) have proved to be useful tools in this subclassification.

Among these selective muscarinic antagonists, racemic hexahydro-difenidol [(R/S)-*1*; Figure 1] has been shown to have high affinity for M_1 receptors in neuronal tissues as well as for M_3 receptors in exocrine glands and smooth muscles, but a much lower affinity for cardiac M_2 receptors (Mutschler & Lambrecht, 1984; Eltze *et al.*, 1988; Lambrecht *et al.*, 1988c; Waelbroeck *et al.*, 1989). The main aim of the present study was to characterize the structural demands, including stereochemical aspects, for potency and selectivity of some chiral acetylenic analogues of hexahydro-difenidol (*1*) (Figure 1). Since a conformationally rigid acetylenic moiety is present in some selective muscarinic agonists such as McN-A-343 (Roszkowski, 1961; Lambrecht *et al.*, 1986; Eltze *et al.*, 1988; Wess *et al.*, 1988) and arecaidine propargyl ester (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Moser *et al.*, 1989), it would be of interest to investigate whether the acetylenic analogues of hexahydro-difenidol are also selective for muscarinic receptor subtypes.

In the last few years, data have accumulated that muscarinic receptors can be differentiated on the basis of their stereoselectivity to chiral antagonists such as procyclidine (Lambrecht & Mutschler, 1986; Tacke *et al.*, 1986; Waelbroeck *et al.*, 1988b), trihexyphenidyl (Lambrecht *et al.*, 1988b; 1989d), phenglutarimide (Lambrecht *et al.*, 1989a), biperiden (Eltze & Figala, 1988) and telenzepine (Eveleigh *et al.*, 1989). Hexahydro-difenidol (*1*) and its analogues hexbutinol (*2*), hexbutinol methiodide (*3*) and *p*-fluoro-hexbutinol (*4*) (Figure 1) possess a centre of chirality and therefore exist in two enantiomers. We took advantage of this by determining the antagonist affinities of the individual enantiomers of these compounds at muscarinic receptor subtypes. The results were compared with those obtained for the selective reference drugs hexahydro-sila-difenidol ($M_3 \geqslant M_1 > M_2$) and *p*-fluoro-hexahydro-sila-difenidol ($M_3 > M_1 > M_2$). The receptors studied were presynaptic M_1 heteroreceptors in rabbit vas deferens (Eltze, 1988; Eltze *et al.*, 1988; Lambrecht *et al.*, 1988a,b), cardiac M_2 receptors present in guinea-pig atria and smooth muscle M_3 receptors present in guinea-pig ileum.

Some of the present results have been briefly presented elsewhere (Feifel *et al.*, 1988; Lambrecht *et al.*, 1989c; Tacke *et al.*, 1989).

Methods

Rabbit isolated vas deferens

Experiments on rabbit isolated vas deferens were carried out according to Eltze (1988) and Eltze *et al.* (1988). Male New Zealand white rabbits (2.5–3.0 kg) were killed by i.v. injection of 120 mg kg^{-1} pentobarbitone sodium. Vasa deferentia were excised, dissected free of connective tissues and divided into four segments of approximately 1.5 cm length. The preparations were set up in 7 ml organ baths containing modified

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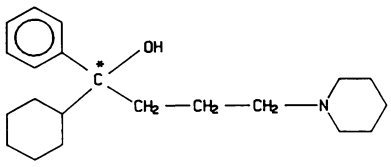
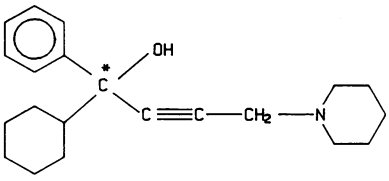
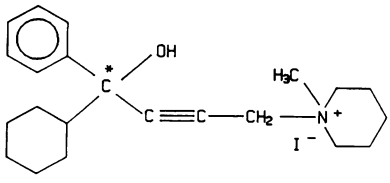


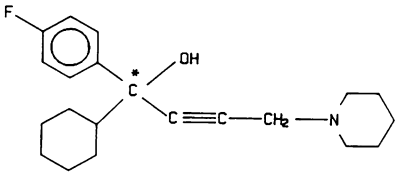
Hexahydro-difenidol (1)



Hexbutinol (2)



Hexbutinol methiodide (3)



p-Fluoro-hexbutinol (4)

Figure 1 Chemical structures of the enantiomers of hexahydro-difenidol (*1*), hexbutinol (*2*), hexbutinol methiodide (*3*) and *p*-fluoro-hexbutinol (*4*). The asterisk denotes the centre of chirality.

Krebs buffer which consisted of (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and (+)-glucose 11.1; 1 μM yohimbine was included to block α_2-adrenoceptors. The bathing fluid was maintained at 31°C and aerated with 95% O_2/5% CO_2. A basal tension of 750 mg was applied and after a 30 min period of initial equilibration isometric twitch contractions were elicited by electrical field stimulation (0.05 Hz, 0.5 ms, 30 V) with platinum electrodes. The contractions were measured isometrically by a force-displacement transducer connected to a Hellige amplifier and a Rikadenki polygraph. These effects were concentration-dependently inhibited by the M_1 receptor agonist 4-(4-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) (Eltze *et al.*, 1988).

Guinea-pig isolated left atria and ileal longitudinal muscle

Guinea-pigs (300–400 g) of either sex were killed by cervical dislocation. The organs required were removed and set up in 6 ml organ baths, under 500 mg tension, in oxygenated (95% O_2/5% CO_2) Tyrode solution (32°C) composed of (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and (+)-glucose 5.6. Arecaidine propargyl ester (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Moser *et al.*, 1989) was used as an agonist. Left atria were paced electrically (2 Hz, 3 ms, 5 V) by means of platinum electrodes. Negative inotropic effects to the agonist were measured as changes in isometric tension. Responses of ileal longitudinal muscle strips (Paton & Zar, 1968) to arecaidine propargyl ester were measured as isotonic contractions. The effects in atria and ileum were recorded as with the rabbit isolated vas deferens.

Antagonist affinities

After a 1 h equilibration period, concentration-response curves were constructed by adding doses of the agonists cumulatively, according to the method of Van Rossum (1963). When these responses were constant, concentration-response curves were repeated in the presence of antagonists. At least three concentrations of antagonists with log intervals of 0.5 were tested 3 to 5 times (see Table 1) in the three tissues. Each concentration of antagonist was allowed to equilibrate for 15 to 30 min (ileum) and 30 min (atrium) in guinea-pig preparations, respectively, and 30 [(S)-isomers] to 60 min [(R)-isomers] in rabbit vas deferens. Preliminary experiments indicated that these intervals were sufficient for equilibration of the antagonist concentrations used. No preparation was exposed to more than three concentrations of antagonists. EC_{50} values of agonists in the absence and presence of antagonists were determined graphically for calculation of dose-ratios. The slopes of the Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were determined by linear regression by the method of least squares. pA_2 values were estimated as the intercept on the abscissa scale by fitting to the data the best straight line with a slope of unity (Tallarida *et al.*, 1979).

Data analysis

All data are presented as means ± s.e.mean of 9–17 experiments. Differences between mean values were tested for statistical significance by Student's t test; $P < 0.05$ was accepted as being significant. Linear regression analyses were carried out by the method of least squares (Tallarida *et al.*, 1979).

Drugs

Pirenzepine dihydrochloride was obtained from Boehringer Ingelheim (F.R.G.). 4-(4-Chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium iodide (4-Cl-McN-A-343) (Nelson *et al.*, 1976), arecaidine propargyl ester (Mutschler & Hultzsch, 1973), racemic hexahydro-sila-difenidol hydrochloride (Tacke *et al.*, 1985), (R)- and (S)-hexahydro-difenidol hydrochloride [(R)-*1*·HCl and (S)-*1*·HCl] (Tacke *et al.*, 1989) as well as (R)- and (S)-hexbutinol [(R)-*2* and (S)-*2*] (Tacke *et al.*, 1989) were synthesized in our laboratories according to the literature. The enantiomeric excess (ee) of the enantiomers of hexahydro-difenidol and hexbutinol was >99.8%, determined by calorimetric analysis as described by Tacke *et al.* (1987). Racemic *p*-fluoro-hexahydro-sila-difenidol hydrochloride was prepared by analogy to the synthesis of hexahydro-sila-difenidol (Tacke *et al.*, unpublished results). The enantiomers of *p*-fluoro-hexbutinol [(R)-*4* and (S)-*4*; enantiomeric purity: ee > 99.8%; calorimetric analysis] were synthesized by analogy to (R)- and (S)-hexbutinol (Tacke *et al.*, 1989). The other chemicals not described under 'Synthetic chemistry' were of reagent grade and were used as purchased.

Synthetic chemistry

The enantiomers of hexbutinol methiodide [(R)-*3* and (S)-*3*] were prepared as follows:

Freshly distilled methyl iodide (18 mmol) was added under an atmosphere of dried nitrogen to a solution of (R)- or (S)-hexbutinol (*2*) (8 mmol) in dry ethanol (50 ml). The reaction mixture was stirred for 3 h at 30°C, dry n-pentane (100 ml) was added, and the mixture was stirred for 1 h at 20°C. Thereafter, the precipitate was collected by filtration and then recry-

Table 1 pA_2 values and slopes of Arunlakshana-Schild plots (in parentheses) for muscarinic antagonists at M_1, M_2 and M_3 receptors

Compound	*Rabbit vas deferens* (M_1)	*Guinea-pig atria* (M_2)	*Guinea-pig ileum* (M_3)
HHSiD[a]	7.92 ± 0.07	6.53 ± 0.05	7.96 ± 0.03
p-F-HHSiD[a]	6.68 ± 0.12	6.01 ± 0.06	7.84 ± 0.03
Hexahydro-difenidol (*1*)			
(R)-*1*	8.71 ± 0.05	7.03 ± 0.06[b]	8.35 ± 0.04[b]
	[*n* = 17; 6]	[*n* = 9; 8]	[*n* = 14; 11]
	(1.26 ± 0.09)*	(0.83 ± 0.30)	(0.92 ± 0.07)
(S)-*1*	5.97 ± 0.04	5.80 ± 0.07[b]	6.07 ± 0.05[b]
	[*n* = 13; 9]	[*n* = 9; 8]	[*n* = 9; 6]
	(0.87 ± 0.10)	(0.96 ± 0.19)	(0.87 ± 0.17)
Hexbutinol (*2*)			
(R)-*2*	8.78 ± 0.05	7.77 ± 0.04[b]	8.78 ± 0.04[b]
	[*n* = 13; 6]	[*n* = 10; 10]	[*n* = 13; 10]
	(1.16 ± 0.11)	(1.09 ± 0.07)	(1.10 ± 0.06)
(S)-*2*	6.75 ± 0.07	6.84 ± 0.05[b]	7.14 ± 0.04[b]
	[*n* = 17; 8]	[*n* = 9; 9]	[*n* = 10; 5]
	(0.84 ± 0.15)	(1.03 ± 0.13)	(0.99 ± 0.07)
Hexbutinol methiodide (*3*)			
(R)-*3*	9.43 ± 0.06	8.62 ± 0.02	8.85 ± 0.04
	[*n* = 9; 5]	[*n* = 13; 13]	[*n* = 17; 16]
	(0.92 ± 0.11)	(1.07 ± 0.03)	(1.14 ± 0.06)
(S)-*3*	7.83 ± 0.05	7.40 ± 0.02	7.26 ± 0.02
	[*n* = 12; 4]	[*n* = 12; 4]	[*n* = 12; 7]
	(0.92 ± 0.07)	(0.91 ± 0.04)	(0.96 ± 0.03)
p-Fluoro-hexbutinol (*4*)			
(R)-*4*	8.08 ± 0.06	6.97 ± 0.04	8.50 ± 0.04
	[*n* = 13; 5]	[*n* = 12; 12]	[*n* = 16; 8]
	(1.31 ± 0.10)*	(0.84 ± 0.06)	(0.84 ± 0.06)
(S)-*4*	6.55 ± 0.08	6.75 ± 0.05	7.57 ± 0.04
	[*n* = 17; 6]	[*n* = 9; 9]	[*n* = 12; 12]
	(0.85 ± 0.18)	(1.03 ± 0.09)	(0.99 ± 0.06)

[a] Data taken from Lambrecht *et al.* (1988a).
[b] Data taken from Tacke *et al.* (1989).
The parameters shown represent the mean ± s.e.mean. The slopes of Arunlakshana-Schild plots were determined by linear regression analysis (Tallarida *et al.*, 1979). pA_2 values were obtained after the unity constraint had been imposed. The numbers of total data points (*n*) and tissues used are given in square parentheses. The slopes shown are not significantly different from unity ($P > 0.05$), except those for (R)-*1* and (R)-*4* at M_1 receptors (marked with an asterisk).
HHSiD = racemic hexahydro-sila-difenidol; p-F-HHSiD = racemic *p*-fluoro-hexahydro-sila-difenidol.

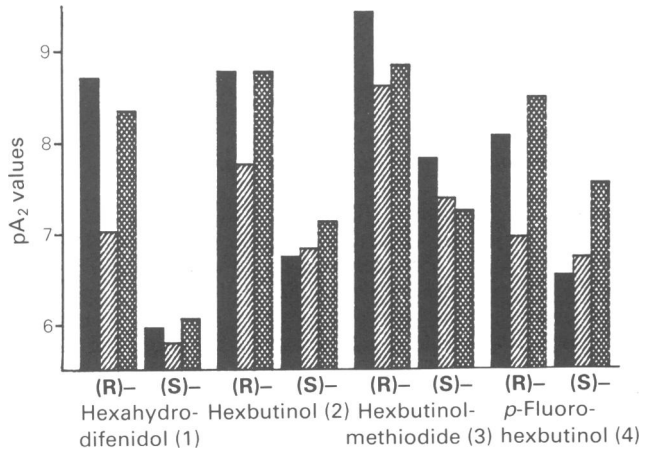


Figure 2 Affinity profiles of the enantiomers of compounds *1–4* at muscarinic M_1 receptors in rabbit vas deferens (solid columns), M_2 receptors in guinea-pig atria (diagonally-hatched columns) and M_3 receptors in guinea-pig ileum (cross-hatched columns).

Table 2 Receptor selectivity and stereoselectivity [(R)/(S)] ratios for chiral muscarinic antagonists

	Receptor selectivity			*Stereoselectivity*		
	M_1/M_2	M_3/M_1	M_3/M_2	M_1	M_2	M_3
Hexahydro-difenidol (*1*)						
(R)-*1*	48	0.44	21			
				550	17	191
(S)-*1*	1.5	1.3	1.9			
Hexbutinol (*2*)						
(R)-*2*	10	1.0	10			
				107	8.5	44
(S)-*2*	0.81	2.5	2.0			
Hexbutinol methiodide (*3*)						
(R)-*3*	6.5	0.26	1.7			
				40	17	39
(S)-*3*	2.7	0.27	0.72			
p-Fluoro-hexbutinol (*4*)						
(R)-*4*	13	2.6	34			
				34	1.7	8.5
(S)-*4*	0.63	10.5	6.6			

The values shown represent the antilogs of the differences between corresponding mean pA_2 values (Table 1) determined at M_1 receptors in rabbit vas deferens as well as at atrial M_2 and ileal M_3 receptors of guinea-pigs.

stallized from ethanol/diethylether. After drying the crystals *in vacuo*, analytically pure products were obtained [characterized by ^{1}H NMR, ^{13}C NMR, FAB MS measurements and calorimetric analysis (data not given) as well as by elemental analyses].

(R)-*3*: $C_{22}H_{32}INO$ (453.4), yield 92%, m.p. 174–175°C, $[\alpha]^{27}_{586} = -3$ (c = 0.5, $CHCl_3$), ee > 99.8%. Found: C, 58.3; H, 7.1; N, 2.9. Calculated: C, 58.28; H, 7.11; N, 3.09. (S)-*3*: $C_{22}H_{32}INO$ (453.4), yield 94%, m.p. 174–175°C, $[\alpha]^{27}_{586} = 3$ (c = 0.5, $CHCl_3$), ee > 99.8%. Found: C, 58.3; H, 7.1; N, 3.1. Calculated: C, 58.28; H, 7.11; N, 3.09.

Results

Twitch contractions of rabbit vas deferens elicited by electrical field stimulation were inhibited by the M_1 receptor agonist, 4-Cl-McN-A-343 (EC_{50} = 250 nM). This effect was concentration-dependently antagonized by the (R)- and (S)-enantiomers of compounds *1–4*. Similarly, all stereoisomers antagonized the negative inotropic responses in guinea-pig atria (EC_{50} = 7 nM) and ileal contractions (EC_{50} = 25 nM) of arecaidine propargyl ester. In the three tissues, parallel shifts of the agonists concentration-response curves without any appreciable changes of basal tension or reduction of maximum responses were obtained and Arunlakshana-Schild plots were linear through the concentration range tested for each antagonist, indicating competitive antagonism. Slopes (Table 1) were not significantly different from unity ($P > 0.05$), except for compounds (R)-*1* and (R)-*4* at M_1 receptors. The pA_2 values of (R)-*1* and (R)-*4* in rabbit vas deferens (8.71 and 8.08, respectively; Table 1) might therefore be regarded as a purely experimental quantity. However, the binding affinities of (R)-*1* and (R)-*4* to M_1 receptors in NB-OK 1 cells (pK_i = 8.6 and 8.1, respectively; M. Waelbroeck, unpublished results) were very similar to that obtained in rabbit vas deferens (Table 1). In these radioligand binding studies, competition curves with (R)-*1* and (R)-*4* did not deviate significantly from results expected for competitive inhibition of [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding.

The (R)- and (S)-enantiomers of compounds *1–4* showed quite wide variations in their affinities for the muscarinic receptor subtypes, their pA_2 values differing by more than three orders of magnitude (Table 1). Introduction of a triple bond into the basic side chain of the parent compound

hexahydro-difenidol (*1*) (→hexbutinol, *2*), quaternization of *2* (→hexbutinol methiodide, *3*) and *p*-fluoro-substitution of the phenyl ring of *2* (→*p*-fluoro-hexbutinol, *4*) changed the affinities of these compounds for M_1, M_2 and M_3 receptors differently. Thus compounds with qualitatively and/or quantitatively different receptor selectivity profiles were obtained (Table 2 and Figure 2).

At each of the three muscarinic receptor subtypes, the (R)-enantiomer of compounds *1–4* was more potent than the (S)-configurated isomer. The difference in potencies between the enantiomers of compounds *1–4* was greatest at the M_1, less at M_3 and least at M_2 receptors (Table 2). The degree of stereoselectivity (Table 2) was also dependent on the structure of the compounds (M_1 and M_3: *1* > *2* > *3* > *4*; M_2: *1* = *3* > *2* > *4*).

Discussion

Structural variations in the (R)- and (S)-hexahydro-difenidol (*1*) molecules led to muscarinic antagonists that exhibited a qualitatively or quantitatively different spectrum of receptor selectivity to the parent stereoisomers (R)-*1* and (S)-*1* (Table 2, Figure 2). These observed selectivities did not appear to be associated in general with high affinity and absolute configuration (Figure 2). For example, (S)-*p*-fluoro-hexbutinol [(S)-*4*] was a relatively weak compound but it had a novel receptor selectivity profile: $M_3 > M_2 \geqslant M_1$.

The results of the present study confirm and extend previous findings that rabbit vas deferens (M_1 receptors) as well as guinea-pig atria (M_2 receptors) and ileum (M_3 receptors) possess different muscarinic receptor subtypes (Eltze, 1988; Eltze *et al.*, 1988; Lambrecht *et al.*, 1988a; Waelbroeck *et al.*, 1989). Furthermore, our findings provide additional evidence that these subtypes can be identified on the basis of their stereoselectivity. There appears to be a consistent trend showing that the degree of stereoselectivity is always greatest at M_1, intermediate at M_3 and lowest at M_2 receptors (Lambrecht & Mutschler, 1986; Tacke *et al.*, 1986; Eltze & Figala, 1988; Lambrecht *et al.*, 1988b; 1989a,c,d).

Receptor selectivity of (R)-enantiomers

(R)-Hexahydro-difenidol [(R)-*1*] was found to be a potent muscarinic antagonist exhibiting high affinity for M_1 receptors in rabbit vas deferens as well as for M_3 receptors in guinea-pig ileum, whereas its affinity for M_2 receptors in guinea-pig atria was lower by factors of 48 and 21, respectively (Table 2, Figure 2). The resulting affinity profile of (R)-*1* ($M_1 \geqslant M_3 > M_2$) is qualitatively very similar to that found for the racemic hexahydro-difenidol (Waelbroeck *et al.*, 1989) and its racemic silicon analogue hexahydro-sila-difenidol (Table 1).

The introduction of a triple bond into the (R)-hexahydro-difenidol molecule [→(R)-hexbutinol; (R)-*2*] increased the affinity for M_3 and M_2 receptors by factors of 2.7 and 5.5, respectively (Table 1), whereas the affinity for M_1 receptors was not significantly different. Thus, (R)-hexbutinol shows a receptor selectivity profile that is qualitatively similar to that of the parent compound (R)-hexahydro-difenidol [(R)-*1*]. However, the selectivity for M_1 and M_3 over M_2 receptors is lower than that of (R)-*1*. This might be explained by differences in the electronic structures and/or the conformational behaviour of (R)-hexahydro-difenidol and (R)-hexbutinol. The triple bond in (R)-hexbutinol should make it more difficult for this molecule to adopt the active conformation at all subtypes, thus creating selectivity (Barlow *et al.*, 1988). On the other hand, the carbon-carbon triple bond might contribute to affinity and thus counteract any selectivity creating effect of rigidity.

N-methylation of (R)-hexbutinol [→(R)-hexbutinol methiodide; (R)-*3*] increased the affinity for M_1 receptors in rabbit vas deferens, as well as for M_2 receptors in guinea-pig atria by factors of 4.5 and 7.1, whereas the affinity for M_3 receptors in guinea-pig ileum was not affected (Table 1). Thus, N-methylation of the tertiary amine (R)-*2* changed the receptor selectivity pattern. The following affinity rank order for (R)-hexbutinol methiodide [(R)-*3*] was observed: $M_1 > M_3 \geqslant M_2$ (Figure 2).

A comparison of the antimuscarinic potencies of (R)-*p*-fluoro-hexbutinol [(R)-*4*] and (R)-hexbutinol [(R)-*2*] outlines the effect of fluoro-substitution in the phenyl ring on antimuscarinic potency. The *p*-fluoro substituent reduced the affinity for the muscarinic receptors up to 6 fold (Table 1). This decrease in affinity was the least pronounced at the ileal M_3 receptors. Thus, as a result (R)-*p*-fluoro-hexbutinol [(R)-*4*] showed a small preference for M_3 over M_1 receptors, but the selectivity of M_3 over M_2 receptors was enhanced (Table 2, Figure 2).

Receptor selectivity of (S)-enantiomers

In general, the (S)-enantiomers of compounds *1–4* were less potent than the corresponding (R)-configurated isomers (Table 1, Figure 2). However, introduction of a triple bond into the hexahydro-difenidol molecule (*1*) as well as quaternization and fluoro-substitution of hexbutinol (*2*) had different effects on affinity for the (S)-isomers in comparison to the (R)-enantiomers at the three muscarinic receptor subtypes. Thus, the (S)-enantiomers showed affinity profiles which were qualitatively and/or quantitatively different from those obtained for the (R)-isomers.

(S)-Hexahydro-difenidol [(S)-*1*] was a very weak muscarinic antagonist showing no muscarinic receptor selectivity (Table 2, Figure 2). Introduction of a triple bond into (S)-hexahydro-difenidol [→(S)-hexbutinol; (S)-*2*] increased the affinity for M_1, M_2 and M_3 receptors by factors of 6, 11 and 12, respectively (Table 1). Thus, (S)-hexbutinol [(S)-*2*] showed at most a 2 fold preference for the ileal M_3 receptors (Table 2, Figure 2). This is different to the situation of the (R)-enantiomers.

N-methylation of (S)-hexbutinol [(S)-*2*] increased the affinity for M_1 and M_2 receptors 12 and 3.6 fold, respectively, whereas the affinity for ileal M_3 receptors was nearly unchanged (Table 1). Thus the receptor selectivity profile of (S)-hexbutinol methiodide [(S)-*3*] is slightly different from that obtained for the (R)-enantiomer (Figure 2).

The influence of fluoro-substitution on potency and selectivity is demonstrated by comparison of (S)-hexbutinol [(S)-*2*] and (S)-*p*-fluoro-hexbutinol [(S)-*4*] (Table 1). Compared to (S)-*2* the fluoro derivative (S)-*4* exhibited a relatively higher affinity ($pA_2 = 7.57$) for M_3 receptors, whereas its antimuscarinic potency at M_1 and M_2 receptors was lower by factors of 10.5 and 6.6, respectively (Table 1). Thus, fluoro-substitution in the *para*-position of the phenyl ring of (S)-hexbutinol [(S)-*2*] enhanced its M_3-selectivity. The receptor selectivity profile of (S)-*p*-fluoro-hexbutinol ($M_3 > M_2 \geqslant M_1$) is also different from that of the corresponding (R)-enantiomer ($M_3 \geqslant M_1 > M_2$) and of *p*-fluoro-hexahydro-sila-difenidol ($M_3 > M_1 > M_2$; Table 1).

Stereoselectivity of muscarinic receptors

It has been suggested (Pfeiffer, 1956) that, with greater potencies of drugs, larger differences in pharmacological effects will be seen between the enantiomers of chiral compounds. The results obtained in this study (Table 1 and 2) do not substantiate the above suggestion and its implications (Lehmann, 1986). For example, at M_1 receptors the affinity constants (pA_2 values) of the more potent (R)-enantiomers decrease in the order (R)-hexbutinol methiodide (9.43) > (R)-hexbutinol (8.78) = (R)-hexahydro-difenidol (8.71) > (R)-*p*-fluoro-hexbutinol (8.08), whereas the stereoselectivity ratios [(R)/(S)] of these chiral compounds are in the order hexahydro-difenidol (550) > hexbutinol (107) > hexbutinol methiodide (40) > *p*-fluoro-hexbutinol (34). Thus, in this series of compounds the stereoselectivity ratio at, e.g. M_1 receptors,

was higher by more than one order of magnitude for hexahydro-difenidol (*1*) than for hexbutinol methiodide (*3*), although compound (R)-*3* was 5 fold more potent than (R)-*1*. A similar lack of correlation between potency of the eutomer and stereoselectivity ratios of enantiomers was obtained at M_2 and M_3 receptors (Tables 1 and 2). The findings of the present study confirm and extend previous results obtained with the enantiomers of biperiden (Eltze & Figala, 1988), trihexyphenidyl (Lambrecht *et al.*, 1988b; 1989d) and phenglutarimide (Lambrecht *et al.*, 1989a).

In 1982, Robert *et al.* stated a corollary of Pfeiffer's rule: "When different receptor subtypes interact with the enantiomers of chiral drugs, their stereoselectivity should increase as a function of affinity of the more potent enantiomer (= eutomer; Lehmann, 1986) for the respective subtypes". However, when the magnitude of receptor subtype stereoselectivity (difference in pA_2 values of the (R)- and (S)-enantiomers = eudismic index; Lehmann, 1986) was plotted against the pA_2 value of the more potent isomer for that particular receptor subtype, a strong correlation (correlation coefficient $r = 0.995$) was only observed for hexahydro-difenidol (Figure 3). The stereoselectivity and the affinity of the eutomer of hexahydro-difenidol was greatest at M_1, intermediate at M_3 and lowest at M_2 receptors. On the other hand the enantiomers of compounds *2–4* did not fulfill the predictions made by Robert *et al.* (1982). However, the interesting finding of this study is that the stereoselectivity ratios of all the chiral compounds *1–4* consistently show the same order: $M_1 > M_3 > M_2$. This implies that the stereochemical demands made by the muscarinic receptor subtypes are different for the enantiomers of compounds *1–4* being most stringent at M_1 receptors. Similar results have been obtained with the enantiomers of telenzepine (Eveleigh *et al.*, 1989), biperiden (Eltze & Figala, 1988), trihexyphenidyl and its methiodide (Lambrecht *et al.*, 1988b) and procyclidine (Lambrecht & Mutschler, 1986; Waelbroeck *et al.*, 1988b).

In conclusion, the present study shows that the antimuscarinic potencies and receptor subtype selectivities of the enantiomers of hexahydro-difenidol (*1*) and the acetylenic analogues *2–4* (Figure 1) depend on different structural parameters including absolute configuration. The M_1, M_2 and M_3 receptors make qualitatively and quantitatively different stereochemical demands for the (R)- and (S)-enantiomers, resulting in different receptor selectivity profiles. It is interesting to note that, of the enantiomers investigated in this study, (S)-*p*-fluoro-hexbutinol shows a novel receptor selectivity profile: $M_3 > M_2 \geqslant M_1$ (Figure 2). There was a variation in stereoselectivity ratios on the three receptor subtypes (Table 2): $M_1 > M_3 > M_2$. These results indicate that stereoselectivity ratios can be successfully used as a parameter to characterize muscarinic receptor subtypes providing information that racemates cannot give. However, the stereoselectivity ratios do not conform to the predictions of the 'classical' Pfeiffer's rule (Pfeiffer, 1956), and that of its corollary (Robert *et al.*, 1982).

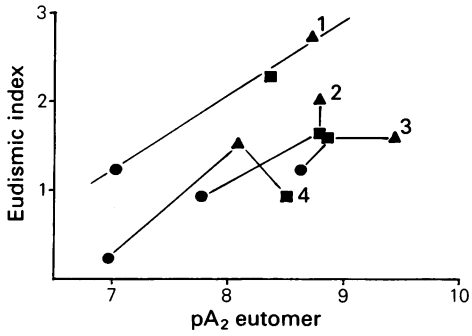


Figure 3 Plot of receptor stereoselectivity [eudismic index (EI) = difference of pA_2 values of the (R)- and (S)-enantiomers; Lehmann, 1986] versus pA_2 values of the more potent isomer [eutomer (EU)] for compounds *1–4* (see Figure 1) at muscarinic M_1 receptors in rabbit vas deferens (▲) as well as at M_2 (●) and M_3 receptors (■) in guinea-pig atria and ileum. A strong linear correlation was only observed for compound *1*: EI = 0.87 (±0.08) pA_2^{EU} − 4.91 (±0.68), $r = 0.995$, s.d. = 0.182, $n = 3$. The correlation coefficient of 0.995 is significant at the $P < 0.05$ level.

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Mediation of bradykinin-induced contraction in canine veins via thromboxane/prostaglandin endoperoxide receptor activation

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1 Canine jugular and femoral veins were studied to determine the possible importance of thromboxane (TXA_2) and prostaglandin endoperoxides (prostaglandin H_2, PGH_2) in mediating bradykinin(BK)-induced contraction.

2 Isolated vein rings incubated in modified Krebs solution contracted to TXA_2/PGH_2 analogs SQ26655 and U44069 with potency of contraction exceeding that for BK. The potency ranking for both veins was SQ26655 > U44069 > BK > $PGF_{2\alpha}$ > TXB_2 ≫ PGD_2.

3 The cyclo-oxygenase inhibitors indomethacin (3×10^{-7} M) and flufenamic acid (10^{-5} M) reduced BK contractions without affecting those induced by noradrenaline (NA).

4 TXA_2/PGH_2 receptor antagonists SQ29548 (10^{-8} M) and BM13177 (10^{-6} M) strongly inhibited BK-induced tension. The action of antagonists was reversible with negligible influence on NA-elicited contraction. Selective removal of endothelium had no effect on BK-induced contraction or the action of the antagonists.

5 The thromboxane synthase inhibitors dazoxiben (10^{-4} M) and CGS 12970 (10^{-5} M) had no significant inhibitory effect on BK-induced tension.

6 These results suggest that in canine jugular and femoral vein, the action of BK is largely dependent upon stimulation of the cyclo-oxygenase pathway to produce PGH_2 and possibly TXA_2, which can activate a smooth muscle TXA_2/PGH_2 receptor to elicit vasoconstriction.

Introduction

Bradykinin (BK) is a potent autacoid released during trauma, anaphylactic and other types of shock, and inflammatory reactions. Although it can exert a hypotensive effect *in vivo* due to arteriolar dilatation, BK has a predominantly constrictive action in many mammalian isolated veins (Tsuru *et al.*, 1976; Gaudreau *et al.*, 1981).

Evidence for endogenous prostaglandin synthesis along the cyclo-oxygenase pathway associated with BK-induced contraction has been obtained in several vascular preparations. However, the net vasoactive influence of the prostaglandins synthesized varies with species and vascular bed. Thus the cyclo-oxygenase inhibitors meclofenamate and indomethacin reduce contraction to BK in human foetal placental arteries (Tulenko, 1981) and bovine mesenteric vein (Wong *et al.*, 1977) respectively, implying that a cyclo-oxygenase product mediates the contraction. Yet in rabbit ear vein (Crouch *et al.*, 1981) and canine intestinal vasculature (Greenberg & Kadowitz, 1982), indomethacin either has no effect or potentiates the response to BK.

Though BK can stimulate production of the vasoconstrictor prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in certain mesenteric veins (Wong *et al.*, 1977; Limas, 1977), few BK-related studies have been done to determine the possible mediator role of the potent cyclo-oxygenase products thromboxane A_2 (TXA_2) and the precursor prostaglandin endoperoxides (PGH_2, PGG_2) (Hamberg *et al.*, 1975a), which interact with the thromboxane/prostaglandin endoperoxide (TXA_2/PGH_2) receptor (Kennedy *et al.*, 1982; Saussy *et al.*, 1985). Endogenous TXA_2 can mediate vasoconstriction to acetylcholine in rabbit pulmonary artery (Altiere *et al.*, 1986) and canine cerebral artery (Shirahase *et al.*, 1987), and to arachidonic acid in rabbit pulmonary artery (Salzman *et al.*, 1980) and intact rat kidney (Sakr & Dunham, 1982). In the perfused hydronephrotic rabbit kidney (a pathophysiological model), BK yields renal vasoconstriction associated with release of TXA_2; this vasoconstriction can be reduced by the thromboxane synthase inhibitor OKY1581 (Kawasaki & Needleman, 1982).

The potential of PGH_2 as an endogenous mediator of agonist-induced vasoconstriction is less well known, despite its ability to constrict vessels (Hamberg *et al.*, 1975b; Goldberg *et al.*, 1977; Coleman *et al.*, 1981). A recent study has suggested that prostaglandin endoperoxides can mediate arachidonic acid-induced vasoconstriction in the rat perfused kidney through the TXA_2/PGH_2 receptor, under conditions where TXA_2 synthesis is inhibited (Quilley *et al.*, 1989).

In this study, we show that the contractile action of BK on 2 canine isolated vein preparations proceeds primarily through activation of the cyclo-oxygenase pathway and subsequent interaction of evoked PGH_2 and possibly TXA_2 with the TXA_2/PGH_2 receptor.

Methods

Dissection of canine veins

Femoral and external jugular veins (4 vessels per dog) were carefully harvested from female mongrel dogs anaesthetized with sodium pentobarbitone (Nembutal, 25 mg kg^{-1} animal weight, injected into the cephalic vein). Supporting tissue was carefully removed and side branches tied off, with care taken to maintain blood flow and not disturb the endothelial surface in the main vessels. The veins were then perfused with saline solution, ligated, excised and transferred to bicarbonate-buffered physiological saline solution (PSS) at 4°C. The dogs were then killed by an intravenous injection of T-61. Veins were stored with daily changes of PSS and were found to give consistent responses over a 3 day period.

In vitro *setup and procedure*

Circular ring segments 2.5 mm wide were cut perpendicular to the vessel axis and were mounted with metal hooks and

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jeweler's chains in individual tissue baths. Each bath was equipped with (1) a micrometer gauge to measure and manipulate tissue length and (2) a Statham isometric force transducer. This latter was connected through a signal amplifier and multiplexer to a Kipp and Zonen strip chart recorder. The mounted tissues were incubated in the baths with 50 ml volumes of PSS bubbled with 95% O_2 – 5% CO_2 at 37°C, pH 7.4. The composition of the PSS was (mM): NaCl 127.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 16.0, $CaCl_2 \cdot 2H_2O$ 2.5, EDTA 0.04 and glucose 10.0. Each mounted vein segment was stretched in 0.4 g increments at 15 min intervals over a 90 min period to a stable preload of 0.8 g.

Concentration-response curves were obtained by the cumulative addition of aliquots of agonist solutions. In a typical experiment, vein rings were assayed with BK to obtain a reproducible control response, then incubated with an antagonist or inhibitor for 30 min before re-assay with the agonist.

Removal of endothelium

In some experiments, the endothelium was selectively removed from mounted jugular and femoral vein rings. The flattened end of a small wooden dowel was carefully rubbed several times over the entire luminal surface of the ring which was then rinsed in PSS. Anatomically adjacent rings with undisturbed endothelium were used as controls.

The presence or absence of endothelium was determined by scanning electron microscopy (SEM). After a set of experiments, some vein rings were fixed in 1% glutaraldehyde while mounted in the tissue baths, then removed for dehydration and critical-point drying. Samples were gold-coated and examined by SEM, which confirmed that the physical denudation was effective in specifically removing endothelium.

Drugs used

Agonists used in this study were: noradrenaline-HCl (NA), prostaglandin D_2 (PGD_2) and thromboxane B_2 (TXB_2) (all from Sigma Chemical Co.); bradykinin (BK, Boehringer-Ingelheim); prostaglandin $F_{2\alpha}$ (dinoprost tromethamine, $PGF_{2\alpha}$; Upjohn Co.); U44069 ((15S)-hydro-9a,11a-(epoxymethano) prosta-5Z, 13E-dienoic acid; donated by Upjohn Co.) and SQ26655 ((1S-(1a,2b(5Z),3a(1E,3s*),4a))-7-(3-(3-hydroxy-1-octenyl)-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptenoic acid; donated by Dr Ogletree, Squibb Research Inst.). Inhibitors and antagonists included: indomethacin and flufenamic acid (Sigma Chemical Co.); SQ29548 ((1S-(1a,2b(5Z),3b,4a))-7-(3-((2-((phenylamino)carbonyl)-hydrazino)methyl)-7-oxabicyclo (2.2.1)hept-2-yl)-5-heptenoic acid; donated by Squibb Research Inst.); BM13177 ((4-(2-benzenesulphonamido)-ethyl)-phenoxyacetic acid; donated by Dr Lefer, Thomas Jefferson School of Medicine); CGS12970 (3-methyl-2(3-pyridyl)-1-indoleoctanoic acid; a gift from Dr Watthey, Ciba-Geigy, Inc.) and dazoxiben-HCl (4-(-2-(1H-imidazol-1-yl)ethoxy) benzoic acid hydrochloride; donated by Dr Urquilla, Pfizer, Inc.).

Stock solutions of SQ26655, U44069, PGD_2, TXB_2, SQ29548 and BM13177 were prepared in ethanol and stored at −20°C. Other stock solutions were made on the day of use: flufenamic acid and indomethacin were dissolved in 10% $NaHCO_3$, CGS12970 was dissolved in 0.1 N NaOH and all remaining agents were prepared directly in buffered PSS. All stock dilutions into PSS were made on the day of use and discarded at the end of the experiment. At the maximum added volumes, none of the solvent vehicles alone had any observable effect on relaxed or NA-constricted jugular or femoral veins.

Statistics

Statistical data are presented as the means ± s.e.mean. Each mean value is the average of data points from at least 3 animals. Data were compared by Student's t test for paired observations. Statistical significance was considered to occur when $P < 0.05$. ED_{50} values were derived by linear interpolation of the normalized dose-response curves for a given agonist.

Results

Contractile responses of canine veins

As shown in Figure 1, TXA_2/PGH_2 analogs SQ26655 and U44069, along with $PGF_{2\alpha}$ and BK, strongly contracted both vein preparations. The maximum responses for the latter 3 agonists were at least 55% of the maximum SQ26655 response which was 3.77 ± 0.40 g ($n = 15$) for jugular and 2.17 ± 0.22 g ($n = 15$) for femoral vein. In comparison, the maximum NA (10^{-5} M) response was 2.38 ± 0.21 g ($n = 40$) for jugular, and 3.11 ± 0.20 g ($n = 37$) for femoral vein. The ED_{50} values for the agonists in Figure 1 were: jugular—SQ26655: 0.22 ± 0.01 nM ($n = 15$), U44069: 1.70 ± 0.03 nM ($n = 8$), BK: 17 ± 1 nM ($n = 35$), $PGF_{2\alpha}$: 159 ± 5 nM ($n = 12$); femoral—SQ26655: 0.26 ± 0.01 nM ($n = 15$), U44069: 2.23 ± 0.05 nM ($n = 10$), BK: 55 ± 2 nM ($n = 22$), $PGF_{2\alpha}$: 501 ± 5 nM ($n = 17$). TXB_2 contracted the veins only at concentrations exceeding 10^{-6} M. PGD_2 (10^{-5} M) yielded negligible contractions in both veins. In order of potency, these agents were ranked: SQ26655

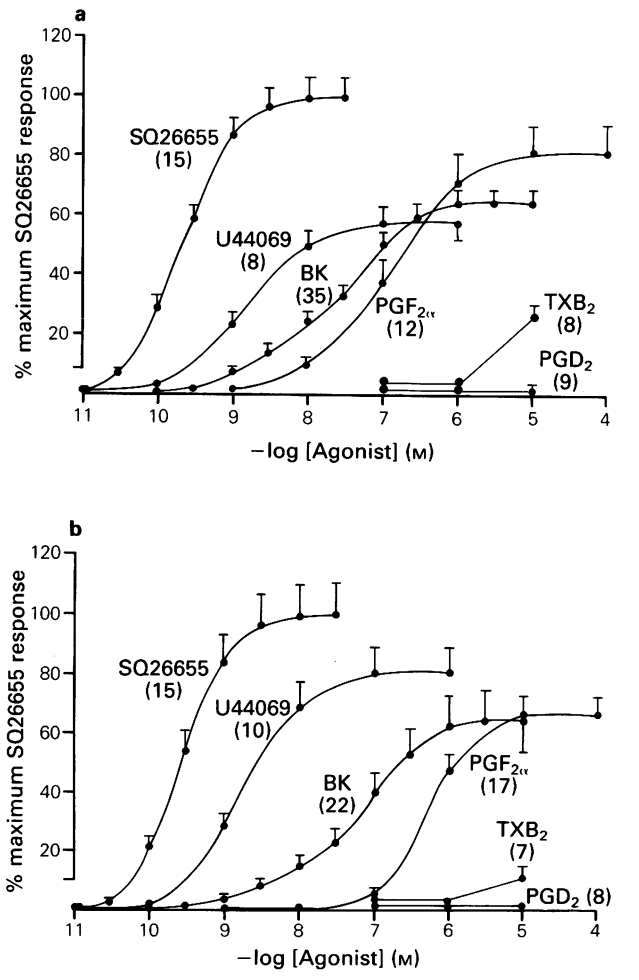

Figure 1 Contractile response of canine jugular and femoral veins to the thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) analogues SQ26655 and U44069, bradykinin (BK), $PGF_{2\alpha}$, TXB_2 and PGD_2. (a) Jugular; (b) femoral vein rings. All concentration-response curves are normalized to the average maximum SQ26655 response for each vein. For Figures 1, 2, 4 and 5A, all values are means with vertical lines showing s.e.mean and numbers in parentheses = number of rings used.

> U44069 > BK > $PGF_{2\alpha}$ > TXB_2 ≫ PGD_2 for both veins. SQ26655 and U44069 were respectively 700–1900 times and 100–200 times more potent than $PGF_{2\alpha}$. Of the above agonists, only the TXA_2/PGH_2 analogues exceeded BK in potency as vasoconstrictors of canine jugular and femoral vein.

Effect of cyclo-oxygenase inhibitors on BK-induced contractions

Canine jugular and femoral vein were tested with 2 inhibitors of the cyclo-oxygenase pathway, indomethacin and flufenamic acid (Flower, 1974), to determine the influence of BK-evoked cyclo-oxygenase products on contraction (Figure 2). Indomethacin (3×10^{-7} M) significantly reduced the maximum BK (10^{-6} M)-induced contractions to 32.5 ± 8.0% ($n = 11$) and 26.0 ± 7.5% ($n = 10$) of control responses in jugular and femoral vein, respectively. Flufenamic acid, a less potent cyclo-oxygenase inhibitor, showed a similar inhibitory action at 10^{-5} M by reducing BK-induced responses in the jugular and femoral vein to 29.1 ± 8.2% ($n = 9$) and 3.1 ± 1.8% ($n = 8$) of their respective control values.

Neither indomethacin nor flufenamic acid at 10^{-5} M had any significant effect on NA (10^{-5} M)-induced contraction (Figure 2). Indomethacin (10^{-5} M) also had negligible effect on contractions to submaximal NA concentrations as well as on contractions induced by U44069 (data not shown). Thus indomethacin and flufenamic acid specifically blocked a major portion of the contraction to BK in jugular and femoral vein, presumably through inhibition of cyclo-oxygenase metabolism.

Antagonism of TXA_2/PGH_2 receptors with SQ29548 and BM13177

SQ29548 is a potent, specific TXA_2/PGH_2 receptor antagonist in platelets and smooth muscle (Ogletree *et al.*, 1985). In canine jugular and femoral vein, SQ29548 (10^{-8} M) shifted the U44069 dose-response curve to the right, as shown by representative tracings in Figure 3a, indicating antagonism at the TXA_2/PGH_2 receptor.

Preincubation with SQ29548 (10^{-8} and 10^{-7} M) also significantly reduced the level of BK-induced contraction. As shown in Figure 4, SQ29548 (10^{-8} M) reduced tension elicited by 10^{-6} M BK to 14.1 ± 3.9% ($n = 9$) and 2.1 ± 1.0% ($n = 8$) of control for jugular and femoral veins respectively. SQ29548 (10^{-7} M) reduced the BK response to less than 10% in both veins.

A second selective TXA_2/PGH_2 receptor antagonist, BM13177 (Stegmeier *et al.*, 1984), likewise significantly antagonized U44069- (Figure 3b) as well as BK-induced contractions (Figure 4) in both vein preparations. BM13177 (10^{-6} M) reduced tension elicited by 10^{-6} M BK to 40.9 ± 6.4% ($n = 8$) and 33.0 ± 7.9% ($n = 7$) of control in jugular and femoral veins respectively. BM13177 (10^{-5} M) yielded further reductions to less than 25%.

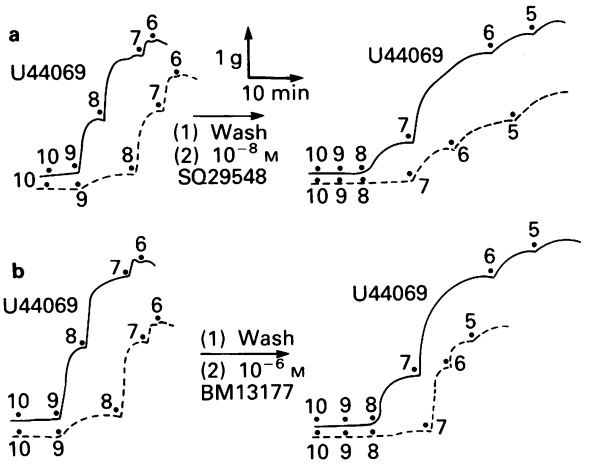

Figure 3 Effect of thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor antagonists SQ29548 and BM13177 on U44069 concentration-response curves in canine jugular and femoral veins, as shown by representative tracings. Numbers indicate cumulative agonist concentrations shown as −log[agonist]. Scale at top applies to all tracings. Jugular: solid lines; femoral: dashed lines. (a) Antagonism of U44069-induced contraction by 10^{-8} M SQ29548. (b) Antagonism of U44069-induced contraction by 10^{-6} M BM13177.

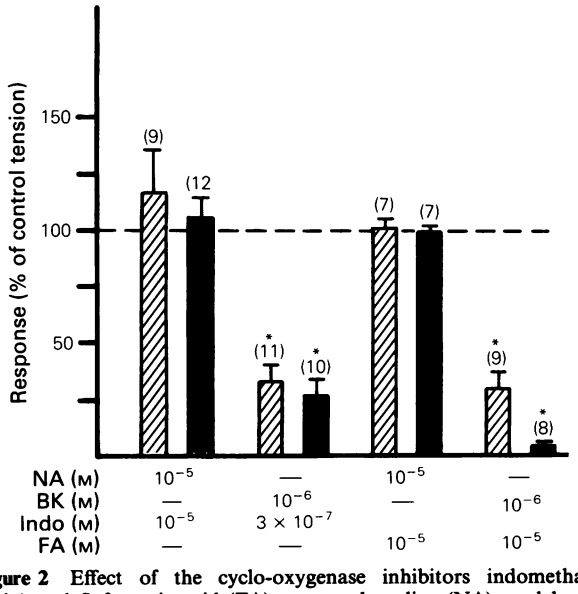

Figure 2 Effect of the cyclo-oxygenase inhibitors indomethacin (Indo) and flufenamic acid (FA) on noradrenaline (NA)- and bradykinin (BK)-induced contractions in canine jugular and femoral vein. The dotted line represents the normalized control response to NA or BK for each group of rings before incubation with a given inhibitor. Columns show contractile responses to NA or BK in presence of inhibitors as a % of control values. Hatched columns—jugular vein; solid columns—femoral vein. *Indicates significant difference ($P < 0.05$) between inhibited and control values.

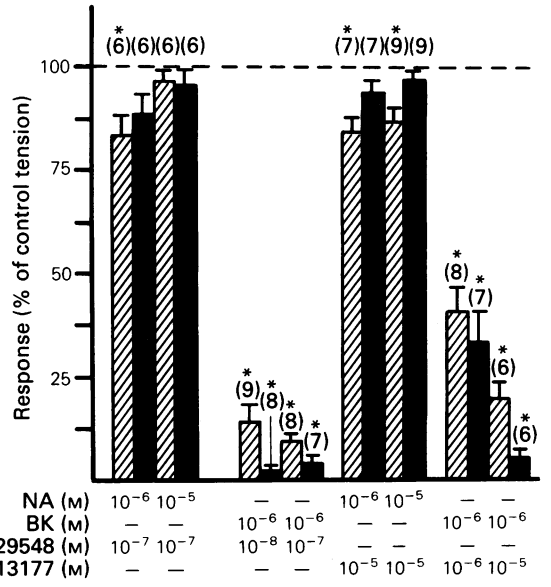

Figure 4 Effect of the thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor antagonists SQ29548 and BM13177 on noradrenaline (NA) and bradykinin (BK)-induced contractions in canine jugular and femoral vein. As in Figure 2, the dotted line represents the normalized control response to NA or BK for each group of rings before incubation with a given antagonist. Columns show responses to NA or BK in the presence of antagonists as a % of control values. Hatched columns—jugular vein. Solid columns—femoral vein. *Indicates significant difference ($P < 0.05$) between inhibited and control values.

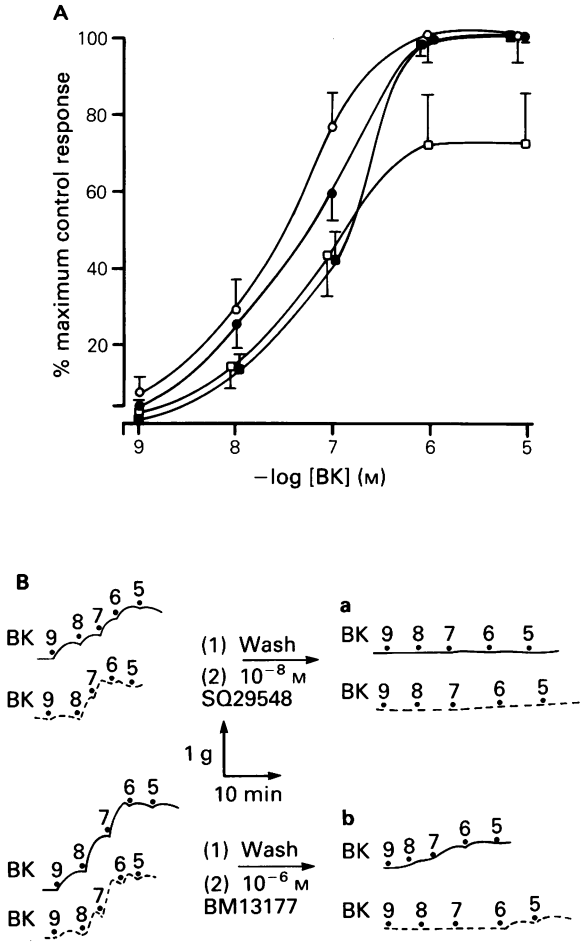

Figure 5 (A) Effect of endothelial removal on canine jugular and femoral vein contraction to bradykinin (BK). Control responses: jugular (●) (9); femoral (■) (11). Responses following endothelial removal: jugular (○) (9); femoral (□) (11). For each vein, all responses are normalized to the maximum control response to BK. See Methods for protocol for removal of endothelium. (B) Inhibitory effect of TXA_2/PGH_2 receptor antagonists 10^{-8} M SQ29548 (a) and 10^{-6} M BM13177 (b) on BK-induced contraction in vein rings denuded of endothelium. Numbers indicate cumulative concentrations shown as −log[BK] in representative tracings. Jugular: solid lines; femoral: dashed lines.

The inhibition by SQ29548 or BM13177 was also reversible. Thirty min after washout of either antagonist, both jugular and femoral veins largely regained their ability to contract to BK. In contrast to their inhibition of BK, high concentrations of SQ29548 (10^{-7} M) or BM13177 (10^{-5} M) had little effect on the NA-induced contraction which retained over 80% of its initial control tension in both veins (Figure 4).

Since $PGF_{2\alpha}$ can interact at TXA_2/PGH_2 receptors, the TXA_2/PGH_2 antagonists might be expected to have some effect against the $PGF_{2\alpha}$-induced contraction. SQ29548 (10^{-8} M) did inhibit contractions to $PGF_{2\alpha}$ (10^{-5} M) in both veins. The extent of inhibition (38.8 ± 3.4% ($n = 4$) for jugular and 43.6 ± 6.4% ($n = 4$) for femoral) was substantially less than that of BK-induced contraction.

Table 1 Effect of thromboxane synthase inhibitors dazoxiben and CGS12970 on bradykinin (BK)-induced contraction in canine jugular and femoral vein

	% control tension†	
*Condition**	*Jugular*	*Femoral*
10^{-5} M Dazoxiben	120.9 ± 8.2	126.6 ± 13.4
10^{-4} M Dazoxiben	90.4 ± 14.4	90.3 ± 16.7
10^{-5} M CGS12970	113.4 ± 10.0	130.5 ± 13.5

Each value is the mean ± s.e.mean of data from at least 3 animals. $n = 5$–6.

* Each ring was initially assayed with 10^{-6} M BK alone, relaxed, then incubated with inhibitor and reassayed with BK. † Values represent BK-induced tension in the presence of inhibitor expressed as % of tension to BK alone according to the formula:

$(Tension_{BK+inhibitor}/Tension_{BK\ alone}) \times 100$.

Influence of removal of the endothelium on BK-induced contractions

To determine the extent of endothelial influence on the contractile action of BK in canine jugular and femoral vein, BK concentration-response curves were obtained from vein rings before and after endothelium removal (see Methods). Removal of endothelium had a negligible effect on the BK response in both veins except for a slight but not-significant inhibition of femoral vein tension at 10^{-6} M and 10^{-5} M BK (Figure 5A). Likewise, endothelium removal had no pronounced effect on the inhibition of BK-induced contraction by SQ29548 (10^{-8} M) or BM13177 (10^{-6} M) in either vein (Figure 5B). Thus, the endothelium appears to exert little influence on the contraction of either vein by BK.

Effect of thromboxane synthase inhibitors on BK-induced contractions

Since a TXA_2/PGH_2 receptor antagonist cannot discriminate between vasoactive effects due to TXA_2 and PGH_2, 2 inhibitors of thromboxane synthase were tested to determine whether TXA_2 alone was responsible for the mediation of BK-induced contraction. The first inhibitor, dazoxiben (Randall *et al.*, 1981), had no significant ($P < 0.05$) inhibitory effects on BK (10^{-6} M)-induced contraction in either vein at 10^{-5} M or 10^{-4} M (Table 1). Similarly, the maximum BK-induced contraction was not reduced by CGS12970 (10^{-5} M), a second more potent thromboxane synthase inhibitor (Ambler *et al.*, 1985) (Table 1). In general, inhibition of thromboxane synthase, and hence of thromboxane production, did not significantly decrease the contractile response to BK in canine jugular and femoral vein.

Discussion

This study has shown that the major component of contraction to BK in 2 canine vein preparations may proceed via stimulation of the TXA_2/PGH_2 receptor. The rank order of agonist potency in both veins, showing the high potency of TXA_2/PGH_2 analogues vis a vis prostanoids such as $PGF_{2\alpha}$ and PGD_2 (Figure 1), resembles that for other vascular preparations shown to possess TXA_2/PGH_2 receptors (Coleman *et al.*, 1981; Tore *et al.*, 1984). This suggests the presence of TXA_2/PGH_2 receptors in both veins according to the classification of Kennedy *et al.* (1982).

Two cyclo-oxygenase inhibitors, indomethacin and flufenamic acid, reduced BK-induced contractions in canine jugular and femoral vein (Figure 2), implying a dependence of contraction on product(s) of the cyclo-oxygenase pathway. Indomethacin was effective at a low concentration of 3×10^{-7} M where non-specific interactions are unlikely (Flower, 1974). Neither inhibitor affected NA-induced contractions, indicating the absence of a general effect on calcium mobilization at the concentrations used.

SQ29548 is a TXA_2/PGH_2 receptor antagonist which selectively binds to TXA_2/PGH_2 receptors (Mihara *et al.*, 1989) and inhibits TXA_2 analogue-induced vascular contraction (Ogletree *et al.*, 1985; Darius *et al.*, 1985; Mihara *et al.*, 1989). It effectively blocked the BK-induced contraction in canine jugular and femoral veins at concentrations as low as 10^{-8} M (Figure 4). Inhibition of BK-induced contraction was likewise

demonstrated with the TXA_2/PGH_2 receptor antagonist BM13177, which at 3×10^{-6} to 3×10^{-5} M inhibits the TXA_2-mimetic U46619 in rabbit aorta (Stegmeier *et al.*, 1984) and rat perfused kidney (Quilley *et al.*, 1989). At 10^{-6} M, BM13177 reduced the BK-induced contraction by over 60%; at 10^{-5} M, inhibition exceeded 80% (Figure 4). These inhibitory effects of SQ29548 and BM13177 were reversible and apparently specific, implying that contraction in both veins due to activation of the BK receptor is largely mediated through the TXA_2/PGH_2 receptor, presumably by PGH_2 and/or TXA_2.

SQ29548 also moderately antagonized the venous contraction induced by $PGF_{2\alpha}$. The possibility cannot be rigorously excluded that a component of this antagonism occurs at $PGF_{2\alpha}$ as opposed to TXA_2/PGH_2 receptors. Nonetheless, the agonist potency rankings in Figure 1 imply that TXA_2/PGH_2 rather than $PGF_{2\alpha}$ receptors predominate in these preparations and mediate the action of $PGF_{2\alpha}$. Radioligand studies in pig aorta have shown that binding of [^{3}H]-U44619 to the TXA_2/PGH_2 receptor can be inhibited by $PGF_{2\alpha}$ (Mihara *et al.*, 1989). This interaction of $PGF_{2\alpha}$ with TXA_2/PGH_2 receptors can account for the SQ29548 antagonism of $PGF_{2\alpha}$ in canine jugular and femoral vein.

Previous studies have shown stimulation of $PGF_{2\alpha}$ synthesis by BK in mesenteric veins (Wong *et al.*, 1977; Limas, 1977), suggesting a possible role for $PGF_{2\alpha}$ in the mediation of BK-induced contraction. However, in vascular preparations containing TXA_2/PGH_2 receptors, $PGF_{2\alpha}$ is significantly less potent than PGH_2 and TXA_2 (Hamberg *et al.*, 1975b; Coleman *et al.*, 1981), the PGH_2 analogue U44069 (Tore *et al.*, 1984) and the TXA_2 mimetic U44619 (Mihara *et al.*, 1989). Similarly, $PGF_{2\alpha}$ as a vasoconstrictor of canine jugular and femoral vein is 10^2–10^3 times less potent than the TXA_2/PGH_2 analogues U44069 or SQ26655. Hence, relatively massive amounts of $PGF_{2\alpha}$ would be required to equal TXA_2 or PGH_2 in the potentiation of venous contraction to BK. Although $PGF_{2\alpha}$ may be synthesized in BK-stimulated canine jugular and femoral veins, its low potency compared to SQ26655, U44069 and BK makes it an unlikely mediator of contraction to BK in either vein. Mediation via PGD_2 is also unlikely in view of the negligible contractile response of either vein to PGD_2 (Figure 1).

Unlike their antagonism of U44069, inhibition of BK-induced contraction by SQ29548 and BM13177 appeared to be non-competitive (see Figure 5B). It could be argued that an excess of BK might generate sufficiently large amounts of endogenous TXA_2 and/or PGH_2 agonist to compete with the antagonists at the TXA_2/PGH_2 receptor and yield competitive inhibition. However, the higher potency of SQ26655 and U44069 versus BK (Figure 1) suggests that the maximum BK response is mediated by relatively small amounts of the endogenous agonists. Under these conditions, saturation of the BK receptor (with 10^{-5} M BK) is unlikely to generate sufficient endogenous agonists to counteract the inhibitory effect of the antagonists at the TXA_2/PGH_2 receptor. This would account for the apparent non-competitive inhibition of BK-induced contraction.

In response to varied pharmacological stimuli, endothelium can produce vasoactive metabolites such as PGI_2, TXA_2 and endothelium-derived relaxing factor (Shirahasi *et al.*, 1987; Gryglewski *et al.*, 1988). Therefore, it was important to establish the degree to which the contractile action of BK in canine jugular and femoral vein was modulated by endothelial factors. Though these veins show moderate endothelium-dependent relaxation to BK if preconstricted with NA, the relaxation is transient with tension regaining or exceeding preconstriction levels within 1 min (Aksoy *et al.*, 1984). In this study, removal of endothelium had no significant ($P < 0.05$) effect on the longer term BK-induced contraction in either vein (Figure 5A), though a slight reduction in the maximum femoral response suggests the marginal influence of an endothelium-derived vasoconstrictor. The action of the TXA_2/PGH_2 antagonists (Figure 5B) was also unaffected by endothelium removal, indicating that their principal site of action is on smooth muscle. Thus, the endothelium contributes little to the steady-state contractile action of BK or the effect of the TXA_2/PGH_2 antagonists.

In contrast to the pronounced antagonism of BK-induced contraction by SQ29548 and BM13177, high levels of the thromboxane synthase inhibitors dazoxiben and CGS12970 had no significant inhibitory effect (Table 1). One possible explanation is that, *in situ*, PGH_2 itself may be capable of contracting both veins. PGH_2 has a longer half-life than TXA_2 (5 min vs 32 s at 37°C and pH 7.4) (Hamberg *et al.*, 1975a), and is a potent vasoconstrictor (Hamberg *et al.*, 1975b; Goldberg *et al.*, 1977; Coleman *et al.*, 1981). The contractile action of accumulated PGH_2 resulting from inhibition of TXA_2 synthesis could therefore mask any reduction in contraction due to reduced TXA_2. Under these conditions, a thromboxane synthase inhibitor might not yield a decrease in the final contractile response to BK even if TXA_2 production were significantly reduced. It could therefore be concluded that both TXA_2 and PGH_2 normally mediated the contraction to BK. Alternatively, the thromboxane synthase inhibitors might be ineffectual because both veins may normally have negligible thromboxane synthase activity during BK-induced contraction. In this case, only PGH_2 would be the principal mediator of the contraction.

In blood platelets, the synthase inhibitor 1-carboxyheptyl imidazole reduces TXA_2 production without affecting arachidonic acid-induced platelet aggregation, implying that PGH_2 is a potential mediator of aggregation (Grimm *et al.*, 1981). In the vasculature, a recent study on rat perfused kidney has shown that the thromboxane synthase inhibitor CGS-13080 does not inhibit arachidonic acid-induced vasoconstriction, despite the demonstrated dependence of this contraction on TXA_2/PGH_2 receptor activation. The authors concluded that PGH_2 can mediate the vasoconstriction in conjunction with TXA_2 (Quilley *et al.*, 1989). *In situ*, PGH_2 may similarly function as a mediator of constriction to BK in certain vessels, either in conjunction with TXA_2 or when TXA_2 production is negligible.

In this study, BK-induced contraction in 2 anatomically distinct canine veins can apparently be mediated through the TXA_2/PGH_2 receptor. The applicability of this mechanism to other vessels remains to be determined. There may be anatomical and species variability in the mediator processes underlying BK-induced contraction due to differences in prostaglandin receptor type and number, as well as in the subtle balance of evoked cyclo-oxygenase products between potent vasoconstrictors (TXA_2 and PGH_2) and vasodilators (PGI_2). These differences would also influence the possible importance of PGH_2 as a mediator itself. Clearly, *in situ*, the role of TXA_2/PGH_2 receptors in the mediation of vascular smooth muscle contraction to BK and other agonists is an area which deserves further study.

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α-Methyldopa induces a naltrexone-insensitive antinociception and hypomotility in rats

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1 This study served to investigate whether endogenous opioid peptides play a role in the putative antinociceptive and the sedative actions of α-methyldopa.

2 In conscious normotensive rats, α-methyldopa induced hypotension, starting around 1 h and reaching a maximum 3–4 h after administration. Pretreatment with naltrexone resulted in an inhibition of α-methyldopa-induced hypotension.

3 α-Methyldopa dose-dependently increased hot plate latency which became evident after a 4 h lag period and reaching a maximum effect at 6 h. The antinociceptive effect of α-methyldopa was not affected by naltrexone.

4 In a small open field, α-methyldopa dose-dependently suppressed locomotion and sniffing behaviour. These effects of α-methyldopa were apparent 1 h after administration and were naltrexone-insensitive.

5 No changes in the level of β-endorphin-like immunoreactivity in plasma and cerebrospinal fluid were observed after administration of α-methyldopa.

6 The results indicate that endogenous opioid peptides are involved in the hypotensive action of α-methyldopa but not in α-methyldopa-induced hypomotility and antinociception.

Introduction

The antihypertensive substance α-methyldopa reduces blood pressure after neuronal uptake and metabolization to α-methylnoradrenaline, an α_2-adrenoceptor agonist, by a centrally mediated effect on the autonomic nervous system (for review see Henning, 1984). The α_2-receptors, that are stimulated by α-methylnoradrenaline, are presumably located in the nucleus tractus solitarii (NTS) (Nijkamp & De Jong, 1975; De Jong & Nijkamp, 1976).

Evidence is accumulating showing that endogenous opioid peptides play a role in the hypotensive mechanism of action of α-methyldopa and clonidine. Kunos and coworkers were the first to report that the opiate receptor antagonist, naloxone, inhibited clonidine-induced hypotension (Farsang & Kunos, 1979) and partially reversed the hypotension induced by α-methyldopa (Farsang *et al.*, 1980) in spontaneously hypertensive rats. In normotensive rats, intracisternal (Van Giersbergen & De Jong, 1988) and intra-NTS (Petty & De Jong, 1984) pretreatment with naloxone resulted in an inhibition of the decrease in blood pressure induced by administration via the same route of α-methyldopa and α-methylnoradrenaline, respectively. Since an antiserum against β-endorphin also inhibited α-methyldopa- (Van Giersbergen *et al.*, 1989b) and clonidine-induced hypotension (Ramirez-Gonzalez *et al.*, 1983; Van Giersbergen *et al.*, 1989a), this opioid peptide might be the endogenous ligand for the opiate receptors that are blocked by naloxone. Microinjection of low doses (pg range) of β-endorphin into the NTS resulted in hypotension (Petty & de Jong, 1982). It was postulated that the release of β-endorphin, probably in the area of the NTS, is a step in the process by which α_2-adrenoceptor agonists reduce blood pressure (Kunos *et al.*, 1981). In fact, both clonidine and α-methylnoradrenaline stimulate the release of β-endorphin-like immunoreactivity (β-ELIR) from brainstem slices of spontaneously hypertensive rats (Kunos *et al.*, 1981). This was an *in vitro* study and, as yet, no data are available on the effects of α-methyldopa on β-ELIR levels in normotensive rats *in vivo*.

It has been suggested that a release of endogenous opioid peptides in the central nervous system is also involved in the antinociceptive action of clonidine (Tchakarov *et al.*, 1985; Mastrianni *et al.*, 1989). Such an action of clonidine has been long known (Schmitt *et al.*, 1973; Paalzow & Paalzow, 1976) and recently it was demonstrated that naloxone inhibited clonidine-induced antinociception in both normotensive and hypertensive rats (Tchakarov *et al.*, 1985). Since α-methylnoradrenaline could also induce a release of β-ELIR (see above), α-methyldopa might cause a naltrexone-sensitive antinociception. At the moment, no data are available on the putative antinociceptive effects of α-methyldopa.

In the present study, using normotensive rats, we investigated, besides α-methyldopa-induced hypotension, the postulated antinociceptive effect and the sedative action of α-methyldopa. Our attention focussed on the question whether endogenous opioid peptides are involved in the observed effects. In addition, the effects of α-methyldopa on the level of β-ELIR in cerebrospinal fluid (CSF) and plasma were studied.

Methods

Animals

Male Wistar rats of an inbred strain (Cpb: WU) weighing between 200 and 250 g were used for the experiments. They were housed under standard conditions (room temperature 21°C, light on from 06 h 00 min till 20 h 00 min) and received food pellets and water *ad libitum*.

Blood pressure measurement

The left femoral artery was cannulated under ether anaesthesia (Van Giersbergen & De Jong, 1988). After surgery, the rats were housed individually. On the days of the experiments, 1 and 3 days after cannulation of the artery, the rats were brought to the experimental room and allowed 30 min to adjust to the environment. Then, the catheter was connected to a Statham pressure transducer (P23AC) and arterial pressure was displayed on a Grass polygraph (model 79C and 7D).

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Heart rate was calculated from the blood pressure recording using a 6 s period of fast chart speed. Basal blood pressure and heart rate were determined during a period of 30 min.

To assess the antagonistic activity of naltrexone on the hypotension induced by α-methyldopa, rats were pretreated with this opiate antagonist (2 mg kg^{-1}, i.p.) or with saline followed 20 min later by the i.p. injection of 200 mg kg^{-1} of α-methyldopa. The animals were used twice. On the second experimental day, 3 days after surgery, the pretreatment was reversed. Thus, the effect of α-methyldopa on blood pressure was tested twice in each animal, once after saline and once after naltrexone. Arterial blood pressure was recorded continuously for 8 h after the administration of α-methyldopa and each 30 min heart rate was determined.

Collection of CSF

For this purpose a stainless steel cannula was implanted into the cisterna magna according to a method developed by Bouman & Van Wimersma Greidanus (1979). Briefly, rats were anaesthetized with Hypnorm (fentanyl 0.2 mg ml^{-1}; fluanison 10 mg ml^{-1}; 1 ml kg^{-1}, i.m.) and the head was fixed in a stereotaxic apparatus. After exposure of the skull, three holes were drilled, two for stainless steel screws and one for the cannula. The cannula was fixed with dental acrylic cement (Biofast D.L., Dental CCO, Richmond, N.Y., U.S.A.). After surgery the rats were housed individually. Each day after cannulation the rats were given a sham i.p. injection, the needle was introduced but no fluid was injected, followed by the collection of approximately 50 μl of CSF using PE-100 tubing attached to a 100 μl syringe. This procedure was followed for two reasons: (1) To keep the cannula patent, and (2) to accustom the rats to this procedure. The experiments in which CSF was collected were carried out 4 and 7 days after the intracisternal cannulation.

Behavioural tests

For behavioural testing the rats were transferred to a sound-attenuated room at least 1 h before the start of the experiment. The actual behavioural testing was performed in all experiments between 14 h 00 min and 18 h 00 min. The animals were used once. To assess the activity of rats they were placed in a circular perspex test cage (diameter 19.5 cm; height 28.5 cm), the bottom of which was divided into 4 sections of equal size. In this small open field locomotion (number of sections explored), rearing, standing on the hind legs, grooming (number of demonstrations) and sniffing (total time) were determined during a 3 min observation period (Van Ree & Wolterink, 1981). The hot plate method (Eddy & Leimbach, 1953) was used to investigate the antinociceptive action of α-methyldopa. The rats were placed on a hot plate which was kept at 54°C and the latency of the first nociceptive response was measured. Licking of the paws or fast stamping was the criterion of the response. Cutoff time in the absence of a response was 30 s. The rats were placed on the hot plate 60 and 30 min before the first injection to determine basal latency, and once after the injection to assess the effects of the administered substance(s).

Protocols

Two groups of rats with an intracisternal cannula were prepared. On the day of the experiment the rats were transported to the quiet experimental room and allowed 1 h to adjust. Then, α-methyldopa (200 mg kg^{-1}, i.p.) or saline was injected. The first group was used to study the effect of α-methyldopa on the CSF level of β-ELIR. At several times after administration (15–240 min) CSF was collected. For each time point a separate group of rats was used and the animals were used twice. On the first day, 4 days after the intracisternal cannulation, the rats received α-methyldopa or saline. This treatment was reversed during a second experiment 3 days later. CSF was collected in ice-chilled tubes, centrifuged at 4°C for 15 min at 2000*g* and the supernatant was stored at −20°C until further processing. In the second group of animals the effect of α-methyldopa on plasma β-ELIR content was determined. At several time points after drug or saline administration (30–240 min) the rats were decapitated and trunk blood was collected in ice-chilled polypropylene tubes containing 0.2 ml 50 mM EDTA. Plasma was prepared by centrifugation at 2000*g* for 10 min at 4°C and stored at −20°C until further processing.

The effects of α-methyldopa (200 mg kg^{-1} i.p.) on small open field behaviour and hot plate latency were tested between 1 and 8 h after injection. A separate group of rats, receiving α-methyldopa or saline, was used for each time point. To construct a dose-response curve rats were treated with α-methyldopa (50–400 mg kg^{-1} i.p.) or saline and behavioural testing was performed 4 h after drug administration.

To test whether the observed behavioural and antinociceptive effects of α-methyldopa were mediated by activation of opiate receptors, the influence of naltrexone was tested. A dose of 2 mg kg^{-1} i.p. of naltrexone was chosen since this dose effectively antagonized both the antinociception induced by morphine as measured on the hot plate (data not shown) and α-methyldopa-induced hypotension (see Results). Naltrexone or saline was administered 30 min before the injection of α-methyldopa or 30 min before the behavioural testing which was performed 4 h after α-methyldopa administration. It has been shown that the dose of α-methyldopa in relation to the dose of antagonist is an important factor in establishing an antagonistic action of naltrexone (Van Giersbergen & De Jong, 1988). Therefore, doses of 100 and 200 mg kg^{-1} of α-methyldopa, that induced reproducible effects, were used in the small open field and hot plate experiments, respectively.

Extraction of β-endorphin-like immunoreactivity from plasma and radioimmunoassay

β-Endorphin and related peptides were extracted and concentrated from plasma according to the method described by Ratcliffe & Edwards (1971) with slight modifications (Barna *et al.*, 1988). The recovery of the extraction procedure was monitored by simultaneous extraction of ^{125}I-labelled β-endorphin and amounted to 82%. Radioimmunoassays were performed as described elsewhere (Barna *et al.*, 1988). The antiserum (B4) was raised in rabbits against human β-endorphin. This antiserum was directed against the (9–16) sequence of the β-endorphin molecule. Synthetic camel β-endorphin was used as standard and iodinated camel β-endorphin as tracer. With the B4 antiserum the following cross-reactivities (expressed as % on mass basis) were obtained: human β-LPH, 39%; camel β-endorphin, 100%; γ-endorphin, 391%; α-endorphin, 170%; α-MSH, 0.7%. Cross-reactivity with ACTH, [Met^5]enkephalin, dynorphin A(1–13), α-methyldopa and α-methylnoradrenaline was <0.2%. All samples were assayed in duplicate.

Drugs

(±)-Naltrexone HCl (Du Pont Pharmaceuticals, Wilmington, Delaware, U.S.A.) and (−)-α-methyldopa (Merck, Sharpe and Dohme, Haarlem, The Netherlands) were dissolved in physiological saline (0.9% NaCl) and injected i.p. in a volume of 1 and 12 ml kg^{-1} body weight, respectively.

Statistical analysis

Values for mean arterial pressure (MAP) and heart rate are shown in Figure 2 as a change from the base-line values. MAP was calculated from the blood pressure recording by the equation:

$$\text{MAP} = \text{diastolic blood pressure} + 2/5\,(\text{systolic} - \text{diastolic blood pressure}).$$

All values presented are means ± s.e. The data shown in Figures 1, 3, and 4, and in the tables are analyzed by one-way

analysis of variance (ANOVA) followed by Student-Newman-Keuls test; Student's t test was used for the other data. A value of P less than 0.05 was considered to indicate a significant difference.

Results

Blood pressure experiment

A dose of 200 mg kg^{-1} of α-methyldopa potently decreased blood pressure as can be seen in Figure 1. This hypotension did not become evident until 2 h after administration and reached a maximum between 3 and 4 h after injection. Blood pressure returned to base-line value at about 8 h after administration (data not shown). The fall in blood pressure was accompanied by a tachycardia which was maximal at 1 h after drug administration and, thereafter, heart rate began to return to the base-line value (Figure 1). Pretreatment with the opiate receptor antagonist naltrexone (2 mg kg^{-1}, i.p.) resulted in a significant inhibition of α-methyldopa-induced hypotension (-7.7 ± 4.5 mmHg as compared to -21.4 ± 1.8 mmHg in saline-treated controls, $P < 0.05$), whereas the tachycardia was not affected by naltrexone (Figure 1). Administration of naltrexone or saline alone did not result in changes in blood pressure or heart rate (data not shown).

Hot plate experiments

Administration of α-methyldopa (200 mg kg^{-1}, i.p.) to male Wistar rats resulted in an increased hot plate latency but the effect did not become evident until 4 h after administration (Figure 2). The maximal response was reached 6 h after drug administration and the effect was still present 8 h after injection (Figure 2). Figure 3 shows a dose-response curve of the antinociceptive action of α-methyldopa. Doses of 50 and 100 mg kg^{-1} tended to increase hot plate latency. This increase became significantly different from the saline-treated rats at a dose of 200 mg kg^{-1} (Figure 3). Treatment of the animals with naltrexone 30 min before the administration of α-methyldopa, or 30 min before the hot plate test, 3.5 h after α-methyldopa, had no influence on α-methyldopa-induced antinociception (Figure 4). The hot plate latency of animals treated with naltrexone and saline did not differ from that of saline-saline-treated controls (Figure 4).

Small open field experiments

Administration of α-methyldopa (200 mg kg^{-1}, i.p.) markedly depressed small open field behaviour. As is illustrated in Figure 5, this dose of α-methyldopa significantly decreased locomotion and sniffing while rearing and grooming were also depressed, but this depression did not reach, in general, statistical significance. These effects of α-methyldopa were already apparent 1 h after drug administration, reached a maximum 3–4 h after injection and lasted for at least 8 h (Figure 5). The behavioural effects of α-methyldopa were dose-dependent. As can be seen in Table 1, a dose of 50 mg kg^{-1} decreased total sniffing time and tended to inhibit locomotion at 4 h after injection. Both parameters were inhibited by the two higher doses of α-methyldopa (100 and 200 mg kg^{-1}). Rearing and grooming were not significantly affected. In contrast to α-methyldopa-induced hypotension, pretreatment with naltrexone (data not shown) as well as treatment with this opiate antagonist 3.5 h after administration of α-methyldopa (Table 2) did not influence the suppression of locomotion and sniffing caused by α-methyldopa. The small open field behaviour remained unchanged after the injection of naltrexone alone (Table 2).

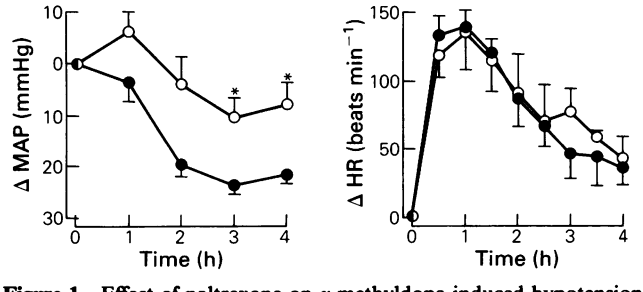

Figure 1 Effect of naltrexone on α-methyldopa-induced hypotension and tachycardia in conscious rats. Naltrexone (○, 2 mg kg^{-1}, i.p.) or saline (●) was injected 20 min before the administration of α-methyldopa 200 mg kg^{-1}, i.p. Mean arterial pressure (MAP, mmHg) was recorded continuously for 4 h and heart rate (HR, beats min^{-1}) was determined once every 30 min. Data represent change from base-line value and are presented as means with s.e. indicated by vertical bars. *$P < 0.05$ (ANOVA followed by Student-Newman-Keuls test) as compared to saline-pretreated controls.

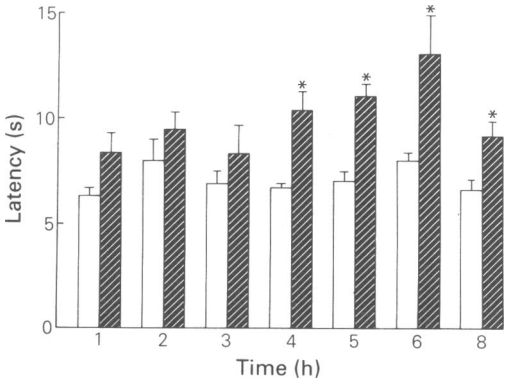

Figure 2 Time course of the antinociceptive action of α-methyldopa. Rats were placed on a hot plate at 54°C, 60 and 30 min before the injection of α-methyldopa (hatched columns, 200 mg kg^{-1}, i.p.) or saline (open columns) to determine the basal response latency and once after drug administration. The latency (s) after treatment is depicted and each column represents mean (with s.e. indicated by vertical bars) of a separate group of 6–7 animals. *$P < 0.05$ (Student's t test) as compared to saline-treated controls.

Effect of α-methyldopa on CSF and plasma β-endorphin-like immunoreactivity levels

At several time points after the administration of α-methyldopa (200 mg kg^{-1}, i.p.), corresponding to the time

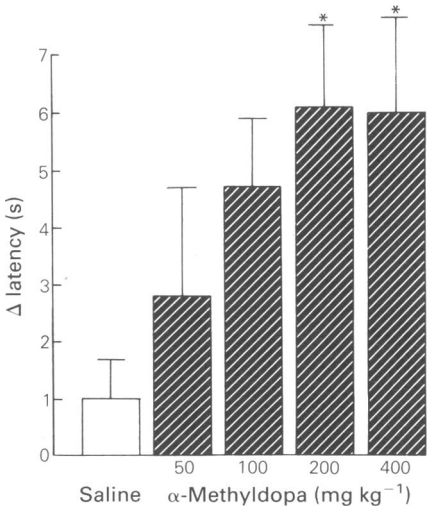

Figure 3 Dose-response curve of the analgesic action of α-methyldopa. Rats were placed on a hot plate at 54°C 60 and 30 min before the injection of α-methyldopa (50–400 mg kg^{-1}, i.p.) or saline to determine the basal response latency and 4 h after drug administration. The change in latency (s) as compared to the basal response was determined and each dose group consisted of 6–7 animals. Data represent change from the basal response latency (s) and are presented as means (s.e. indicated by vertical bars). *$P < 0.05$ (ANOVA followed by Student-Newman-Keuls test) as compared to saline-treated controls.

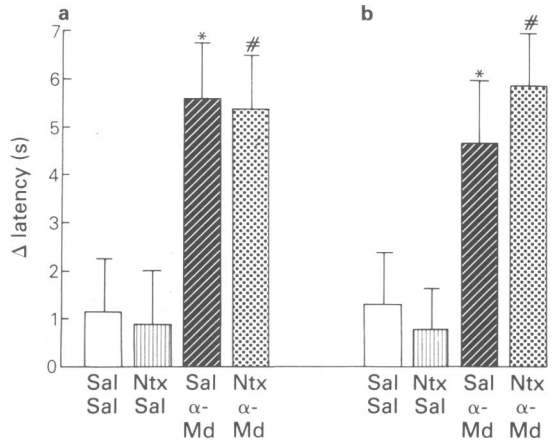

Figure 4 Effect of naltrexone on the antinociceptive action of α-methyldopa. Rats were treated with naltrexone (Ntx, 2 mg kg^{-1}, i.p.) or saline (Sal) 30 min before the injection of α-methyldopa (α-Md, 200 mg kg^{-1}, i.p.) or saline (a), or with naltrexone or saline 3.5 h after α-methyldopa (b), and tested on the hot plate 4 h after α-methyldopa administration. Basal hot plate latencies were determined 60 and 30 min before the first injection. The latency (s) to the first response was determined and each group consisted of 6–7 animals. Data represent the change from the basal response latency and are presented as means (s.e. shown by vertical bars). $^{*,\#}P < 0.05$ (ANOVA followed by Student-Newman-Keuls test) as compared to saline- or naltrexone-treated controls, respectively.

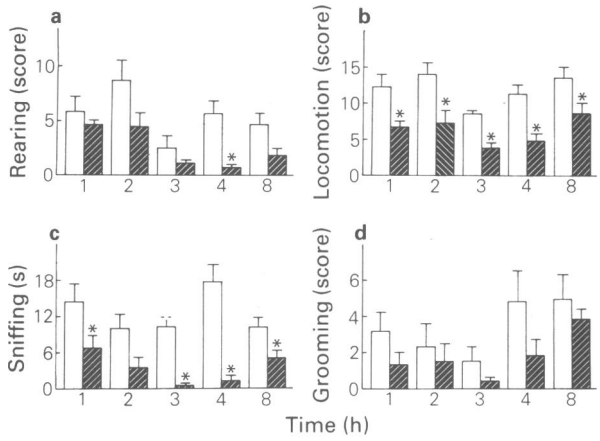

Figure 5 Time course of the behavioural effects of α-methyldopa. Naive rats were treated with α-methyldopa (hatched columns, 200 mg kg^{-1}, i.p.) or saline (open columns) and were placed in a small open field. During 3 min, (a) rearing, (b) locomotion, (d) grooming and the time spent on (c) sniffing (s) were scored. Data are presented as means, each column represents a separate group of 6–7 animals (vertical bars show s.e.). $^{*}P < 0.05$ (Student's t test) as compared to saline-treated controls.

curve of the hypotensive effect of α-methyldopa, the concentration of β-ELIR in both plasma and CSF was determined. Neither in the first hour, 15, 30, 45 and 60 min after administration, when the interaction between α-methyldopa and the endogenous opioids is suspected to take place (Van Giersbergen & De Jong, 1988), nor at the time point of the maximal fall in blood pressure, 3–4 h after drug administration, was the level of β-ELIR in CSF affected by α-methyldopa (data not shown). The concentration of β-ELIR in CSF ranges from 221–587 pg ml^{-1} in the saline-treated controls. Throughout the study, the concentration of β-ELIR in plasma was lower than in CSF (119 ± 3.6 pg ml^{-1} as compared to 352 ± 20.9 pg ml^{-1}). α-Methyldopa had no significant effect on the concentration of β-ELIR as measured in plasma 60, 90, 120 and 240 min after administration of α-methyldopa (data not shown). The level of β-ELIR in plasma tended to be increased by α-methyldopa 30 min after the injection of the antihypertensive drug (data not shown).

Discussion

The present data indicate that activation of opiate receptors does not play a role in the hypomotilic and antinociceptive action of α-methyldopa. On the other hand, naltrexone was able to inhibit α-methyldopa-induced hypotension which confirms earlier findings (Van Giersbergen & De Jong, 1988) of an involvement of endogenous opioids in the cardiovascular effects of this centrally acting antihypertensive drug.

Pretreatment with naltrexone resulted in an inhibition of the fall in blood pressure after i.p. administration of α-methyldopa, whereas the accompanying tachycardia was not affected by the opiate antagonist. Earlier studies have shown that intracisternal administration of opiate antagonists and of a β-endorphin antiserum inhibited the hypotension and bradycardia induced by α-methyldopa administered via the same route (Van Giersbergen & De Jong, 1988; Van Giersbergen *et al.*, 1989b). In contrast to central administration, peripheral injection of α-methyldopa causes tachycardia which gradually returns to the base-line value. This tachycardia is probably the result of a direct action of α-methyldopamine, a metabolite of α-methyldopa, on the heart (Van der Maas *et al.*, 1986) and endogenous opioids do not mediate this response, as shown by the lack of effect of naltrexone. Since the combination of the dose of naltrexone and the dose of α-methyldopa is a critical factor in demonstrating a naltrexone-α-methyldopa interaction (Van Giersbergen & De Jong, 1988), it is important to note that the doses of these substances used in the present study do reveal such an interaction with respect to blood pressure.

To our knowledge this is the first time that an antinociceptive action of α-methyldopa has been reported. The lag period before the appearance of the increase in hot plate latency might be the reason why an antinociceptive effect of α-methyldopa has not been reported earlier. The antinociceptive effect of α-methyldopa is dose-dependent over a dose-range similar to the range of doses that induce hypotension. However, the time course of the antinocicepetive response appears to be different from that of the cardiovascular events, suggesting different underlying mechanisms. This notion is further supported by the observation that naltrexone did not influence α-methyldopa-induced antinociception whereas it inhibited α-methyldopa-induced hypotension. These data, therefore, indicate that endogenous opioid peptides are not involved in the antinociceptive action of α-methyldopa.

Table 1 Dose-response relationship of the behavioural suppressive effect of α-methyldopa (50–200 mg kg^{-1}, i.p.)

Treatment	*Dose* (mg kg^{-1})	*Locomotion* (score)	*Sniffing* (s)	*Rearing* (score)	*Grooming* (score)
Saline		12.1 ± 1.6	11.5 ± 2.6	3.4 ± 1.3	1.6 ± 0.7
α-Methyldopa	50	9.5 ± 1.4	3.4 ± 1.3*	3.6 ± 1.3	4.3 ± 1.0
α-Methyldopa	100	7.8 ± 0.9*	1.4 ± 0.5*	1.9 ± 0.4	1.4 ± 0.4
α-Methyldopa	200	5.8 ± 1.1*	0.3 ± 0.2*	2.0 ± 0.7	1.3 ± 0.6

Rats were tested in a small open field 4 h after administration of α-methyldopa. Data are presented as means ± s.e. ($n = 8$). $^{*}P < 0.05$ as compared to saline-treated controls (ANOVA followed by Student-Newman-Keuls test).

Table 2 Effect of naltrexone (2 mg kg^{-1}, i.p.) administered 30 min before the small open field test on the behavioural response to α-methyldopa (100 mg kg^{-1})

Treatment	*Dose* (mg kg^{-1})	*Locomotion* (score)	*Sniffing* (s)	*Rearing* (score)	*Grooming* (score)
Saline + saline		12.8 ± 1.0	17.4 ± 1.4	5.0 ± 1.2	3.2 ± 0.9
Naltrexone + saline	2	11.7 ± 0.8	21.3 ± 3.0	5.3 ± 0.8	1.7 ± 1.7
Saline + α-Methyldopa	100	7.8 ± 0.8*	2.7 ± 1.2*	3.0 ± 1.0	3.8 ± 1.7
Naltrexone + α-Methyldopa	2 100	7.0 ± 0.9†	0.8 ± 0.3†	2.5 ± 0.7	1.8 ± 0.5

Rats were tested in a small open field 4 h after administration of α-methyldopa. Data are presented as means ± s.e. ($n = 6$).
*$P < 0.05$ as compared to saline-saline-treated controls.
†$P < 0.05$ as compared to naltrexone-saline-treated controls (ANOVA followed by Student-Newman-Keuls test).

Several studies have demonstrated an antinociceptive action of clonidine in mice (Schmitt *et al.*, 1973) and other animal species (Skingle *et al.*, 1982), and in man (Tamsen & Gordh, 1984; Coombs *et al.*, 1985). This effect of clonidine appears to be centrally mediated (Skingle *et al.*, 1982). In the literature the evidence for a clonidine-naloxone interaction regarding antinociception is controversial. Several reports show no effect of naloxone (Paalzow & Paalzow, 1976; Fielding *et al.*, 1978; Dennis *et al.*, 1980) whereas in others naloxone inhibited the antinociceptive effect of clonidine (Lin *et al.*, 1980; Tchakarov *et al.*, 1985; Nakamura *et al.*, 1988; Mastrianni *et al.*, 1989). In part, these controversial results may be explained by the use of high doses of clonidine, that are in excess of the dose range that induces hypotension, which might mask a possible involvement of endogenous opioids. Tchakarov *et al.* (1985) demonstrated in rats that naloxone inhibited the antinociceptive response to low doses but not to a high dose of clonidine. On the other hand, a great variety of pain tests have been employed in which various neuronal pathways are involved. It is possible that in one pathway opioids play a role whereas in another one an opioid component is absent (Watkins & Mayer, 1982).

The outcome of the hot plate test might be influenced by the sedative action of α-methyldopa. Such an action is well documented (Sjoerdsma, 1963; Clineschmidt *et al.*, 1980) and, therefore, the effects of α-methyldopa on the behaviour of rats in a small open field were investigated. Already 1 h after administration, α-methyldopa significantly decreased motor activity as shown by a lower locomotion score. The time-effect relationship of α-methyldopa-induced hypolocomotion is in agreement with an earlier observation (Clineschmidt *et al.*, 1980). Although locomotion is disturbed the first 3 h after the injection of α-methyldopa, the rats displayed a latency on the hot plate during this period not different from that of the saline-treated controls. Therefore, the sedative action of α-methyldopa appears to contribute little to the antinociceptive effect. Clineschmidt *et al.* (1980) demonstrated using α-adrenoceptor antagonists and FLA-63, a dopamine-β-hydroxylase inhibitor, that α-methyldopa reduces locomotor activity via the formation of α-methylnoradrenaline and subsequent stimulation of α_2-receptors. In contrast to its hypotensive effect, the hypolocomotive action of α-methyldopa is naltrexone-resistent. In addition, the hypothermia induced by α-methyldopa in conscious rats, that might play a role in the hypolocomotive mechanism of action of α-methyldopa, is also resistant to treatment with naltrexone (Sitsen & Nijkamp, 1984). Together, these results indicate that endogenous opioid peptides are not involved in the behavioural effects of α-methyldopa.

At present, the site(s) of action through which α-methyldopa induces analgesia and hypolocomotion are not known. α-Methylnoradrenaline acts on α_2-adrenoceptors, probably located in the NTS, to induce hypotension (Nijkamp & De Jong, 1975). Conway *et al.* (1979) demonstrated that in the NTS, but not in several other medullary and hypothalamic nuclei, after administration of 200 mg kg^{-1} of α-methyldopa to rats the accumulation and disappearance of α-methylnoradrenaline correlates with the time course of the hypotensive effect. The accumulation and disappearance of α-methyldopa and its metabolites, and the α-methyldopa-induced changes in the levels of endogenous neurotransmitters, might be factors which determine the time course of an effect of α-methyldopa. For example, the time-effect relationship of the antinociceptive effect of α-methyldopa, which is characterized by a 4 h lag period, could be determined by the above mentioned factors at the site of action.

Endogenous opioid substances are present in CSF (Kiser *et al.*, 1983; Jackson *et al.*, 1985). The concentration in CSF of pro-opiomelanocortin-derived peptides like β-endorphin appears to reflect release from the brain (Akil *et al.*, 1978; Hosobuchi *et al.*, 1979) and is not influenced by changes in the concentration of these peptides in plasma (Kalin *et al.*, 1987; Barna *et al.*, 1988). Administration of α-methyldopa to conscious rats had no effect on the concentration of β-ELIR in either plasma or CSF at various time points after drug administration. An increase in plasma β-ELIR has been implicated in the hypotensive mechanism of clonidine (Farsang *et al.*, 1984). However, in other studies hypotensive doses of clonidine did not affect (Levin *et al.*, 1986) or lowered (Yasunari *et al.*, 1985) the level of plasma β-ELIR. Therefore, a change in the concentration of β-ELIR in plasma appears not to be a step in the mechanism via which clonidine lowers blood pressure. This notion is supported by the present results showing no effect of a hypotensive dose of α-methyldopa on the level of β-ELIR in plasma. The lack of an effect of α-methyldopa on the CSF β-ELIR concentration might suggest that α-methyldopa does not induce release of β-endorphin in the brain *in vivo*. However, an α-methyldopa-induced increased release of β-endorphin in a restricted brain area, such as the NTS, which is not reflected by a change in CSF, may still occur.

In summary, α-methyldopa induces hypotension, antinociception and hypomotility without affecting the level of β-ELIR in either plasma or CSF. Naltrexone inhibited α-methyldopa-induced hypotension but not antinociception and hypomotility. Therefore, it is concluded that α-methyldopa induces hypotension, antinociception and hypomotility via (at least in part) different mechanisms. Endogenous opioid peptides play a role in the hypotensive action of α-methyldopa, whereas they appear not to be involved in the sedative and antinociceptive effects induced by α-methyldopa.

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Suppression by neuropeptide Y of capsaicin-sensitive sensory nerve-mediated contraction in guinea-pig airways

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1 In the present study we have examined whether neuropeptide Y (NPY) interferes with non-adrenergic, non-cholinergic nerve-mediated contractions and relaxations in the guinea-pig airways. In these experiments we have used ring preparations of bronchi and trachea, incubated in the presence of atropine, propranolol and indomethacin (each 1 μM).

2 The contractile response to electrical stimulation of non-adrenergic, non-cholinergic nerve fibres was suppressed by NPY and NPY 13–36 in a concentration-dependent manner, these agents having similar inhibitory potencies. NPY caused a more complete inhibition than the C-terminal fragment.

3 NPY affected neither the basal tension nor the substance P-evoked contraction in the bronchi and trachea and did not interfere with nerve-mediated, non-adrenergic relaxation in the trachea.

4 On the basis of these results, it is suggested that NPY may act on the terminals of sensory neurones in the airways to prevent antidromic, excitatory neurotransmission by inhibiting transmitter release.

Introduction

Neuropeptide Y (NPY) is a peptide containing 36 amino acids. It has a wide distribution in the peripheral nervous system. It is present in many sympathetic neurones where it is co-localized with noradrenaline (NA). NPY causes vasoconstriction *per se* and enhances NA-evoked vasoconstriction (for a review see Edvinsson *et al.*, 1987). The effects of NPY are mediated via postjunctional receptors, tentatively referred to as Y_1-receptors (Wahlestedt *et al.*, 1986). In addition, NPY suppresses the stimulated release of NA from sympathetic nerve terminals. This effect is prejunctional and mediated via Y_2-receptors which can be activated by the C-terminal 13–36 fragment of NPY, which is not recognized by the Y_1-type receptor (Wahlestedt *et al.*, 1986; 1987).

In the tracheobronchial wall, NPY fibres are fairly numerous around arteries, seromucous glands and in the smooth muscle (Uddman *et al.*, 1984). NPY has been shown to suppress parasympathetic nerve activity in the trachea (Grundemar *et al.*, 1988; Stretton & Barnes, 1988), uterus (Stjernquist *et al.*, 1983), heart (Potter, 1985) and ileum (Garzon *et al.*, 1986). Electrical field stimulation of guinea-pig bronchi elicits nerve-mediated contractions, that consist of an atropine-sensitive and an atropine-resistant component (Grundström *et al.*, 1981). The latter response is mediated by excitatory non-adrenergic, non-cholinergic (excitatory NANC) nerves. NA is known to suppress these contractions by an action on prejunctional α_2-adrenoceptors (Grundström *et al.*, 1984; Grundström & Andersson, 1985). Electrical field stimulation of the guinea-pig trachea elicits nerve-mediated relaxations that also can be divided into two components. Part of the response is sensitive to antagonists at β-adrenoceptors and to adrenergic neurone-blocking agents. The remaining component is mediated by inhibitory non-adrenergic, non-cholinergic (inhibitory NANC) nerves (Coburn & Tomita, 1973; Grundström *et al.*, 1981).

The purpose of the present sutdy was to see whether NPY interferes with excitatory or inhibitory NANC neurotransmission in the guinea-pig airways.

Methods

Male guinea-pigs weighing 300–600 g were used. The lungs including the trachea and the bronchi were removed and placed in Krebs solution equilibrated with 95% O_2 and 5% CO_2. Ring segments, 2–3 mm in length, were obtained from the upper part of the main bronchi and from the lower part of the trachea. The preparations were mounted on special holders (Grundström *et al.*, 1981) for measurement of isometric tension and placed in a 5 ml organ bath containing Krebs solution at 37°C. After an equilibration period of 60–90 min a resting tension of 1.5–3 mN was attained. Platinum wire electrodes were placed on either side of the preparation and a Grass S88 stimulator was used to produce electrical field stimulation. Monophasic square waves of 1 ms duration at 40 V and 2 Hz were used. The pulse trains had a duration of 30 s and were delivered once every 15 min when contractions were studied and once very 5 min when relaxations were studied. The buffer solution contained propranolol 1 μM and atropine 1 μM. Indomethacin 1 μM was added because it improves the reproducibility of the contractile response to electrical stimulation (Szolcsányi & Barthó, 1982). The peptides were added cumulatively with each concentration increment being given 10–12 min before the next stimulated contraction.

The pD_2 or $-\log(EC_{50})$ values were estimated from the concentration-response curves by fitting the logistic function (Parker & Waud, 1971) to the experimental data by means of non linear regression analysis (Marquardt, 1963). Data are given as mean values ± s.e.mean except when pD_2 values are presented; these are instead accompanied by 95% confidence limits. Statistical significance were tested by means of analysis of variance.

The following drugs were used: indomethacin, capsaicin and tetrodotoxin (TTX) were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.; porcine NPY and substance P (SP) (Peninsula, Belmont, CA, U.S.A.); porcine NPY 13-36 (Ferring AB, Malmö, Sweden); atropine sulphate (ACO, Solna, Sweden); propranolol hydrochloride (Du Pont Meda, U.S.A.); idazoxan (Reckitt & Colman, Hull, England).

The Krebs solution had the following composition (mM): NaCl 122, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, KH_2PO_4 1.2 and glucose 5.5. Indomethacin was dissolved in 5% $NaHCO_3$ in distilled water to a concentration of 1 mM. All other drugs were dissolved in and diluted with distilled water.

Results

Electrical field stimulation of guinea-pig bronchial rings evoked a rapid twitch, followed by a more long-lasting contraction with a duration of 10–15 min. Addition of atropine

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(1 μM) abolished the rapid response. Addition of TTX (1 μM) or capsaicin (1 μM) virtually abolished the atropine-resistant contraction (Figure 1).

In subsequent studies, the preparations were allowed to equilibrate for 90 min before a series of atropine-resistant contractions were elicited; the second contraction (which amounted to 5.0 ± 0.9 mN, $n = 10$) in a series was set as 100%. With indomethacin in the bath the non-cholinergic contractions were reproducible for at least 3 h.

Neither NPY nor NPY 13–36 had any effect on the basal tension. Electrically induced atropine-resistant contractions were inhibited by NPY and NPY 13–36 in a concentration-dependent manner (Figures 2 and 3).

Maximally effective concentrations of NPY and NPY 13–36 reduced the responses to 17.5 ± 3.4% and to 68.5 ± 3.4% of control contractions, respectively. Both peptides showed a similar inhibitory potency; the estimated pD_2 values are shown in Table 1. Pretreatment with 1 μM idazoxan, a selective α_2-adrenoceptor antagonist (Doxey *et al.*, 1983), did not affect the NPY-evoked suppression of the electrically evoked contractile response ($n = 5$). As expected, idazoxan (1 μM) suppressed the NA-evoked inhibition of the stimulated atropine-resistant contractions (not shown in figure). SP, the proposed neuromessenger in excitatory NANC fibres induced a contraction (at a concentration of 1 μM) which roughly matched the electrically evoked, atropine-resistant response. The SP-evoked contractions were reproducible and the second response following the equilibration period was used as control. NPY (0.3 μM) did not affect these responses (Figure 4).

The possibility that NPY might suppress the inhibitory NANC neurotransmission of guinea-pig airways was also

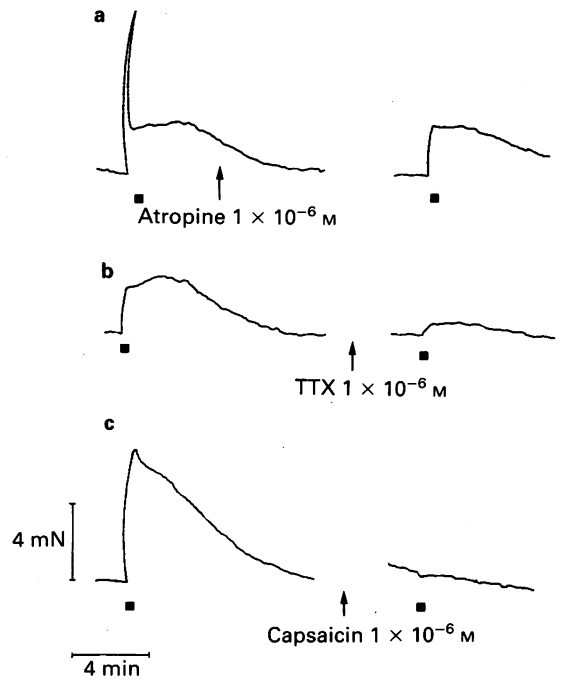

Figure 1 Guinea-pig isolated bronchial ring: electrical field stimulation (EFS), (1 ms, 40 V and 2 Hz) indicated below the recordings, evokes a rapid twitch and a slow contraction. (a) Exposure to atropine (10 min) blocks the twitch but is without effect on the slow contraction. (b) EFS on an atropinized preparation evokes a slow contraction that is abolished by adding tetrodotoxin (TTX) to the bath (30 min). (c) The contractile effect of EFS on an atropinized preparation is abolished by adding capsaicin to the bath (60 min).

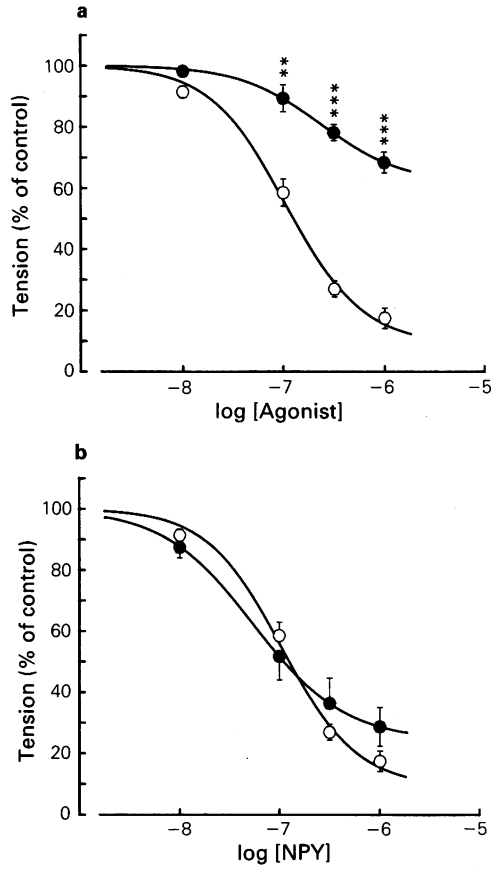

Figure 3 Guinea-pig isolated bronchial ring: log concentration-response curves for the inhibitory effects of neuropeptide Y (NPY) and NPY 13–36 on the electrically induced non-cholinergic contractions. In (a) the effects of NPY (○) and NPY 13–36 (●) are shown. In (b) the effect of pretreatment with 1 μM idazoxan (●) on NPY-induced inhibition is shown; the NPY control curve (○) is the same as in (a). The significance of the differences between the observations of the NPY control curve and the other two curves was tested by analysis of variance. Significances are denoted by ** = $P < 0.01$ and *** = $P < 0.001$. Vertical bars denote s.e.mean ($n = 4$–6).

Table 1 Estimated pD_2-values for the inhibitory effect of neuropeptide Y (NPY) and NPY 13–36 on electrically induced non-cholinergic contraction of guinea-pig bronchial rings

Substances	pD_2	*(95% confidence limits)*	*n*
NPY	7.0	(6.8–7.2)	6
NPY 13–36	6.6	(6.0–7.3)	4
NPY + idazoxan	7.3	(6.8–7.7)	6

n = number of experiments.

Figure 2 Guinea-pig isolated bronchial ring: a representative recording illustrating the effect of neuropeptide Y (NPY) on the nerve-mediated contractions of bronchi in the presence of atropine (1 μM) and propranolol (1 μM). Indomethacin (1 μM) was also present throughout. Electrical field stimulations (parameters as in Figure 1) are indicated above the recording.

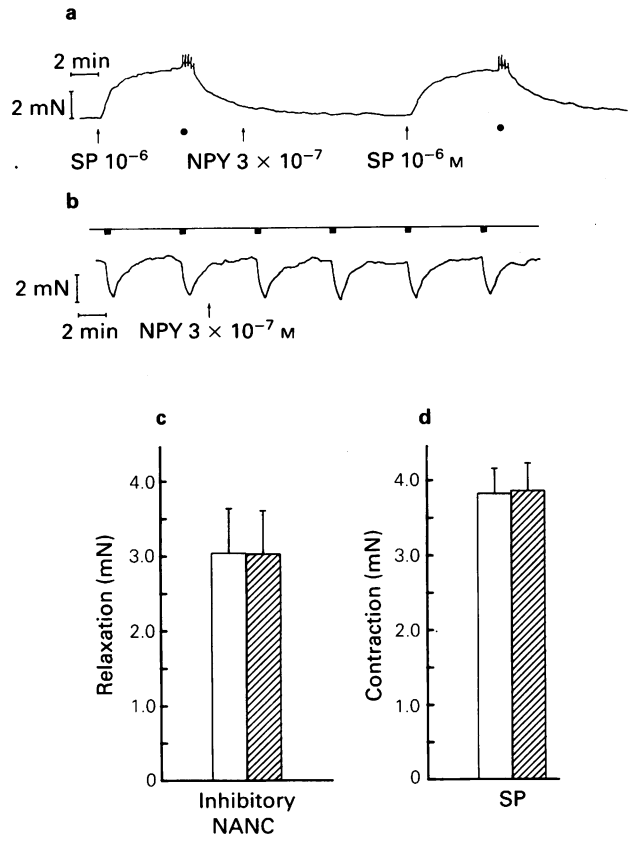

Figure 4 (a) Nerve-mediated non-adrenergic, non-cholinergic (NANC) relaxation of guinea-pig trachea before and after addition of neuropeptide Y (NPY). (b) Substance P (SP)-evoked contraction of the bronchi before and after incubation of NPY. (● indicates rinsing of the bath). NPY had no effect on either SP-induced contractions or on electrically evoked relaxations. (c and d) Data summarizing the results of all experiments performed as in (a) and (b), respectively ($n = 5$). Vertical lines give s.e.mean. Open columns = control responses; hatched columns = responses observed after treatment with NPY (3×10^{-7} M).

investigated. The tracheal preparations were equilibrated in the presence of propranolol, atropine and indomethacin and were contracted with 0.3 μM histamine which gave a stable tension of 18 ± 4.4 mN ($n = 5$). Electrical field stimulation then elicited reproducible relaxations. The relaxation preceding the addition of NPY was set as 100 and the second relaxation after the addition of NPY was used to evaluate the possible effect. The non-adrenergic relaxations were unaffected by 0.3 μM NPY (Figure 4).

Discussion

NPY has a number of effects outside the sympathetic neuroeffector junction. It has for instance been shown to inhibit nerve-mediated (cholinergic) contractions of the isolated vagus-nerve trachea preparation (Grundemar *et al.*, 1988) and of tracheal ring preparations (Stretton & Barnes, 1988). The results of the present study suggest that NPY is capable of suppressing not only parasympathetic activity in the guinea-pig airways but also neurotransmission in excitatory NANC nerve fibres. It is quite likely that these nerve fibres are identical with sensory nerves of the C-fibre type, which employ SP and/or related tachykinins as transmitters. The contractile response evoked by these fibres upon electrical stimulation is inhibited by tachykinin antagonists (Lundberg *et al.*, 1983; Leander *et al.*, 1984) and the response is abolished by pretreatment with capsaicin (Figure 1; Lundberg & Saria, 1982) (for a recent review see Holtzer, 1988). NPY 13–36 was also effective, although less so than the whole molecule. NPY had no effect on basal tension or on SP-evoked contractions and NPY had no effect on the inhibitory NANC neurotransmission in the trachea. The present data indicate therefore that NPY acts on the sensory nerve terminals, rather than on the terminals of NANC inhibitory neurones or on the smooth muscle cells of the airways.

In the central nervous system there is evidence for an interaction between NPY and α_2-adrenoceptors. It has been suggested that NPY upregulates α_2-adrenoceptors in the medulla oblongata in the rat (Agnati *et al.*, 1983). Idazoxan inhibits NPY-induced suppression of spontaneous locomotor activity in the rat (Heilig *et al.*, 1988). Like NPY, NA inhibits electrically stimulated, sensory nerve-mediated, atropine-resistant contractions in the guinea-pig bronchi (Grundström *et al.*, 1984). However, NA acts via α_2-adrenoceptors on the sensory nerve terminals. The results of the present study indicate that the action of NPY on sensory neurotransmission in the airways does not involve α_2-adrenoceptors.

NPY seems to act on both cholinergic and NANC (sensory) excitatory nerves in the airways. The inhibitory NANC-nerves of the airways are not affected. The involvement of excitatory nerves in asthmas is a subject of considerable interest (See Andersson & Grundström, 1987; Nadel & Barnes, 1987). The possibility that nerve-mediated obstruction of the airways can be prevented by interfering with NPY-receptors or α_2-adrenoceptors, may offer new approaches for treatment.

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The effects of novel vasodilator long chain acyl carnitine esters in the isolated perfused heart of the rat

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1 The effects of palmitoyl carnitine (PC) and novel derivatives were examined on the isolated Langendorff perfused heart of the rat.

2 Bolus injections of PC (1–300 nmol) produced coronary constriction accompanied by a cumulative irreversible depression of contractility.

3 Prior storage of PC in chloroform containing 2% ethanol in heat-sealed ampoules resulted in production of the ethyl ester of the compound (PCE). This compound was isolated and also synthesized (P1E). In contrast to PC, both PCE and P1E exhibited potent vasodilator activity.

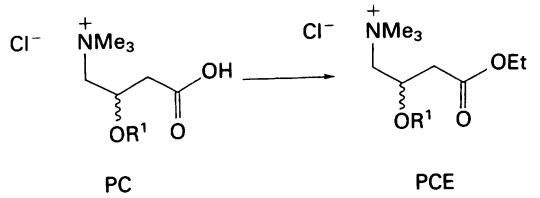
4 Increasing the fatty acid chain length from palmitoyl to stearoyl resulted in a significant reduction in coronary dilator activity of the ester compound, whereas different ester groups did not affect the vasodilator action appreciably. Complete removal of the fatty acid chain abolished all vascular effects at the doses used.

5 The vasodilatation produced by these acyl carnitine esters was comparable to that produced by several known vasodilator drugs including verapamil, cromakalim, amyl nitrate and iloprost; however, the duration of the vasodilator response was more prolonged with the carnitate derivatives.

Introduction

Accumulation of long chain acyl carnitines occurs in the ischaemic myocardium (Liedtke *et al.*, 1978; Corr *et al.*, 1984) and these fatty acid metabolites exert detrimental electrophysiological, biochemical and mechanical effects on the heart (Adams *et al.*, 1979; Knabb *et al.*, 1988; Nakaya & Tohse, 1986). They have also been reported to be calcium channel activators (Spedding & Mir, 1987) and are thought to be the mediators of the increase in α-adrenoceptor numbers seen in the ischaemic myocardium (Corr *et al.*, 1981; Heathers *et al.*, 1987; Allely & Brown, 1988). Much of the existing work has been carried out using Purkinje fibres, myocytes or subcellular fractions whilst there have been few studies on perfused hearts or blood vessels. There is a report on the interaction between palmitoyl carnitine and α-adrenoceptor-mediated responses in the rat caudal artery (Ugwu *et al.*, 1987) and we have previously shown that palmitoyl carnitine exerts both constrictor and dilator actions on the coronary circulation of the rat (Criddle *et al.*, 1987). However, we now attribute most of this activity to a derivative of the parent compound.

In our previous experiments palmitoyl carnitine had been stored for convenience in chloroform, on the assumption of it being an inert solvent, in heat-sealed ampoules. However, chloroform contains 2% ethanol included as stabilizer to prevent phosgene formation. We have subsequently established that the ethanol reacts with palmitoyl carnitine to produce the ethyl ester of the compound (Figure 1). The structure has now been confirmed by chemical and spectroscopic analysis and unambiguous synthesis of the compound. Following this serendipitous discovery, further pharmacological investigations of this novel vasodilator agent and its derivatives have been carried out on the Langendorff isolated perfused heart of the rat.



PC PCE

Figure 1 Conversion of palmitoyl carnitine (PC) to its ethyl ester (PCE) on storage in $CHCl_3$ (2% ethanol) in heat-sealed ampoules (R^1 = palmitoyl).

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Methods

Hearts from male Wistar rats (University of Bath strain) were perfused by the Langendorff technique at a constant flow of 10 ml min^{-1} at 37°C with a modified Krebs-Henseleit solution containing 5.9 mM K^+, gassed with 95% O_2, 5% CO_2. Following a 15 min stabilization period, the perfusate was changed to one containing 3.2 mM K^+ which raises coronary tone. Perfusion pressure changes were monitored with a Bell and Howell pressure transducer connected to a side arm. Developed tension under a resting tension of 2 g was recorded with a Devices isometric transducer attached to the apex of the heart; the signal from this transducer was also used to trigger an instantaneous rate meter. All recordings were made on a Devices M19 recorder. Drug injections were made in volumes of less than 100 μl via a side arm situated 3 cm from the aortic valves.

The following drugs were used; (±)-palmitoyl carnitine, sodium palmitate, atropine sulphate (Sigma), amyl nitrate (BDH), cromakalim (Beechams Pharmaceuticals), verapamil hydrochloride (Abbott) and iloprost (Schering). All novel compounds were synthesized in our laboratories by W.B.W. (University of Bath). Drugs were prepared in 0.9% saline, except cromakalim and sodium palmitate which were first dissolved in ethanol and then diluted in saline; at the final concentration used the ethanol was without effect on the coronary circulation.

Statistical analysis of the differences between means was assessed by a non-paired Student's *t* test.

Results

Palmitoyl carnitine exhibited a dose-related vasoconstrictor action on the coronary vascular bed of the rat (Figure 2). In addition, it caused a cumulative irreversible depression of

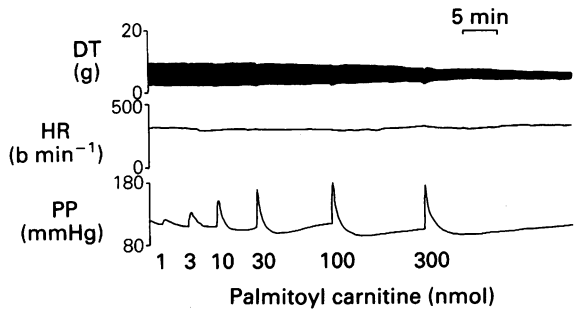


Figure 2 Effects of palmitoyl carnitine (1–300 nmol) on developed tension (DT), heart rate (HR) and perfusion pressure (PP) in the isolated Langendorff perfused heart of the rat.

myocardial contractility with no concomitant effect on heart rate ($n = 4$). This profile of activity was observed for the other acyl carnitines tested, myristoyl(C14) and stearoyl(C18), whilst carnitine (50–250 nmol) had no effect on coronary vascular tone ($n = 4$) (data not shown).

In contrast, the synthetic ethyl ester of palmitoyl carnitine (P1E) exhibited a completely different profile of action from that of the parent compound. We now observed a dose-related vasodilatation that was both rapid in onset and sustained at higher doses ($n = 4$) (Figure 3). A maximal response was produced by 10 nmol P1E; higher doses produced a smaller fall in perfusion pressure preceded in several cases by a transient vasoconstriction possibly reflecting a non-specific effect of the drug. The decreases in perfusion pressure elicited by P1E were dependent on the basal tone of the preparation i.e. the greater the resting perfusion pressure the larger the vasodilator response. Small rises in resting tension were observed on addition of bolus injections of P1E and at no time was any effect on heart rate observed. The vasodilator activities of the synthetic ester P1E and the 'chloroform-produced' equivalent PCE were compared (Figure 4) providing pharmacological confirmation of previous chemical and spectroscopic analyses suggesting that palmitoyl carnitine was being esterified to P1E while undergoing storage in chloroform in heat-sealed ampoules.

Having established the identity of the novel vasodilator compound, structural variations of the molecule were investigated for pharmacological activity, alterations of chemical groups R^1 and R^2 being made (Table 1). Figures 5 and 6 show the effect of increasing the fatty acid chain length by 2 carbon atoms from palmitoyl to stearoyl on the vasodilator activity. Both the maximal dilatation elicited, and the rate at which the

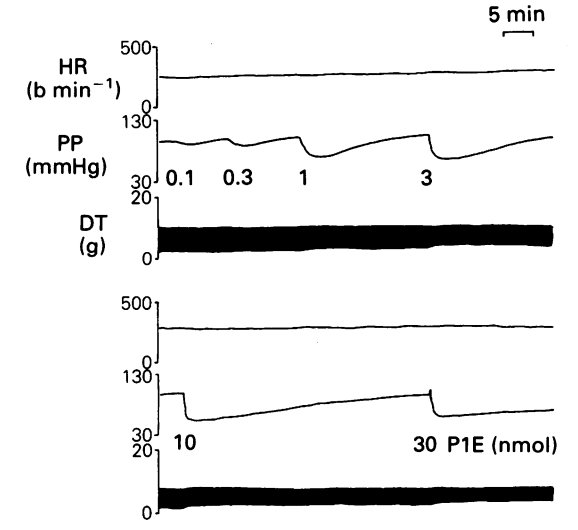


Figure 3 Effect of P1E (0.1–30 nmol) on heart rate (HR), perfusion pressure (PP) and developed tension (DT) in the isolated Langendorff perfused heart of the rat.

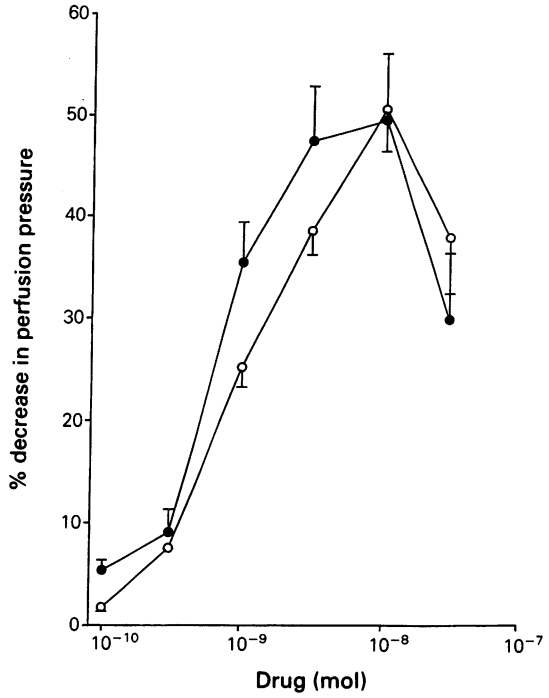


Figure 4 A comparison of the effects of the 'chloroform-produced' ethyl ester of palmitoyl carnitine (PCE, ○), [100% = 102.5 ± 5.7 mmHg, $n = 4$] and the synthetic ester (P1E, ●), [100% = 95.8 ± 3.5 mmHg, $n = 4$] on perfusion pressure in the isolated Langendorff perfused heart of the rat, expressed as % decrease from basal values. Vertical bars represent s.e.mean.

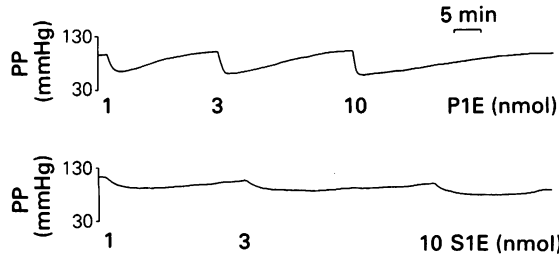
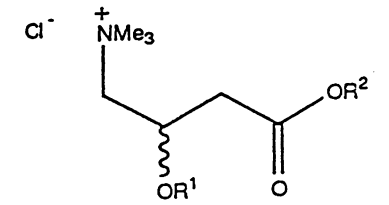


Figure 5 Effects of P1E (1–10 nmol) and S1E (1–10 nmol) on perfusion pressure (PP) in the isolated Langendorff perfused heart of the rat.

Table 1 Guide to the structure and coding of synthetic compounds



Code	R^1	R^2
P1M	$CO(CH_2)_{14}Me$	Me
P1E	$CO(CH_2)_{14}Me$	Et
P1P^i	$CO(CH_2)_{14}Me$	Pr^i
S1E	$CO(CH_2)_{16}Me$	Et
1P^i	H	Pr^i

decrease in perfusion pressure occured were smaller with S1E than the P1E (Figure 6). Complete removal of the fatty acid moiety (1P^i) abolished any effect on coronary vascular tone (1–300 nmol, $n = 3$) (data not shown).

Substitution of the ethyl ester group with methyl (P1M) or isopropyl (P1P^i) groups did not alter the vasodilator activity

of the compounds as markedly as altering the fatty acid chain, although a significant reduction in the response elicited by P1M compared to P1E or P1P^i was observed with some doses (Figure 7).

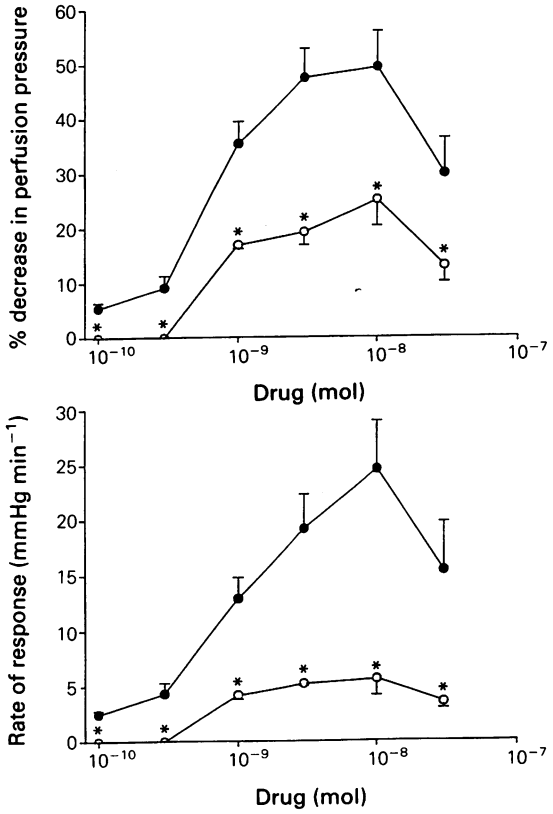


Figure 6 Effects of P1E (●), [100% = 95.8 ± 3.5 mmHg, $n = 4$], and S1E (○), [100% = 86.8 ± 9.0 mmHg, $n = 4$] on perfusion pressure in the rat isolated Langendorff heart, expressed both as % decrease from basal values and as rate of response. Vertical bars represent s.e.mean (*$P < 0.05$).

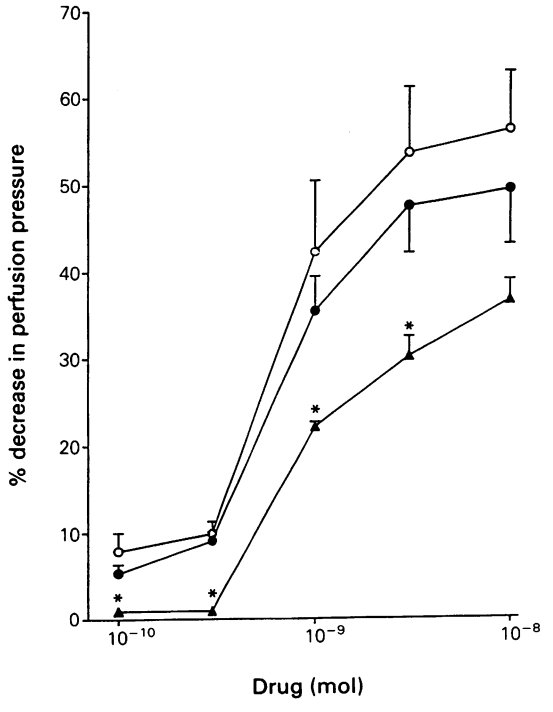


Figure 7 Effects of P1M (▲), [100% = 92.5 ± 6.7 mmHg, $n = 4$], P1E (●), [100% = 95.8 ± 3.5 mmHg, $n = 4$] and P1P^i (○), [100% = 104.0 ± 5.0 mmHg, $n = 4$] on perfusion pressure in the isolated Langendorff perfused heart of the rat, expressed as % decrease from basal values. Vertical bars represent s.e.mean (*$P < 0.05$ compared with P1P^i and P1E).

A comparison of the synthetic carnitates with known vasodilators amyl nitrate, verapamil, cromakalim and iloprost is shown in Figure 8. Vasodilatations of similar magnitude to those elicited by the known agents were produced by the novel compounds at comparable doses; however the duration of responses was greater with the acyl carnitine esters.

High concentrations of palmitic acid relax potassium depolarized guinea-pig taenia-coli preparations (Spedding & Mir, 1987). In order to rule out the possibility that P1P^i was being broken down to release palmitate, and that this was responsible for mediating the coronary relaxation seen with P1P^i, the effects of palmitate were examined in the rat isolated heart. In contrast to its effect in the potassium depolarized taenia preparation, bolus injections of sodium palmitate (1–30 nmol) produced dose-dependent vasoconstrictions in the rat coronary circulation ($n = 4$); at the higher doses the vasoconstrictor response to palmitate was followed by a vasodilator component which was smaller and less well maintained than that seen with smaller doses of P1P^i.

In view of the structural similarities between acetylcholine and the acyl carnitine esters it was possible that these compounds could be acting as muscarinic agonists on endothelial cells to release endothelial derived relaxant factor (Vanhoutte & Miller, 1985). However, in the rat isolated heart the coronary vascular effects of acetylcholine (1–10 nmol) were completely abolished by atropine (5–50 nM) while these concentrations of atropine had no effect on the vasodilator action of P1P^i. Also, the major effect of acetylcholine on the rat coronary circulation is to produce vasoconstriction which is sometimes preceded by a small inconsistent vasodilator response, unlike the long-lasting vasodilatation seen with the acyl carnitine esters.

Discussion

We have shown in the rat isolated heart that bolus injections of palmitoyl carnitine cause constriction of the coronary circulation associated with an irreversible depression of myocardial contractility. Alteration of the carboxyl group of the molecule to an ester group produces compounds with an opposite action on the coronary vasculature.

In common with the acyl carnitines from which they were derived, the novel esters are amphiphiles i.e. they have both hydrophobic and hydrophilic groups. Incorporation of such compounds into membranes alters ionic permeability (Levitsky & Skulachev, 1972; Corr *et al.*, 1984) and this property may underlie their mechanism of action. However, in view of the opposing actions of acyl carnitines and their esters on coronary vascular tone, such a non-specific membrane effect appears an unlikely explanation of their mode of action. It has recently been shown that palmitoyl, myristoyl and lauroyl carnitines caused damage of Langendorff perfused rat hearts as measured by enzyme leakage and that there was a 20 fold difference in potency between palmitoyl and lauroyl car-

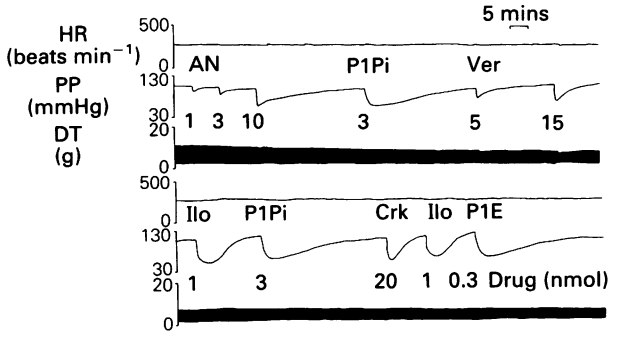


Figure 8 Comparison of the effects of P1E and P1P^i with amyl nitrate (AN), verapamil (Ver), iloprost (Ilo) and cromakalim (Crk) on heart rate (HR), perfusion pressure (PP) and developed tension (DT) in the isolated Langendorf perfused rat heart.

nitines in this respect (Sercu et al., 1986). We have observed differences in the activities of the carnitine esters depending on the functional groups present, most noticeably the saturated fatty acid chain. These structure-activity relationships of P1E and related compounds, may therefore indicate a specific receptor mechanism underlying the effects of acyl carnitines and their esters.

It has been shown that in K^+ depolarized smooth muscle and chick myocytes, palmitoyl carnitine resembles Bay K 8644, the dihydropyridine calcium channel activator (Spedding & Mir, 1987; Patmore et al., 1989). Palmitoyl carnitine also causes displacement of bound tritiated calcium antagonists in rat cortex and it has been suggested that acyl carnitines may be endogenous modulators of calcium channel function (Spedding & Mir, 1987). In addition, effects of palmitoyl carnitine have been reported on mitochondrial calcium handling in rat heart (Baydoun et al., 1988). The possibility arises therefore that carnitine esters may be exerting opposite effects to acyl carnitines regarding calcium modulation in the cell.

The fact that in the rat heart the coronary response to acetylcholine is primarily one of vasoconstriction and the vasodilator action of $P1P^i$ was not inhibited by atropine rules out the possibility that $P1P^i$ was acting on endothelial cell muscarinic receptors to release endothelial derived relaxant factor. It is also unlikely that the vasodilatation seen with these compounds was due to the release of their fatty acid component. Over the dose range 0.1–10 nmol, $P1P^i$, P1M and P1E, all of which are palmitoyl carnitine derivatives, produced vasodilatation (Figure 7), while similar doses of palmitate produced a vasoconstrictor response with a secondary vasodilator component only becoming apparent at doses of 10 nmol or more.

In conclusion, the pharmacological profile of the compounds we have synthesized shows that relatively small changes in the acyl carnitine structure can have a profound effect on the vascular activity, changing them from vasoconstrictors to vasodilators. As yet, the precise mechanism whereby the novel carnitine esters elicit a profound and sustained relaxation of the coronary vasculature is unclear; however, further study of these compounds is continuing and may provide a fuller understanding of the actions and role of acyl carnitines in the regulation of vascular tone.

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Heterogeneity of α_2-adrenoceptors in rat cortex but not human platelets can be defined by 8-OH-DPAT, RU 24969 and methysergide

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1 Saturation experiments indicated that [^{3}H]-yohimbine binding was specific, saturable and labelled a single population of sites in rat cerebral cortex (K_d 5.3 ± 0.9 nM, B_{max} 121 ± 10 fmol mg^{-1} protein) and human platelets (K_d 0.7 ± 0.1 nM, B_{max} 152 ± 10 fmol mg^{-1} protein).

2 The α_2-adrenoceptor antagonists, yohimbine, rauwolscine, WY 26703, idazoxan and BDF 6143 displaced [^{3}H]-yohimbine binding to each tissue in a simple manner, with high affinity and Hill slopes close to unity.

3 The α_1-adrenoceptor agonist, oxymetazoline and the antagonist prazosin inhibited the binding of [^{3}H]-yohimbine to rat cortex in a complex manner consistent with an interaction at more than one site. However, indoramin and WB 4101 only appeared to interact with one site. In contrast, in human platelets, all antagonists gave rise to monophasic displacement curves with Hill slopes close to unity suggesting a single site of interaction.

4 The 5-hydroxytryptamine (5-HT) receptor ligands, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT), RU 24969, and methysergide inhibited the binding of [^{3}H]-yohimbine to rat cortex with high and low affinity, consistent with an interaction with two populations of binding sites. However, inhibition of [^{3}H]-yohimbine binding to human platelets suggested a single site of interaction. The low affinity of 5-HT, 5-carboxyamidotryptamine (5-CT) and dipropyl-5-CT indicated that [^{3}H]-yohimbine was not labelling a 5-HT$_1$-like site in rat cortex.

5 The ability of 8-OH-DPAT, RU 24969 and methysergide in addition to prazosin and oxymetazoline to differentiate [^{3}H]-yohimbine binding provides additional pharmacological evidence for heterogeneity within rat cortical α_2-adrenoceptors. However, if the two sites in rat cortex that are differentiated by the 5-HT ligands represent α_{2A}- and α_{2B}-adrenoceptor subtypes as defined by prazosin and oxymetazoline, then they do not correspond to the population of sites in human platelets. As receptor classification should be linked to affinity of drugs rather than tissue distribution, the current classification of α_2-adrenoceptor subtypes does not appear to be satisfactory.

Introduction

Amino acid sequence of the α_2-adrenoceptor (Kobilka *et al.* 1987) and molecular weight determination (Lanier *et al.*, 1988) have identified structurally distinct α_2-adrenoceptor subtypes. Cloning and gene expression studies revealed that the gene for the human platelet α_2-adrenoceptor was localised on chromosome 10 (Kobilka *et al.*, 1987) and was distinct from an α_2-adrenoceptor subtype gene localised on chromosome 4 (Regan *et al.*, 1988).

A number of radioligand binding studies have indicated that heterogeneity exists within α_2-adrenoceptors. This heterogeneity has been shown to occur between species (Feller & Bylund, 1984; Alabaster *et al.*, 1986; Dickinson *et al.*, 1986) and within species (Nahorski *et al.*, 1985; Hamilton *et al.*, 1988). The α_2-adrenoceptor antagonists [^{3}H]-yohimbine and [^{3}H]-rauwolscine bind with lower affinity to rodent tissue compared to non-rodent tissue (Bylund, 1985). Competition experiments have indicated that the affinities and rank order of a number of compounds to displace the binding of [^{3}H]-yohimbine at α_2-adrenoceptors differ between rodent and non-rodent species (Kawahara & Bylund, 1985). The α_1-adrenoceptor antagonist prazosin has been shown to be more potent as an inhibitor of [^{3}H]-yohimbine binding to α_2-adrenoceptors in certain rodent compared to non-rodent tissue, whereas oxymetazoline is more potent in non-rodent tissue (Cheung *et al.*, 1982; Bylund, 1985). Thus α_2-adrenoceptor subtypes have been differentiated on the basis of affinity for prazosin; the α_{2A} subtype has low affinity for prazosin whereas the α_{2B} has higher affinity (Cheung *et al.*, 1982; Petrash & Bylund, 1986). According to this definition, the α_2-adrenoceptor in human platelets is consistent with the α_{2A} subtype (Cheung *et al.*, 1982), whereas the α_2-adrenoceptor in neonatal rat lung tissue is of the α_{2B} subtype (Latifpour *et al.*, 1982). Rat cerebral cortex and corpus striatum appear to contain approximately equal amounts of α_{2A} and α_{2B} subtypes (Bylund, 1985).

In this study we have examined the displacement of [^{3}H]-yohimbine binding to human platelets and rat cerebral cortex by prazosin, oxymetazoline and a number of compounds known to have affinity for α-adrenoceptors and for 5-HT$_1$-like receptors.

Methods

Membrane preparation

Male Sprague-Dawley rats (180–200 g) were stunned, decapitated and the cerebral cortex dissected from the other brain regions over ice. Tissues were homogenised in 20 volumes of 50 mM Tris HCl, 5 mM EDTA, pH 7.4 with a polytron PT 10 tissue disruptor (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in the original volume of assay buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 7.4). Membranes were washed twice by repeated centrifugation at 48,000 *g* for 15 min at 4°C. The final pellet was resuspended in assay buffer at an approximate protein level of 1 mg ml^{-1}. Membranes were stored under liquid nitrogen

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Table 1 [^{3}H]-yohimbine binding to rat cortex and human platelets

	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Rat cortex	5.3 ± 0.9	121 ± 10
Human platelets	0.7 ± 0.1	152 ± 10

Saturation experiments were carried out over the [^{3}H]-yohimbine concentration range 0.1–8 nM for platelets and 0.1–15 nM for rat cortex. Each value represents the meant ± s.e.mean of three determinations.

until used in the binding assay. Human platelet membranes were prepared and binding carried out as described by Cheung *et al.* (1982).

Saturation experiments

Membranes (400–600 μg protein) and [^{3}H]-yohimbine, (0.1–15 nM for rat cortex; 0.1–8 nM for human platelets) were incubated to equilibrium in 50 mM Tris HCl buffer, pH 7.4 containing 0.5 mM EDTA. Incubations were carried out for 30 min at 25°C in a final volume of 0.5 ml. Bound ligand was separated from free by vacuum filtration over GF/B filters on a Brandell cell harvester. The filters were washed twice for 10 s with incubation buffer and the bound radioactivity determined by liquid scintillation counting. Non-specific binding at each free ligand concentration was determined in the presence of 10 μM phentolamine.

Equilibrium binding parameters (K_d and B_{max}) were obtained by the iterative non-linear least square curve fitting programme 'ligand' (Munson & Rodbard, 1980).

Competition experiments

Competition experiments were carried out in the presence of 1.5 nM [^{3}H]-yohimbine and various drugs over the concentration range 1 mM to 1 pM in a final volume of 0.5 ml. Conditions for the competition assays were identical to those described for the saturation experiments. Non-specific binding was defined by 10 μM phentolamine and represented between 25–30% total binding in rat cortex and 20% in human platelets. Each determination was carried out in duplicate.

Computer analysis of displacement curves

Inhibition of specific binding of [^{3}H]-yohimbine by the drug was analysed to estimate the IC_{50} (concentration of drug displacing 50% of specific binding). The inhibitory constant (K_i) was calculated from the IC_{50} by the equation of Cheng & Prusoff (1973). Binding isotherms from displacement studies were analysed by a non-linear least square parametric curve fitting programme capable of iterative curve fitting to a single or two-site model. The programme provided a sum of squares error for a single or two site model (Michel & Whiting, 1988). The single site and two site models for each isotherm were compared by the differential F value defined by the following equation;

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/(df_2)}$$

where SS_1 is the sum of squares error for the single site, SS_2 is the sum of squares error for the two-site model, df_1 is the degrees of freedom for the single site model and df_2 the degrees of freedom for the two-site model (Munson & Rodbard, 1980; Petrash & Bylund, 1986). A two-site fit was assumed to be significantly better than a single site fit if the determined F value had a $P < 0.05$.

Protein level was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the protein standard. All data were tested for significance by Student's parametric two tailed *t* test and significance taken at $P < 0.05$.

Chemicals and drugs

[^{3}H]-yohimbine (89 Ci mmol^{-1}) was purchased from Du Pont, U.K. Drugs were obtained from the following sources; 5-hydroxytryptamine creatinine sulphate (5-HT) and oxymetazoline HCl from Sigma; rauwolscine HCl from Inverbeff; WY 26073 (N-methyl-N-(1,3,4,6,7,11b-hexahydro-2H-benzo-αaβ-quinolizin 2-yl)-s-butanesulphonamide hydrochloride) and indoramin HCl from Wyeth; idazoxan HCl from Reckitt & Colman; WB 4101 (2-[2,6-dichloro(N-beta chloroethyl-N-methyl)-4 methylamino] phenylimino-2-imidazoline dihydrochloride) from Ward Blenkinsop; 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide) from Merrell Dow; RU 24969 (5-methoxy-3-(1,2,3,6, tetrahydropyridin-4-yl)-IH-indole) from Roussel; methysergide bimaleate from Sandoz; prazosin HCl from Pfizer. BDF 6143 (4-chloro-2-[2-imidazoline-2-ylamino]-isoindoline hydrochloride); 5-CT (5-

Table 2 Inhibition of [^{3}H]-yohimbine binding to human platelet and rat cortical membranes by α-adrenoceptor and 5-HT receptor ligands

	Rat cortex			*Human platelets*	
	pK_i		*nH*	pK_i	*nH*
Yohimbine	7.87 ± 0.07		0.89 ± 0.09	9.10 ± 0.07	1.03 ± 0.02
Rauwolscine	7.86 ± 0.06		0.97 ± 0.02	8.98 ± 0.03	1.08 ± 0.01
WY 26703	7.94 ± 0.07		1.02 ± 0.03	ND	
Idazoxan	7.67 ± 0.06		1.00 ± 0.05	8.30 ± 0.02	0.94 ± 0.01
BDF 6143	8.96 ± 0.04		1.03 ± 0.03	8.96 ± 0.06	1.00 ± 0.01
Prazosin	8.10 ± 0.30	5.94 ± 0.08	(50:50)	6.05 ± 0.09	0.93 ± 0.03
Oxymetazoline	8.25 ± 0.02	6.92 ± 0.13	(60:40)	8.40 ± 0.04	1.03 ± 0.04
Indoramin	5.09 ± 0.04		0.98 + 0.05)	5.90 ± 0.03	1.11 ± 0.03
WB 4101	7.24 ± 0.08		0.89 + 0.07	8.40 ± 0.04	1.01 ± 0.02
8-OH-DPAT	6.43 ± 0.07	4.97 ± 0.14	(40:60)	6.66 ± 0.07	0.97 ± 0.02
RU 24969	7.30 ± 0.11	5.20 ± 0.06	(40:60)	7.12 ± 0.10	0.97 ± 0.02
Methysergide	7.17 ± 0.09	5.34 ± 0.12	(45:55)	7.05 ± 0.11	0.95 ± 0.04
5-HT	<4.00			<4.00	
5-CT	<5.5			ND	
DP-5-CT	<5.5			ND	

The affinity values obtained for the displacement of specific binding of 1.5 nM [^{3}H]-yohimbine from rat cortical and human platelet membranes. Prazosin, oxymetazoline, 8-OH-DPAT, RU 24969 and methysergide displaced [^{3}H]-yohimbine binding to rat cortical membranes with Hill slopes (nH) that could be resolved into high and low affinity components. Values in parentheses represent the proportion of high to low affinity sites differentiated by the ligands. The pIC_{50} values for the unresolved curves were, prazosin 7.1 ± 0.10, oxymetazoline 7.7 ± 0.01, 8-OH-DPAT 5.70 ± 0.09, RU 24969 6.08 ± 0.07 and methysergide 6.16 ± 0.10. Each value represents the mean ± s.e.mean of at least four experiments. ND, not determined.

carboxyamidotryptamine maleate) and DP-5-CT (dipropyl-5-carboxyamidotryptamine hydrobromide) were synthesized by Syntex. All other chemicals were of the highest purity commercially available.

Results

Saturation analysis of [^{3}H]-yohimbine binding indicated that it was specific, saturable and bound to a single population of high affinity sites in rat cerebral cortex and human platelets. The affinity and density of α_2-adrenoceptors in rat cortex and human platelets are shown in Table 1.

The affinities of a number of drugs were determined at α_2-adrenoceptors in rat cortex and human platelets. The α_2-adrenoceptor antagonists yohimbine, rauwolscine. WY 26703, idazoxan and BDF 6143 displaced [^{3}H]-yohimbine binding to human platelets and rat cerebral cortical membranes with high affinity and with Hill slopes not significantly different from unity. The α_1-adrenoceptor antagonist indoramin and the mixed α_1/5-HT$_{1A}$ ligand WB 4101 gave rise to simple displacement curves in human platelets and rat cerebral cortical membranes, consistent with a single site of interaction. The α_1-adrenoceptor antagonist prazosin and the agonist, oxymetazoline gave rise to biphasic displacement curves indicating the possibility of two classes of binding sites. Computer analysis of the binding isotherms indicated that prazosin and oxymetazoline displaced [^{3}H]-yohimbine binding to rat cortex with high and low affinity and that these sites existed in approximately equal proportions. Differentiation of [^{3}H]-yohimbine binding by prazosin and oxymetazoline into a high and low affinity site was not apparent in human platelets. Prazosin inhibited [^{3}H]-yohimbine binding to human platelets with low affinity whereas oxymetazoline displayed high affinity (Table 2).

Inhibition of [^{3}H]-yohimbine binding to rat cortical membranes by the 5-HT agonist, 8-OH-DPAT, the mixed 5-HT$_{1A/1B}$ agonist RU 24969 and the mixed 5-HT$_1$ and 5-HT$_2$ ligand methysergide gave rise to biphasic competition curves. Computer analysis statistically resolved the binding data into a two-site fit for each of the compounds (Table 2). In contrast, displacement of [^{3}H]-yohimbine binding to human platelet α_2-adrenoceptors was monophasic, consistent with an interaction at a single site. The affinities of the 5-HT ligands at human platelet α_2-adrenoceptors corresponded to their respective values at the high affinity site on rat cortical membranes. The high affinity sites differentiated by 8-OH-DPAT, RU 24969 and methysergide are not minor components of [^{3}H]-yohimbine binding. They represent between 40 and 45% of specific [^{3}H]-yohimbine binding to rat cortical membranes. [^{3}H]-yohimbine binding to human platelet and rat cortical α_2-adrenoceptors was unaffected by 5-HT at concentrations up to 100 μM.

Methysergide, in the presence of 100 nM prazosin, no longer displaced [^{3}H]-yohimbine binding to rat cortex in a biphasic manner (Figure 1). Analysis of the displacement curves indicated low affinity binding and a Hill slope close to unity, a finding consistent with an interaction at a single site. Similarly, the high affinity component of 8-OH-DPAT and RU 24969 binding was abolished by prazosin (Table 3), suggesting

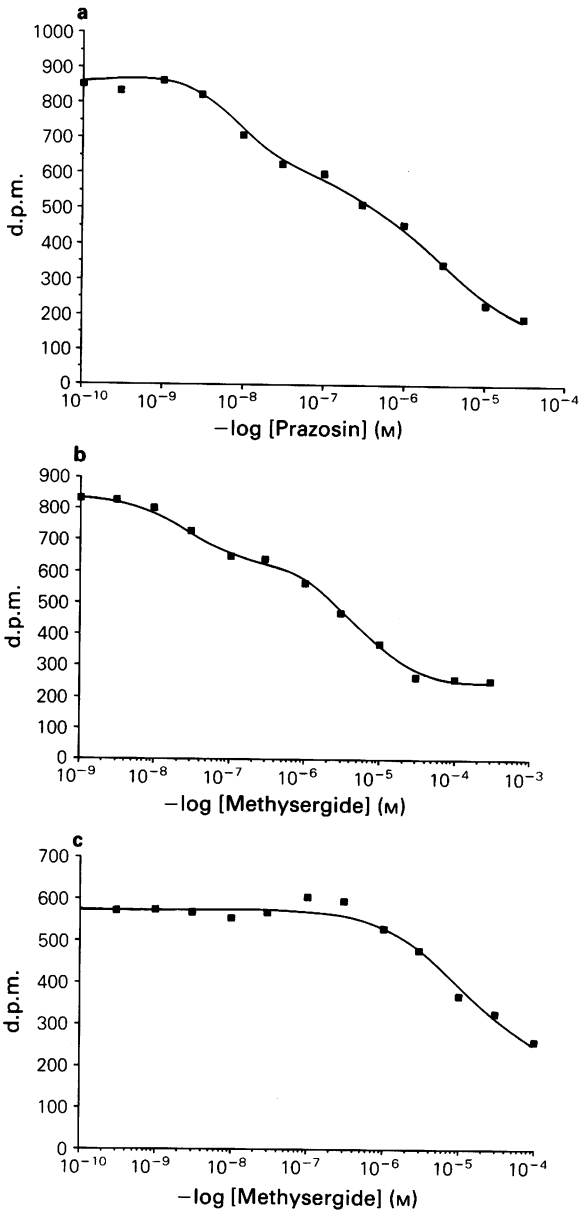

Figure 1 Displacement of [^{3}H]-yohimbine binding to rat cerebral cortical membranes by prazosin (a) and by methysergide in the absence (b) and presence (c) of 100 nM prazosin. Computer assisted curve fitting on the prazosin displacement curve (a) demonstrated that a two-site fit was significantly better ($P < 0.01$) than a one-site fit. (1 site, SS$_1$ 9483; 2 site, SS$_2$ 3684; F value 6.29). In the absence of prazosin (b), computer assisted curve fitting showed a two-site fit was significantly better ($P < 0.01$) than a one-site fit for the displacement of [^{3}H]-yohimbine by methysergide. (1 site, SS$_1$ 11212, d.f. 10; 2 site, SS$_2$ 4342, d.f. 8: F value 6.32). Prazosin decreased specific [^{3}H]-yohimbine binding by approximately 40%. Methysergide, in the presence of prazosin (c), inhibited [^{3}H]-yohimbine binding with a Hill slope of unity and showed a one-site fit. The data describe a single experiment performed in duplicate.

Table 3 [^{3}H]-yohimbine binding to rat cortical and human platelet membranes in the presence of 100 nM prazosin

	pK_i			
	Rat cortex	*nH*	*Human platelets*	*nH*
Oxymetazoline	8.07 ± 0.03	1.00 ± 0.04	8.56 ± 0.11	0.93 ± 0.07
8-OH-DPAT	5.07 ± 0.03	0.97 ± 0.04	6.34 ± 0.04	1.02 ± 0.06
RU 24969	5.38 ± 0.04	0.89 ± 0.07	6.84 ± 0.06	0.98 ± 0.03
Methysergide	5.38 ± 0.04	0.94 ± 0.05	7.09 ± 0.10	1.03 ± 0.06

Binding of 1.5 nM [^{3}H]-yohimbine to rat cortical and human platelet membranes was carried out in the presence of 100 nM prazosin. The affinity values were obtained from the displacement of the remaining specific binding to human platelet and rat cortical membranes. Specific binding of [^{3}H]-yohimbine to platelet membranes was unaffected by prazosin. Each value represents the mean ± s.e.mean of at least four experiments. nH represents the Hill slope of the displacement curves.

Table 4 Effect of GTP on the affinity values for α_2-adrenoceptor binding sites in rat cortical membranes

	pIC_{50}	
	$-GTP$	$+GTP$
Adrenaline	7.63 ± 0.05	5.95 ± 0.01*
Noradrenaline	6.81 ± 0.05	6.12 ± 0.05*
Prazosin	7.10 ± 0.10	7.05 ± 0.10
8-OH-DPAT	5.70 ± 0.09	5.70 ± 0.08
RU 24969	6.08 ± 0.07	5.90 ± 0.10
Methysergide	6.16 ± 0.10	5.90 ± 0.10

The affinity values were obtained for specific [^{3}H]-yohimbine (1.5 nM) binding to rat cortical membranes in the presence (+) and absence (−) of 0.1 mM GTP. The results are expressed as pIC_{50} since the compounds showed Hill slopes that were significantly ($P < 0.001$) different from unity. Values shown are the mean ± s.e.mean of at least three experiments. Statistical significance relative to the value in the absence of GTP, *$P < 0.001$.

that the site showing high affinity for the 5-HT$_1$ ligands and prazosin were likely to be identical. In contrast, oxymetazoline in the presence of prazosin, still retained high affinity for [^{3}H]-yohimbine binding but this time gave rise to monophasic displacement curves. Thus the site displaying high affinity towards oxymetazoline is different from the sites showing high affinity towards prazosin and the 5-HT$_1$ ligands. The inclusion of 100 nM prazosin was repeated in human platelets. Prazosin had no significant effect on the affinity of the 5-HT$_1$ ligands. The affinity of each ligand, even in the presence of prazosin, was significantly higher at the α_2-adrenoceptors on human platelets than at the prazosin-insensitive site in rat cortex.

A possible explanation for the complex displacement curves obtained with prazosin, oxymetazoline and the 5-HT$_1$ ligands is that they act as agonists at one or both of the [^{3}H]-yohimbine binding sites. Displacement studies were repeated in the presence of 0.1 mM guanosine 5'-triphosphate (GTP). The potency of adrenaline and noradrenaline to displace [^{3}H]-yohimbine binding from cortical membranes was reduced significantly in the presence of GTP (Table 4). In contrast GTP failed to alter the inhibition curves for prazosin, 8-OH-DPAT, methysergide and RU 24969.

Discussion

Prazosin has been shown previously to inhibit [^{3}H]-yohimbine binding to rat cerebral cortex in a biphasic manner, with an approximate 40 fold separation between the high (pK_i 8.2) and low (pK_i 6.6) affinity site (Bylund, 1985). The affinity of prazosin at each site and the proportion of the subtypes identified in this study, is in agreement with the findings of Bylund (1985) and supports the view that multiple α_2-adrenoceptor subtypes exist in rat cerebral cortex.

The results of this study also demonstrated that the 5-HT receptor ligands, 8-OH-DPAT, RU 24969 and methysergide, but not 5-HT, 5-CT or DP-5-CT, inhibited the binding of [^{3}H]-yohimbine to rat cortex in a complex manner. Computer analysis of the displacement curves demonstrated that each drug inhibited [^{3}H]-yohimbine binding with high and low affinity. Binding of [^{3}H]-yohimbine to the high affinity site differentiated by these ligands was inhibited by 100 nM prazosin. Under these conditions 8-OH-DPAT, RU 24969 and methysergide no longer displaced [^{3}H]-yohimbine binding to rat cortical membranes with high and low affinity. The resulting monophasic displacement curves were of low affinity. Oxymetazoline, in the presence of 100 nM prazosin, also displayed monophasic displacement curves, but unlike the 5-HT$_1$ ligands retained high affinity towards the binding site labelled by [^{3}H]-yohimbine. Based on the α_2-adrenoceptor subtype classification proposed by Bylund (1985) the [^{3}H]-yohimbine binding site that displayed high affinity towards the 5-HT$_1$ ligands would correspond to the α_{2B}-adrenoceptor subtype (high affinity for prazosin), whereas the site that displayed low affinity towards the 5-HT$_1$ ligands would equate with the α_{2A}-adrenoceptor subtype (low affinity for prazosin). Occlusion experiments were repeated with oxymetazoline instead of prazosin, to determine if the 5-HT$_1$ ligands retained only high affinity towards the [^{3}H]-yohimbine binding site in rat cerebral cortex. Oxymetazoline was found to be unsuitable in this type of experiment, as the difference in affinity of oxymetazoline at the prazosin sensitive and insensitive sites was not sufficient to allow a concentration to be chosen which would selectively block one site. Occlusion experiments to prevent [^{3}H]-yohimbine binding to α_{2A}-adrenoceptors must await the identification of more selective ligands than oxymetazoline. The ability of 8-OH-DPAT, RU 24969 and methysergide to differentiate [^{3}H]-yohimbine binding provides additional pharmacological evidence that [^{3}H]-yohimbine labels with high affinity more than one site in rat cortex.

Human platelet α_2-adrenoceptors have been labelled with [^{3}H]-yohimbine (Motulsky *et al.*, 1980; Daiguji *et al.*, 1981). Saturation experiments indicate the presence of a single population of high affinity α_2-adrenoceptors (Boon *et al.*, 1983; Kerry *et al.*, 1984). The low affinity displayed by prazosin towards the [^{3}H]-yohimbine binding site in human platelets is the basis of the suggestion that platelets contain the α_{2A}-adrenoceptor subtype (Cheung *et al.*, 1982). In the present study a paradox is evident. First, the low affinity displayed by prazosin indicated that the platelet site can be designated as α_{2A}, as demonstrated by others (Cheung *et al.*, 1982), whereas the affinities of the 5-HT$_1$ ligands at the α_2-adrenoceptor in human platelets were closer to the site previously considered to be the α_{2B}-adrenoceptor subtype in rat cortex. The inclusion of 100 nM prazosin had no effect on the specific [^{3}H]-yohimbine binding to human platelets or on the affinity of the 5-HT$_1$ ligands for this site, thus confirming the α_{2A}-adrenoceptor nature of this site. The failure to define clearly the nature of the sites may relate to species differences with the existence of several sub-classes of sites. The human platelet α_2-adrenoceptor may itself be somewhat atypical. The dissociation constant for [^{3}H]-yohimbine binding was considerably lower in human platelets than in rat cortex. At present the affinities of the 5-HT$_1$ ligands are not entirely consistent with a simple α_{2A}- and α_{2B}-adrenoceptor classification.

Evidence for [^{3}H]-yohimbine binding to more than one site was apparent only from displacement studies. Saturation experiments, over the [^{3}H]-yohimbine concentration range 0.1 to 15 nM, failed to demonstrate binding to more than one site in rat cortex. Analysis of the binding isotherms indicated that [^{3}H]-yohimbine labelled a uniform population of binding sites. [^{3}H]-yohimbine has been shown to label a single population of binding sites in rat (Cheung *et al.*, 1982; Rouot *et al.*, 1982) and human cortex (Petrash & Bylund, 1986). The failure to identify more than one population of binding sites in saturation studies implies either that [^{3}H]-yohimbine binds with equal affinity to more than one site or that the difference in affinity between sites is not sufficient to be resolved by the analysis programmes used. In human caudate nucleus, a tissue suggested to contain two α_2-adrenoceptor subtypes, saturation experiments, although giving rise to curvilinear Rosenthal plots, failed to demonstrate that the binding of [^{3}H]-yohimbine was significantly better as a two-site than a one-site fit (Petrash & Bylund, 1986).

Saturation studies carried out over a higher [^{3}H]-yohimbine concentration range (0.2 to 80 nM) than that used in this study (0.1–15 nM) have demonstrated binding to a second site in rat cortex (Michel & Whiting, 1984). In addition to [^{3}H]-yohimbine, [^{3}H]-rauwolscine at high concentrations has been shown to bind in a biphasic manner to rat cortical membranes (Diop *et al.*, 1983). Binding isotherms generated over the [^{3}H]-rauwolscine concentration range 0.25–50 nM were consistent with binding to a high and low affinity site (Broadhurst & Wyllie, 1986). The finding that [^{3}H]-rauwolscine binding to the low affinity site was inhibited by 300 nM spiroperidol led

Broadhurst & Wyllie (1986) to conclude that [^{3}H]-rauwolscine, in addition to binding to α_2-adrenoceptors, also labelled 5-HT_2 receptors. It is unlikely that either of the [^{3}H]-yohimbine binding sites differentiated by prazosin or the 5-HT_1 ligands are 5-HT_2 receptors. Inclusion of 300 nM spiroperidol did not alter the affinity of prazosin or the 5-HT ligands, 8-OH-DPAT, RU 24969 and methysergide for the high or low affinity sites in rat cortex (data not shown). Recent studies have shown that 5-HT can displace [^{3}H]-rauwolscine binding from a non α_2-adrenoceptor site in human cortex with nanomolar affinity (Convents *et al.*, 1988; 1989) and that [^{3}H]-rauwolscine may label 5-HT_{1A} receptors in rat cerebral cortex (Broadhurst *et al.*, 1988). Yohimbine has also been shown to possess high affinity (59 nM) towards the 5-HT_{1D} binding site identified in non-rodent brain (Heuring & Peroutka, 1987). The low affinity of 5-CT and DP-5-CT and the failure of 5-HT, at concentrations up to 100 μM, to displace [^{3}H]-yohimbine binding to rat cortical membranes argues against either of the sites being 5-HT_1-like in nature. The displacement of [^{3}H]-yohimbine binding to rat cortical membranes by 5-HT was also studied under conditions in which 5-HT_1-like sites are labelled by [^{3}H]-5-HT (50 mM Tris HCl buffer, pH 7.4 containing 5.7 mM ascorbic acid, 4 mM $CaCl_2$ and 10 μM pargyline). The affinity of 5-HT was not increased under these conditions (data not shown), indicating that the low affinity of 5-HT is the result of a failure to displace [^{3}H]-yohimbine and not from either metabolism or oxidation of 5-HT. The ability of WY 26703, idazoxan and BDF 6143, in addition to rauwolscine and yohimbine, to displace [^{3}H]-yohimbine binding to cortical membranes with high affinity, is consistent with the radioligand labelling an α_2-adrenoceptor. The α_2-adrenoceptor subtype with 'high' affinity for prazosin is not related to the α_1-adrenoceptor, as this receptor displays a further 250 times higher affinity for prazosin (Petrash & Bylund, 1986).

Evidence indicates that α_2-adrenoceptor agonists form a ternary complex with the receptor and inhibitory guanine-nucleotide protein (Gi) resulting in a high affinity state of the receptor (Hoffman *et al.*, 1982; Bylund & U'Prichard. 1983). Guanine nucleotides act by inhibiting the formation/or destabilizing the receptor-guanine nucleotide protein complex to a low affinity receptor state. In this study, the potency of adrenaline and noradrenaline to displace [^{3}H]-yohimbine from its binding site was reduced significantly in the presence of 0.1 mM GTP. In contrast the displacement curves for 8-OH-DPAT, RU 24969 and methysergide were unaffected by GTP, suggesting that different affinity states or agonist interactions are unlikely to account for the biphasic displacement curves obtained with the 5-HT ligands. It has been shown previously that the biphasic displacement of [^{3}H]-yohimbine binding to rat brain by prazosin does not involve negative cooperativity or different affinity states of the α_2-adrenoceptor (Bylund, 1985). It remains to be determined if 8-OH-DPAT, RU 24969 and methysergide act at an allosteric binding site influencing α_2-adrenoceptor binding rather than competing directly at the site itself.

The results of this study provide additional evidence that human platelets contain a single population of α_2-adrenoceptors whereas rat cerebral cortex contains a mixed population of α_2-adrenoceptor subtypes. The ability of 8-OH-DPAT, RU 24969 and methysergide, in addition to prazosin and oxymetazoline, to differentiate [^{3}H]-yohimbine binding provides additional pharmacological evidence for heterogeneity within rat brain α_2-adrenoceptor binding sites and thus possibly within functional α_2-adrenoceptors. Sequencing studies have indicated that the 5-HT_{1A} receptor is closely related to the structural core for adrenoceptors (Fargin *et al.*, 1988) and it is therefore to be expected that there will be some overlap in binding affinities for the 5-HT_{1A} receptor and subtypes of α_2-adrenoceptor. If the two sites defined in rat cortex by the 5-HT ligands represent the α_{2A}- and α_{2B}-adrenoceptor subtypes, then they do not correspond to the population of sites that exists on human platelets. Alternatively, if the human platelet adrenoceptor is considered to be the α_{2A}-adrenoceptor subtype, then the subtypes defined in rat cortex, by the 5-HT ligands, do not fit into the current classification for α_2-adrenoceptor subtypes.

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Effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive potassium channels in insulin-secreting cells

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1 The whole-cell patch-clamp technique has been used to investigate the effects of pinacidil, RP 49356 and nicorandil on membrane potential and adenosine 5'-triphosphate (ATP)-sensitive K^+ channel currents in the insulin-secreting cell line RINm5F. Interactions between pinacidil, RP 49356, nicorandil, diazoxide and ATP have been studied in excised outside-out membrane patches and open-cells.

2 In RINm5F whole-cells (current-clamp mode) continually exposed to glucose, pinacidil, RP 49356 and nicorandil, at concentrations greater than 100 μM, consistently reversed the effects of the sugar by repolarizing the membrane and terminating voltage-gated Ca^{2+} spike-potentials.

3 The actions of pinacidil, RP 49356 and nicorandil on membrane potential may be explained by their effects on the opening of ATP-sensitive K^+ channels, since all three compounds activated channels in whole-cells (voltage-clamp mode), excised outside-out membrane patches and open-cells, at concentrations greater than 100 μM. Below 100 μM the actions of pinacidil, RP 49356 and nicorandil were weak and inconsistent.

4 The mechanism of channel activation appears to depend on the presence of cytosolic ATP, since in its absence, pinacidil, RP 49356 and nicorandil (> 100 μM) had either no effects or inhibited K^+ channels.

5 Pinacidil, nicorandil and RP 49356 (200–500 μM) also appeared to open K^+ channels inhibited by quasi-physiological concentrations of ATP (4 mM) and ADP (1 mM). However, in comparison to diazoxide their effects were weak.

6 Since pinacidil, RP 49356 and nicorandil have been shown to have effects on smooth muscle preparations at concentrations of 30 μM and below, the data presented in this study may have important implications for any prospective therapeutic application of the drugs, since at the concentrations required to relax smooth muscle cells, hyperglycaemia will be avoided.

Introduction

The pharmacological control of adenosine 5'-triphosphate (ATP)-sensitive potassium (K_{ATP}) channels in insulin-secreting cells, first identified by Cook & Hales (1984), has important implications for the therapeutic treatment of insulin regulation disorders (Cook, 1988), since these channels govern the transmembrane potential of pancreatic β-cells (Petersen & Findlay, 1987; Petersen & Dunne, 1989). Glucose metabolism closes K^+ channels found to be open in intact cells, thus initiating a membrane depolarization (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986) which is functionally required for the activation of voltage-gated L-type calcium channels (Velasco *et al.*, 1988; Velasco & Petersen, 1989), responsible for Ca^{2+} spike potentials (Matthews & Sakamoto, 1985) and an increase in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Wollheim & Biden, 1987; Dunne *et al.*, 1990b). Tolbutamide mimics the effects of glucose on these cells since it is a selective inhibitor of K_{ATP} channels (Sturgess *et al.*, 1985; Dunne *et al.*, 1987). The sulphonamide diazoxide, on the other hand, reverses the effects of the sugar, causing hyperglycaemia (Henquin *et al.*, 1982), through the activation of channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989).

The actions of the putative antihypertensive compounds nicorandil and pinacidil are also thought to involve novel modulatory effects on K^+ channels (Cook, 1988; Quast & Cook, 1989), and the vasorelaxant RP 49356 (Mondot *et al.*, 1988) has been shown to have a direct effect in opening K_{ATP} channels in cardiac myocytes (Escande *et al.*, 1988; Thuringer & Escande, 1989). Due to the current interest in the pharmacological regulation of K^+ channels and the fact that pinacidil has recently been shown to inhibit glucose-induced insulin release from pancreatic β-cells, by a mechanism involving an increase in the rate of ^{86}Rb efflux (Lebrun *et al.*, 1988), my aim was to investigate the effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive K^+ channels in insulin-secreting cells using the patch-clamp technique.

Methods

Cell isolation and maintenance

All experiments were carried out on the clonal insulinoma cell line RINm5F, maintained as previously described (Wollheim *et al.*, 1988; Dunne *et al.*, 1989).

Patch-clamp experiments and analysis

Eletrophysiological investigations of insulin-secreting cells were carried out using the patch-clamp technique (Hamill *et al.*, 1981). Single-channel current recordings from excised outside-out membrane patches, as well as whole-cell current- and voltage-clamp experiments, were made with K^+-rich solution in the pipette and Na^+-rich solution in the bath. Permeabilized or open-cell experiments (Dunne *et al.*, 1986), in which the plasma membrane is perforated by saponin (0.05%) in contact with the plasma membrane outside the area from which the single-channel current recording is made, were carried out with Na^+-rich solution in the pipette and K^+-rich solution in the bath. Patch-clamp pipettes (Type 101PB, Ceebee Glass, Denmark) had a final resistance of between 5 and 10 MΩ when filled. Exchange from control to test solution was achieved manually under visual control through a series of outlet pipes (Dunne et *al.*, 1987; 1988a,b).

All whole-cell and single-channel current data were prefiltered at 3 kHz (low pass) (Kemo, UK) and stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis. Changes in potassium channel open-state probability were quantified from pre-recorded stretches of data lasting between 15 and 20 s and are expressed as a fraction of the pre-control level of activity (100%), as previously described (Dunne *et al.*, 1989; Dunne, 1989). This particular method of quantification was preferred to that of expressing a finite value of open-probability, since the definitive number of operational K_{ATP} channels in a particular patch of membrane is often unknown.

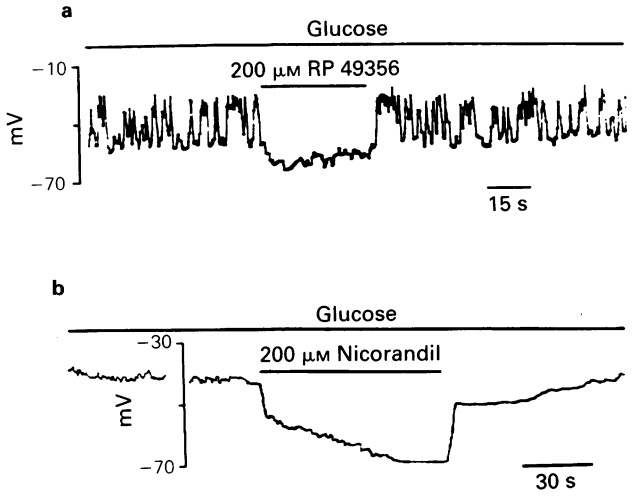


Figure 1 RP 49356 (a) and nicorandil (b) modify the glucose (5 mM)-induced electrical activity of RINm5F cells. Both records come from separate RINm5F whole-cells (currents-clamp mode). The experiment shown in (a) began 600 s after formation of the whole-cell, whereas the one shown in (b) began 480 s after initiation of the intracellular recording and 450 s after the run-down of Ca^{2+} spike-potentials. Both records started 40 s after the addition of glucose to the bathing solution.

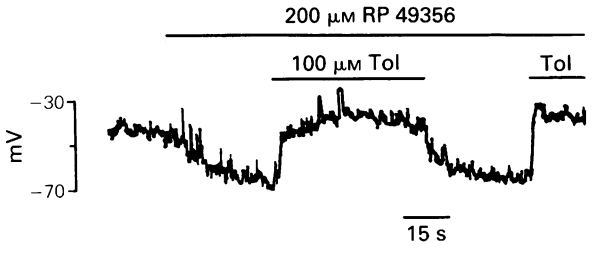


Figure 2 Tolbutamide (Tol) reversed the effects of RP 49356 on the glucose (5 mM)-induced electrical activity of a single RINm5F cell. The voltage recording began 400 s after initiation of the intracellular recording, 200 s after the run-down of Ca^{2+} spike-potentials and 50 s after the addition of glucose to the bathing solution.

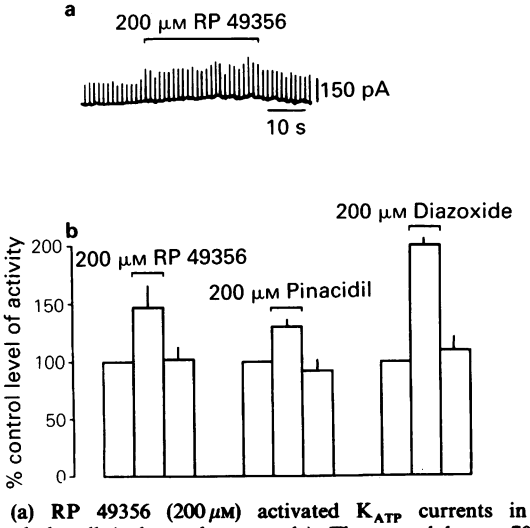


Figure 3 (a) RP 49356 (200 μM) activated K_{ATP} currents in a RINm5F whole-cell (voltage-clamp mode). The record began 700 s after initiation of the intracellular recording and 200 s after the addition of glucose (5 mM) to the bath, which reduced the K_{ATP} current to 41% of the control level of activity (not shown). (b) Shows a quantitative analysis of the effects of 200 μM concentrations of RP 49356, pinacidil and diazoxide on whole-cell K_{ATP} currents. All data have been expressed as a percentage of the pre-test level of activity (100%) (i.e. in the presence of 5 mM glucose), from 4, 4 and 7 separate cells, and includes the data presented in (a). In (b) each column represents the mean and vertical bars show s.e.mean.

Changes in whole-cell K_{ATP} currents, generated with depolarizing voltage pulses of 10/20 mV from a holding potential of −60 mV (100 ms duration, 1 pulse every 0.8 s) are also expressed as a percentage of the pre-test value (100%). All records presented were directly photographed from either the oscilloscope screen or from the pen-recording trace (Devices, UK). In all single-channel current records shown, upward deflections represent outward current flow (i.e. from the inside to the outside of the membrane patch).

Media

The standard extracellular Na^+-rich solution used throughout these experiments contained (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13 and HEPES 10. When the solution was used in the bath (whole-cell and excised outside-out membrane patches), 2 mM $CaCl_2$ was added, whereas when it was used to fill the patch-clamp pipette (open-cell experiments) $CaCl_2$ was omitted and 0.5 mM EGTA added. The pH of all Na^+-rich solutions was adjusted to 7.2 with NaOH. The standard intracellular K^+-rich solution contained (mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, EGTA 0.5 and HEPES 10. ATP 1 mM was added to this solution when it was used to fill patch pipettes (whole-cell and outside-out membrane patch experiments). The pH of all K^+-rich solutions was adjusted to 7.2 with KOH. Stock solutions of RP 49356 (N-methyl (pyridyl-3)-2 tetrahydrothiopyranne carbothioamide-2-t-oxide-1-r), nicroandil (Rhone-Poulenc Ltd, UK), and pinacidil ((±)-2-cyano-1-(4-pyridyl)-3-(1,2,2-trimethyl-propyl) guanidine monohydrate) (Leo Laboratories, UK) were prepared in 70% ethanol (vol/vol), whereas stock solution of tolbutamide (Sigma, UK) and diazoxide (Glaxo Research Ltd., UK) were prepared in dimethylsulphoxide (DMSO). The maximal concentrations of DMSO (1%) and ethanol (2%) used in these experiments had no effects on K^+ channels (Dunne *et al.*, 1987; Dunne *et al.*, 1990a). The osmolality of all solutions was 290 ± 5 mosm kg^{-1}. All experiments were carried out at room temperature (22–25°C).

Results

The effects of pinacidil, RP 49356 and nicorandil on membrane potential

The actions of pinacidil, RP 49356 and nicorandil on the transmembrane potential of RINm5F cells were investigated using the whole-cell current-clamp variation of the patch-clamp technique. In total 42 cells were used in this study. On average the spontaneous membrane potential, estimated within seconds of beginning the intracellular recording, was found to be −62 ± 2 mV (n = 42) (mean ± s.e.mean). Twenty eight of these cells were exposed to glucose (5–10 mM) added directly to the bath solution, 37 times. Glucose initiated (i) a depolarization of the membrane, found to be on average 22 ± 2 mV (n = 37) and (ii) caused the generation of spike-potentials. The effects of 200 μM RP 49356 on the glucose-induced electrical activity of a single RINm5F whole-cell is shown in Figure 1a. In the continued presence of the sugar, RP 49356 repolarized the membrane which led to the termination of spike-potentials. Of the 28 cells exposed to glucose in this particular series of experiments, only 3 were able to sustain Ca^{2+}-spikes for longer than 15 min. In the remaining 25 cells, the average duration of spike-potentials was found to be 302 ± 36 s. One such experiment is illustrated in Figure 1b, which also shows that 200 μM nicorandil reversed the effects of glucose by repolarizing the membrane.

Similar effects to those seen in Figure 1 were found for pinacidil, RP 49356 and nicorandil, at concentrations between 200 and 500 μM, in 4 (4/4 applications), 8 (9/10 applications) and 4 (4/4 applications) separate cells, respectively. At concentrations below 100 μM the effects of pinacidil, RP 49356 and nicorandil were weak and inconsistent (n = 7 separate cells).

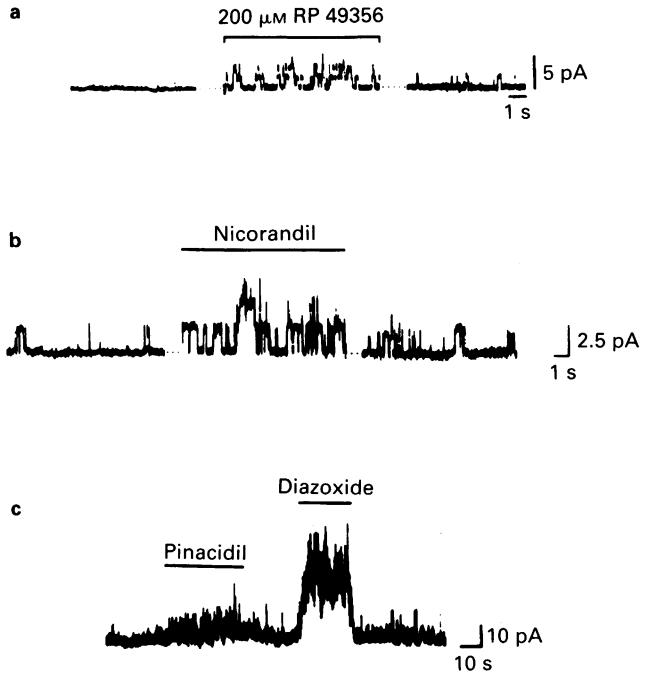


Figure 4 RP 49356 (200 μM) (a), nicorandil (200 μM) (b), pinacidil (200 μM) (c) and diazoxide (200 μM) (c) open ATP-sensitive K^+ channels in excised outside-out membrane patches. All 3 current traces came from separate cells.

Since the effects of glucose on insulin-secreting cells are initiated by changes in the gating of K_{ATP} channels, interactions between pinacidil, RP 49356, nicorandil and tolbutamide were investigated. Figure 2 shows that in the continued presence of glucose, 200 μM RP 49356 repolarized the membrane. When tolbutamide (100 μM) was then added in the presence of both glucose and RP 49356, a sharp, sustained depolarization of the cell was initiated. Removal of tolbutamide repolarized the membrane, which was followed by a renewed depolarization upon readmission of the sulphonylurea to the bath. This result was confirmed in a further 3 separate cells (4/5 attempts), and also seen for pinacidil (200 μM) ($n = 4$ cells) and nicorandil (200 μM) ($n = 3$ cells).

Pinacidil, RP 49356 and nicorandil enhance K_{ATP} currents

The effects of pinacidil, RP 49356 and nicorandil on whole-cell K_{ATP} currents were studied under voltage-clamp conditions. Outward K_{ATP} currents, elicited by 10 to 20 mV depolarizing voltage pulses from a holding potential of −60 mV (100 ms duration, 1 pulse every 0.8 s), were inhibited by glucose (5–10 mM) to, on average, 35 ± 9% of the control level of activity (not shown) due to the closure of K_{ATP} channels (Dunne *et al.*, 1990c) and enhanced by pinacidil (200 μM), RP 49356 (200 μM) (Figure 3a), nicorandil (200 μM) ($n = 3$ separate cells) and diazoxide (200 μM) in the continued presence of the sugar. A quantitative analysis of the effects of pinacidil ($n = 4$ cells), RP 49356 ($n = 4$ cells) and diazoxide ($n = 7$ cells) is shown in Figure 3b.

Single K_{ATP} currents from excised outside-out membrane patches, with 1 mM ATP added to the cytosolic face of the membrane, were also found to be activated by RP 49356 (200–

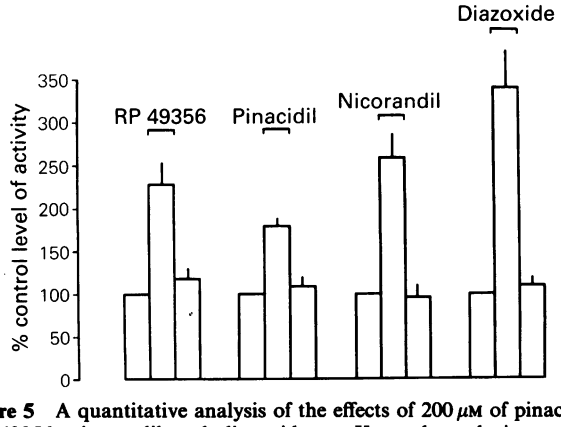
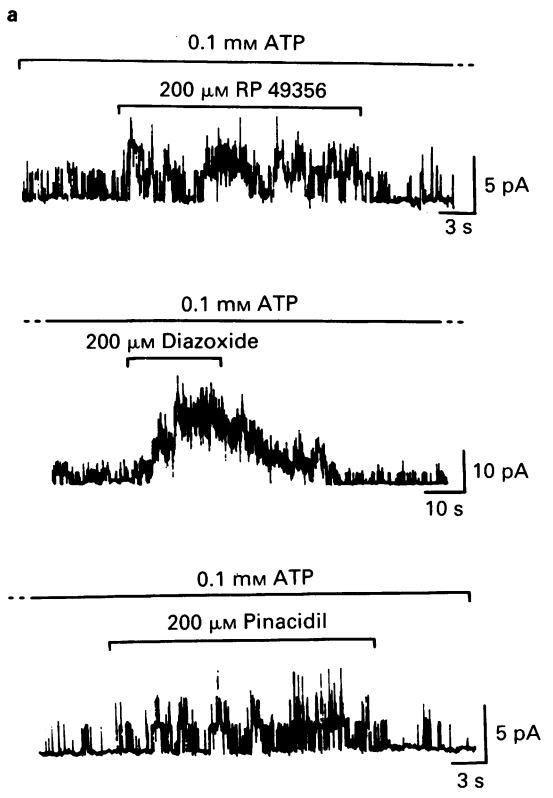


Figure 5 A quantitative analysis of the effects of 200 μM of pinacidil, RP 49356, nicorandil and diazoxide on K_{ATP} channels in excised outside-out membrane patch experiments. Average current values for the effects of RP 49356, pinacidil, nicorandil and diazoxide have been expressed as a percentage of the control level of activity (100%) from 10, 9, 5 and 8 separate cells, respectively, and the data shown in Figure 4 have been included. Each column represents the mean and vertical bars show s.e.mean.



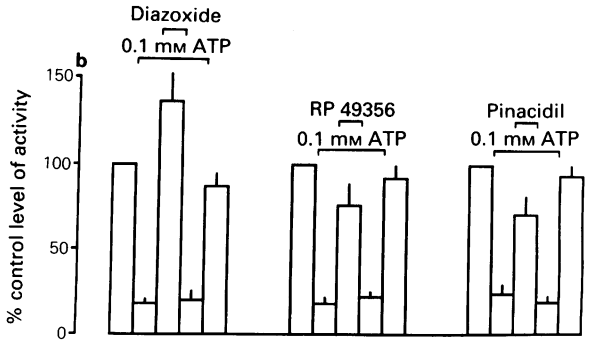


Figure 6 RP 49356, diazoxide and pinacidil open K^+ channels inhibited by 0.1 mM ATP. The data shown (a) are from the same RINm5F open-cell. The intervals between the end of the first record and the beginning of the second and the end of the second and the start of the third are 10 and 30 s, respectively. (b) Shows a quantitative analysis of the effects of 200 μM of diazoxide, RP 49356 and pinacidil on K_{ATP} channels inhibited by ATP (0.1 mM), and includes the experiments shown in (a). Average current values for the effects of diazoxide ($n = 5$ open-cells), RP 49356 ($n = 4$) and pinacidil ($n = 5$) have been expressed as a percentage of the control level of activity (100%). Vertical bars show s.e.mean.

500 μM) (Figure 4a), nicorandil (200–500 μM) (Figure 4b) and pinacidil (200–500 μM) (Figure 4c). A quantitative analysis of the effects of these compounds along with diazoxide (100 μM) is shown in Figure 5.

Opening of ATP-sensitive K^+ channels by pinacidil, RP 49356 and nicorandil requires cytosolic ATP

The open-cell recording configuration was used to study the mechanism by which pinacidil, RP 49356 and nicorandil open ATP-sensitive K^+ channels. Figure 6a shows 3 continuous current recordings taken from the same RINm5F open-cell. Following cellular perforation with 0.05% saponin (not shown) the activity of K^+ channels was reduced by adding 0.1 mM ATP to the inside of the plasma membrane. In the continued presence of ATP, 200 μM concentrations of RP 49356, diazoxide and pinacidil were all found to activate channels reversibly (note that the vertical scale in the centre panel has been compressed to accommodate the larger effect of diazoxide). A quantitative analysis of this and an additional 4 separate open-cell experiments, where direct comparisons were made between pinacidil (4/4 applications), RP 49356 (6/6 applications) and diazoxide (16/16 applications), has been presented in Figure 6b. Nicorandil (200 μM) was also found to open K^+ channels inhibited by 0.1 mM ATP in 3 separate cells (3/3).

When added to the inside of the membrane in the absence of ATP, the effects of pinacidil, RP 49356 and nicorandil were mixed. Out of 8 applications of pinacidil to 7 separate cells; 1 attempt activated channels, 1 addition had no effects whereas 6 applications were found to reduce the K_{ATP} current (Figure 7a). Similar inhibitory effects were found for both RP 49356 and nicorandil on 5/7 and 4/8 occasions in 6 and 4 separate cells, respectively. Inhibition was apparently not associated with any significant change in the frequency of channel opening, but was characterised by a decrease in the single-channel current amplitude (Figure 7).

Interactions between the putative K^+ channel modulators and ATP and ADP have also been studied. Figure 8a shows a typical experiment in which direct comparisons were made between the activator effects of 500 μM RP 49356, 500 μM pinacidil and 100 μM diazoxide on K^+ channels inhibited by 4 mM ATP and 1 mM ADP, whereas Figure 8b shows the quantitative analysis of this and an additional 6 separate open-cell experiments. Nicorandil was also found to open channels inhibited by ATP and ADP in 3 separate cells (n = 3/3 applications).

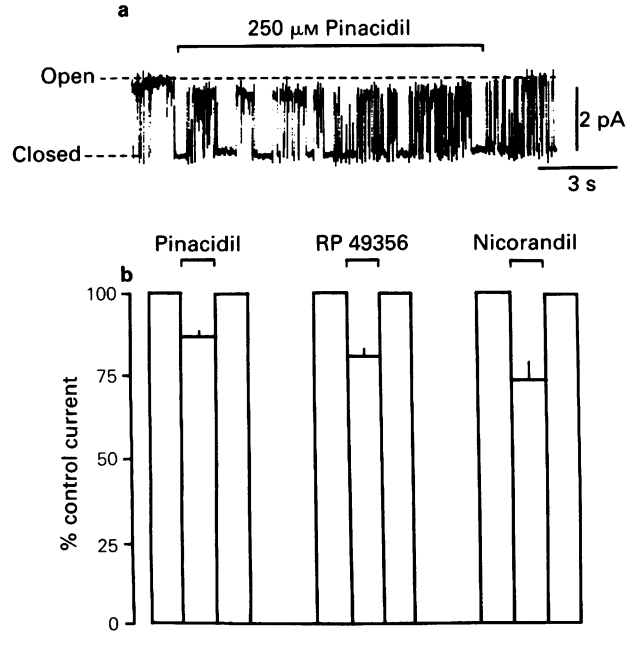


Figure 7 (a) Inhibition of K_{ATP} channels by pinacidil when added to the cytosolic face of the membrane in the absence of ATP. (b) A quantitative analysis of the effects of pinacidil, RP 49356 and nicorandil on K_{ATP} channels in the absence of internal ATP. Average data have been expressed as a percentage of the pre-control current value (100%), from 4, 4 and 4 separate open-cells, respectively, and include the data shown in (a). Vertical bars show s.e.mean.

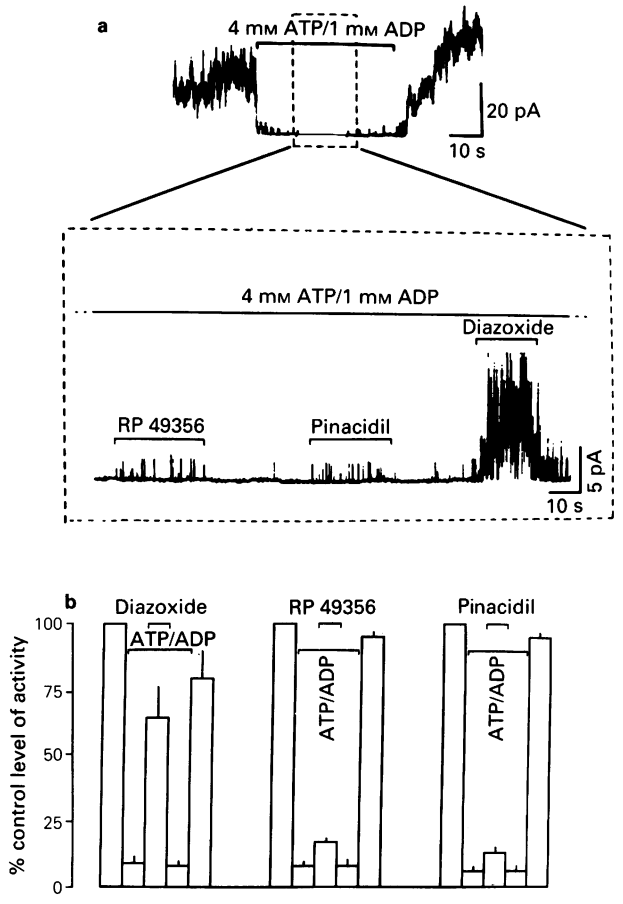


Figure 8 Pinacidil, RP 49356 and diazoxide open K^+ channels inhibited by 4 mM ATP and 1 mM ADP. (a) A typical continuous current trace, recorded from a RINm5F open-cell, whereas (b) illustrates the quantitative analysis of the effects of 500 μM concentrations of RP 49356 and pinacidil and 100 μM diazoxide on K_{ATP} channels inhibited by ATP (4 mM) and ADP (1 mM), and includes the experiments shown in (a). Average current values for the effects of diazoxide (n = 6 open-cells), RP 49356 (n = 4) and pinacidil (n = 4) have been expressed as a percentage of the control level of activity (100%). Vertical bars show s.e.mean.

Discussion

The patch-clamp technique has been used in the present study to investigate the effects of novel K^+ channel modulator compounds on ATP-sensitive K^+ channels in insulin-secreting cells. The effects of glucose on RINm5F cells (Ribalet *et al.*, 1988; Dunne *et al.*, 1990b) were found to be abolished by pinacidil, RP 49356 and nicorandil (Figure 1). All three compounds were found to enhance the flow of current through ATP-sensitive K^+ channels in whole-cells (Figure 3), excised outside-out membrane patches (Figures 4 and 5) and open-cells (Figures 6 and 8). Since carbohydrate-metabolism and tolbutamide-evoked inhibition of K_{ATP} channels in these cells results in the initiation of a depolarization of the membrane, the effects of pinacidil, RP 49356 and nicorandil on these channels may explain why, in whole-cells, each of the drugs repolarized the membrane and abolished spike-potentials. In total, 89% of the cells used in these experiments were unable

to generate Ca^{2+}-spikes for longer than 15 min. The disappearance of spike-potentials is probably related to the run-down of Ca^{2+} channels in the whole-cell, as has been found for a number of other cell types (Byerly & Hagiwara, 1982; Fenwick *et al.*, 1982; Katayama *et al.*, 1985).

The mechanism of K^+ channel activation was studied in permeabilized open-cells and outside-out membrane patches. These data suggest: (i) that internal ATP is required in order to observe channel activation regularly and (ii) that pinacidil, RP 49356 and nicorandil will open channels inhibited by ATP and ADP at concentrations present in intact cells (Wollheim *et al.*, 1988; Dunne *et al.*, 1988b). Qualitatively, the effects of the novel K^+ channel modulators are very similar to those of diazoxide, a hyperglycemia-inducing sulphonamide that has been used clinically to treat certain forms of insulinomas of the pancreas (Altsuzler *et al.*, 1977), due to its ability to open K_{ATP} channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989). However, in comparison to the effects of diazoxide, the actions of pinacidil, RP 49356 and nicorandil were weak (Figures 3, 4, 5, 6 and 8), suggesting that they are unlikely to replace the sulphonamide as effective hyperglycemia-inducing agents.

The experiment shown in Figure 7 is particularly interesting, since not only does it suggest thst ATP is obligatory for the actions of pinacidil, RP 49356 and nicorandil, but the data may indicate that each of the compounds will inhibit channels in the absence of ATP. The inhibition of K^+ channels was not characterized by any significant change in open-state probability but rather by a decrease in the single-channel current amplitude. Interactions between ATP and diazoxide have been investigated in some detail. Dunne *et al.* (1987) first concluded that internal ATP was required for diazoxide to open channels and that competitive interactions between ATP (ATP^{4-}) and diazoxide were apparent. More recently, both Dunne (1989) and Kozlowski *et al.* (1989) showed that the actions of diazoxide are dependent upon protein phosphorylation of either the channel or a related control unit. These conclusions were reached by the observations that (i) in the presence of non-hydrolyzable forms of ATP ($ATP\gamma S$, AMP-PNP and AMP-PCP) the activator effects of diazoxide were lost (Dunne, 1989; Kozlowski *et al.*, 1989) and (ii) in the complete absence of internal Mg-ATP, diazoxide actually closed channels (Kozlowski *et al.*, 1989). The activation of K^+ channels in Mg^{2+}-free solutions shown by Dunne *et al.* (1987) probably results from the presence of 3 μM Mg^{2+} in the test solution, which presumably provides enough Mg-ATP complex to augment the effects of the drug. In insulin-secreting cells cromakalim, which has little influence on channels below 100 μM (Kozlowski *et al.*, 1989, Dunne *et al.*, 1990a,b), is an effective modulator of channel gating at higher concentrations (Dunne *et al.*, 1990a,b). Since, like diazoxide, the actions of cromakalim are also dependent on protein phosphorylation (Dunne *et al.*, 1990a), it is interesting to speculate that a similar mechanism of channel activation may underlie the effects of pinacidil, RP 49356 and nicorandil.

In conclusion, the data presented in this study suggest that pinacidil, RP 49356 and nicorandil open ATP-sensitive K^+ channels in insulin-secreting cells, both from the inside and the outside of the plasma membrane. In the presence of glucose, K^+ channel activation results in a repolarization of the membrane and the termination of Ca^{2+} spike-potentials. This would then lead to an attenuation of the secretagogue-induced rise in $[Ca^{2+}]_i$, the key internal regulator of insulin secretion (Wollheim & Biden, 1987) and an inhibition of insulin release, as has been found for pinacidil (Lebrun *et al.*, 1988). However, since: (i) in a number of preparations, vaso-relaxant effects of nicorandil and pinacidil have been regularly observed at concentrations below 10 μM (Bray *et al.*, 1987; Cook *et al.*, 1988; Hermsmeyer, 1988; Videbaek *et al.*, 1988; Wilson *et al.*, 1988) and (ii) RP 49356 has been shown to open K_{ATP} channels in cardiac myocytes at concentrations around 30 μM (Thuringer & Escande, 1989), at these particular doses, the compounds will have little effect on β-cell electrical activity. This could be important for any possible therapeutic application of pinacidil, RP 49356 and nicorandil as effective antihypertensive drugs, since at the concentrations required to relax smooth muscle cells, hyperglycemia would be avoided.

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Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and α,β-methylene ATP

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1 Strength-duration curves for threshold mechanical responses to single transmural stimuli were identical for rat and guinea-pig detrusor. In both species atropine had no effect on the curves, but the curves were shifted to the right by nerve blockade with tetrodotoxin (TTX), and by blockade of P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP).

2 With short duration pulses of 50 V and less, the responses were nerve-mediated. Increase in either the strength or duration of the stimulus caused direct muscle stimulation, resistant to blockade with atropine, TTX and α-β-MeATP.

3 The shape of the contractile response to a single nerve stimulus varied from tissue to tissue. The responses could be mono-, bi-, or multiphasic. Bi- or multiphasic responses were normally seen in tissues which were spontaneously active. The multiphasic nature of the response was enhanced by factors which increased the excitability of the cells and was reduced by factors which decreased the excitability.

4 The frequency-response curves in the rat are similar to those previously obtained in the guinea-pig. Atropine suppresses the high frequency response by 25%, with little effect at low frequencies, whereas desensitization of P_2-purinoceptors with α,β-MeATP suppresses the responses maximally at low frequencies but still by 75% at high frequencies. A combination of both drugs eliminates the nerve-mediated responses.

5 It is concluded that the response to a single nerve stimulus is mediated by a non-cholinergic transmitter, through activation of P_2-purinoceptors. The possibility that simultaneous release of acetylcholine can modify the excitability of cells and thus the configuration of the response to a single stimulus is discussed.

Introduction

Contractile activity can be initiated in the smooth muscle of the urinary bladders of most mammals by stimulation of the parasympathetic nerves, or by field stimulation of intrinsic excitatory nerves in smooth muscle strips. The contractile response has, however, long been known to be partially resistant to blockade with atropine (Langley & Anderson, 1985; Henderson & Roepke, 1934; Ursillo & Clark, 1956). In the guinea-pig, Burnstock and his colleagues have provided evidence that the non-cholinergic excitatory transmitter is adenosine 5'-triphosphate (ATP) (Burnstock *et al.*, 1978a,b), and it has also been shown (Kasakov & Burnstock, 1983; Mostwin, 1986; Brading, 1987) that the contractile response of guinea-pig detrusor strips to transmural stimulation can be blocked by a combination of atropine and desensitization of the P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP). Atropine has little effect on the contractile response at low frequencies of stimulation, whereas desensitization of the P_2-purinoceptors is most effective at low frequencies of stimulation.

Electrophysiological studies on rabbit and guinea-pig detrusor (Hoyle & Burnstock, 1985; Fujii, 1988) have demonstrated that excitatory junction potentials can be elicited by single transmural nerve stimuli, and that they are unaffected by atropine, and blocked in the presence of α,β-MeATP. Application of ATP causes depolarization of the cell membranes, whereas acetylcholine contracts the tissues at concentrations that have little effect on the membrane potential, and only a modest effect on spike frequency. It seems likely in the guinea-pig, that the contractile response to a single stimulus of the intrinsic nerves is largely due to the excitatory junction potential initiating synchronous action potentials in the tissue, whereas the cholinergic response is largely initiated through pharmaco-mechanical coupling, and is more effective when acetylcholine is released by repetitive stimulation.

Recently, studies in rat detrusor strips have suggested that the response to a single nerve stimulus may often be biphasic, the second phase being preferentially blocked by atropine at a concentration of 3×10^{-6} M (Maggi *et al.*, 1985; Bhat *et al.*, 1989). Bhat *et al.* (1989) also demonstrated that the second phase is preferentially blocked by lowering extracellular Ca and by Ca channel blockers, findings that would not be expected for a drug acting primarily through pharmaco-mechanical coupling. In fact the authors showed that the response to bath-applied acetylcholine was less susceptible to Ca antagonists than the atropine-sensitive component of the neurogenic response.

To clarify this, we have compared the contractile responses of strips of rat and guinea-pig smooth muscle, recording the effects of atropine, α,β-MeATP and tetrodotoxin (TTX) on strength-duration curves and frequency-response curves. We have also examined the shape of the contractile response to single nerve stimuli. Our results suggest that the behaviour in both species is very similar, and that the slower components of the contractile response are more dependent on the level of excitability of the tissue than they are on activation of muscarinic receptors.

Methods

Rats and guinea-pigs of either sex were stunned by a blow to the head, and bled. The urinary bladder was removed, and opened by a ventral incision from the urethra to the dome. With the aid of a dissecting microscope, the mucosa was removed from the smooth muscle and strips of detrusor of about 1 mm by 10 mm containing parallel muscle bundles were prepared. The strips were mounted in small chambers (0.2 ml capacity, Brading & Sibley, 1983) and continuously superfused with Krebs solution (35–37°C) at a flow rate of approximately 1 ml min^{-1}. Recessed platinum ring electrodes,

1 cm apart, were used for stimulation. The apparatus allowed six strips to be studied simultaneously. Tension was measured isometrically on Pioden transducers and the output fed to a six channel Watanabe pen recorder. The strips were initially placed under 0.5 g resting tension, and allowed to equilibrate for at least an hour.

Krebs solution contained (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1, $CaCl_2$ 2.5 and glucose 11. Solutions were equilibrated with 97% O_2, 3% CO_2. Drugs used were atropine sulphate and carbachol, (B.D.H.), TTX and α,β-MeATP (Sigma).

Strength-duration curves were constructed by recording the voltage needed at each duration of stimulus to produce a just noticeable contraction of the tissue. After obtaining a control curve, the tissues were incubated in one of the three drugs, TTX, atropine or α,β-MeATP for at least 10 min and the procedure repeated in the continuous presence of the drug. For the frequency-response curves, a pulse duration of 0.05 ms and strength of 50 V was chosen, and the tissues were stimulated with 5 s trains of pulses at increasing frequencies, leaving 10 min between each stimulation. The responses were expressed as a percentage of a control response to a 10 s exposure to 10^{-4} M carbachol.

Results are expressed as the mean ± s.e.mean.

Results

Strength-duration curves

The strength-duration curves were remarkably similar in the two species, as illustrated in Figures 1 and 2. In the presence of 5×10^{-7} M atropine, a concentration which virtually abolished the tissue response to 10^{-4} M carbachol, the strength-duration curves were unaltered. In the presence of 1.6×10^{-6} M TTX the curves were shifted upwards and to the right.

Preliminary experiments in which muscle strips were continuously perfused with Krebs solution containing 10^{-5} M α,β-MeATP revealed that this concentration of drug did not abolish the contractile response of the muscle strips to short applications of 10^{-4} M α,β-MeATP, therefore a concentration of 10^{-4} M was used, which completely desensitized the P_2-purinoceptors. On application of the drug in this dosage, the tissues contracted, and then relaxed back to the baseline and did not respond to a further 10 s application of 10^{-2} M α,β-MeATP. The strength-duration curves in the presence of this drug were also shifted upwards and to the right. In the guinea-pig (Figure 1), α,β-MeATP shifted the curves significantly more than did TTX, suggesting that either the drug may have some additional effect on the excitability of the smooth muscle, or that in the presence of TTX, high voltages can release some transmitters from the nerve terminals. In the rat, the number of experiments was too small to show any significant difference between the effects of TTX and α,β-MeATP.

The response to single shocks

The shape of the contractile response to a single shock varied considerably from preparation to preparation, and also depended on the parameters of the stimulus used and the length of time since the preparation had been set up.

Correlation with spontaneous activity One of the most important determinants of the shape of the response seemed to be the degree of spontaneous activity shown by the preparation. In the majority of cases where there was no spontaneous activity, the contractile response elicited by a single stimulus was simple in shape, and showed only a single component. When spontaneous activity was seen, the response to a single stimulus was normally bi- or multiphasic, although in the guinea-pig monophasic responses were seen in some tissues

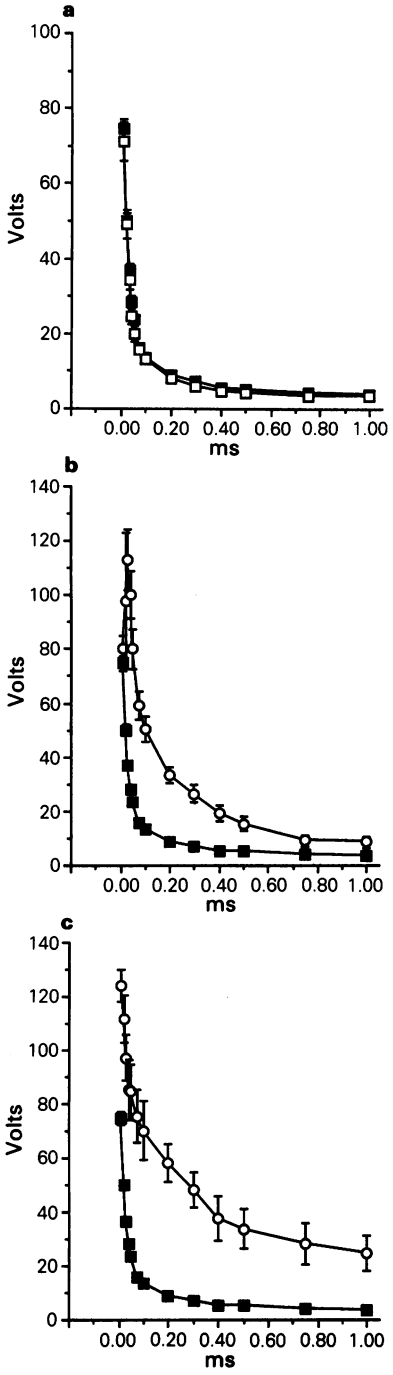

Figure 1 Strength-duration curves for threshold mechanical responses to single transmural electrical stimuli in strips of guinea-pig detrusor. In each panel (■) indicate control responses. (a) The effect of muscarinic receptor blockade with 5×10^{-7} M atropine. (b) The effect of blocking action potentials in the nerves with 1.6×10^{-6} M tetrodotoxin (TTX). (c) The effect of desensitization of P_2-purinoceptors with 10^{-4} M α,β-methylene ATP (α,β-MeATP). Note the lack of effect of atropine, and the fact that the curves are shifted further by α,β-MeATP than by TTX. Points show mean, s.e.mean (n = 11–34) shown by vertical bars where larger than the symbols.

that had relatively low frequency spontaneous activity. Since the rat tissues showed spontaneous activity more frequently than the guinea-pig tissues (this occurred in 90% of rat strips and only 60% of guinea-pig strips, n = 21 and 15 respectively), bi- and multiphasic responses were more commonly seen in rat strips. When spontaneous contractions occurred in the guinea-pig, they were always of a lower frequency than in the rat, and often disappeared during the course of the experi-

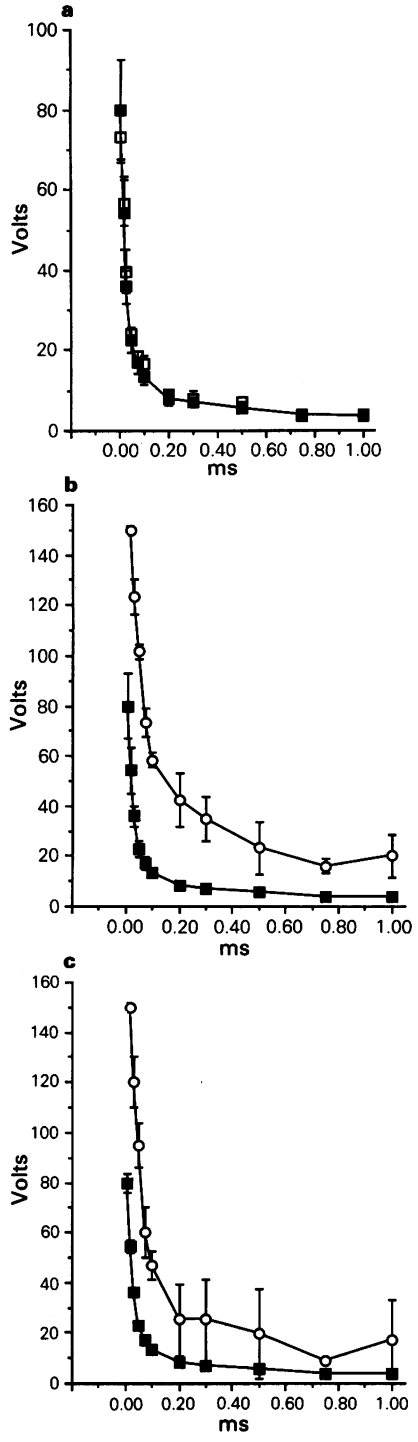

Figure 2 Strength-duration curves for threshold mechanical responses to single transmural electrical stimuli in strips of rat detrusor. In each panel (■) indicate control responses. (a) The effect of muscarinic receptor blockade with 5×10^{-7} M atropine. (b) The effect of blocking action potentials in the nerves with 1.6×10^{-6} M tetrodotoxin. (c) The effect of desensitization of P_2-purinoceptors with 10^{-4} M α,β-methylene ATP. Note the lack of effect of atropine. Points show mean, s.e.mean ($n = 2$–6) shown by vertical bars where larger than the symbols.

ment. In this case, the response to a single stimulus usually changed from being bi- or multiphasic to being monophasic.

Effect of alteration of stimulus parameters The effect on the magnitude of the contractile response of increasing the pulse duration at constant voltage of 50 V and increasing voltage at a constant pulse duration of 0.05 ms, is shown in Figure 3. With a 50 V stimulus, there was no response at a pulse width

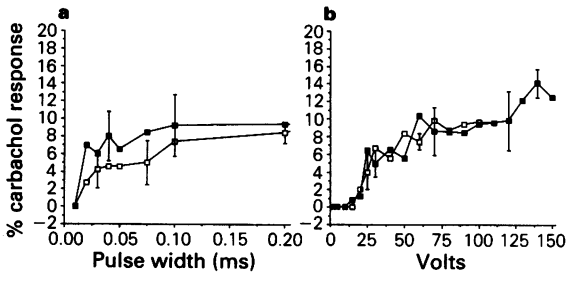

Figure 3 The effect on the contractile response of guinea-pig (■) and rat (□) detrusor strips of (a) increasing the pulse width of single 50 V transmural stimuli, and (b) increasing the stimulus strength of single 0.05 ms stimuli. Points are means of three tissues. Representative s.e.mean are shown.

of 0.01 ms. A response occurred at 0.02 ms, but in the guinea-pig there was little increase in the size of contraction on further increasing the stimulus width (Figure 3a). In the rat the size appeared to reach a plateau at about 0.03 ms, and then rose again progressively as the width increased. When a stimulus width of 1 ms was reached (not shown in Figure 3a), the contractile response of the rat had reached an amplitude of 16.1% of the maximum carbachol response, whereas the guinea-pig response was still only 9.5%. Examples of the shape of the responses are shown in Figure 4, along with the effects of atropine (3×10^{-7} M), TTX (1.6×10^{-6} M) and α,β-MeATP (10^{-4} M). With pulse durations of over 0.1 ms, the increase in amplitude in the rat was clearly caused by a slow component which was superimposed on the nerve-evoked response. This was insensitive to TTX, α,β-MeATP and atropine, and probably caused by direct excitation of the smooth muscle cells. In the guinea-pig the direct muscle response was

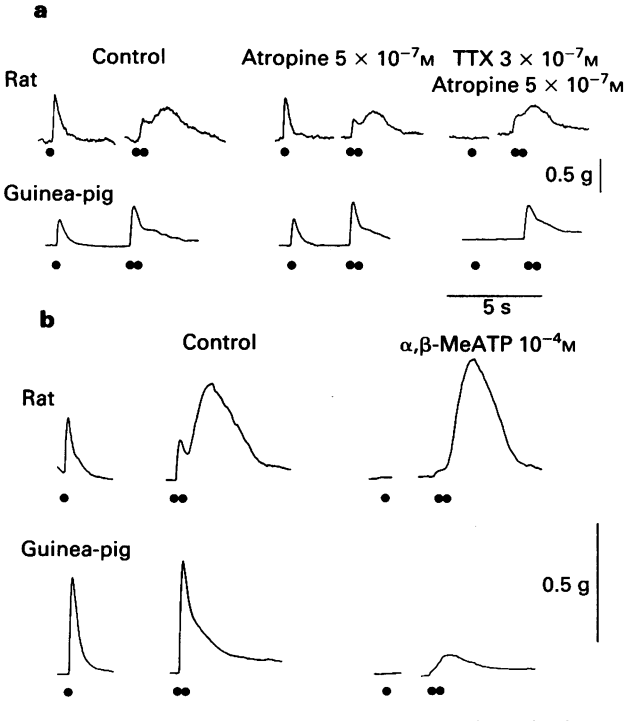

Figure 4 The contractile response of rat and guinea-pig detrusor strips to single stimuli. (a) The effects of atropine and tetrodotoxin (TTX). (b) The effects of desensitization of the P_2-purinoceptors with α,β-methylene ATP (α,β-MeATP). (●) Single shock of 0.05 ms, 50 V; (●●) single shock of 1 ms, 50 V. Note that in the rat, TTX- and α,β-MeATP-resistant response (direct muscle stimulation) appears as a separate slow component of the contraction and often reduces the size of the nerve-mediated component, whereas in the guinea-pig, it adds to the first component, and also contributes to a slower relaxation.

small and it was not clearly separated in time from the nerve evoked response. When the voltage was increased at a constant pulse width of 0.05 ms increases in response amplitude were produced that were indistinguishable in the two species (Figure 3b). Responses occurred at a threshold of about 20 V, were relatively constant between 25 and 50 V and then increased. This increase was again caused by direct stimulation of the smooth muscle cells. The response had the same characteristics as the response brought in at increasing pulse widths. It was resistant to TTX, α,β-MeATP and atropine. This direct muscle stimulation was not seen with the electrode configuration and stimulus parameters used by Bhat *et al.* (1989) or Maggi *et al.* (1985).

Effect of drugs For the investigation of the effect of drugs on nerve-mediated contractile responses, a standard stimulus of 0.05 ms and 50 V was used. Figure 4 shows that the response in both species was completely abolished by TTX (3×10^{-7} M) and by desensitization of the P_2-purinoceptors with α,β-MeATP (5×10^{-7} M). Figure 5 shows two responses of a strip of guinea-pig muscle before and after atropine (5×10^{-7} M), in which there was no obvious effect of the drug. However, because of the great variability of the responses with time, it was difficult to quantify the effects of atropine precisely and in some cases a small decrease in the size of the response was seen, although this could have been a time-dependent phenomenon. If the dose of atropine was increased to 3×10^{-6} M there was a clear decrease in the spontaneous activity of many of the tissues and there was always a decrease in the nerve-mediated response and a supression of the bi- or multiphasic activity as shown in Figure 6. It seems likely that this dose of atropine may have some non-specific inhibitory effect on the tissue.

Bi- or multiphasic responses could always be turned into monophasic responses by giving a second stimulus immediately after the first response had decayed, as shown in Figure 7. This also occurred in the presence of 5×10^{-7} M atropine. The multiphasic nature of the response became more pronounced when the spontaneous activity of the tissue was

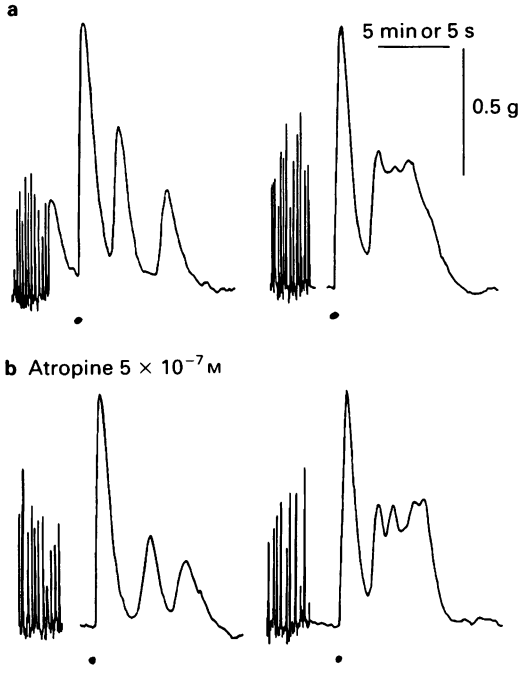

Figure 5 The effect of 5×10^{-7} M atropine on the contractile response to single stimuli (at ●, 0.05 ms, 50 V) in the guinea-pig. In this animal there was considerable spontaneous activity, and the response was multiphasic and appeared in two configurations. Atropine had little effect at this concentration. Sweep speed at faster rate for traces showing effect of electric stimulation.

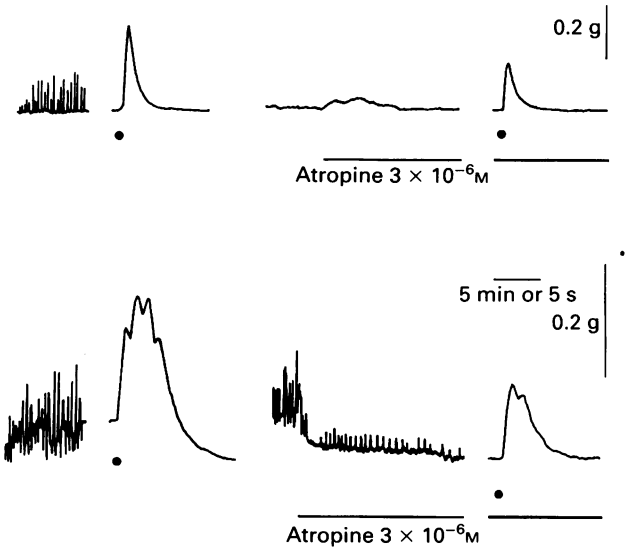

Figure 6 The effect of 3×10^{-6} M atropine on spontaneous activity and the response to single stimuli (at ●, 0.05 ms, 50 V) in guinea-pig and rat (top) detrusor. Note the reduction (bottom) of the spontaneous activity (in the guinea-pig the synchronous phasic activity was abolished), and the reduction in the amplitude of the contractile response. Sweep speed at faster rate for traces showing effect of electrical stimulation.

increased by application of TEA (1 mM), as shown in Figure 8, consistent with the results of Maggi *et al.* (1985).

Frequency-response curve

Figure 9 shows the frequency-response curve for rat detrusor strips. Five-second trains of pulses of 0.05 ms duration and 50 V were used. The maximum response of the tissues to transmural nerve stimulation was about 70% of the response to 10^{-4} M carbachol, in comparison to just over 80% in the guinea-pig (Brading, 1987). In this experiment, 10^{-6} M atropine was used for comparison with the guinea-pig data, and at this concentration there is a small, but not significant

Figure 7 The effect of a second stimulus given immediately after the response to a first stimulus (0.05 ms, 50 V) in the rat detrusor strip (stimulus marked ●). The response of two strips taken from different rats is shown. Note that the second response is a simple monophasic response.

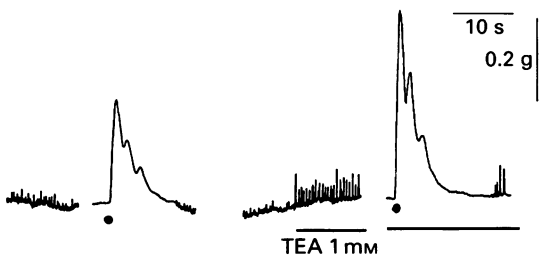

Figure 8 The effect of 1 mM tetraethylammonium (TEA) on the spontaneous activity and evoked response (single stimulus at ●, 0.05 ms, 50 V) of a strip of rat detrusor. Response in normal Krebs solution is shown on the left.

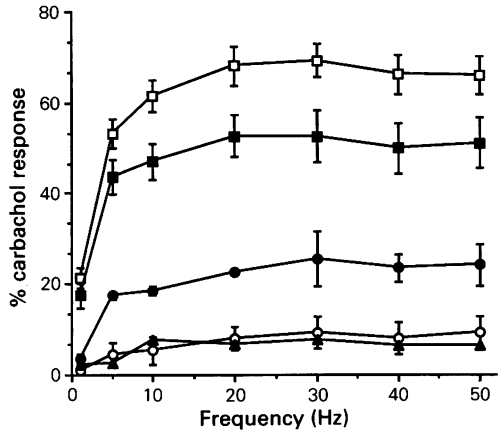

Figure 9 Frequency-response curves for rat detrusor strips: 0.05 ms, 50 V stimuli were applied at increasing frequencies for 5 s, every 10 min. Responses are mean with s.e.mean shown by vertical bars of 4–6 strips. (□) Control; (■) atropine 10^{-6} M; (●) α,β-methylene ATP (α,β-MeATP) 10^{-4} M; (○) atropine + α,β-MeATP; (▲) tetrodotoxin 1.6×10^{-6} M. Note the large atropine-resistant response, the greater effect of atropine at high frequencies of stimulation and the proportionally greater effect of desensitization of the P_2-purinoceptors with α,β-MeATP at the lower frequencies. The responses in the combined presence of atropine and α,β-MeATP are not significantly different from those in TTX.

reduction in response to stimulation at 1 Hz. As can be seen from the figure, atropine had its maximum effect on the response at frequencies above 20 Hz, and caused about 25% reduction of the contraction. The same was true in the guinea-pig (Brading, 1987). In contrast, desensitization of the P_2-purinoceptors with 10^{-4} M α,β-MeATP completely abolished the response of the tissue to a single stimulus of this duration and intensity in all tissues tested (see Figure 4), almost completely abolished the response at 1 Hz and depressed the contractile response by 73–77% at frequencies above 10 Hz. A smaller concentration of α,β-MeATP (5×10^{-6} M, not shown) depressed the response by 34% at 1 Hz, and by progressively smaller amounts until 40 Hz, when it had no effect. This again was very similar in the guinea-pig, suggesting that at this concentration not all of the purinoceptors are desensitized. The contractile responses in the combined presence of 10^{-4} M α,β-MeATP and 10^{-6} M atropine, were not significantly different from the responses in the presence of TTX, and in both cases it was noted that there was no contractile response during the stimulation period but the small contraction recorded occurred after the stimulus had ended.

Discussion

The results described in this paper confirm that in the rat, as in the guinea-pig, the contractile response of the detrusor muscle to transmural nerve stimulation consists of two components, which can be activated by different frequencies of stimulation, and that one part of the response is mediated through muscarinic receptors and the other part probably through P_2-purinoceptors. This duality of excitatory innervation has been found in all mammals studied with the exception of some old world monkeys and man (Craggs & Stephenson, 1985; Sibley, 1984), although there have been some reports (e.g. Husted *et al.*, 1983) of a non-cholinergic component in the human bladder. It has also been found, however, that the responses to low frequencies of stimulation are very resistant to atropine (see Brading, 1987), the cholinergic component of contraction appearing as the frequency of stimulation is increased. The suggestion has recently been made that in the rat, the contractile response to a single transmural nerve stimulus also has two components, the second of which may be mediated by activation of muscarinic receptors (Maggi *et al.*, 1985; Bhat *et al.*, 1989). Bhat and his colleagues have shown that the second component is somewhat more susceptible to blockade with Ca-channel blocking drugs, suggesting that Ca entry through voltage-sensitive Ca channels may be involved. Interestingly however, they found that the response to bath-applied acetylcholine was less sensitive to Ca antagonists.

It has recently been demonstrated that the response to muscarinic receptor activation in the guinea-pig detrusor does not involve significant depolarization of the cell membrane, whereas the response to ATP and to single nerve stimuli both involve marked depolarization of the membrane (Fujii, 1988). If the situation is similar in the rat, then it would be expected that the second component of the contraction, if it were indeed cholinergic, would be more resistant to Ca-antagonists than the early non-cholinergic response. We have therefore compared the contractile responses of the rat with those of the guinea-pig detrusor to transmural stimulation.

The strength-duration curves we have recorded are similar to those obtained in the rabbit and the pig by Sibley (1984). The curves are shifted to the right in the presence of TTX, suggesting that the contractile response at threshold voltages is caused by an action potential in the transmural nerves releasing an excitatory transmitter. The predominant transmitter is clearly not acetylcholine, since atropine has no effect on the strength-duration curves in either the rat or the guinea-pig. The transmitter seems to work through P_2-purinoceptors, since desensitization of these with α,β-MeATP also shifts the curves to the right, eliminating the response of the tissue to nerve-mediated stimuli. This alone suggests that acetylcholine is not playing a significant role in contractile response to single stimuli, since, in the guinea-pig at least, α,β-MeATP is specific in its actions, and does not affect the tissue response to acetylcholine (Fujii, 1988).

The responses to transmural stimulation which persist in the presence of TTX and α,β-MeATP are presumably due to direct activation of the smooth muscle cells, and it is interesting to note that with sufficient voltage, the cells can be activated by very short pulses. In the rat, direct muscle stimulation with a single pulse can produce a contractile response considerably larger than the maximum nerve-mediated response, whereas in the guinea-pig there is only a relatively small increment. The fact that the strength-duration curve in human tissues is not affected by TTX, is consistent with the lack of a non-cholinergic excitatory transmitter in man.

We found it difficult to study the effect of drugs on the shape of the contractile response to a single nerve stimulus, because of its variability.

We would agree with Maggi *et al.* (1985) and Bhat *et al.* (1989) that atropine at 3×10^{-6} M suppresses the response and that this effect is more marked on the second component where present, but this is a very high concentration of atropine and probably lowers the excitability of the tissue, as shown by a reduction in the spontaneous activity. At the more selective concentration of 5×10^{-7} M (in which the response to 10^{-4} M carbachol was virtually abolished) it was no longer clear that the second response was selectively suppressed. In some instances the whole response was somewhat reduced by this dose of atropine, although this might have been a time-dependent depression and in some instances the response was little affected or even bigger. These results are not sufficiently clear to allow us to rule out a minor contribution of acetylcholine to the later components of the response. However, since it is clear from the frequency-response curves that the cholinergic component becomes more significant with repetitive stimulation, we looked at the effect of giving two pulses close together. In this instance, the results are extremely clear cut. Giving the second stimulus immediately after the first response had decayed to baseline, invariably results in the second response being monophasic, even if the response to the first stimulus was multiphasic. This does not support a role

for acetylcholine in the later contractions. It is probable that the smooth muscle cell membranes are less excitable for a short while after the stimulus.

Our experiments suggest that the shape of the response to a single nerve stimulus is strongly dependent on the excitability of the tissue. It is closely correlated with the degree of spontaneous activity of the tissue, and the shape becomes simpler as the spontaneous activity decreases. In the experiments carried out by Maggi *et al.* (1985), the tissue was continuously stimulated at 0.01 Hz, which seems to suppress spontaneous activity, and in the paper by Bhat *et al.* (1989) no reference is made to the spontaneous activity. In agreement with Maggi *et al.* (1985) we find that the size of the single response and the amplitude of the secondary components are increased in the presence of tetraethylammonium, but we also show that this drug increases the spontaneous activity of the tissue.

We feel that the most likely explanation for the later components of the contractile response to single nerve-mediated stimuli, may be repetitive firing of action potentials triggered by an excitatory junction potential resulting from activation of P_2 purinoceptors and that the number of action potentials and the size of the contractile response to them may depend to a large extent on the excitability of the tissue. It is possible that simultaneous release of acetylcholine does modify the excitability of the tissue, either at the level of the cell membrane, or by release of intracellular calcium bringing the cells nearer to their contractile threshold. In the rabbit, the response to a single nerve stimulus consists of a fast atropine-resistant excitatory junction potential with superimposed spikes, followed by a slower depolarization which is blocked by atropine (Creed *et al.*, 1983; Fujii, 1988). However, this secondary depolarization is not evident in the guinea-pig (Fujii, 1988) under normal conditions, and similar experiments have not been carried out on rat bladder. In the rabbit (Sibley, 1984), the cholinergic component of the contractile response at high frequencies is larger than in the rat and guinea-pig (up to 40% as compared with 25% in the rat and guinea-pig).

If the role of acetylcholine in the response to a single nerve stimulus is to modify the excitability of the cells, then this could explain the difference seen by Bhat and his colleagues (1989) in the susceptibility of the response to bath-applied acetylcholine and the second component of the response to single stimuli to Ca-antagonist drugs, and the lowering of extracellular calcium.

In conclusion, the excitatory innervation of the rat and guinea-pig detrusor is very similar. The predominant innervation is non-cholinergic, and the cholinergic component of the contractile response is most marked with repetitive stimulation at frequencies of 10 Hz and above, when it can generate 25% of the contraction. The non-cholinergic transmitter is dominant in the response to single nerve stimuli, the size being unaffected by blockade of muscarinic receptors, but blocked by desensitization of the P_2-purinoceptors with α,β-MeATP. The shape of the response is influenced by the excitability of the tissue, and this may be modified by the cholinergic component.

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Relationships between tumour necrosis factor, eicosanoids and platelet-activating factor as mediators of endotoxin-induced shock in mice

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1 The toxicity of intravenous recombinant human tumour necrosis factor (rhTNF), a TNF fragment ($TNF_{114-130}$), endotoxin and combinations of rhTNF or $TNF_{114-130}$ were tested in mice. Neither rhTNF nor $TNF_{114-130}$ was lethal alone, but when combined with a non-lethal dose of endotoxin, rhTNF provoked dose-dependent mortality, as did higher doses of endotoxin alone.

2 Both the toxicity and the vasopermeability changes induced by endotoxin alone were blocked by the platelet-activating factor (PAF) antagonist BN52021, indomethacin or the dual cyclo-oxygenase/lipoxygenase inhibitor BW755C.

3 The lethality of the combined low dose endotoxin/rhTNF challenge was unaffected by pretreatment with BN52021, indomethacin or BW755C, or by treatment at 6 h intervals with BN52021 or BW755C.

4 The results of these studies suggest that TNF, a putative, early mediator of septic or endotoxin shock, cannot by itself mimic all of the effects of bacterial endotoxin in the model used in this study. Apparently, TNF works synergistically with other mediators whose release is stimulated by endotoxin.

5 The results also suggest that the mechanism of shock production by the rhTNF/endotoxin combination in mice is not dependent on the early stimulation of eicosanoid or PAF synthesis by rhTNF.

Introduction

Among the endogenous mediators of shock and lethality induced by i.v. injection of bacterial endotoxin, tumour necrosis factor (TNF), eicosanoids and platelet-activating factor (PAF) have been the focus of considerable recent attention. Pretreatment of experimental animals with TNF antibodies (Tracey *et al.*, 1987) or antagonists of thromboxane A_2 (Olanoff *et al.*, 1985). peptido-leukotrienes (Hagmann & Keppler, 1982) or PAF (Terashita *et al.*, 1985; Handley *et al.*, 1988) can block many of the pathophysiological sequelae of i.v. endotoxin or live *Escheria coli* injection. Further, i.v. injection of TNF (Tracey *et al.*, 1986; Kettelhut *et al.*, 1987; Satomi *et al.*, 1988), PAF (Lanara *et al.*, 1982; Myers *et al.*, 1983a) or thromboxane agonists (Myers *et al.*, 1983b) can, under some circumstances, produce shock and death.

Results of early studies of i.v. recombinant human TNF (rhTNF) suggested that this macrophage-derived cytokine was a primary mediator of endotoxin shock, and that it alone was capable of producing all of the major pathophysiology associated with endotoxaemia (Tracey *et al.*, 1986). As TNF can stimulate the release of lipid mediators such as PAF and eicosanoids from some cell types (Roubin *et al.*, 1987; Sun & Hsueh, 1988), the hypothesis has been developed that endotoxin-induced shock and lethality is largely mediated by the initial release of TNF from macrophages, followed by release of PAF and eicosanoids from various tissues, leading to the associated platelet activation, bronchoconstriction, hypotension, increased vascular permeability and eventually death. In fact, some studies have demonstrated that rhTNF induces shock and death in mice and rats which can be prevented by prior administration of cyclo-oxygenase inhibitors (Kettelhut *et al.*, 1987; Satomi *et al.*, 1988).

In contrast to studies which show a clear shock-producing effect of rhTNF alone, we have been unable to produce shock by i.v. injection of even large doses of rhTNF in either rats or mice (A.K. Myers & R.M. Price, unpublished results). Others have published similar findings (Rothstein & Schreiber, 1988), and suggest that a possible factor enhancing rhTNF toxicity in some studies is contamination by endotoxin, although this cannot account for TNF toxicity in all studies. However, under circumstances in which rhTNF displays no toxicity, simultaneous administration of low dose endotoxin leads to death in mice (Rothstein & Schreiber, 1988).

In fact, many recent studies demonstrate synergistic relationships between TNF and bacterial lipopolysaccharide (Rothstein & Schreiber, 1988; Sun & Hsueh, 1988) or other cytokines such as interleukin-1 (Ruggiero & Baglioni, 1987; Waage & Espevik, 1988). Based on these and our own observations, we hypothesized that TNF alone is only minimally toxic in healthy animals and that interaction with other mediators is an essential component of its role in endotoxaemia. In the present study, we investigated the lethality in mice of i.v. rhTNF and a TNF fragment, alone and in combination with bacterial endotoxin, and compared the efficacy of various drugs in blocking the effects of endotoxin and TNF.

Methods

Animals

Female CD-1 mice weighing 18 to 20 g were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and assigned randomly to the various groups below. Mice were anaesthetized by sodium amytal injection (100 mg kg^{-1}, i.p.) before challenge (below) with rhTNF, endotoxin or related compounds via a surgically exposed jugular vein. After completion of experiments, animals were killed by an overdose of pentobarbitone (i.p.).

Toxicity studies

Challenges consisted of i.v. endotoxin (*E. coli* serotype 0127:B8, Sigma), rhTNF (Cetus Corp., Emeryville, CA, U.S.A.), the TNF fragment, $TNF_{114-130}$ (Pennisula Laboratories, Belmont, CA, U.S.A.) or a solution containing both rhTNF and lipopolysaccharide (LPS). Biological activity of our rhTNF samples was periodically confirmed in an L929 cell cytotoxicity assay (data not shown), with no loss of activity observed over the period in which these experiments were

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performed. Doses of the challenges are indicated below. In the mortality studies, each group consisted of at least 12 mice except where indicated below. Survival of animals was followed for 7 days in the studies presented in Figure 1. In later studies, based on these results, the observation period was limited to 48 h. Because death only rarely occurred after 24 h, 24 h mortality was compared between groups by the Chi-square statistic.

Vasopermeability studies

In further experiments, haematocrit measurements were made just before and 15 min after endotoxin challenge. Blood for the basal haematocrit was collected from a small incision in a rear foot pad; the final haematocrit sample was obtained from freely flowing blood from an incision in the exposed jugular vein. The latter method is necessary for collection of blood from mice in the shock state. The change in haematocrit was compared between various experimental and control groups by Student's t test for independent means. Each group consisted of at least 9 mice. Hematocrit was also determined in preliminary studies before and at various intervals after rhTNF and endotoxin challenge. However, at no time after this challenge was a consistent, large rise in haematocrit observed, so the experiment was not pursued.

Drug treatments

Some groups of mice in the experiments below were treated with the PAF antagonist (3-(1,1-dimethylethyl) hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7α (epoxymethano)-1H, 6αH-cyclopenta [c] furo (2,3b) [3',2':3,4) cyclopenta-(1,2-d]furan-5,9,12(4H)-trione) (BN 52021) (Braquet *et al.*, 1985), the cyclo-oxygenase inhibitor indomethacin (Sigma Chemical Co., St. Louis, MO, U.S.A.), or the combination cyclo-oxygenase/lipoxygenase inhibitor (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) (BW 755C) (Higgs *et al.*, 1979) 30 to 45 min before challenge with rhTNF or endotoxin. Doses for these drugs were 40 mg kg^{-1}, 10 mg kg^{-1} and 40 mg kg^{-1}, respectively, and were based on preliminary dose-finding studies in which they were tested against endotoxin lethality. All three drugs were dissolved in dimethyl sulphoxide (DMSO) at a concentration such that the i.p. injection volume was 2.5 μl g^{-1} body weight. Controls for these experimental groups received i.p. DMSO, 2.5 μl g^{-1} body weight. Each specific drug treatment group had a corresponding control group. In an additional experiment, the effects of repeated doses of BW 755C, BN 52021 or the DMSO vehicle were tested, with the first injection 30 to 45 min before challenge, and subsequent treatments at 6 h intervals for one day.

Results

Toxicity of rhTNF, $TNF_{114-130}$ and endotoxin

Among the tested compounds, only endotoxin produced mortality by itself (Figure 1). The response was dose-dependent, with an LD_{50} of approximately 25 mg kg^{-1}. In contrast, neither rhTNF nor the synthetic TNF fragment, $TNF_{114-130}$, produced mortality at doses of up to 0.4 and 5.0 mg kg^{-1}, respectively. Mice injected with these compounds recovered normally from anaesthesia and appeared healthy for the entire 7 day observation period.

The addition of a dose of endotoxin (0.24 mg kg^{-1}), which by itself was well below the endotoxin dose-mortality curve, had a profound potentiating effect on rhTNF toxicity. This combination of rhTNF with a constant, low dose of endotoxin produced death with an LD_{50} of approximately 0.15 mg kg^{-1} rhTNF combined with 0.24 mg kg^{-1} endotoxin (Figure 1). Unlike rhTNF, $TNF_{114-130}$, 5 mg kg^{-1}, was not toxic in combination with this dose of endotoxin.

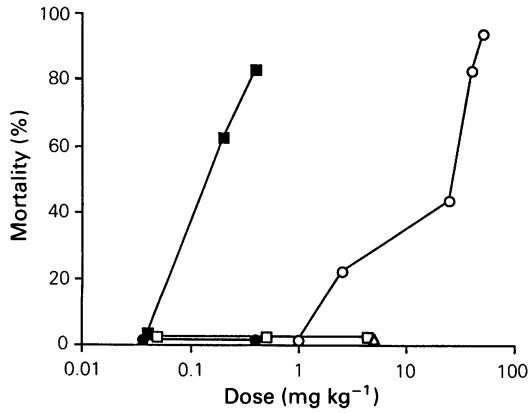

Figure 1 Lethality of i.v. endotoxin (○), recombinant human tumour necrosis factor (TNF) (●), $TNF_{114-130}$ (□), and low dose endotoxin (0.24 mg kg^{-1}) in combination with TNF (■) or $TNF_{114-130}$ in mice (△). Neither TNF nor $TNF_{114-130}$ produced any mortality in the absence of endotoxin; the TNF fragment, at the one dose tested, did not provoke death in combination with low dose endotoxin. Each data point represents mortality in a group of at least 12 mice.

A notable difference was observed in time of death in these comparative studies. With the approximate LD_{80} for the combination challenge, 0.4 mg kg^{-1} rhTNF/0.24 mg kg^{-1} endotoxin, 88% of the deaths occurred four or more hours after challenge. In contrast, 82% of deaths provoked by the approximate LD_{80} of endotoxin (40 mg kg^{-1}) occurred in less than four hours after challenge.

Effects of drugs on endotoxin-induced mortality and haematocrit changes

When mice were pretreated with doses of BN 52021, indomethacin or BW 755C as stated in the Methods, mortality induced by an approximate LD_{80} of endotoxin was significantly inhibited (Figure 2), with both the PAF antagonist and the joint cyclo-oxygenase/lipoxygenase inhibitor providing 100% protection. Similarly, the rise in haematocrit following endotoxin LD_{80} administration was significantly attenuated by each of the three drugs (Figure 3).

Effects of drugs on the toxicity of a combination of rhTNF and low dose endotoxin

The rhTNF/low dose endotoxin combination (0.4 mg kg^{-1} rhTNF/0.24 mg kg^{-1} endotoxin) which resulted in approximately 80% mortality (Figure 1), was equiactive in the pre-

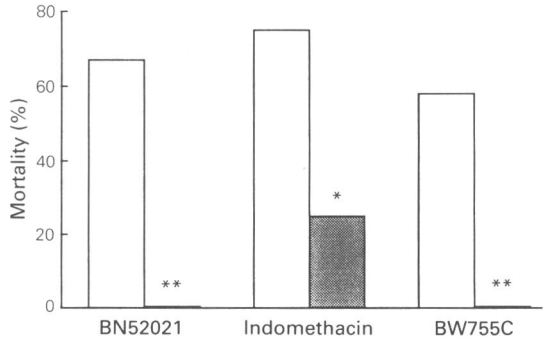

Figure 2 Effects of the PAF antagonist BN 52021, the cyclo-oxygenase inhibitor indomethacin, and the dual cyclo-oxygenase/lipoxygenase inhibitor BW 755C on 24 h mortality induced by an approximate LD_{80} challenge of i.v. endotoxin. Each drug treatment group and corresponding control group consisted of at least 12 mice. Open columns, control groups; shaded columns, drug pretreated groups. *$P < 0.05$; **$P < 0.01$ compared to the control group.

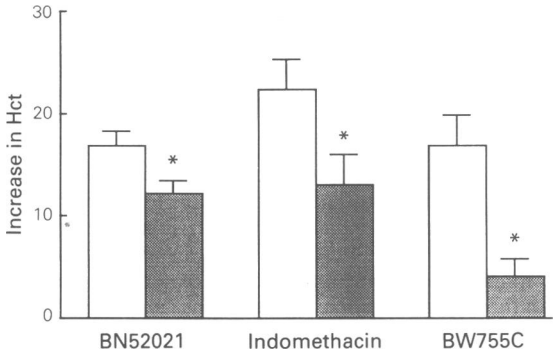

Figure 3 Rise in haematocrit (Hct) 15 min following an approximate LD_{80} endotoxin challenge in groups of mice pretreated with BN 52021, indomethacin, BW 755C or vehicle. Each treatment and the corresponding control group consisted of 9 mice. Open columns, control groups; shaded columns, drug pretreated groups. $*P < 0.05$ compared to the control group.

sence or absence of BN 52021, indomethacin or BW 755C pretreatment (Figure 4). Repeated administration of BW 755C or BN 52021, before challenge and at 6 h intervals after challenge, did not improve survival after rhTNF/low dose endotoxin (Table 1). The rhTNF/low dose endotoxin combination produced only a variable and modest rise in haematocrit compared to the lethal doses of endotoxin alone (data not shown). Based on this lack of a substantial rise in haematocrit, effects of indomethacin, BW 755C and BN 52021 were not examined.

Discussion

Under some circumstances, i.v. TNF can be highly toxic, producing lethal shock in both mice (Satomi *et al.*, 1988, Waage & Espevik, 1988) and rats (Tracey *et al.*, 1986; Kettelhut *et al.*, 1987). In our laboratories, and in others (Rothstein & Schreiber, 1988), rhTNF alone has failed to provoke lethality. Rothstein & Schreiber (1988) attributed the discrepancy to possible contamination of some rhTNF preparations by bacterial endotoxin, and showed that death could be produced by rhTNF when given simultaneously with low doses of endotoxin. Our studies confirm their experimental result. They failed to observe any lethality of rhTNF, even at 40 μg i.v. per mouse, but addition of a non-lethal dose of endotoxin resulted in dose-dependent death following rhTNF exposure. Interestingly, Waage & Espevik (1988), who found TNF to be toxic to mice at high doses, also observed great potentiation of TNF toxicity by interleukin-1 (IL-1), another cytokine which might be released by macrophages during sepsis. Waage *et al.* (1989) also correlated septic deaths in humans with increased release of IL-1 and IL-6.

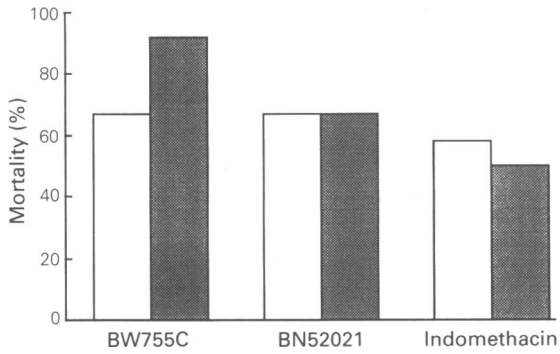

Figure 4 Effects of BN 52021, indomethacin and BW 755C on 24 h mortality induced by an approximate LD_{80} challenge of combined i.v. recombinant human tumour necrosis factor (0.4 mg kg^{-1}) and low dose endotoxin (0.24 mg kg^{-1}). Each drug treatment group and corresponding control group consisted of at least 12 mice. Open columns, control groups; shaded columns, drug pretreated groups.

Table 1 Effects of BN52021 and BW755C given in repeated doses at 6 h intervals, on mortality induced by combined recombinant human tumour necrosis factor (0.4 mg kg^{-1}) and low dose endotoxin (0.24 mg kg^{-1}).

Treatment	*Number of animals*	*Mortality (%)* 12 h	24 h
Control	7	14	86
BN52021	6	33	67
BW755C	6	33	100

Conflicts concerning the toxicity of TNF might also be attributed to a variety of other factors, including species and strain differences, condition of animals and differences in TNF preparations besides endotoxin level. It is also possible that the previous exposure of rodents to immunological challenges such as viral or bacterial infection or tumours might duplicate the priming effect of endotoxin. In addition, most studies of TNF toxicity in rodents, including the present one, have utilized rhTNF, which is more readily available than murine TNF. The experiments of Waage & Espevik (1988) involved murine recombinant TNF, and thus the one study of murine TNF-induced death in rodents suggests that it alone can produce lethal shock. Whether this result is specific to particular experimental conditions, as is apparently the case for rhTNF, is an important issue which should be addressed in further experiments and has obvious implications with respect to the current study.

The questions regarding TNF toxicity are also important from the standpoint of the role of TNF as a primary mediator of endotoxin-induced shock, capable of mimicking all of the major effects of endotoxin. Besides the apparently low toxicity of rhTNF alone in these experiments, other aspects of our studies suggest that TNF is probably not by itself a central, early mediator of endotoxin shock and demonstrate substantial differences between the pathophysiological effects of the two substances. First, rhTNF, even in combination with low dose endotoxin, was not capable of producing the rapid death which follows high dose endotoxin injection. Most of the mortality was expressed 4 or more hours after i.v. rhTNF/endotoxin challenge, whereas high dose endotoxin was lethal in most animals before this time. Second, rhTNF/low dose endotoxin-induced death was not blocked by previous treatment with indomethacin, BW 755C or the PAF antagonist, in contrast to the efficacy of these drugs against the effects of endotoxin. Apparently, endotoxin, directly or indirectly, stimulates the production of lipid mediators, which participate in its toxicity. Whether or not the rhTNF/endotoxin combination stimulates a similar, early production of PAF and eicosanoids, the toxicity of this combination is not dependent on such an event. The rapid, large elevation in haematocrit which follows endotoxin challenge was also mediated by PAF and eicosanoids, but a similar large rise in haematocrit was not seen with rhTNF/endotoxin. Further, Kiener *et al.* (1988) found that both toxic and non-toxic forms of Lipid A (the biologically active part of the structure of endotoxin) elicited similar increases in TNF secretion in mice. However, such increases in serum TNF levels were not uniformly associated with toxicity, leading the authors to suggest that 'although it is essential, TNF alone is not necessarily sufficient to induce septic shock in mice'.

In addition to the issue regarding the precise nature of the role of TNF in endotoxin shock, the question of mediation of the effects of TNF by PAF and eicosanoids is also of interest. Under those circumstances in which rhTNF alone produces lethal shock, its actions are probably mediated in part by lipid mediators, since some lipoxygenase and cyclo-oxygenase inhibitors are protective (Kettelhut *et al.*, 1987; Satomi *et al.*, 1988). In our studies, rhTNF-induced death is not dependent on an early stimulation of lipid mediator production as occurs following endotoxin administration. Because it is quite pos-

sible that the BN 52021, BW 755C and indomethacin pretreatments had lost efficacy by the time of death, we also tested the effects of repeated dosing at 6 h intervals with two of these drugs which were most efficacious against endotoxin. Neither BN 52021 nor BW 755C displayed any protective action with repeated dosing against the combination of rhTNF and endotoxin. The main purpose of our experiments was to provide a basis for comparing the acute toxicity of rhTNF and endotoxin, and our results demonstrate a clear divergence in mice between the toxicity of endotoxin as opposed to rhTNF, the time of death following the challenge and the effects of the pretreatments.

The synthetic TNF fragment, $TNF_{114-130}$ was included in these studies because we wished to determine whether this fragment, which is commercially available and represents a highly conserved region of TNF, could produce effects similar to rhTNF and could thus be used to model TNF effects. Its failure to cause any obvious pathophysiology, alone or in combination with endotoxin, suggests that at least *in vivo*, a larger or different portion of the peptide is required for TNF activity.

Based on these results, we propose that rhTNF toxicity is minimal in healthy, uncompromised rodents. The role of TNF in endotoxin-induced shock, while obviously important, probably involves synergism with other mediators, including interleukin 1, which is known to act synergistically with TNF in some systems (Ruggiero & Baglioni, 1987; Waage & Espevik, 1988). The pathophysiology of high dose endotoxin exposure apparently differs from that of rhTNF in terms of rapidity of onset and degree of dependence on early production of lipid mediators. We suggest that high dose, i.v. endotoxin-induced shock might differ from other models of sepsis in this regard, in that the cardiopulmonary alterations and lethality can be provoked in a time frame which might not allow the full production and interaction of all of the many mediators of sepsis, but instead relies heavily on a particular class of mediators, the eicosanoids and PAF.

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Opioid receptor ligands in the neonatal rat spinal cord: binding and *in vitro* depression of the nociceptive responses

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1 Opioid receptors in the neonatal rat spinal cord have been characterized by measurements of ligand binding to crude membrane fractions and by functional tests on the nociceptive spinal response in a spinal cord-tail preparation *in vitro*.

2 There were high affinity binding sites for [^{3}H]-[D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin (DAGOL), [^{3}H]-U69593, and [^{3}H]-ethylketocyclazocine (EKC) on spinal cord membranes from neonatal rats. Hill slopes for binding of [^{3}H]-DAGOL and [^{3}H]-U69593 were close to unity. The Hill slope for binding of [^{3}H]-EKC was less than unity, even after its interactions at μ-receptors had been blocked with 100 nM unlabelled DAGOL. Binding sites for [^{3}H]-[D-Pen^2, D-Pen^5]enkephalin (DPDPE) could not be detected.

3 In competition assays U50488 was as potent as PD117302 and U69593 in competition for either [^{3}H]-U69593 or [^{3}H]-EKC binding sites. Hill slopes for a range of competing ligands at [^{3}H]-DAGOL or [^{3}H]-U69593 sites were close to unity. Hill slopes for competition at [^{3}H]-EKC sites were less than one.

4 In the spinal cord-tail preparation from neonatal rats, opioid receptor agonists depressed spinal nociceptive responses evoked by application of capsaicin or heat to the tail. The order of potency was DAGOL > U69593 = PD117302 > morphine > U50488 = [D-Pen^2, L-Pen^5]enkephalin (DPLPE).

5 The antagonist naloxone was about equally potent against DAGOL, morphine and DPLPE, and about ten times less potent against U69593 and PD117302. The effects of U50488 were much less sensitive to blockade by naloxone than the effects of PD11703 or U69593. The κ antagonist, nor-binaltorphimine was equipotent against all three κ agonists.

6 The absence of δ-binding sites, and the low potency and relatively high sensitivity to naloxone suggest that DPLPE could be working at μ-receptors in the neonatal rat spinal cord.

7 The binding assays show that U50488 has the same affinity as PD117302 and U69593 for κ-receptors, yet it was less effective in the depression of nociceptive responses. This may be because U50488 has a relatively low efficacy at κ-receptors. It is possible that at high concentrations U50488 activates receptors not affected by other κ-ligands. These additional receptors may be non-opioid receptors (hence the insensitivity to naloxone), or they could be a subtype of κ-opioid receptor.

Introduction

There are at least three types of opioid receptor, μ, δ and κ, in the adult mammalian brain (Moskowitz & Goodman, 1984; Mansour *et al.*, 1987) and spinal cord (Zarr *et al.*, 1986; Traynor & Wood, 1987). Functional studies made in adult spinal cord support the involvement of μ- (Schmauss & Yaksh, 1984) and δ- (Porecca *et al.*, 1984; Rodriguez *et al.*, 1986) opioid receptors in spinal antinociception. Evidence for the involvement of κ-receptors is less clear, as κ-ligands are inactive in some tests (Schmauss & Yaksh, 1984; Leighton *et al.*, 1988), but active in others (Schmauss & Yaksh, 1984; Porreca *et al.*, 1987). The study of drug effects on the spinal cord *in vivo* can be complicated by penetration into the brain or systemic redistribution of drugs given by the intrathecal route (McQuay *et al.*, 1989). One way round this is by use of functionally relevant models maintained *in vitro*.

In the neonatal rat, cutaneous nociceptors are well developed and have functional characteristics similar to those found in adult animals (Fitzgerald, 1985; 1987). These nociceptors respond to a variety of noxious peripheral stimuli (Yanagisawa & Otsuka, 1984; Fitzgerald, 1985; 1987) and nocifensor responses have been measured *in vivo* with behavioural tests (see McDowell & Kitchen, 1987 for references). A preparation from the neonatal rat has been developed in which the spinal cord with the tail attached can be maintained for prolonged periods *in vitro* (Yanagisawa & Otsuka, 1984; Yanagisawa *et al.*, 1984). The activation of polymodal nociceptors in the tail can be measured by recording the depolarization produced in a spinal ventral root. Nociceptive mechanisms therefore are amenable to analysis *in vitro*. Nociceptive responses were depressed in this preparation by administration of opioids to the spinal cord (Yanagisawa & Otsuka, 1984; Yanagisawa *et al.*, 1984; Dray & Perkins, 1986), though the specific opioid receptors involved have not been studied. μ- and κ-receptors have been found in binding studies with neonatal rat brain (Spain *et al.*, 1985; McDowell & Kitchen 1987), but sites on neonatal spinal cord were not measured. It seemed important, therefore, to determine the types of opioid receptors present in the neonatal spinal cord, and to evaluate their function in the antinociceptive effects of opioids. Some preliminary findings from this study have already been presented (Dray *et al.*, 1989).

Methods

Ligand binding

Spinal cords were removed from newborn or one day old rats and homogenized in 10 volumes of 50 mM Tris-HCl, pH 7.4. A crude membrane fraction was isolated by centrifuging the homogenate at 17000*g* for 20 min. Membranes were resuspended in Hanks balanced salt solution, buffered to pH 7.4 with HEPES and left on ice for 30 min to allow dissociation of any endogenous opioids. They were then centrifuged as above and washed twice in Tris buffer. Finally the membrane preparation was resuspended in Tris to give approximately 0.3 mg protein ml^{-1}. Use of Tris buffer without additional ions is standard in measurement of opioid binding, because binding of agonists can be reduced quite significantly in the presence of physiological ions (see for example Childers *et al.*, 1979; James & Goldstein, 1984). Tris was used in this

study, firstly, to avoid potential reductions in binding and, secondly, to allow direct comparisons with other binding studies. The binding assay mixture contained 250 μl of membrane suspension plus appropriate radioligands (and unlabelled ligands where necessary) in a total volume of 500 μl of Tris buffer. Samples were incubated at room temperature for 60 min, filtered through Whatman GF/C glass fibre filters, washed three times with 5 ml of ice-cold Tris buffer and counted. Saturable binding was the difference between binding in the absence and presence of 10 μM naloxone. [^{3}H]-[D-Ala^2, MePhe^4, Gly(ol)^5]enkephalin ([^{3}H]-DAGOL), [^{3}H]-[D-Pen^2, D-Pen^5]enkephalin (DPDPE) and [^{3}H]-U69593 were used as selective μ-, δ- and κ-ligands respectively. [^{3}H]-ethylketocyclazocine (EKC) was also used to label κ-receptors after its binding to μ sites was blocked with 100 nM unlabelled DAGOL. DPDPE was used as the δ-ligand in the binding assays because radiolabelled [D-Pen^2, L-Pen^5]enkephalin (DPLPE, which had been used in the functional assays) is not available. DPLPE and DPDPE are very similar in affinity and selectivity for μ-, δ- and κ-receptors (James & Goldstein, 1984). In competition assays, radiolabelled ligands were used at a concentration of 1 nM.

Functional studies

The spinal cord and attached tail were removed from one day old rats following decapitation. The most superficial layer of the skin was removed from the distal four fifths of the tail with fine forceps. This procedure exposed cutaneous fibres and their endings, and allowed penetration of drugs. Damage to deeper skin layers and tissue severely compromised chemical sensitivity. The spinal cord and tail were superfused separately with a physiological salt solution (composition mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10) at 24°C and gassed with 95% O_2/5% CO_2. The activation of peripheral fibres in the tail by peripheral stimuli was assessed by measuring the depolarization in a spinal ventral root (L_3–L_5). This was recorded by a low impedance extracellular glass micropipette, filled with electrolyte, placed in an electrolyte-filled well which contained the selected ventral root. The ventral root potential was recorded d.c. with respect to the spinal cord which was earthed. Signals were amplified by conventional methods and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder. Noxious stimuli consisted of a brief (10 s) exposure of the tail to a submaximal concentration of capsaicin (0.4–0.6 μM) or a 10 s exposure to superfusate heated to 48°C. Innocuous stimulation consisted of lightly brushing the tail (10 s) with a fine sable-hair paint-brush. Peripheral stimuli were repeated sequentially at intervals of 15 min.

In preliminary studies, the spinal cord was treated with opioids for up to 60 min, then the peripheral stimuli were tested. The effect of opioids stabilized after 15 min exposure. This presumably reflects the time taken for the opioid to equilibrate in the tissue. Hence, in routine tests of the effects of opioid agonists, each ligand was administered in the spinal cord superfusate for at least 15 min before the effect of peripheral stimulation was tested. The concentration of each opioid was then increased to obtain a cumulative dose-effect relationship against capsaicin and heat-evoked responses. The agonist EC_{50} was obtained from a plot of percentage inhibition of the ventral root response against opioid dose. At least four concentrations of each opioid were tested in these experiments with one concentration producing a maximal effect. In the studies with opioid antagonists a similar protocol was used. Thus cumulative concentrations (at least three) of antagonist were administered in the presence of a fixed concentration of the agonist (usually twice the EC_{50} concentration). Antagonist potency was assessed by determining the concentration of antagonist (IC_2) required to reduce the effects of a 2 × dose of agonist to that of a single dose. The IC_2 value was obtained from a plot of % response against dose of antagonist. No assumptions were made concerning the kinetics of agonist/antagonist interactions.

Materials

[^{3}H]-DAGOL (41 Ci mmol^{-1}) and [^{3}H]-DPDPE (31 Ci mmol^{-1}) were from Amersham International p.l.c., [^{3}H]-ethylketocyclazocine (EKC, 24 Ci mmol^{-1}) and [^{3}H]-U69593 (60 Ci mmol^{-1}) were from New England Nuclear. [D-Ala^2, MePhe^{4}Gly(ol)^5] enkephalin (DAGOL) and DPDPE were from Peninsula Labs, San Carlos CA; *trans*-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl] benzacetamine methane sulphonate (U50488H) and (5,7,8)-(±)-N-methyl-N-(7-(1-pyrrolidinyl-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide (U69593) were from Upjohn, Kalamazoo, Michigan; (±)-*trans*-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzo[b]-thiophene-4-acetamide hydrochloride (PD117302) was a gift from Dr J. Hunter, Park-Davis Research Unit, Cambridge; nor-binaltorphimine (nor-BNI) was from Research Biochemicals Inc; naloxone hydrochloride was from Sigma Chemical Company, Poole, Dorset; Hanks balanced salt solution was from Gibco Ltd., Paisley, Scotland.

Results

Ligand binding experiments

Parameters for the saturable binding of opioids to membranes from neonatal rat spinal cord are presented in Table 1. In these experiments μ-binding sites were labelled with [^{3}H]-DAGOL, and κ-sites with [^{3}H]-U69593 or [^{3}H]-EKC. Binding of [^{3}H]-EKC to μ-sites was blocked by including 100 nM DAGOL in all samples. As before (Dray *et al.*, 1989), we found approximately four times more μ- than κ-sites. There were also approximately twice as many κ-like binding sites for [^{3}H]-EKC than for [^{3}H]-U69593. Saturable binding sites for the δ-ligand [^{3}H]-DPDPE could not be detected. Slopes of Hill plots for [^{3}H]-DAGOL and [^{3}H]-U69593 binding were close to unity. The Hill plot for binding of [^{3}H]-EKC to nominally κ-sites had a slope significantly less than one.

Results of competition experiments are shown in Table 2. Unlabelled DAGOL and naloxone competed for [^{3}H]-DAGOL sites much more effectively than the κ-ligands U50488, U69593, PD117302 and nor-BNI, or the delta-ligand DPDPE (Table 2A). DPDPE did have a low, but measurable affinity for μ-sites, consistent with a weak agonist activity at this receptor (Dray *et al.*, 1989).

Table 1 Parameters for binding of opioid ligands to membranes of neonatal rat spinal cord

Radioligand	K_d (nM)	B_{max} (pmol mg^{-1} protein)	*Hill slope*
[^{3}H]-DAGOL	0.94 ± 0.06	0.22 ± 0.04	0.92 ± 0.03
[^{3}H]-U69593	2.3 ± 0.60	0.055 ± 0.006	0.92 ± 0.03
[^{3}H]-EKC*	0.80 ± 0.19	0.11 ± 0.02	0.72 ± 0.07
[^{3}H]-DPDPE	Not detectable		

Saturable binding was measured as described in Methods. Parameters were estimated by non-linear regression on the binding isotherm, assuming a single type of non-interacting binding sites. Hill slopes are from linear regression on Hill plots.

* Binding of [^{3}H]-ethylketocyclazocine ([^{3}H]-EKC) was measured in the presence of 100 nM unlabelled [D-Ala^2, MePhe^4, Gly(ol)^5] enkephalin (DAGOL), to prevent interaction at μ-sites. Data are means ± s.e.mean from four to six experiments.

[^{3}H]-DPDPE = [^{3}H]-[D-Pen^2, D-Pen^5] enkephalin.

Table 2 Competition for opioid binding sites in neonatal rat spinal cord membranes

Competer	*Radioligand* [^3H]-*DAGOL*	[^3H]-*U69593*	[^3H]-*EKC**
A *IC_{50} values (nM)*			
DAGOL	1.0 ± 0.21	400 ± 12	> 1000
U50488	> 1000	0.86 ± 0.06	18 ± 4.4
U69593	> 1000	0.82 ± 0.46	24 ± 10
PD117302	900 ± 52	0.49 ± 0.04	31 ± 10
Nor-BNI	78 ± 7.0	0.78 ± 0.10	5.8 ± 2.2
Naloxone	2.1 ± 0.07	3.5 ± 0.10	120 ± 46
DPDPE	620 ± 14	> 1000	> 1000
B *Hill slopes*			
DAGOL	0.92 ± 0.03	0.88 ± 0.11	—
U50488	—	1.0 ± 0.04	0.86 ± 0.08
U69593	—	0.87 ± 0.08	0.63 ± 0.07
PD117302	—	0.98 ± 0.10	0.79 ± 0.04
Nor-BNI	1.0 ± 0.03	0.98 ± 0.15	0.49 ± 0.10
Naloxone	0.88 ± 0.01	0.96 ± 0.17	1.0 ± 0.09
DPDPE	1.2 ± 0.10	—	—

IC_{50} values (the concentration of competing ligand required to reduce saturable binding of the radioligand by 50%) and Hill slopes were estimated by linear regression on Hill plots of the competition data, and are presented as means ± s.e.means from 3 to 6 experiments.
* Measurements with [^{3}H]-EKC were made in the presence of 100 nM DAGOL to prevent interaction at μ-sites. In the competition assays, saturable binding was at least 70% of total binding.
Nor-BNI = nor-binaltorphimine; for key to other abbreviations see Table 1.

The κ-ligands were much more potent than either DAGOL or DPDPE in competition for [^{3}H]-U69593 sites. There were no large differences in affinity for these sites between κ-ligands. Notably U50488 was similar to U69593 and PD117302 in this assay. The antagonist nor-BNI had about 100 fold higher affinity for κ- than for μ-sites, confirming previous assessments of its selectivity (Takemori *et al.*, 1988).

Once binding to μ-receptors had been blocked with a high concentration of unlabelled DAGOL, neither DAGOL nor DPDPE competed for the remaining [^{3}H]-EKC binding sites. Affinities of the κ-ligands for these sites were generally lower than affinities for [^{3}H]-U69593 binding sites. Again there was no difference between U50488 and the other κ-ligands. Naloxone showed considerably lower affinity for the [^{3}H]-EKC sites than for the [^{3}H]-U69593 sites.

Hill plots for competition against either [^{3}H]-DAGOL or [^{3}H]-U69593 all had slopes close to one. This was not found for sites labelled with [^{3}H]-EKC. Notably nor-BNI and U69593 had Hill slopes considerably less than one in competition for these sites.

Spinal-cord/tail experiments

Reproducible ventral root responses were obtained following stimulation of peripheral fibres with innocuous (brush) or noxious (heat or capsaicin) stimuli (Figure 1). Superfusion of the spinal cord with opioid agonists consistently produced a dose-related depression of capsaicin and heat evoked responses (Figures 1–3). The EC_{50} values are shown in Table 3. Overall the rank order of potency was DAGOL > U69593 = PD117302 > morphine > U50488 = DPLPE. Each opioid was more potent against the capsaicin-evoked response than against responses evoked by heat (Table 3). In some experiments (2 for U50488; 2 for U69593; 3 for P117302) where doses of κ-agonists $\leq$ 1 nM were tested an initial enhancement of nociceptive responses was observed (Figure 3a). This phenomenon was not quantified or studied in detail, but similar observations have been made *in vivo*, following intrathecal administration of κ-ligands (Knox & Dickenson, 1987). Subsequent higher agonist doses always produced a depression of the nociceptive response.

The antagonist naloxone, was equally potent against the same opioid when compared in tests in which either capsaicin or heat was used to evoke the nociceptive response. The naloxone IC_2 concentration was lowest (most potent) against the μ-agonists DAGOL (Figure 1b) and morphine, and the δ-

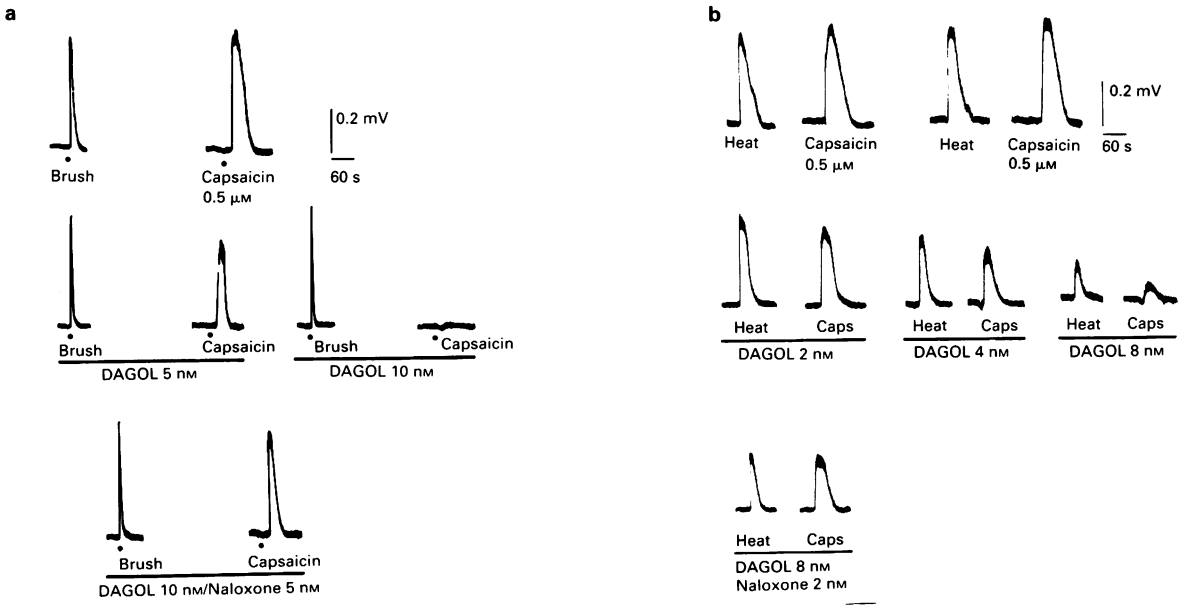

Figure 1 The dose related depression of nociceptive spinal responses by [D-Ala^2, MePhe^4, Gly(ol)^5] enkephalin (DAGOL) and the antagonism of these effects by naloxone. (a) The reproducible responses to capsaicin (Caps) and innocuous brush. DAGOL at 5 and 10 nM selectively attenuated the nociceptive responses to capsaicin in a naloxone reversible manner. (b) Top traces show the reproducible spinal ventral root depolarization produced by stimulation of peripheral nociceptive fibres with heat (48°C) or capsaicin (0.5 μM). The middle traces show the attenuation of the nociceptive responses by cumulative spinal superfusion with DAGOL. Note that the capsaicin-evoked response appears to be attenuated preferentially. In the bottom traces superfusion with 2 nM naloxone reduced the depressant effect of 8 nM DAGOL to that produced by 4 nM DAGOL. This was the approximate IC_2 concentration of naloxone. Drugs administered (indicated by the bar below the trace) in the spinal superfusate were allowed to equilibrate for at least 15 min before stimuli were repeated. The calibration bars are as indicated.

Table 3 Potency comparisons of opioids agonists against spinal responses evoked by peripheral stimulation with capsaicin or heat (48°C)

		ED_{50} (nM)	*Naloxone* IC_2 (nM)	*nor-BNI* IC_2 (nM)
A	*Against capsaicin*			
	Morphine	56	10	—
	DAGOL	2.8	5.8	>500
	DPLPE	120	7.6	—
	U50488	120	>500	50
	U69593	5.5	50	63
	PD117302	4.8	50.2	60
B	*Against heat*			
	Morphine	150	11	—
	DAGOL	7.8	7.0	>500
	DPLPE	200	8.3	—
	U50488	270	500	37
	U69593	25	53	50
	PD117302	24	64	42

ED_{50} values were obtained from plotting % depression of the nociceptive response against drug concentration. Three to 5 concentrations were used for each estimate in each of 6–10 experiments. The IC_2 values for naloxone and nor-BNI express the concentration of antagonist required to reduce the effect of a 2× dose of opioid agonist to that of a single dose ($n = 4$–6 for each determination). For each determination at least three doses of antagonist were used, and the IC_2 value was interpolated from a plot of % reduction in agonist effect against antagonist dose.
For key to abbreviations used see Tables 1 and 2.

agonist DPLPE (Figure 2a). Naloxone also consistently antagonised the effect of U69593 and PD117302 (Figure 3a) though approximately ten fold higher concentrations were required (Table 3). In most experiments the naloxone IC_2 concentration against U50488 was greater than 500 nM, arbitrarily chosen as the upper concentration limit for routine testing (Figure 2b, Table 3).

Freshly prepared solutions of nor-BNI consistently antagonised the effects of U50488 (8 of 10 experiments), U69593 (7 of 7 experiments, Figure 3b) and PD117302 (8 of 8 experiments) and similar IC_2 concentrations were found whether heat or capsaicin was used as the stimulus (Table 3). The effect of DAGOL (4 of 4 experiments) was not reduced by nor-BNI superfused on the spinal cord up to a concentration of 500 nM.

Discussion

We have shown that there are functional μ- and κ-opioid receptors in neonatal rat spinal cord, and that activation of either of these receptors depresses the ventral root response evoked by noxious peripheral stimuli. μ-Receptors were identified with the selective agonist DAGOL (Handa *et al.*, 1981) and by showing that the effects of DAGOL were readily reversed by naloxone. κ-Receptors were identified with the selective agonists U69593 (Lahti *et al.*, 1985) and PD117302 (Clark *et al.*, 1988). The actions of the κ compounds were blocked by naloxone, but about ten times higher concentrations were needed than for blockade at μ-receptors. The ten fold difference in sensitivity to naloxone betweeen μ- and κ-receptors is well established in other tissues, for example the guinea-pig ileum and the mouse vas deferens (Lord *et al.*, 1977; Goldstein & James, 1984). The actions of U69593 and PD117302 were also blocked by the κ-selective antagonist nor-BNI, which was ineffective against DAGOL. These findings are in keeping with both *in vitro* (Birch *et al.*, 1987; Portoghese *et al.*, 1987; Takemori *et al.*, 1988) and *in vivo* data (Sandler *et al.*, 1988; Takemori *et al.*, 1988; Tortella *et al.*, 1989) showing nor-BNI to be a highly selective κ-ligand. However, other studies suggest considerable difficulties in using this compound *in vivo*, as it exhibits effects only after a considerable delay and shows poor selectivity (Birch *et al.*, 1987).

A third well documented κ-agonist, U50488 (Von Voigtlander *et al.*, 1983), gave anomalous results. It was less potent than expected and very high concentrations of naloxone were

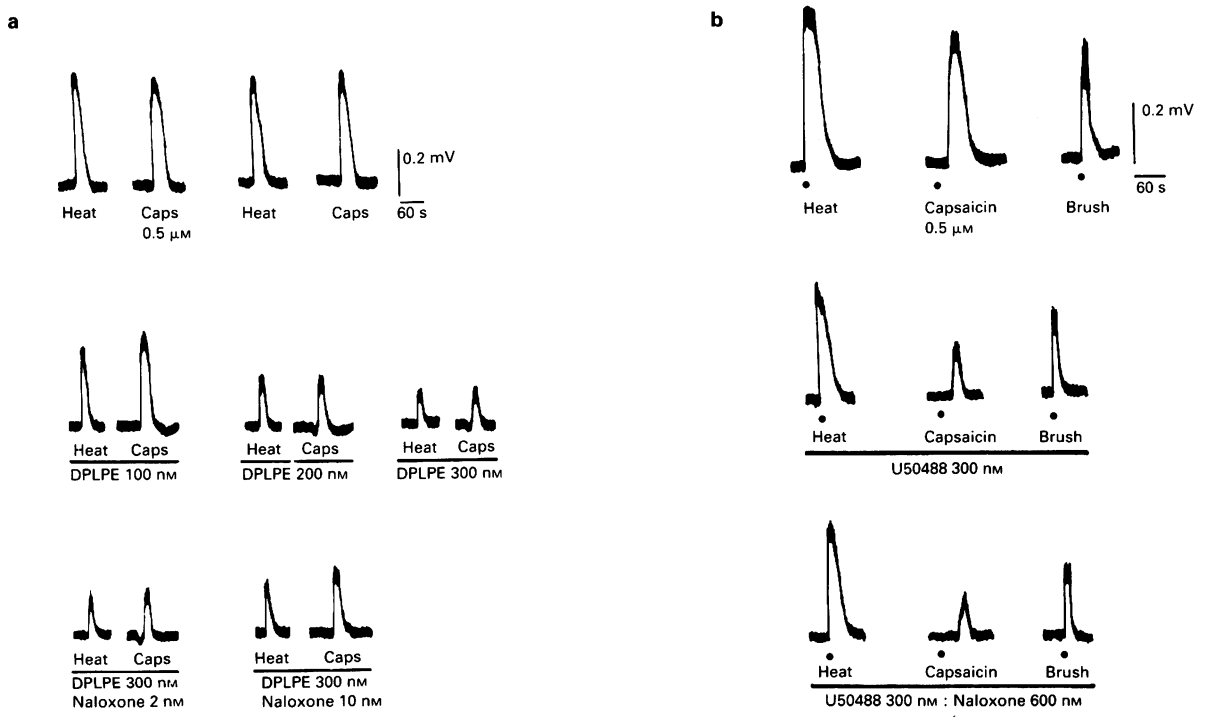

Figure 2 Effects of [D-Pen^2, L-Pen^5]enkephalin (DPLPE) and U50488 on the spinal nociceptive responses. (a) Illustrates the dose-related depression of spinal responses by the δ-receptor ligand DPLPE (middle trace). The bottom traces show examples of the dose-related antagonism of DPLPE (300 nM) by cumulative doses of naloxone. (b) Shows the non-selective depression of nociceptive responses and innocuous brush responses produced by 300 nM U50488. The effect of U50488 was not influenced by superfusion with 600 nM naloxone.

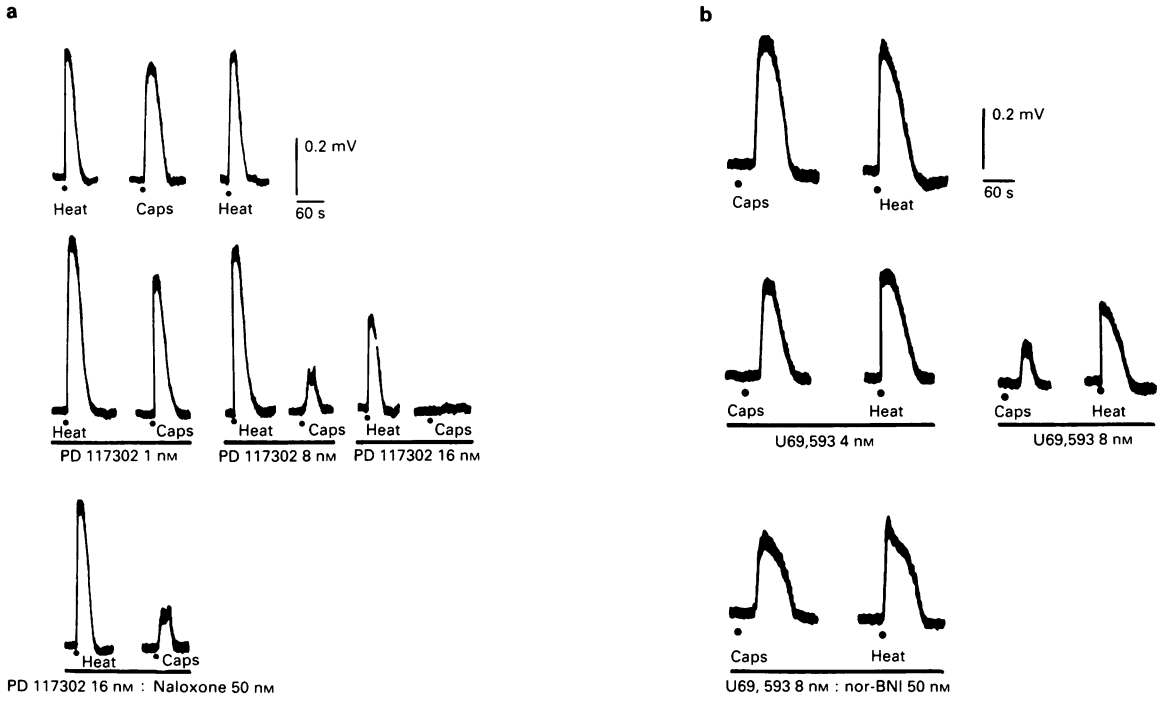

Figure 3 Dose-related depression of the noiciceptive spinal response by κ-agonists and the effects of antagonists. (a) The top traces show control responses to heat (48°C) and capsaicin (Caps, 0.5 μM) administered to the tail. The middle traces show that superfusion of the spinal cord with PD117302 (indicated by the bar below the traces), for at least 15 min before peripheral stimuli were retested, produced a dose-related depression of the nociceptive response. The response evoked by capsaicin was more substantially depressed than that evoked by heat, and completely abolished by 16 nM PD117302. Note that the lowest dose of PD117302 (1 nM) produced an enhancement of the nociceptive responses. The bottom traces show antagonism of the PD117302 effect by concomitant superfusion of the spinal cord with naloxone (50 nM). At this concentration the effect of the 16 nM PD117302 was reduced to approximately that of 8 nM. The calibration bars are as indicated. (b) The top trace shows control responses to capsaicin and heat. Superfusion of the spinal cord with cumulative doses of U69593 produced a dose-related depression of the nociceptive responses (middle traces). A concomitant spinal superfusion of nor-binaltorphimine (nor-BNI, 50 nM) antagonized the effect of U69593, reducing the effect of 8 nM U69593 to that normally seen with 4 nM.

required to reverse its effects. The κ-antagonist nor-BNI was effective against U50488 at the same concentrations as for other κ-agonists. These results suggest that U50488 may be acting on a different population of receptors from U69593 and PD117302.

DPLPE, which is normally a δ-selective agonist (Mosberg *et al.*, 1983), also depressed the ventral root response, again at comparatively high concentrations. The actions of DPLPE were as sensitive to naloxone as those of DAGOL and morphine. In other systems, δ-agonists are about ten times less sensitive to naloxone than μ-agonists (Lord *et al.*, 1977; Goldstein & James, 1984). The low potency and relatively high sensitivity to naloxone are consistent with DPLPE acting at μ-receptors in neonatal rat spinal cord, where binding assays suggest that there are no δ-sites (see below).

Our binding data are consistent with single types of binding sites for [^{3}H]-DAGOL and [^{3}H]-U69593, in that Hill slopes for binding isotherms and competition assays were close to one. Hill slopes for [^{3}H]-EKC binding and for competition assays with [^{3}H]-EKC were less than one. There were more sites for [^{3}H]-EKC than for [^{3}H]-U69593, and the affinities of κ-ligands for sites labelled with [^{3}H]-EKC were lower than those for [^{3}H]-U69593 sites. These results suggest that [^{3}H]-EKC labels more than one type of binding site and that at least some of the [^{3}H]-EKC sites have a low affinity for U69593. Zukin *et al.* (1988) have described subtypes of κ-receptors in rat and guinea-pig brain based on heterogeneity of [^{3}H]-EKC binding. In their model, κ_1-sites bind PD117302, U69593 and U50488 with reasonable affinities. κ_2-sites have lower affinities than κ_1-sites for U50488 and PD117302, and do not bind U69593 even at high concentrations (up to 10 μM). Sites for [^{3}H]-U69593 in neonatal rat spinal cord could correspond to the κ_1-receptors proposed by Zukin and colleagues (1988). We could not identify the additional EKC sites as Zukin's κ_2-receptors because although the competition assays showed that U69593 had a relatively low affinity for these sites, so too did U50488 and PD117302. We have no evidence that heterogeneity of [^{3}H]-EKC binding sites represents heterogeneity of κ-receptors. The pitfalls of using non-selective radioligands in conjunction with unlabelled blocking ligands in these kind of experiments have been emphasized recently by Traynor (1989).

We conclude from the binding and functional assays that μ- and κ-receptors are present and are involved in the modulation of nociception in neonatal rat spinal cord. In fact, spinal antinociceptive actions of κ-ligands are most easily demonstrated in neonatal and young animals (Allerton *et al.*, 1989; Dickenson & Sullivan, personal communication), while in the adult, spinal mechanisms are relatively insensitive to κ-opioids (Knox & Dickenson, 1987). The reasons for this are unclear. It is possible that κ-receptors are involved in spinal developmental processes, and may be functionally less significant in adults. The binding assays provided no evidence for δ-sites. As discussed above, in the functional assays the naloxone sensitivity of DPLPE effects suggests that this normally δ-selective ligand can act at μ-sites in tissues that lack δ-receptors.

U69593 and PD117302 are κ-agonists with high potency and efficacy. Although U50488 bound with high affinity to κ-receptors, its potency as an antinociceptive agent was surprisingly low. We suggest that U50488 may have a relatively low efficacy at κ-receptors. The difference in IC_2 values for naloxone between U50488 and the other κ-agonists could then be explained if at the high concentration of U50488 required (because of the low efficacy) to activate κ-receptors, other receptors were also activated. These would have to be relatively insensitive to naloxone and unaffected by either U69593 or PD117302 at the concentrations that we used. Others have also described effects of U50488 that are not sen-

sitive to naloxone or the opioid antagonist 16-methylcyprenorphine, and have concluded that U50488 can act at non-opioid receptors (Hayes *et al.*, 1988; Leff & Dougal, 1989). Our data suggest that there may be a population of naloxone-insensitive receptors for U50488 in neonatal rat cord, but that these sites are still sensitive to nor-BNI. If nor-BNI is highly selective for κ-opioid receptors (and does not interact with non-opioid receptors) then these additional sites for U50488 would represent a subtype of κ-receptor.

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Effects of B-HT 920 on nigrostriatal and mesolimbic dopamine systems in normosensitive and supersensitive rats

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1 B-HT 920, a D_2 dopamine receptor agonist, was tested for its ability to exert presynaptic actions in normosensitive rats, and for possible postsynaptic actions in rats made 'supersensitive' to apomorphine.

2 In normosensitive rats, B-HT 920 (0.01–0.3 mg kg^{-1}, i.p.) increased dopamine concentrations and lowered metabolite levels to a similar extent in all four terminal regions examined (medial prefrontal cortex, olfactory tubercle, nucleus accumbens, caudate-putamen). Analogous effects were seen for 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid.

3 Rats which received bilateral 6-hydroxydopamine (6-OHDA) infusions into the caudate-putamen showed signs of postsynaptic dopamine receptor activation (stereotyped behaviour) in response to B-HT 920 (0.1 and 1.0 mg kg^{-1}, i.p.) and to apomorphine (0.2 mg kg^{-1}, s.c.). Similarly, B-HT 920 (0.1 mg kg^{-1}) induced contralateral circling in rats that had received unilateral 6-OHDA infusions into the medial forebrain bundle; the rate of circling increased gradually over several weeks.

4 In contrast, bilateral 6-OHDA infusions into the nucleus accumbens resulted in a supersensitive (locomotor stimulant) response to a low dose of apomorphine (0.1 mg kg^{-1}, s.c.), but not to B-HT 920 (0.01 and 0.1 mg kg^{-1}).

5 In intact rats, withdrawal of chronic haloperidol treatment induced behavioural supersensitivity to apomorphine but not to B-HT 920.

Introduction

B-HT 920 (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo- [4, 5-d]azepine) was originally identified as an α_2-adrenoceptor agonist (Kobinger & Pichler, 1981). However, at low doses it displays selective dopamine autoreceptor agonist properties, with a pharmacology resembling that of 3(−)PPP, TL-99 and EMD 23,448 (Anden *et al.*, 1982; 1983a,c). Thus, in normosensitive animals, systemic administration of B-HT 920 reduces A9 and A10 cell firing (Eriksson *et al.*, 1985; Clark & Chiodo, 1988) and attenuates dopamine synthesis and release in terminal areas (Anden *et al.*, 1982; 1983a,b,c). Possible pharmacological actions of B-HT 920 on non-catecholaminergic systems have received little attention.

Anden *et al.*, (1983b) have reported that B-HT 920 reduces dopamine synthesis with greater potency and efficacy in mesolimbic terminal regions (particularly in olfactory tubercle) than in caudate-putamen. A preferential mesolimbic action was also suggested by behavioural experiments, in which B-HT 920 potently inhibited spontaneous locomotor activity but did not induce catalepsy (Ahlenius & Hillegart, 1986). However, two recent reports cast doubt on this conclusion. Dopamine release, assessed by brain dialysis in freely moving rats, was reduced by B-HT 920 with similar potency and effectiveness in nucleus accumbens and dorsal striatum (Imperato *et al.*, 1988), and in addition, neuronal activity was reduced with similar potency though somewhat slower recovery in mesolimbic than in nigrostriatal dopaminergic neurones (Clark & Chiodo, 1988). Thus, at present, it is unclear whether the presynaptic actions of B-HT 920 are selective for mesolimbic neurones.

B-HT 920 has little if any activity at D_1 dopamine receptors but acts potently at D_2 receptors (Jennewein *et al.*, 1986; Hinzen *et al.*, 1986). When given alone to normosensitive animals, B-HT 920 does not produce behavioural signs of postsynaptic dopamine receptor activation; thus, it fails to induce stereotypy or locomotor stimulation (Anden *et al.*, 1982; 1983c), and does not induce circling in rats with unilateral striatal ibotenate lesions or in hemitransected animals (Hinzen *et al.*, 1986; Arnt & Perregaard, 1987). However, when coadministered with a selective D_1 agonist (SKF 38393), B-HT 920 produces intense stereotyped behaviour, consistent with a postsynaptic dopamine action within the striatum (Meltzer *et al.*, 1988; Johansen *et al.*, 1988). In the presence of SKF 38393, B-HT 920 does not appear to increase place-to-place locomotor activity (Meltzer *et al.*, 1988), suggesting that an analogous activation of postsynaptic mesolimbic dopamine receptors does not occur, even though D_1-enabled electrophysiological actions of B-HT 920 have been demonstrated on neurones within the nucleus accumbens (Johansen *et al.*, 1988). Taken together, these findings suggest that the postsynaptic dopaminergic behavioural actions of B-HT 920 in normosensitive animals, when enabled by concurrent D_1 receptor activation, are preferentially expressed via the nigrostriatal system.

Following the destruction of dopaminergic neurones with 6-OHDA, B-HT 920 readily displays behavioural signs of postsynaptic activation when given alone (Hinzen *et al.*, 1986). This has been shown in the unilateral 6-OHDA lesion model in rats, and also in a primate model of Parkinson's disease (MPTP-treated rhesus monkey), where administration of B-HT 920 reversed parkinson-like symptoms and restored normal behaviour (Hinzen *et al.*, 1986). Thus B-HT 920 appears to act preferentially at dopamine receptors made supersensitive through loss of dopaminergic input. At present, there is little or no experimental data bearing on the possible postsynaptic effects of B-HT 920 at 'supersensitive' dopamine receptors in the mesolimbic system, which is also frequently compromised in Parkinson's disease (Fibiger, 1984).

The mechanism whereby postsynaptic effects of B-HT 920 become unmasked following denervation are unknown, but is believed to reflect an uncoupling of D_1 and D_2 stimulus-effector mechanisms following the loss of dopaminergic tone (Clark & White, 1987). It was therefore of interest to examine

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the effects of chronic D_2 receptor blockade on subsequent responses to B-HT 920, in behavioural tests designed to examine nigrostriatal and mesolimbic activation.

The experiments described below had several objectives: (1) to compare the effects of B-HT 920 on nigrostriatal and mesolimbic dopamine metabolism in normosensitive, freely-moving rats; (2) to compare the effects of B-HT 920 on dopamine and 5-hydroxytryptamine (5-HT) metabolism, as a test of pharmacological selectivity; (3) to examine the behavioural effects of B-HT 920 following nigrostriatal and/or mesolimbic denervation; and (4) to study the effects of B-HT 920 in animals made supersensitive to dopamine agonists after withdrawal of chronic neuroleptic treatment.

Methods

Male Long-Evans hooded rats (Charles River) were housed two or four per cage (depending on the experiment) in a room illuminated from 08 h 00 min to 20 h 00 min, with free access to food and water. Rats were adapted to laboratory conditions for at least two weeks prior to behavioural testing.

Surgery

Rats were pretreated with pargyline HCl 50 mg kg^{-1} i.p., 30 min before surgery. In Experiment 2, rats also received desmethylimipramine (DMI) 25 mg kg^{-1} i.p., 30 min before surgery. Rats were anaesthetized with chloral hydrate 400 mg kg^{-1} i.p. and placed in a stereotaxic frame (David Kopf, Tujunga, CA) with the tooth bar positioned 3.9 mm below the interaural line. A small burr hole was made in the skull above each injection site. Injection of 6-OHDA solution or vehicle (control subjects) was made into the brain via 30 gauge stainless steel cannulae attached by polyethylene tubing to 5 μl Hamilton syringes driven by a syringe pump (Sage Instruments, Cambridge, MA). In Experiment 2, injections were given at 1 μl min^{-1}, with 1 min delay before cannula withdrawal. To improve anatomical specificity, slower injections were used subsequently (2 μl in 10 min plus 5 min delay in Experiment 3). After cannula removal, the wound was closed with stainless steel clips (Autoclips, Becton, Dickinson and Co., USA).

For caudate-putamen infusions, 6-OHDA was administered bilaterally at two sites per side (interaural A +10.4, L ±2.2, V +4.9, and A +8.9, L ±3.3, V +4.7). Infusions into the medial forebrain bundle were at interaural A +5.9, L +2.3, V +2.2. For greater accuracy at nucleus accumbens sites, stereotaxic coordinates were derived from the mean of two systems based respectively on interaural (IA) zero and bregma; coordinates were A_{IA} +10.9/A_{Bregma} +1.7, L ±1.5, V_{IA} +2.7/V_{Bregma} −7.3. 6-Hydroxydopamine HBr was made up in vehicle consisting of 0.1 mg ml^{-1} ascorbic acid in 0.9% saline solution, kept on ice, and shielded from light. 6-OHDA was given as 8 μg base in 2 μl per site, except into the MFB (Experiment 3, 4 μg in 2 μl). Control rats received vehicle.

Locomotor activity

Five circular (61 cm diameter) activity cages (BRS/LVE), each transected by two perpendicular coplanar arrays of three infrared photobeams, were used to measure locomotor activity. Photocell beam interruptions occurring more than 500 ms apart were recorded with a NOVA IV (Data General) minicomputer equipped with MANX (GC Controls) software and interface. During testing, a light-proof lid was placed over each activity cage. Prior to each test session, rats were placed in a holding cage for 50 min, in order to habituate to the test room. Unless otherwise stated, each rat then received an injection of drug or saline, was replaced in the holding cage, and then introduced to the test cage 60 s after injection. The test session was started 60 s later and was of 20–120 min duration, depending on the experiment. As far as possible, equal numbers of rats from each experimental condition were tested in each cage. All behavioural tests were carried out between 09 h 00 min and 17 h 00 min.

Behavioural observation and stereotypy rating

Observation cages comprised a wire grid floor (40 × 45 cm) with plexiglass sides (45 × 45 cm). An oblique mirror permitted viewing from below the cage floor. Rats were kept in a holding cage for 15 min before injection and then placed individually in observation cages immediately after injection. General observations of behaviour and stereotypy ratings were made for 2 min at 10, 20 and 30 min after injection. The observer was blind with respect to surgical treatment and drug treatment. During this time, a brief description of posture and movements was recorded. Stereotypy was continuously rated during the observation period, as follows (modified from Kelly *et al.*, 1975): 0 – asleep or stationary; 1 – active; 2 – predominantly active with bursts of stereotyped sniffing and/or rearing; 3 – stereotyped activity, predominantly sniffing and rearing, over a large area of the cage; 4 – stereotyped behaviour maintained in one location; 5 – stereotyped behaviour in one location with bursts of gnawing and/or licking; 6 – continual gnawing or licking of grid floor. During each observation period, one stereotypy score was allocated to each rat, corresponding to the behavioural category occupying the most time.

Circling behaviour

The locomotor test cages were used (see above). Subjects were kept in a holding cage for 15 min before drug injection. For tests with B-HT 920, rats were placed individually in observation cages immediately after injection, and rotational behaviour (complete 360 degree rotations to left or right) scored for 2 min periods at 20, 40 and 60 min (Experiment 3).

Determination of brain amines

Rats were killed by cervical dislocation and the brain was rapidly removed and frozen. One millimetre thick coronal sections of forebrain were taken with a freezing microtome, and major dopaminergic terminal areas (medial prefrontal cortex – mPFCx, olfactory tubercle – OT, nucleus accumbens – NACC, caudate-putamen – CP) were dissected, as previously described (Clarke *et al.*, 1988a). For the determination of amines and metabolites, tissue samples from left and right sides were combined, weighed, and disrupted by sonication. Samples were analyzed by high pressure liquid chromatography with electrochemical detection, as described in detail elsewhere (Clarke *et al.*, 1988a). The following monoamines and metabolites were assayed: dopamine, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindolacetic acid (5-HIAA).

Drugs

Drugs and suppliers were as follows: 6-hydroxydopamine HBr, pargyline HCl, and apomorphine HCl (Sigma Chemical Co., St Louis, MO), chloral hydrate (BDH, Toronto, Ont), desmethylimipramine (Merrel Dow Research Institute, Cincinnati, OH), haloperidol HCl (McNeil), B-HT 920 (Boehringer-Ingelheim KG, Ingelheim, FRG). Chloral hydrate, pargyline, B-HT 920 and desmethylimipramine were given i.p.; apomorphine was administered s.c. into the flank. Unless otherwise stated, drugs were dissolved in 0.9% saline and given in a volume of 1 ml kg^{-1}. Ascorbic acid (0.1 mg ml^{-1}) was added to 6-OHDA and apomorphine to inhibit oxidation. For oral administration, haloperidol 10 mg ml^{-1} was dissolved in 0.5% lactic acid solution and stored at 4°C in the dark. Aliquots of this stock solution were diluted to final concentrations of 0.02 mg ml^{-1} (haloperidol) and 0.001% (lactate vehicle), and presented in opaque drinking bottles. Every other day, bottles

were weighed to determine fluid intake, and drinking solutions were changed. Drug doses refer to the salt, except for apomorphine and haloperidol (expressed as base).

Data analysis

Stereotypy scores occurring at a given dose of drug were analyzed for differences between experimental groups by the Mann-Whitney test (Tallarida & Murray 1981). Locomotor activity scores were assessed by univariate and multivariate analysis of variance (SPSS/PC+ software, SPSS Inc., Chicago). The following between-subjects factors were analyzed: Dose (for comparisons across different levels of dose, including saline), Lesion (6-OHDA vs. vehicle), and Group (haloperidol- vs. vehicle-withdrawn). Dose-dependent effects refer to linear trends across absolute values of dose. Each rat served as its own control, where appropriate. The within-subjects factors were: Drug, for comparisons across different drug treatments, and Time, referring to changes occurring within individual test sessions (divided into six equal periods of 10 or 20 min). In order to avoid positive biasing of the F test, which can occur in repeated measures designs, nonsignificant results refer to a conventional F test, whereas a significant result refers to a more conservative F test (Huyhn-Feldt; SPSS/Pc+ software). As in previous studies (Clarke *et al.*, 1988a,b), the drug effect was defined as the difference between drug and saline test scores. Comparisons between control (i.e. saline-treated) and other drug groups were made with the Dunnett multiple range test. Other comparisons were made by Student's t test. Probability values are 2-tailed.

Procedures

Experiment 1: Acute effects of B-HT 920 on locomotor activity and on dopamine and 5-hydroxytryptamine metabolism in normosensitive rats Forty-four rats were used, weighing 260–330 g. They were randomly allocated to five groups for drug testing: saline ($n = 12$), B-HT 920 0.01, 0.03, 0.1, 0.3 mg kg^{-1} ($n = 8$ for each dose). After injection, each rat was replaced in the holding cage, then tested for locomotor activity from 20–40 min post-injection. Each rat was killed for neurochemical analysis immediately after its locomotor test session.

Experiment 2: Behavioural effects of B-HT 920 following bilateral administration of 6-hydroxydopamine into caudate-putamen or nucleus accumbens Forty rats were used, weighing 270–335 g at surgery. Rats were randomly allocated to the following groups for surgery (treatment/site): 6-OHDA/CP ($n = 12$), Vehicle/CP ($n = 8$), 6-OHDA/NACC ($n = 12$), Vehicle/NACC ($n = 8$). Shortly after surgery, several rats died (3 in 6-OHDA/CP group, 2 in Vehicle/CP group). Rats in the 6-OHDA/CP group became aphagic and were tube-fed a mixture of powdered milk and sucrose water. From the day of surgery to the first behavioural test, this group gained 2.2 ± 9.4 g (mean $\pm$ s.e.mean), whereas the controls gained 26.8 ± 5.1 g.

Starting 10 days after surgery, and continuing every other day, rats in the two CP groups received four tests of stereotyped behaviour, once each with saline, apomorphine 0.2 mg kg^{-1}, B-HT 920 0.1 and 1.0 mg kg^{-1}, given in random order. During the same period of days, rats in the two NACC groups received four locomotor tests, following the administration of saline, apomorphine 0.1 mg kg^{-1}, B-HT 920 0.01 and 0.1 mg kg^{-1}, again presented in random order. Rats were habituated for 50 min in holding cages, then tested for 60 min after injection. The dose of apomorphine was selected so as to depress activity in intact rats but to increase activity in dopamine-depleted rats (Clarke *et al.*, 1988b). Five days after the final test, rats were killed for neurochemical analysis.

Experiment 3: Drug-induced circling behaviour following unilateral 6-hydroxydopamine injection into the medial forebrain bundle The above experiment was repeated with the following modifications: (1) 6-OHDA was administered into the MFB, in order to deplete both nigrostriatal and mesolimbic terminal regions of dopamine; (2) tests were conducted for a longer period after B-HT 920 administration and post-surgery; and (3) the possible effects of repeated testing and repeated administration of B-HT 920 were examined.

Twenty rats (290–355 g) received an infusion of 6-OHDA (4 μg in 2 μl) into the MFB and were randomly allocated to one of three groups: the BHT/BHT group ($n = 8$) was tested with B-HT 920 0.1 mg kg^{-1} on days 1, 3, 7, 14 and 35 after surgery; the Sal/BHT group ($n = 8$) was treated in the same way, except that saline replaced B-HT 920 on the first three tests; the third group (No test/BHT, $n = 4$) was tested with the same dose of B-HT 920, but only on the final day. Circling was counted at 20, 40 and 60 min after injection. Forty-two days after surgery, rats were killed for neurochemical analysis.

Experiment 4: Effects of chronic haloperidol treatment on subsequent behavioural responses to B-HT 920 and apomorphine Forty-eight rats were used, weighing 350–445 g on the first behavioural test. In broad outline, the experiment was conducted in two consecutive stages: (1) chronic treatment followed by tests of drug-induced stereotypy, then (2) the same chronic treatment, followed by tests of drug-induced locomotion. In stage 1, rats were randomly allocated to two groups, receiving chronic oral haloperidol ($n = 24$) or vehicle ($n = 24$) for a period of three weeks. Drinking fluids were then replaced with tap water, and 3 and 4 days later, rats received two stereotypy tests. Each group of 24 rats was randomly divided into three subgroups ($n = 8$), and each rat was then tested once with saline and once with either B-HT 920 0.1 mg kg^{-1}, B-HT 920 1.0 mg kg^{-1}, or apomorphine 0.2 mg kg^{-1}, depending on the subgroup. Saline and drug tests were conducted in a counterbalanced order within each subgroup. Stereotypy was rated at 10, 20 and 30 min after injection.

Stage 2 started three days later. The same groups were re-established on oral haloperidol or vehicle for 3 weeks and then withdrawn, as before. Three days later, each group was randomly divided into three subgroups ($n = 8$), and subjects were tested for locomotor activity with saline, or B-HT 920 0.025 mg kg^{-1}, or B-HT 920 0.1 mg kg^{-1}, depending on subgroup. On the next day, equal numbers of rats in each subgroup were randomly reassigned for testing with apomorphine 0.05 or 0.1 mg kg^{-1}. The locomotor testing procedure was intended to emphasize any stimulant effects present. Rats were therefore habituated to the testing apparatus for 40 min, removed for injection and immediately returned for a test session of 120 min.

Results

Experiment 1: Acute effects of B-HT 920 on locomotor activity and on dopamine and 5-hydroxytryptamine metabolism in normosensitive rats.

Locomotor activity was reduced by B-HT 920 in a dose-dependent way (mean effect of Dose: $F = 39.9$, d.f. 4, 38, $P < 0.0001$; linear trend of Dose: $F = 96.3$, d.f. 1, 38, $P < 0.0001$). Photocell counts (mean $\pm$ s.e.mean) in the groups treated with saline and B-HT 920 (0.01–0.3 mg kg^{-1}) were, respectively, 386.5 ± 18.1, 333.9 ± 28.8, 194.0 ± 16.4, 146.3 ± 16.2, and 112.5 ± 14.9. This depressant effect reached significance at 0.03 mg kg^{-1} (Dunnett's test $P < 0.01$).

Regional concentrations of dopamine, 5-HT and metabolites in control (saline-treated) rats are shown in Table 1. B-HT 920 significantly increased dopamine concentrations in all regions except mPFCx (mean effect of Dose: $F > 2.88$, d.f. 4, 38, $P < 0.05$–0.005; Figure 1). This increase was significantly dose-related in NACC and CP (linear trend on dose: $F > 16.0$, d.f. 1, 39, $P < 0.001$; Figure 1), with a similar but non-significant trend in OT ($P < 0.06$). Significant effects were

Table 1 Regional concentrations* of dopamine (DA), 5-hydroxytryptamine (5-HT) and metabolites in control rats (Experiment 1)

Region	*DA*	*HVA*	*DOPAC*	*5-HT*	*5-HIAA*
mPFCx	0.70 ± 0.05	0.76 ± 0.04	0.50 ± 0.02	2.30 ± 0.08	1.75 ± 0.07
OT	39.7 ± 1.8	3.15 ± 0.10	11.3 ± 0.4	5.00 ± 0.15	2.41 ± 0.09
NACC	68.1 ± 3.4	7.86 ± 0.32	20.8 ± 0.9	2.73 ± 0.17	2.76 ± 0.06
CP	79.7 ± 2.4	8.17 ± 0.30	17.4 ± 0.6	1.64 ± 0.08	2.11 ± 0.07

* Concentrations (mean ± s.e.mean, $n = 12$) in picomoles per milligram of wet tissue.
HVA = homovanillic acid; DOPAC = dihydroxyphenylacetic acid; 5-HIAA = 5-hydroxyindolacetic acid; mPFCx = medial prefrontal cortex; OT = olfactory tubercle; NACC = nucleus accumbens; CP = caudate putamen.

reached at 0.03 to 0.1 mg kg^{-1}. B-HT 920 reduced dopamine metabolite concentrations in a dose-related way in all four areas (linear trend of Dose for DOPAC: $F > 6.33$, d.f. 1, 38, $P < 0.005$–0.0001; for HVA, $F > 29.7$, d.f. 1, 38, $P < 0.0001$), significant effects generally starting at the lowest dose (0.01 mg kg^{-1}). B-HT 920 appeared to alter concentrations of dopamine and its metabolites to a similar extent in all three subcortical areas examined.

As shown in Figure 1, B-HT 920 tended to elevate 5-HT concentrations, and this effect was significant in mPFCx, NACC and CP (main effect of Dose: $F = 2.61$, d.f. 4, 39, $P < 0.05$–0.005). B-HT 920 tended to reduce 5-HIAA concentrations in all four areas, but this was only significant in OT (main effect of Dose: $F = 3.88$, d.f. 4, 38, $P < 0.01$; linear trend $F = 9.94$, d.f. 1, 38, $P < 0.005$).

Experiment 2: Behavioural effects of B-HT 920 following bilateral administration of 6-hydroxydopamine into caudate-putamen or nucleus accumbens

Stereotypy Stereotypy scores did not vary appreciably between the three post-injection intervals, and only the observations from the middle period (20 min post-injection) are discussed. In saline tests, CP lesioned animals had significantly lower stereotypy scores than sham-operated controls ($P < 0.05$; Table 2), and they were generally less active. At the doses tested, B-HT 920 produced appreciable stereotypy in CP lesioned rats but not in controls. Thus, significantly higher scores were observed in the lesioned group than in the control group at the lower dose of B-HT 920 ($P < 0.05$) and at the higher dose ($P < 0.02$). The higher dose (1.0 mg kg^{-1}) appeared more effective than the lower (0.1 mg kg^{-1}). In control subjects, the lower dose tended to induce frequent yawning ($n = 3/6$) and prostration ($n = 3/6$), whereas the higher dose produced prostration ($n = 6/6$) with a marked absence of sniffing. In CP lesioned rats, B-HT 920 tended to produce continuous sniffing, especially at the higher dose; by 30 min after injection, prostration was uncommon even at the high dose ($n = 2/9$). Apomorphine induced stereotypy in both groups, and this was more intense in lesioned rats, reflected by higher stereotypy ratings ($P < 0.02$; Table 2).

Infusion of 6-OHDA into the CP resulted in a widespread depletion of dopamine not only in CP (95%), but also in NACC (73%) and OT (56%), compared to sham-operated control subjects. In the control group, the absolute concentrations (mean ± s.e.mean) of dopamine in the CP, NACC and OT were, respectively: 101.7 ± 1.8, 89.3 ± 1.9, and 58.8 ± 2.5 pmol mg^{-1} wet tissue. Concentrations of 5-HT were not significantly altered by the lesion; group mean tissue concentrations of 5-HT in lesioned rats ranged between 96% (OT) and 116% (CP) of those in the sham-operated group.

Locomotor activity Saline test activity did not differ between NACC lesion and control groups (main effect of Lesion:

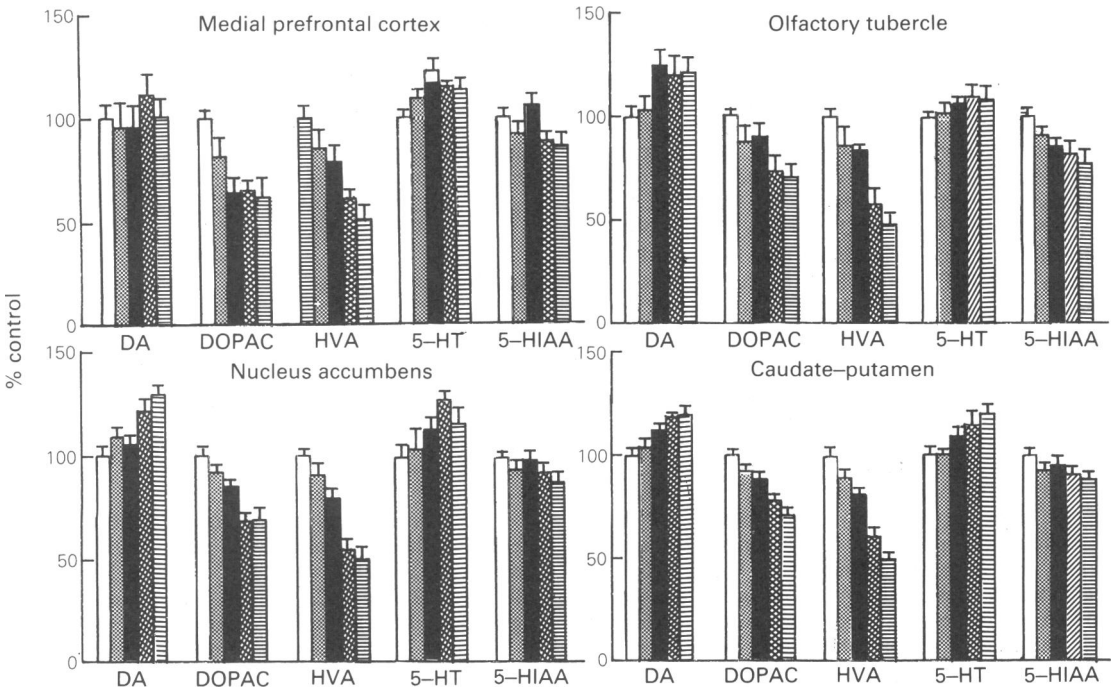

Figure 1 Effects of B-HT 920 on dopamine (DA), 5-hydroxytryptamine (5-HT) and metabolite concentrations in medial prefrontal cortex, olfactory tubercle, nucleus accumbens and caudate-putamen (Experiment 1). Regional concentrations in rats receiving B-HT 920 ($n = 8$ per group) are expressed as a percentage of the corresponding mean value in control (saline-tested) subjects ($n = 12$): Open column, control; stippled column, B-HT 0.01 mg kg^{-1}; solid column, 0.03 mg kg^{-1}; hatched columns 0.1 mg kg^{-1}; horizontally lined columns 0.3 mg kg^{-1}. Columns show mean values with s.e.mean indicated by vertical bars. The control values are shown in Table 1. Rats were killed for analysis 40 min after i.p. injection.

Table 2 Stereotypy scores (Experiments 2 and 4)*

Rating	n	*Saline* 0	1	2	3	4	5	*B-HT 920* (0.1 mg kg^{-1}) 0	1	2	3	4	5	*B-HT 920* (1.0 mg kg^{-1}) 0	1	2	3	4	5	*Apomorphine* (0.2 mg kg^{-1}) 0	1	2	3	4	5
Experiment 2																									
Control	6		6					3	3					6							1	1	2	2	
CP 6-OHDA	9	6	3					1	2			4	2	2			2	4	1				1	4	4
Experiment 4																									
Control	24	2	19	3																					
Haloperidol	24	2	21	1																					
Control	8							5	3					8									7	1	
Haloperidol	8							1	5	1		1		6	2									4	4

* Each rat was allocated a stereotypy score corresponding to the predominant category of behaviour displayed within the observation period (20–22 min after injection). The number of rats exhibiting a given stereotypy score is shown for each group. The rating scale is described in the text. CP = caudate-putamen; 6-OHDA = 6-hydroxydopamine.

$F = 1.25$, d.f. 1, 15, $P > 0.2$; Lesion × time: $F = 0.31$, d.f. 5, 75, $P > 0.9$). Mean (± s.e.mean) saline activity scores (0–60 min) were 516.5 ± 34.5 and 583.9 ± 47.7 for control and NACC lesion groups, respectively. MANOVA revealed significant Lesion × Drug ($F = 6.86$, d.f. 2, 27, $P < 0.01$) and Lesion × Drug × Time interactions ($F = 4.66$, d.f. 6, 84 $P < 0.05$). Both doses of B-HT 920 reduced locomotor activity in lesioned rats as well as in controls; the higher dose was more effective. The depressant effect of the higher dose of B-HT 920 appeared to be attenuated by the lesion in the latter part of the session (Figure 2), but overall (0–60 min), the effect of this dose did not differ significantly between surgery groups ($t = 2.00$, d.f. 15, $P > 0.05$). In contrast, apomorphine depressed locomotor activity only in controls, and increased activity in lesioned animals (Figure 2).

Infusion of 6-OHDA into the NACC depleted dopamine in NACC by 62%, and modest losses were seen in CP (19%) and OT (35%). In the control group, the absolute concentrations (mean ± s.e.mean) of dopamine in the CP, NACC and OT were, respectively: 99.5 ± 2.3, 80.3 ± 4.9, and 47.9 ± 1.1 pmol mg^{-1} wet tissue. Concentrations of 5-HT were not significantly altered by the lesion; group mean tissue concentrations of 5-HT in lesioned rats ranged between 98% (CP) and 111% (NACC) of those in the sham-operated group.

Experiment 3: Drug-induced circling behaviour following unilateral 6-hydroxydopamine injection into the medial forebrain bundle

Circling behaviour did not alter appreciably across the three observation periods (20, 40 and 60 min post-injection), and the data were pooled. Between 3 and 7 days after surgery, B-HT 920 began to have a measurable effect (Figure 3). The ipsiversion shown by rats tested with saline was reduced (day 3) or replaced by contraversion (day 7) in rats tested with B-HT 920. The response to B-HT 920 at 35 days did not appear to be significantly affected by repeated testing with or without the drug. The rate of contralateral turning in rats tested with B-HT 920 gradually increased over successive tests, averaging approximately 4 turns per min on the final test.

The extent of dopamine depletion induced by 6-OHDA was very similar in all three groups, the loss (compared to the unoperated side) ranging from 95–96% (OT), 96–99% (NACC), and 99% (CP). The mean tissue concentrations of dopamine on the unoperated side were similar across these three groups, ranging as follows: 98.9–103.0 (CP), 79.6–84.0 (NACC), and 46.9–50.2 pmol mg^{-1}. As in experiment 2, concentrations of 5-HT were not significantly altered by the lesion; group mean tissue concentrations of 5-HT on the lesioned side ranged between 89–95% compared to the unoperated side.

Experiment 4: Effects of chronic haloperidol treatment on subsequent behavioural responses to B-HT 920 and apomorphine

Haloperidol intake By the end of the first three-week period of chronic treatment, mean daily fluid intake was 42 ml in controls and 28 ml in rats drinking haloperidol solution, corresponding to a drug intake of approximately 1.4 mg kg^{-1} day^{-1}. At the end of the second period, drug intake averaged 1.2 mg kg^{-1} day^{-1}.

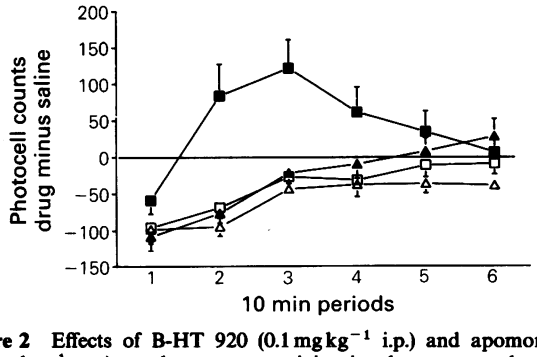

Figure 2 Effects of B-HT 920 (0.1 mg kg^{-1} i.p.) and apomorphine (0.1 mg kg^{-1} s.c.) on locomotor activity in sham-operated and 6-hydroxydopamine (6-OHDA) accumbens-lesioned rats (Experiment 2). The vertical axis shows the group mean (s.e.mean indicated by vertical bars) difference of photocell counts between drug and saline tests. (△) Sham operated, B-HT 0.1 mg kg^{-1}; (▲) 6-OHDA, B-HT 0.1 mg kg^{-1}; (□) sham operated, apomorphine 0.1 mg kg^{-1}; (■) 6-OHDA, apomorphine 0.1 mg kg^{-1}. Saline test activity did not differ significantly between control and lesion groups (see Results). The effect of B-HT 920 was indistinguishable in the two groups of rats; apomorphine decreased activity in controls but increased activity in lesioned subjects.

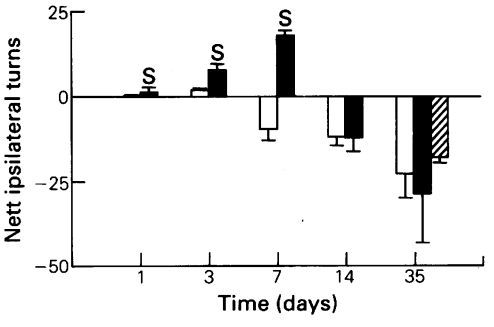

Figure 3 Emergence of ipsilateral circling in response to B-HT 920 (0.1 mg kg^{-1} i.p.) in unilaterally medial forebrain bundle lesioned rats (Experiment 3). Rats received a unilateral infusion of 6-hydroxydopamine into the medial forebrain bundle. Group 1 (open columns) was tested on days 1, 3, 7, 14 and 35 days post-surgery with B-HT 920; Group 2 (solid columns) was tested with saline on days 1, 3 and 7, and subsequently with B-HT 920. Group 3 (hatched column) was tested only on day 35, with B-HT 920. S = saline test. The columns show the mean number of turns (ipsilateral minus contralateral) in a total observation period of 6 min; s.e.mean indicated by vertical bars.

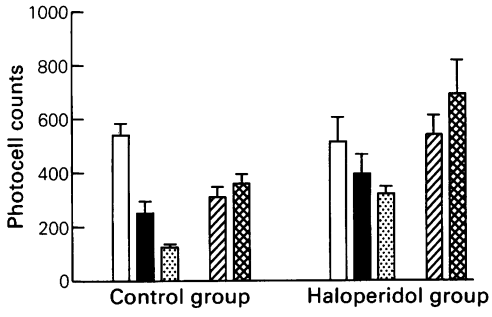

Figure 4 Effects of B-HT 920 and apomorphine on locomotor activity in vehicle- and haloperidol-withdrawn rats (Experiment 6). Three days after a period of chronic haloperidol or vehicle (control) treatment, subjects were tested 0–120 min after injection of saline (open columns), B-HT 920 0.025 mg kg^{-1} (solid columns) or 0.1 mg kg^{-1} (stippled columns) i.p. ($n = 8$ per group). On the next day, rats were tested with apomorphine 0.05 mg kg^{-1} (hatched columns) or 0.1 mg kg^{-1} (cross-hatched columns) s.c. ($n = 12$ per group). Columns show mean values with s.e.mean indicated by vertical bars.

Stereotypy As in Experiment 2, stereotypy scores varied little between post-injection intervals, and data from 20 min post-injection are discussed. In saline tests, there were few signs of stereotypy, and the haloperidol-withdrawn group did not differ significantly from the control group that had not received haloperidol ($P > 0.1$; Table 2). In control subjects, neither dose of B-HT 920 produced stereotypy, and immobility was commonly seen (Table 2). When tested with the lower dose of B-HT 920, haloperidol-withdrawn rats were generally more mobile than the control subjects, and as a result, they obtained significantly higher stereotypy ratings than controls ($P = 0.025$). However, haloperidol-withdrawn rats displayed few signs of stereotypy when tested with either dose of B-HT 920. In contrast, withdrawal from chronic haloperidol treatment clearly increased apomorphine-induced stereotyped behaviour, and this is reflected by significantly higher stereotypy ratings ($P < 0.001$; Table 2).

Locomotor activity On the first test day, saline activity scores (0–120 min) did not differ significantly between control and haloperidol-withdrawn groups (mean ± s.e.mean: 540.4 ± 41.9, 514.8 ± 91.5, respectively). B-HT 920 reduced activity in both groups. This depressant effect appeared attenuated in haloperidol-withdrawn animals, a suggestion which was not supported by statistical analysis (Dose × Group: $F = 2.14$, d.f. 3, 29, $P > 0.1$; Dose × Group × Time: $F = 0.82$, d.f. 10, 195, $P > 0.5$; Figure 4). In contrast, the two chronic treatment groups differed significantly in locomotor scores during tests with apomorphine (main effect of Group: $F = 14.3$, d.f. 1, 42, $P < 0.001$), and there was no significant difference between the two doses (main effect of Dose: $F = 1.82$, d.f. 1, 42, $P > 0.1$; Dose × Group: $F = 0.46$, d.f. 1, 42, $P > 0.5$).

Discussion

In normosensitive rats, B-HT 920 increased regional concentrations of dopamine and decreased those of its major metabolites, consistent with previous evidence indicating autoreceptor agonist activity (Anden *et al.*, 1982; 1983a,b,c; Eriksson *et al.*, 1985; Clark & Chiodo, 1988). However, these effects were strikingly similar both in potency and efficacy in mesolimbic (olfactory tubercle, nucleus accumbens) and nigrostriatal terminal regions, contrary to previous evidence suggesting a preferential effect in mesolimbic areas (Anden *et al.*, 1983b). The present results support and extend a recent study employing intracranial microdialysis, where B-HT 920 was found to reduce extracellular dopamine concentrations to the same extent in dorsal striatum as in nucleus accumbens (Imperato *et al.*, 1988). In addition, they suggest that B-HT 920 also potently reduces dopamine outflow in the medial prefrontal cortex. Given the apparent lack of selectivity for the mesolimbic dopamine system, it is perhaps surprising that B-HT 920 markedly reduces locomotor activity but fails to induce catalepsy, an index of nigrostriatal dopaminergic transmission (Ahlenius & Hillegaart, 1986).

Analogous to its effects on dopaminergic indices, B-HT 920 tended to elevate concentrations of 5-HT whilst lowering those of its metabolite 5-HIAA. The similarity of effect in all four regions examined suggests that B-HT 920 may directly affect 5-hydroxytryptaminergic neurones, possibly acting as an autoreceptor agonist or by inhibiting monoamine oxidase.

B-HT 920 has D_2 but not D_1 dopamine receptor agonist properties (Jennewein *et al.*, 1986). Following prolonged depletion of dopamine, the behavioural effects of D_2 agonists no longer require an enabling action by concurrent D_1 receptor activation (Clark & White, 1987). Thus, in rats unilaterally lesioned with 6-OHDA, B-HT 920 induces contralateral rotation which is independent of D_1 receptor blockade (Hinzen *et al.*, 1986). In the present study, lesions of the nigrostriatal system induced by 6-OHDA produced clear evidence of postsynaptic dopamine receptor activation by B-HT 920 (stereotypy). In contrast, although 6-OHDA lesions directed at the mesolimbic system reduced the locomotor depressant effect of B-HT 920, presumably as a result of the removal of dopaminergic terminals, these lesions did not reveal signs of postsynaptic activation induced by B-HT 920 (locomotor stimulation). The basis for this difference is not clear, especially since both lesions resulted in supersensitive postsynaptic responses to apomorphine. It should be noted that in rats tested for stereotypy, residual dopamine in the target tissue (caudate-putamen) was depleted to a greater extent (95%) than was the target tissue in rats tested for locomotor activity (NACC, 62% loss). Since B-HT 920 markedly reduces dopamine release *in vivo* (Imperato *et al.*, 1988), it is possible that postsynaptic agonist effects of this drug may be masked in partially-lesioned animals by a concomitant reduction of presynaptically-released dopamine. The results of the rotation experiment (Experiment 3) are also consistent with this interpretation. Infusion of 6-OHDA into the medial forebrain bundle, which produced massive loss of dopamine in both nigrostriatal and mesolimbic terminal regions, revealed significant contraversive turning in response to B-HT 920; such behaviour requires postsynaptic dopamine receptor activation in both caudate-putamen and accumbens (Pycock, 1980).

Experiments performed in unlesioned rats (Imperato *et al.*, 1988) suggest that the release of dopamine from presynaptic terminals is inhibited more profoundly and with shorter latency by B-HT 920 than by apomorphine at the doses used in the present experiments. Such a difference could possibly help to explain why apomorphine stimulated locomotor activity in 6-OHDA lesioned rats whereas B-HT 920 did not (Experiment 2), and would also account for the absence of B-HT 920-induced stereotypy or locomotor stimulation in haloperidol-withdrawn animals that were demonstrably supersensitive to low doses of apomorphine (Experiment 4).

Other explanations of the present findings should also be carefully considered. An uncoupling of D_1 and D_2 receptor mechanisms appears to occur in denervated animals (Clark & White, 1987). The failure of B-HT 920 to show postsynaptic dopamine agonist properties in the chronic haloperidol-treated animals suggests that this procedure for producing dopamine receptor supersensitivity possibly does not lead to an uncoupling of D_1 and D_2 receptors. Consistent with the present findings, others have concluded that denervation and dopamine receptor blockade produce receptor supersensitivity by fundamentally different mechanisms (e.g. Staunton *et al.*, 1982; Breese *et al.*, 1987).

When given alone, B-HT 920 produces few signs of postsynaptic dopamine receptor stimulation in normosensitive

animals. However, 'low level' stereotyped behaviour has been unmasked by coadministration of certain α_2-noradrenoceptor antagonists (Johansen *et al.*, 1988; but see also Hinzen *et al.*, 1986). It therefore appears that the α_2-agonist properties of B-HT 920 can inhibit the behavioural expression of postsynaptic dopamine receptor stimulation. The present experiments indicate that in rats lesioned with 6-OHDA, the expression of stereotyped behaviour does not require noradrenoceptor blockade.

Following unilateral nigrostriatal depletion with 6-OHDA, the mixed D_1/D_2 agonist apomorphine induces rapid contralateral turning within one or two days, and maximal circling rates are reached within a very few days (Ungerstedt, 1971; Staunton *et al.*, 1982). Locomotor stimulant effects of apomorphine following bilateral mesolimbic destruction have a similar rapid onset (Arnt & Hyttel, 1984; Clark *et al.*, 1985). In contrast, the D_2-selective agonist pergolide, as well as compounds regarded as selective autoreceptor agonists (EMD 23448 and (−)-3-PPP) begin to show signs of postsynaptic activation over a longer period of time (Arnt & Hyttel, 1984; Arnt, 1985). In the present study, the contraversive tendency displayed by B-HT 920 emerged in a gradual fashion, consistent with the hypothesis (Arnt, 1985) that postsynaptic D_1 and D_2 receptors become uncoupled over a two week period following denervation in rats.

In summary, the conditions under which B-HT 920 displays postsynaptic dopamine receptor activation remain incompletely understood. The present results suggest that any therapeutic potential of B-HT in Parkinson's disease may be limited to cases of severe dopaminergic degeneration.

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A bradykinin $(BK)_1$ receptor antagonist blocks capsaicin-induced ear inflammation in mice

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1 The effect of various peptide antagonists on capsaicin-induced (250 μg per ear) ear inflammation has been examined.

2 Co-administration of the substance P (SP) antagonist [D-Pro^2,D-Trp^{7,9}]SP at 100 and 300 μg per ear with capsaicin markedly attenuated oedema, whereas a vasopressin antagonist was ineffective.

3 Using the same scheme, the mixed BK_2 and BK_1 bradykinin (BK) antagonist NPC 567 (D-Arg[Hyp^3, D-Phe^7]BK) did not inhibit oedema at 100 μg per ear, but did inhibit at a higher dose (300 μg). The BK_1 antagonist [Leu^8,desArg^9]BK produced significant inhibition at both doses.

4 When BK was used to induce ear inflammation (30 μg per ear), the SP antagonist inhibited ear oedema. Both BK receptor subtype antagonists inhibited inflammation with the BK_1 being more potent than the BK_2 antagonist.

5 These results suggest that BK_1 along with BK_2 receptors are located on capsaicin-sensitive fibres, where they may modulate the degree of neurogenic inflammation.

Introduction

It is well known that capsaicin specifically stimulates primary sensory pain fibres to release substance P (SP) and other tachykinins (Nagy *et al.*, 1981; Jancso *et al.*, 1985; Hoover, 1987). The release of SP, an 11 amino acid neuropeptide, by capsaicin and other noxious stimuli may be involved in the generation of local inflammatory responses via an axon reflex (Lembeck & Holzer, 1979). There is further evidence that bradykinin (BK) will also release SP from capsaicin-sensitive nerves (Butler & Hammond, 1980; Hakanson *et al.*, 1987; Geppetti *et al.*, 1988). BK itself is a nonapeptide involved in many biological processes. It is released during tissue damage, causing increased vascular permeability, vasodilatation, and pain (Regoli & Barabe, 1980). Since there appear to be at least two different types of receptors involved in mediating BK effects in tissues (Regoli & Barabe, 1980; Bouthillier *et al.*, 1987), it was of interest to determine which receptor subtype, BK_1 or BK_2, is involved in the control of neurogenic inflammation.

In most tissues BK acts on BK_2 receptors which are sensitive to BK and kallidin (Lys-BK) much more than to any other kinin. As originally noted by Regoli & Barabe (1980), vascular smooth muscle develops responsiveness to the normally inactive metabolites, desArg^{9}BK, and desArg^{10}kallidin. This subset of BK receptors has been termed type 'BK_1'. Further studies have shown that enhancement of BK_1 receptor induction *in vitro* is produced by exposure to lipopolysaccharide *in vivo* (Bouthillier *et al.*, 1987). In these experiments, development of a BK_1 receptor response requires several hours to be detectable.

Capsaicin-induced neurogenic inflammation in mouse ears has a rapid onset (unpublished data). Thus, this model of inflammation presented a simple assay to test whether the BK receptor controlling SP release was of the stable BK_2 or the inducible BK_1 subtype.

Methods

Groups of 6–10 male albino mice, 25–30 g (Charles River, NY) were anaesthetized with isoflurane. Vehicle or capsaicin (25 mg ml^{-1}) or capsaicin plus peptide was applied by a pipette to the right ear, 5 μl to both the dorsal and ventral surface. Animals were anaesthetized 30 min later, and killed by cervical dislocation. Both the right (treated) and left (untreated) ear were removed by cutting horizontally across the indentation at the base of the ear. Weights were determined to the nearest tenth of a mg.

The percentage inhibition of oedema formation was calculated as follows: the weight of the untreated ear was subtracted from the treated ear in each group (vehicle, irritant, drug). The mean irritant oedema was obtained by subtracting the average vehicle control ear weight from the average capsaicin-treated ear weight. The % inhibition of oedema by peptides was calculated by subtracting the average vehicle oedema value from each ear weight in the drug group, and that mean was divided by the difference between the irritant and vehicle groups. The resultant value was subtracted from one and multiplied by 100.

Capsaicin and peptides were dissolved in ethanol. For BK-induced oedema, BK was prepared with captopril, each at concentrations of 3 mg ml^{-1} to achieve a dose of 30 μg per ear. All peptides and peptide antagonists were dissolved in dimethyl sulphoxide. Peptides and drugs for these studies came from the following sources: capsaicin (Fluka, N.Y.); BK, [Leu^8,desArg^9]BK, [D-Pro^2,D-Trp^{7,9}]SP, and [1-mercaptocyclohexyl]acetyl-Tyr[Me]Arg^8-vasopressin (Sigma, St. Louis, MO.). NPC 567 (Arg[Hyp^3,D-Phe^7]BK) was synthesized at NOVA.

Statistical analysis was performed by Student's *t* test for unpaired observations.

Results

Inhibition of capsaicin oedema

Depending on the type of receptor antagonist, quantitatively different degrees of inhibition of capsaicin-induced oedema (250 μg per ear) were produced (Figure 1). At a concentration of 100 μg per ear, the SP antagonist, [D-Pro^2,D-Trp^{7,9}]SP inhibited inflammation by 42%, which was not significantly altered by increasing the dose to 300 μg per ear (52%). For comparison, an arginine vasopressin (AVP) receptor antagonist had no effect at 100 μg per ear. We tested the effects of BK by using both a mixed BK_1 and BK_2 receptor antagonist, NPC 567, and a specific BK_1 receptor antagonist, [Leu^8, desArg^9]BK. At 100 μg per ear, NPC 567 did not produce any appreciable inhibition of oedema, but at 300 μg it did cause a significant, 34%, inhibition of inflammation. On the other

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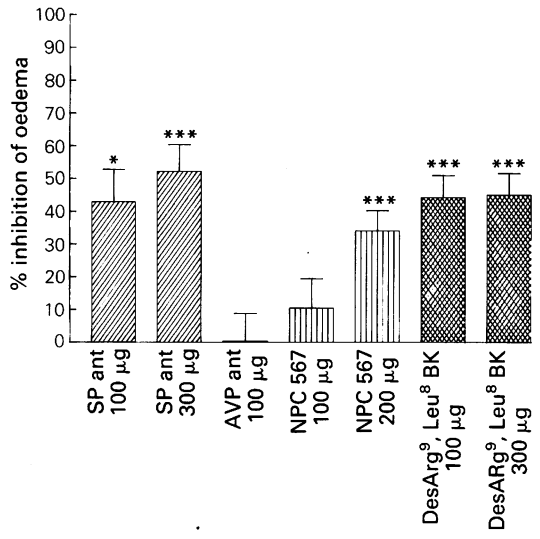

Figure 1 Inhibition of capsaicin oedema by various peptides. A dose of 250 μg capsaicin per ear produced a 36 $\pm$ 1.0% (n = 6) increase in ear weight. The substance P antagonist (SP ant) was [D-Pro^2,D-Trp^{7,9}] bradykinin (BK) and AVP ant = arginine vasopressin antagonist. Peptides were topically co-administered with capsaicin (250 μg) and oedema measured 30 min later. Values represent the mean of 20–30 mice, and vertical bars show s.e.mean. Statistically significant differences are represented as follows: *P < 0.05, ***P < 0.001.

hand, the BK_1 antagonist caused 40% inhibition at 100 μg per ear, and non-significantly increased it to 45% at a dose of 300 μg.

BK-induced oedema

We investigated whether kinins can cause ear oedema, and the ability of various peptide antagonists to inhibit this inflammation (Figure 2). BK (30 μg) was a potent stimulant of inflammation when applied to the ear, the response was effectively inhibited (131%) by a single dose (300 μg) of NPC 567 applied to the ear. [Leu^8,desArg^9]BK produced a lower effect (27%) at 300 μg per ear (data not shown). The SP antagonist (100 μg) was as effective against BK-induced oedema (58%) as it was against capsaicin-induced oedema (Figure 1: 52%).

The dose-response relationship between oedema and the concentration of NPC 567 and [Leu^8,desArg^9]BK was investigated. In Figure 2, [Leu^8,desArg^9]BK is clearly shown to be a more potent inhibitor of oedema than NPC 567. In fact, the BK_1 antagonist reached a maximum of 100% inhibition at 30 μg per ear (Figure 2), then began to become less effective at higher concentrations (data not shown). Figure 2 demonstrates that the mixed BK_2 and BK_1 receptor antagonist, NPC 567, inhibited about 50% of the oedema produced, which is similar to the effect of [Leu^8,desArg^9]BK at low concentrations. At high concentrations of both antagonists there was a greater than 100% inhibition. This stems from the inhibitory effect on oedema produced by the DMSO vehicle alone. The peptide antagonist did not affect basal (non-vehicle) control ear weight. The linear transformation of the data indicated that the ED_{50} for NPC 567 was 16.2 μg per ear, whereas [Leu^8,desArg^9]BK had an ED_{50} of 2.0 μg per ear. Thus, based on these results, the existence of endogenous BK_1 receptors are implied.

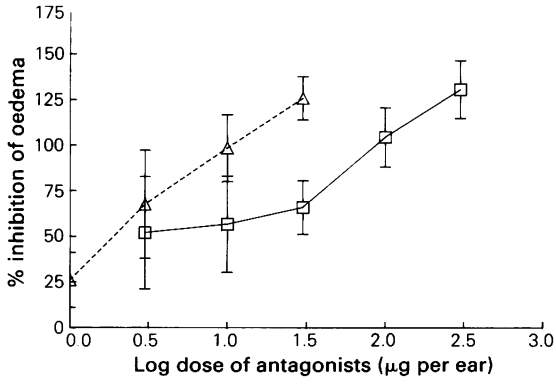

Figure 2 Dose-response curves for the inhibition of bradykinin-induced oedema by BK_1- and BK_2-receptor antagonists. BK itself produced 48 $\pm$ 5.1% (n = 6) oedema. The ED_{50} for NPC 567 (□) was 16.2 μg per ear and that for [Leu^8,desArg^9]BK (△) 2.0 μg per ear. Each point represents the mean of 10 mice and vertical lines show s.e.mean.

Discussion

Co-administration of a BK_1 receptor antagonist, [Leu^8, desArg^9]BK with capsaicin reduced neurogenic inflammation. Another peptide, D-Arg[Hyp^3,D-Phe^7]BK, which is a partial BK_2 receptor antagonist (Regoli *et al.*, 1986), was less effective at blocking this type of inflammation. That the SP antagonist, [D-Pro^2,D-Trp^{7,9}]SP, was effective at inhibiting capsaicin-induced oedema suggests that SP release by capsaicin may be the primary mediator of inflammation in this model.

Moreover BK-induced inflammation in the mouse ear was also strongly inhibited by the same SP antagonist. Others have shown that the intravenous injection of an SP antagonist reduces BK-induced vascular permeability (Shibata *et al.*, 1986). The effects of these peptide antagonists were specific. For example, a vasopressin receptor antagonist did not inhibit oedema formation induced by capsaicin. These results suggest that both BK_1 and BK_2 receptors are able to modify the capsaicin-induced release of SP, and thus influence the development of neurogenic inflammation.

BK_1 receptors have characteristically been shown not to be present in normal tissue, but synthesized upon tissue injury (Regoli & Barabe, 1980). This can also be seen when rabbits are treated intravenously with endotoxin where BK_1 receptor-mediated hypotension is seen 6 h after treatment (Bouthillier *et al.*, 1987). Capsaicin-induced inflammation, as measured by an increase in ear weight, is already maximal at 30 min post application (data not shown). This suggests that BK_1 receptors are normally present on sensory fibres in the mouse ear. BK and its kininase II metabolite desArg^{9}BK can then act at their respective receptors to amplify the inflammatory process further. In fact, BK has been shown to have a synergistic effect with SP on vascular permeability and the pain response in the mouse paw (Shibata *et al.*, 1986).

Thus, these data demonstrate the endogenous presence of BK_1 receptors on capsaicin-sensitive nerves without the *de novo* synthesis of these receptors with tissue injury. In the ear they appear to regulate the degree of neurogenic inflammation. Further studies are in progress to measure directly BK_1 receptors in ear tissue and elsewhere, by using radioligand binding techniques with desArg^{10}kallidin as the ligand.

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The involvement of 5-hydroxytryptaminergic and dopaminergic mechanisms in the eating induced by buspirone, gepirone and ipsapirone

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1 The roles of 5-hydroxytryptamine (5-HT) and dopamine systems in mediating the increased feeding induced by buspirone, gepirone and ipsapirone were investigated.

2 All three compounds induced dose-dependent increases in food intake when administered subcutaneously to free feeding rats. Buspirone was effective over a narrower dose range than either gepirone or ipsapirone, and the maximal effect observed was smaller than the effects elicited by gepirone and ipsapirone.

3 Depletion of brain 5-HT with parachlorophenylalanine (PCPA) prevented the effects of equi-effective doses of gepirone (2.5 mg kg^{-1}) and ipsapirone (2.5 mg kg^{-1}), but failed to prevent buspirone (1 mg kg^{-1})-induced eating. Thus buspirone does not appear to interact with 5-HT systems to elicit feeding.

4 Gepirone (0.2 μg) and ipsapirone (0.04 and 0.2 μg) increased food intake when injected into the dorsal raphé nucleus (DRN), presumably by inhibiting the activity of DRN 5-hydroxytryptaminergic afferents. Buspirone (0.04–5 μg) was ineffective when injected into the DRN.

5 Pretreatment with haloperidol (0.1 mg kg^{-1}, 30 min) significantly attenuated the effects of equi-effective doses of buspirone, gepirone and ipsapirone, indicating that these drugs interact with dopaminergic systems to increase feeding.

6 Previously it has been shown that each of these drugs increases striatal dopamine activity. Increased dopaminergic neurotransmission in the striatum induces a general behavioural activation, which under certain conditions facilitates feeding. It is possible that this mechanism underlies the behavioural effects of buspirone, gepirone and ipsapirone. The effects of gepirone and ipsapirone probably involve an indirect action to inhibit the activity of DRN 5-hydroxytryptaminergic afferents, whereas buspirone interacts directly with dopaminergic systems.

Introduction

The centrally acting 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) induces a variety of behavioural effects when administered peripherally to rats. These effects include the 5-HT motor syndrome (Tricklebank *et al.*, 1984), increased consumption of hypertonic saline (Cooper & Desa, 1987), anxiolytic-like activity (reviewed by Dourish, 1987; Traber & Glaser, 1987) and antidepressant-like activity (Kennett *et al.*, 1987; Cervo *et al.*, 1988). In free-feeding rats 8-OH-DPAT increases food intake (Dourish *et al.*, 1985), and the underlying mechanism of this particular response has received considerable experimental attention.

Depletion of brain 5-HT by parachlorophenylalanine (PCPA) (Dourish *et al.*, 1986a) or 5,7-dihydroxytryptamine (Bendotti & Samanin, 1986) blocks 8-OH-DPAT-induced eating, indicating that presynaptic elements of 5-hydroxytryptaminergic neurotransmission are required for the expression of this behaviour. Electrophysiological studies have shown that 8-OH-DPAT markedly inhibits the activity of 5-hydroxytryptaminergic neurones, notably those arising from the dorsal raphé nucleus (DRN) (Sprouse & Aghajanian, 1987). This effect may occur from an agonist action at receptors which normally regulate impulse flow in DRN neurones, and which are located in the somatodendritic region of the cells (Aghajanian *et al.*, 1987). That the DRN is important in mediating 8-OH-DPAT-induced eating is shown by the finding that microinjection of 8-OH-DPAT into the DRN elicits a feeding response comparable to that observed after peripheral injection (Bendotti & Samanin 1986; Hutson *et al.*, 1986; Fletcher & Davies, 1989). A consequence of this decreased activity of DRN neurones is a reduction in 5-HT turnover in various forebrain regions (Hutson *et al.*, 1986; Hamon *et al.*, 1988; Hjorth & Magnusson, 1988). Thus, it is suggested that 8-OH-DPAT elicits feeding by reducing 5-hydroxytryptaminergic neurotransmission arising from the raphé nuclei. This is in keeping with the current views of the role of 5-HT in controlling feeding behaviour (Blundell, 1984). Since injection of 8-OH-DPAT into the DRN also induces anxiolytic-like (Higgins *et al.*, 1988) and antidepressant-like (Cervo *et al.*, 1988) actions, this mechanism is also thought to underlie these effects of 8-OH-DPAT.

Buspirone and its structural analogues, gepirone and ipsapirone represent a group of non-benzodiazepine anxiolytic compounds which exhibit many of the same properties of 8-OH-DPAT. All three compounds are 5-HT$_{1A}$ agonists (Peroutka, 1985; 1986; McMillen *et al.*, 1987) which reduce 5-HT turnover (Hjorth & Carlsson, 1982; McMillen *et al.*, 1987; Hamon *et al.*, 1988) by inhibiting the activity of DRN neurones (VanderMaelen *et al.*, 1986; McMillen *et al.*, 1987; Sprouse & Aghajanian, 1987). In behavioural tests these drugs, like 8-OH-DPAT, show anxiolytic activity (reviewed by Dourish, 1987), increase the consumption of hypertonic saline (Cooper & Desa, 1987) and increase food intake (Dourish *et al.*, 1986b; Gilbert & Dourish, 1987; Gilbert *et al.*, 1988; Neill & Cooper, 1988). It is possible that buspirone, gepirone and ipsapirone act via the same mechanism as 8-OH-DPAT to exert their behavioural effects. However, there is a lack of direct experimental evidence showing that 5-hydroxytryptaminergic neurones, and particularly those arising from the DRN, are involved in mediating the behavioural effects of buspirone, gepirone and ipsapirone. Several differences in the behavioural effects of these compounds have been noted. For example, all

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four compounds, including 8-OH-DPAT, block the fear-potentiated startle reaction (Davis *et al.*, 1988; Mansbach & Geyer, 1988), but in the case of buspirone 5-hydroxytryptaminergic systems are not involved (Davis *et al.*, 1988). Buspirone has been shown also to affect unpunished responding in pigeons, whereas gepirone and 8-OH-DPAT do not (Barrett *et al.*, 1988). Additionally these compounds differ in their ability to elicit the 5-HT syndrome (Smith & Peroutka, 1986). In regard to the effects of these drugs on feeding, gepirone induces a strong feeding response, whereas ipsapirone appears only to be weakly active (Gilbert & Dourish, 1987). Together these results suggest that a single common mechanism may not underlie the behavioural effects of these drugs.

The present experiments were undertaken to examine the mechanism(s) involved in mediating the effects of buspirone, gepirone and ipsapirone on feeding. Specifically, the effects of these drugs were investigated in PCPA-treated rats to examine whether 5-hydroxytryptaminergic systems are required for the actions of these drugs. In order to determine the possible importance of the DRN, the effects of these drugs on feeding were examined following direct injection into the DRN. Previously it has been suggested that 8-OH-DPAT-induced eating is mediated ultimately by an increase in nigrostriatal dopamine activity (Fletcher, 1987; Montgomery *et al.*, 1988; Fletcher & Davies, 1989). Therefore, the possible involvement of a dopaminergic mechanism in the effects of buspirone, gepirone and ipsapirone was assessed by investigating the actions of these drugs in rats pretreated with the dopamine receptor antagonist haloperidol.

Methods

Male Sprague-Dawley rats weighing 270–420 g at the start of the experiments were used. They were housed individually in hanging wire-mesh cages in a temperature-controlled room (22 ± 1°C) under a 12 h light-dark cycle (lights on at 08 h 00 min). Food and water were available at all times. All animals were handled for several minutes on at least three occasions before the experiments began.

Procedure

On test days the procedure was identical in all experiments. One hour before drug injection fresh food was placed on the floor of each animal's cage. This procedure together with the frequent handling minimizes the amount of food consumed under control conditions during testing. Each rat was then removed from the home cage, injected and returned to the home cage, together with a weighed amount (approximately 25–35 g) of fresh food. One hour later food intakes were determined by reweighing to the nearest 0.1 g the remaining food plus any spillage collected on paper towels placed beneath the cages.

Effects of peripherally injected buspirone, gepirone and ipsapirone on food intake

Three separate groups of rats were used in three different experiments to examine the effects of various doses of peripherally administered buspirone ($n = 8$), gepirone ($n = 8$) and ipsapirone ($n = 8$) on food intake over a 1 h period. The first group was tested on different occasions following an injection of 0.3125, 0.625, 1.25, 2.5 or 5 mg kg^{-1} buspirone hydrochloride (Bristol-Myers, Wallingford, CT) or 0.9% saline; the second group was injected with 0.3125, 0.625, 1.25, 2.5 or 5 mg kg^{-1} gepirone hydrochloride (Bristol Myers) or saline; the third group was tested with 0.625, 1.25, 2.5, 5 and 10 mg kg^{-1} ipsapirone hydrochloride (Miles Inc., West Haven, CT) or saline. For each experiment a repeated measures design was used, with each rat receiving every dose of the appropriate test drug, and saline in a randomized order, with at least 3 drug-free days between successive tests. All drugs were injected subcutaneously (s.c.) in a volume of 1 ml kg^{-1}.

Effects of PCPA on feeding induced by buspirone, gepirone and ipsapirone

Three groups of rats were used in three different experiments to examine the effects of PCPA treatment on the eating induced by 1 mg kg^{-1} buspirone ($n = 7$), 2.5 mg kg^{-1} gepirone ($n = 7$) and 2.5 mg kg^{-1} ipsapirone ($n = 6$). The drug treatment protocol was identical for each experiment. Before the experiments each rat was administered 3 ml kg^{-1} distilled water via a stomach tube. Then, 3–4 days later 1 h food intakes were determined on two consecutive days following injection (s.c.) with saline or the appropriate test drug. On each of these days approximately half of the rats received saline, and the remainder the test drug. Four days later each rat was administered 400 mg kg^{-1} parachlorophenylalanine (PCPA) ethyl ester (Sigma Chemical Co., St. Louis, MI) dissolved in distilled water (3 ml kg^{-1}) via a stomach tube. On the third and fourth days after PCPA treatment, 1 h food intakes were determined, again following injection (s.c.) with saline or the test drug as described above. This dose and time schedule of PCPA treatment depletes whole brain 5-HT content by approximately 90% (Fletcher, 1989).

Effects of dorsal raphé nucleus injection of buspirone, gepirone and ipsapirone on food intake

Three groups of rats were used to examine the effects of buspirone, gepirone and ipsapirone on food intake following injection into the DRN.

Surgery Before these experiments each rat (weighing 270–320 g) was anaesthetized with 40–50 mg kg^{-1} sodium pentobarbitone (Nembutal, Abbott Laboratories) and placed in a stereotaxic frame. A 22 g stainless steel guide cannula (Plastic Products, Roanoke, VA) cut to 15 mm in length was implanted, aimed at a site 4 mm above the DRN. With the incisor bar set at 3.3 mm below interaural zero the following coordinates were used (relative to interaural zero): AP + 1.2 mm, Lat − 1.6 mm, V + 7.8 mm according to the atlas of Paxinos & Watson (1985). The cannula was lowered at an angle of 20° to the vertical to avoid damaging the sagittal sinus. Each cannula was secured by acrylic cement which was held in place by 4 stainless steel screws drilled into the skull. A 28 g stainless steel stylet served to keep the cannula patent. Animals were allowed at least 7 days to recover before testing began; during this period each rat was handled frequently and familiarized with the injection procedure.

Drug delivery At the time of drug injection each rat was removed from the home cage and lightly restrained by hand. Drugs were injected into the DRN through a glass capillary tube (70–90 μm diameter) connected to a manually operated 5 ml Hamilton syringe via a length of plastic tubing. The glass capillary was housed inside a 15 mm length of stainless steel tubing, and could be extended 4 mm beyond the guide cannula to penetrate the DRN (See Azami *et al.*, 1980 for full details of the microinjection assembly). Each drug solution was delivered in a volume of 0.5 μl (in a sterile 0.9% saline vehicle) over a period of 1 min, and the needle was left in place for a further 30 s.

One group of rats ($n = 8$) was tested following injection of saline, 0.2, 1 and 5 μg of buspirone HCl; a second group ($n = 9$) received injections of saline, 0.04, 0.2, 1 and 5 μg gepirone HCl; the third group ($n = 9$) received injections of saline, 0.04, 0.2, 1 and 5 μg ipsapirone HCl. A minimum of 3 days elapsed between successive drug treatments and doses were administered in a randomised order. Following completion of these studies all rats were tested after administration of 1 μg

8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) HBr (RBI, Wayland, MA).

Verification of injection sites After completion of the experiments all rats were anaesthetized with Nembutal, injected with 0.5 μl fast green dye into the DRN, and perfused intracardially with 4% formalin. The brain was removed and standard histological procedures were used to determine the injection sites. Results from animals with injection sites located outside the DRN were not used in the final analysis.

Effects of haloperidol on feeding induced by buspirone, gepirone and ipsapirone

Three groups of rats were used to examine the effects of haloperidol on the eating induced by 1 mg kg^{-1} buspirone ($n = 8$), 2.5 mg kg^{-1} gepirone ($n = 6$) and 2.5 mg kg^{-1} ipsapirone ($n = 6$). The drug treatment protocol was identical in each experiment. Animals were first injected s.c. with 0.1 mg kg^{-1} haloperidol (RBI) or its vehicle, 0.1 M tartaric acid (adjusted to pH 6 with 2 N NaOH) in a volume of 1 ml kg^{-1}. Thirty minutes later the animals were injected s.c. with the appropriate test drug or saline. Food intakes were determined 1 h later as described above. Repeated measures designs were used in each experiment, with every rat receiving all 4 possible drug combinations. Animals were tested first with saline and the appropriate drug, given in a counterbalanced order, following the vehicle pretreatment, and subsequently, in a counterbalanced order, following haloperidol treatment. At least 3 days elapsed between successive drug treatments.

Results

Effects of peripherally injected buspirone, gepirone and ipsapirone on food intake

The effects on food intake of peripheral injection of buspirone, gepirone and ipsapirone are shown in Figure 1. One way analyses of variance showed significant main effects of buspirone ($F(5,35) = 5.5$, $P < 0.001$), gepirone ($F(5,35) = 7.7$, $P < 0.001$) and ipsapirone ($F(5,35) = 5.7$, $P < 0.001$) doses. Thus, each drug produced dose-dependent increases in food intake. In the case of buspirone food intake was increased by doses of 0.625 and 1.25 mg kg^{-1} only; further increases in dose did not elicit eating above control levels. For gepirone and ipsapirone an increase in dose resulted in progressive increments in food intake across the entire dose ranges tested. It can be seen also that the eating responses induced by the highest doses of gepirone and ipsapirone were somewhat greater than the maximal increase in food intake induced by buspirone.

Effects of PCPA on feeding induced by buspirone, gepirone and ipsapirone

Figure 2 illustrates the effects of 5-HT depletion produced by PCPA on the increased food intake induced by equi-effective doses of buspirone, gepirone and ipsapirone. Results were analysed by separate two-way analyses of variance with PCPA treatment and drug treatment as factors. Significant main effects of buspirone ($F(1,6) = 25.7$, $P < 0.003$), gepirone ($F(1,6) = 28.1$, $P < 0.002$) and ipsapirone ($F(1,5) = 51.3$, $P < 0.001$) confirmed that overall each drug increased food intake relative to saline treatment. More importantly, significant interactions between PCPA treatment and buspirone ($F(1,6) = 6.9$, $P < 0.05$), gepirone ($F(1,6) = 25.9$, $P < 0.013$) and ipsapirone ($F(1,6) = 51.3$, $P < 0.001$) were found. Tests of simple main effects further confirmed that each drug significantly enhanced food intake relative to saline injection under the vehicle pretreatment condition ($F(1,6) = 19.7$, 42.8 and

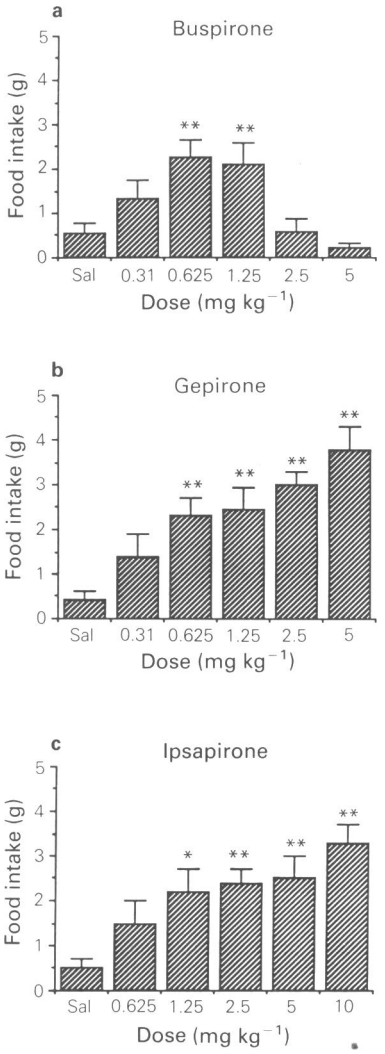

Figure 1 The effects of different doses of (a) buspirone, (b) gepirone, (c) ipsapirone, and saline (Sal) on food intakes. Columns represent mean intakes (g) over 1 h and bars show s.e.mean. *$P < 0.05$, **$P < 0.01$—significantly different from saline, Dunnett's test.

$F(1,5) = 42.3$, respectively, all $P < 0.005$). The PCPA treatment significantly attenuated the eating induced by gepirone ($F(1,6) = 22.6$, $P < 0.01$) and ipsapirone ($F(1,5) = 26.4$, $P < 0.004$) but not buspirone ($F(1,6) = 1$, $P > 0.3$). In all three experiments a slight increase in food intake was observed in animals treated with PCPA and injected with saline.

Effects of DRN injection of buspirone, gepirone and ipsapirone on food intake

The effects of different doses of buspirone, gepirone and ipsapirone, and a single dose of 8-OH-DPAT, on food intake following direct injection into the DRN are shown in Figure 3. The data, including responses to 8-OH-DPAT, were analysed by one-way analysis of variance for each experiment. In the buspirone study a significant treatment effect was observed ($F(4,28) = 10.7$, $P < 0.0001$), but this was due entirely to the increase in food intake observed with 1 μg 8-OH-DPAT. None of the doses of buspirone significantly altered food intake. In an additional group of ten rats injected with saline and 0.04 μg buspirone no effect on food intake was observed (saline, 0.3 ± 0.2 g, 0.04 μg buspirone, 0.5 ± 0.3 g, $t(9) = 0.5$, $P > 0.1$). Significant treatment effects were observed also in the gepirone ($F(5,40) = 3.6$, $P < 0.01$) and ipsapirone ($F(5,40) = 3.98$, $P < 0.01$) studies. Gepirone was effective only at a dose of 0.2 μg, while ipsapirone increased food intake at 0.04

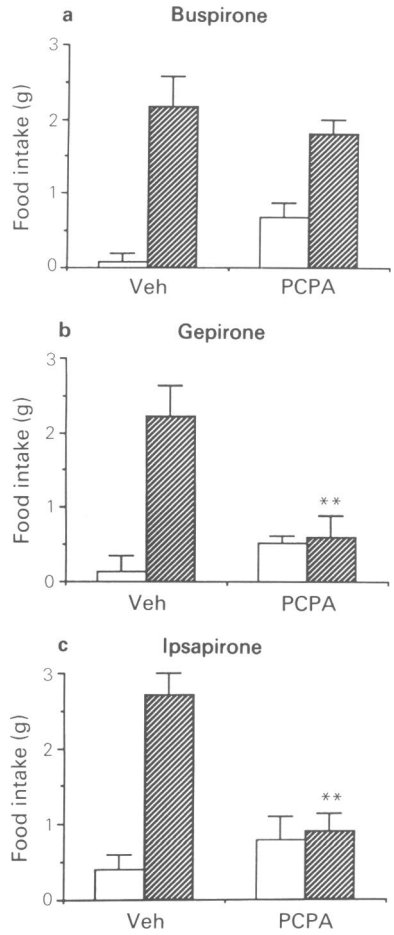

Figure 2 The effects of parachlorophenylalanine (PCPA) treatment (400 mg kg^{-1}) and its vehicle (Veh) on eating induced by (a) 1 mg kg^{-1} buspirone, (b) 2.5 mg kg^{-1} gepirone, (c) 2.5 mg kg^{-1} ipsapirone (hatched columns) and saline (open columns). Columns represent mean food intakes (g) recorded over 1 h and bars show s.e.mean. $^{**}P < 0.01$—significantly different from vehicle-buspirone treatment.

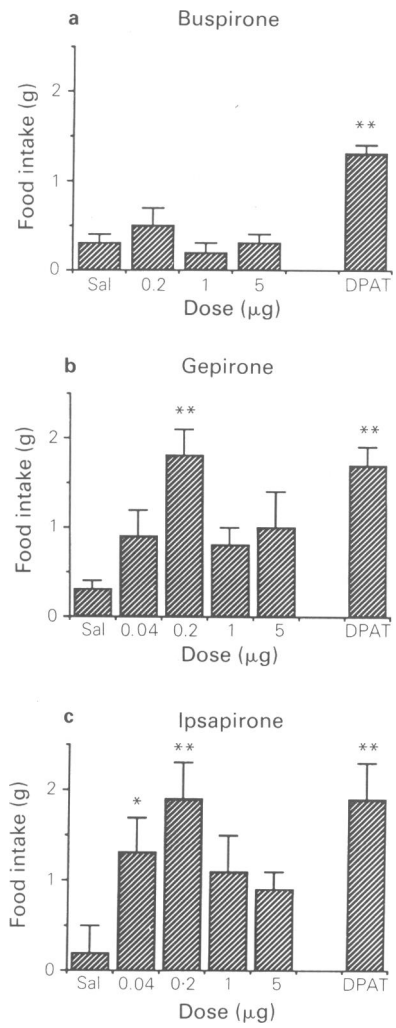

Figure 3 The effects of different doses of (a) buspirone, (b) gepirone, (c) ipsapirone and of saline (Sal) and 1 μg (8-hydroxy-2-(di-n-propylamino) tetralin (DPAT) on food intake. Columns represent mean intakes (g) over 1 h and bars show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$—significantly different from saline, Dunnett's test.

and 0.2 μg. Both groups of animals responded significantly to 1 μg 8-OH-DPAT.

Effects of haloperidol on feeding induced by buspirone, gepirone and ipsapirone

The effects of 0.1 mg kg^{-1} haloperidol on feeding elicited by 1 mg kg^{-1} buspirone, 2.5 mg kg^{-1} gepirone and 2.5 mg kg^{-1} ipsapirone are shown in Figure 4. Results were analysed by separate two-way analyses of variance with haloperidol and drug treatment as factors. Significant main effects of buspirone ($F(1,7) = 13.5$, $P < 0.01$), gepirone ($F(1,5) = 19.5$, $P < 0.01$) and ipsapirone ($F(1,5) = 19.7$, $P < 0.01$) showed that overall each drug increased food intake relative to the saline treatment. Significant interactions were observed between buspirone and haloperidol ($F(1,7) = 12.5$, $P < 0.01$) and gepirone and haloperidol ($F(1,5) = 27.2$, $P < 0.01$). Tests of simple main effects confirmed that buspirone ($F(1,7) = 22.9$, $P < 0.002$) and gepirone ($F(1,5) = 81.8$, $P < 0.001$) enhanced food intake relative to saline treatment under the vehicle pretreatment schedule. Haloperidol significantly reversed the effects of buspirone ($F(1,7) = 18.8$, $P < 0.01$) and gepirone ($F(1,5) = 40.7$, $P < 0.01$). The ipsapirone and haloperidol interaction narrowly failed to reach significance ($F(1,5) = 5.9$, $P < 0.06$), but *post-hoc* comparisons (Newman-Keul's, $\alpha = 0.05$) showed that haloperidol significantly attenuated the effect of ipsapirone. The dose of haloperidol used does not affect feeding elicited by injection of noradrenaline into the paraventricular nucleus of the hypothalamus (Davies & Fletcher, 1989; Fletcher & Davies, 1989). Therefore, the effects observed in these experiments do not appear to result from an impairment of the motor responses required for eating.

Discussion

Subcutaneous injection of buspirone, gepirone and ipsapirone increased food intake in free feeding rats, consistent with previous findings (Dourish *et al.*, 1986b; Gilbert & Dourish, 1987; Gilbert *et al.*, 1988; Neill & Cooper, 1988). Examination of the dose-response relationships for each of these drugs suggests possible differences between the actions of buspirone on the one hand, and gepirone and ipsapirone on the other. Thus, both gepirone and ipsapirone were effective over a wider dose range than was buspirone, and both produced a larger eating response than buspirone. This greater effect of gepirone, at least in comparison to 8-OH-DPAT, was noted by Gilbert & Dourish (1987). In this case a possible explanation for the difference is that at high doses eating induced by 8-OH-DPAT is disrupted by the occurrence of the 5-HT syndrome (Tricklebank *et al.*, 1984), whereas eating induced by gepirone (and ipsapirone) is not, since these compounds are weaker elicitors of the 5-HT syndrome (Smith & Peroutka, 1987). The occurrence of symptoms of the 5-HT syndrome may also explain the weaker action of buspirone on food intake, but it is also clear that different neurochemical mechanisms underlie the eating induced by buspirone, and by gepirone and ipsapi-

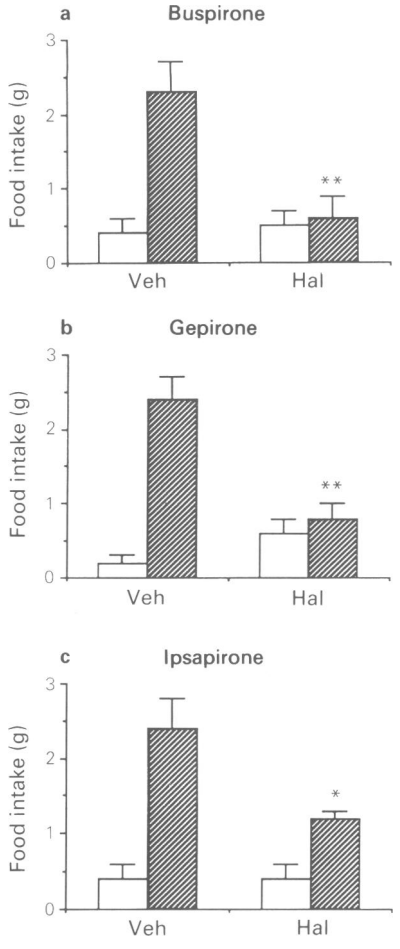

Figure 4 The effects of haloperidol (Hal) treatment (0.1 mg kg^{-1}, 30 min) and its vehicle (Veh) on eating induced by (a) 1 mg kg^{-1} buspirone, (b) 2.5 mg kg^{-1} gepirone, (c) 2.5 mg kg^{-1} ipsapirone (hatched columns) and saline (open columns). Columns represent mean food intake (g) recorded over 1 h and bars show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$—significantly different from corresponding vehicle-pretreated condition.

rone, and this may also contribute to the differences in the magnitude of the eating responses.

Depletion of 5-HT with the tryptophan hydroxylase inhibitor PCPA abolished the eating induced by equi-effective doses of gepirone and ipsapirone, but did not modify buspirone-induced eating. These results demonstrate that gepirone and ipsapirone elicit eating by interacting with 5-hydroxytryptaminergic neural systems, but that buspirone does not. Treatment with PCPA abolishes 8-OH-DPAT-induced eating (Dourish *et al.*, 1986a), and an important brain 5-hydroxytryptaminergic site in mediating the effect of 8-OH-DPAT is the DRN (Bendotti & Samanin, 1986; Hutson *et al.*, 1986; Fletcher & Davies, 1989). This site is also important in the actions of gepirone and ipsapirone, since both drugs elicited eating when injected into the DRN. The magnitude of the effects induced by both drugs appeared lower following DRN injection, compared to subcutaneous injection. This may indicate that subcutaneously injected gepirone and ipsapirone act at sites in addition to the DRN to elicit feeding. One such site may be the median raphé nucleus (MRN) since 8-OH-DPAT increases eating following injection into the MRN (Bendotti & Samanin, 1986; Hutson *et al.*, 1986).

Iontophoretic application of 8-OH-DPAT, ipsapirone (Sprouse & Aghajanian, 1987) and gepirone (McMillen *et al.*, 1987) inhibits DRN neural activity, and local injection of 8-OH-DPAT (Hjorth & Magnusson, 1988) and ipsapirone (Hamon *et al.*, 1988) into the DRN reduces 5-HT turnover in forebrain regions. On the basis of these results, together with the observations that DRN injection of 8-OH-DPAT, gepirone and ipsapirone elicits eating, it is reasonable, at this stage, to postulate that reduced 5-HT function arising from the DRN contributes to the increased food intake observed with 8-OH-DPAT, gepirone and ipsapirone. Buspirone also inhibits the activity of DRN neurones (VanderMaelen *et al.*, 1986) and decreases 5-HT turnover when administered peripherally (Hjorth & Carlsson, 1982; Fuller & Perry, 1988). It is perhaps surprising then that buspirone failed to induce eating when injected into the DRN, even though it has been shown previously that 0.04 and 0.2 μg of buspirone alter behaviour in tests of anxiolytic activity (Higgins *et al.*, 1988). However, this lack of effect of buspirone on feeding is consistent with the finding that the effect of peripherally injected buspirone is not affected by 5-HT depletion with PCPA. Higgins *et al.* (1988) found that buspirone did not affect 5-HT turnover when injected into the DRN at behaviourally active doses, and did so only marginally at a dose of 5 μg. This relatively small effect of buspirone on 5-HT turnover may explain the lack of effectiveness of buspirone in eliciting feeding following DRN injection. These results may also imply that buspirone is not as effective at inhibiting 5-HT neural activity in conscious, behaving animals, as it is in electrophysiological studies involving anaesthetized animals. It is known that anaesthesia can affect markedly the responsiveness of DRN neurones to psychoactive drugs (Trulson & Trulson, 1983). Of particular relevance here is the finding that the α_1-antagonist WB-4101, which like buspirone, gepirone, ipsapirone and 8-OH-DPAT has a high affinity for the 5-HT$_{1A}$ binding site (Peroutka, 1986), totally suppresses DRN activity in anaesthetized cats, but is considerably less effective in conscious cats (Heym *et al.*, 1984). Since the behavioural effects of this class of compounds are often interpreted with reference to their actions on neuronal activity in anaesthetized animals, this issue of drug activity in anaesthetized versus unanaesthetized animals deserves further investigation.

Injection of gepirone and ipsapirone into the DRN induced feeding only at low doses; higher doses failed to increase feeding. Similar biphasic effects related to dose have been observed in tests of anxiolytic activity following DRN injection of 5-HT$_{1A}$ agonists (Higgins *et al.*, 1988). These authors suggest that possible effects of these drugs on hypoactivity, cardiovascular function and/or thermoregulation may be responsible for the disappearance of behavioural effects at high doses (Higgins *et al.*, 1988). Such effects may also adversely affect the expression of eating at higher doses of gepirone and ipsapirone.

Haloperidol pretreatment attenuated the eating induced by peripherally administered buspirone, gepirone and ipsapirone. This dose of haloperidol has been shown previously to attenuate eating induced by DRN injection of 8-OH-DPAT, without affecting the ability of the animals to perform the eating response (Davies & Fletcher, 1989; Fletcher & Davies, 1989). These results suggest the involvement of dopaminergic mechanisms in mediating the increased feeding induced by these compounds. This possibility is supported further by the findings that peripheral injection of buspirone (Wood *et al.*, 1983; Fuller & Perry, 1988), gepirone (McMillen *et al.*, 1987) and both peripheral and DRN injection of ipsapirone (Hamon *et al.*, 1988) increase striatal dopamine turnover. 5-Hydroxytryptaminergic systems exert an inhibitory influence over nigrostriatal dopamine neurones, and a reduction in 5-HT activity increases the functional activity of these nigrostriatal neurones (Dray *et al.*, 1978). Given that 5-HT$_{1A}$ agonists inhibit the activity of 5-hydroxytryptaminergic DRN neurones (VanderMaelen *et al.*, 1986; McMillen *et al.*, 1987; Sprouse & Aghajanian, 1987), it is likely that the effects of gepirone and ipsapirone on dopamine turnover, and on feeding, result from a removal of the inhibitory influence of DRN efferents on nigrostriatal dopamine activity. This 5-HT-dopamine interactive mechanism cannot account for the effects of peripherally injected buspirone on feeding, since PCPA failed to block this response, and buspirone did not

elicit eating following DRN injection. However, low doses of buspirone increase dopamine turnover in the striatum via a direct blockade of presynaptic dopamine autoreceptors (McMillen & Mattiace, 1983; McMillen *et al.*, 1987), which may be sufficient to induce feeding. The diminution of the feeding response at higher doses of buspirone may then arise because of increased postsynaptic receptor blockade, which will tend to normalize or reduce the net functional activity of striatal dopamine synapses (Wood *et al.*, 1983).

Feeding can be elicited by several other manipulations which increase dopaminergic activity, including tail-pinch (Antelman & Szechtman, 1975), low doses of D-amphetamine injected peripherally or into the striatum (Winn *et al.*, 1982) and a selective dopamine D_2 agonist (Martin-Iverson & Dourish, 1988). These manipulations also increase chewing and oral motor activities. It is though that these effects result from a dopamine-mediated behavioural activation or arousal which is expressed as eating when food is the most salient stimulus in the animal's environment. Eating thus appears to be stimulus bound. Previously 8-OH-DPAT has been shown to elicit chewing and oral motor activities, effects which are attenuated by haloperidol (Fletcher, 1987), although these effects are not necessarily observed when palatable foods are available (Dourish *et al.*, 1988). Thus, the effects of 8-OH-DPAT may be stimulus bound, and this may apply also to the effects of buspirone, gepirone and ipsapirone. Future experiments should therefore be concerned with the behavioural specificity of the eating responses induced by these compounds. To date many of the reported changes in behaviour induced by 5-HT_{1A} agonists involve increases rather than decreases in behavioural output (see Introduction). Given the importance of the nigrostriatal dopamine pathway in general behavioural arousal and activation (Robbins & Everitt, 1982; White, 1986) together with the present results, the possibility that these behavioural effects reflect task-specific behavioural activation warrants investigation.

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Vascular effects of helodermin, helospectin I and helospectin II: a comparison with vasoactive intestinal peptide (VIP)

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1 Helodermin, helospectin I and helospectin II, peptides recently isolated from the salivary gland venom of *Heloderma suspectum*, were compared to vasoactive intestinal peptide (VIP) with respect to effects on systemic blood pressure and on isolated femoral arteries in the rat.

2 They all reduced blood pressure in a dose-dependent manner; helodermin was less effective than VIP. However, at doses higher than 1 nmol kg^{-1} all four peptides reduced blood pressure to about the same extent.

3 The half-life of the hypotensive effect of VIP was longer than that of helodermin and the helospectins.

4 VIP and helodermin were equally potent in relaxing femoral arteries precontracted with phenylephrine or prostaglandin $F_{2\alpha}$.

5 Helospectin I and II relaxed phenylephrine-contracted vessels to the same extent as VIP but with a lower potency.

6 Addition of VIP 1 μM to preparations exposed to helodermin 1 μM or to either of the helospectins did not produce a further relaxation.

7 The findings indicate that VIP, helodermin and helospectin I and II have a similar profile of action and therefore may act on a common receptor.

Introduction

Helodermin, helospectin I and helospectin II are 35 aa-, 38 aa- and 37 aa-peptides, respectively, isolated from the salivary gland venom of the lizard *Heloderma suspectum* (Hoshino *et al.*, 1984; Parker *et al.*, 1984). They are all structurally similar to the neuropeptide vasoactive intestinal peptide (VIP) (Table 1). Some biological actions of helodermin resemble those of VIP. Both peptides activate adenylate cyclase in plasma membranes of the rat pancreas (Vandermeers *et al.*, 1984) but only helodermin induces amylase secretion (Konturek *et al.*, 1989). Helodermin-like peptides occur in high concentrations in the thyroid C cells and in the noradrenaline containing cells of the adrenal medulla in many mammals (Sundler *et al.*, 1988; Grunditz *et al.*, 1989; Bjartell *et al.*, 1989). In the dog, intra-arterial infusion of helodermin, like VIP, causes a dose-dependent increase in femoral blood flow and intravenous injection produces systemic hypotension and tachycardia (Naruse *et al.*, 1986; Konturek *et al.*, 1989). In the present study, vascular effects of helodermin and the helospectins in the rat were compared to those of VIP on systemic blood pressure and on isolated distal femoral arteries.

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Methods

Animals

Sprague-Dawley rats of either sex, weighing 250–300 g, were used in all experiments.

In vivo *experiments*

Rats were anaesthetized by intraperitoneal administration of 0.5 ml ketamine (50 mg ml^{-1}) (Ketalar, Parke & Davis) and 0.2 ml xylazin (20 mg ml^{-1}) (Rompun, Bayer). The left jugular vein and right femoral artery were cannulated with polyethene catheters for administration of the peptides and for continuous recording of systemic blood pressure via a Statham P23 pressure transducer, connected to a Grass model 7 polygraph. Experimentation was started when the blood pressure had stabilized at 110–130 mmHg. The peptides were infused slowly in a volume of 100 μl, followed by washing the catheter with 100 μl saline.

In vitro *experiments*

Rats were killed by decapitation. The distal part of the femoral artery (0.2 mm diameter) was removed and cut into

Table 1 Comparison of the amino acid sequence of vasoactive intestinal peptide (VIP) with those of helodermin and the helospectins

VIP (human, porcine)

1 5 10 15 20 25

H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-*

Helodermin (*Heloderma suspectum*)

1 5 10 15 20 25 30 35

H-S-D-A-I-F-T-Q-Q-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-A-S-I-L-G-S-R-T-S-P-P-P-*

Helospectin I + (II) (*Heloderma suspectum*)

1 5 10 15 20 25 30 35

H-S-D-A-T-F-T-A-E-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-E-S-I-L-G-S-S-T-S-P-R-P-P-S-(S)-*

The last amino acid in parentheses belongs to the helospectin sequence. Identical residues are underlined. * = NH_2.

1–2 mm long segments. The preparations were transferred to a thermostated bath (37°C), containing Krebs solution of the following composition (in mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.0, NaH_2PO_4 1.2 and glucose 6.0. The solution was bubbled continuously with 95% O_2 and 5% CO_2, giving a pH of 7.4. The vascular segments were mounted on two L-shaped stainless steel wires (0.1 mm diameter), one of which was connected to a force-displacement transducer (Grass Instrument FT 03C) for continuous tension recording on a Grass polygraph (Högestätt *et al.*, 1983). The resting tension was gradually adjusted to 2–4 mN. The contractile capacity was examined by adding an isotonic 60 mM potassium Krebs solution (NaCl replaced by KCl); these contractions served as an internal control. Concentration-response curves were constructed for each drug; drugs were added in a cumulative manner. Relaxation was studied in preparations precontracted by phenylephrine (3 μM) or prostaglandin $F_{2\alpha}$ (3–10 μM). These concentrations induced contractions that were 60–80% of the maximum response for each agent.

Statistics

pEC_{50} values for the peptides were determined graphically by linear interpolation. The results are expressed as mean values ± s.e.mean; *n* refers to the number of animals or arteries examined. Statistical analysis of the results was performed by means of Student's *t* test and by the two way analysis of variance. Statistical significance was accepted when $P < 0.05$.

Drugs

Phenylephrine hydrochloride (PhE) (Sigma, U.S.A.), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Amoglandin, Astra, Sweden) were provided in ampoules. VIP, helodermin, helospectin I and helospectin II were obtained from Peninsula, Belmont, CA, U.S.A. The drugs were dissolved in and/or diluted with 0.9% saline.

Results

In vivo *experiments*

Like VIP, helodermin, helospectin I and II reduced blood pressure in a dose-dependent manner (Figure 1). Helodermin was less effective than VIP in the low dose range. At doses higher than 1 nmol kg^{-1}, the blood pressure was reduced to about the same extent by all four peptides. The half-life of the hypotensive effect, expressed as the interval between injection and the time when 50% of the effect remained, was longer for VIP than for the other peptides (Figure 2a).

In vitro *experiments*

PhE and $PGF_{2\alpha}$ induced concentration-dependent contractions of similar potency. The pEC_{50} values (denoting the negative logarithm of the drug concentration (M) inducing half maximum contraction) for $PGF_{2\alpha}$ ($n = 5$) and PhE ($n = 5$) were 5.52 ± 0.03 and 5.61 ± 0.06 and the corresponding E_{max} values (denoting the maximum contraction for each drug) were 11.0 ± 1.4 mN and 14.0 ± 0.9 mN, respectively. VIP and helodermin relaxed the precontracted arterial segments with similar potency (Figure 3, Table 2). However, the maximum relaxation evoked by helodermin was less than that evoked by

Table 2 Relaxation by vasoactive intestinal peptide (VIP), helodermin, helospectin I and helospectin II of phenylephrine-contracted rat femoral arteries

	n	pEC_{50}	E_{max}
VIP	10	7.32 ± 0.09	70 ± 4
Helodermin	7	7.53 ± 0.07	51 ± 7*
Helospectin I	8	6.82 ± 0.13*	72 ± 4
Helospectin II	6	6.93 ± 0.05**	68 ± 4

pEC_{50} denotes the negative logarithm of the peptide concentration (M) producing half maximum relaxation. E_{max} denotes the maximum relaxation in %. Asterisks indicate statistical significance for differences between VIP and the other peptides; *$P < 0.05$; **$P < 0.01$.

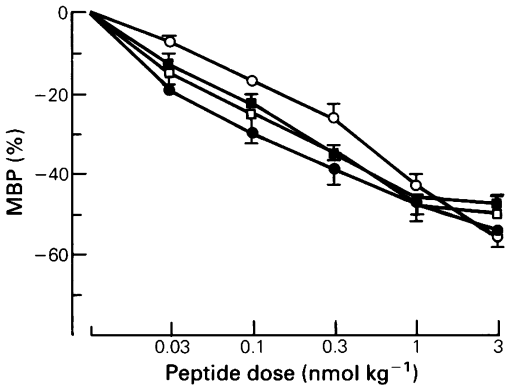

Figure 1 Dose-response curves for the hypotensive effect of vasoactive intestional peptide (VIP, ●, $n = 5$), helodermin (○, $n = 5$), helospectin I (■, $n = 6$) and helospectin II (□, $n = 5$) in anaesthetized rats. Mean blood pressure (MBP) before injection of peptides was 120 ± 2.4 mmHg, ($n = 21$). By using the two way analysis of variance, it was observed that the dose-response curves to VIP and helodermin were significantly different, $P < 0.001$.

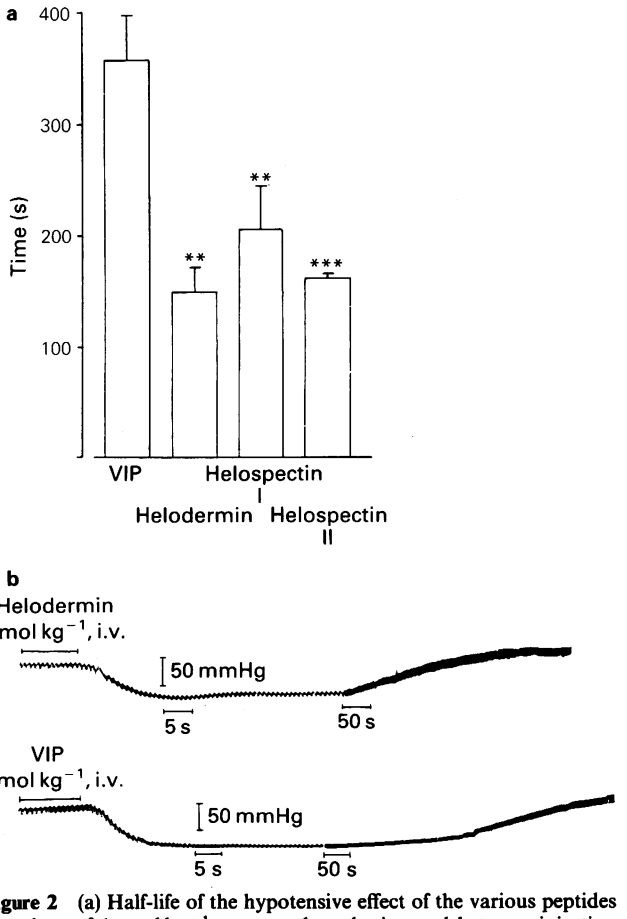

Figure 2 (a) Half-life of the hypotensive effect of the various peptides at a dose of 1 nmol kg^{-1}, expressed as the interval between injection and the time when 50% of the hypotensive effect remained. The statistical significance for the difference between the effects of vasoactive intestional peptide (VIP) and those of the other peptides is indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (b) The effects of i.v. injections of VIP and helodermin on continuous recordings of rat systemic blood pressure.

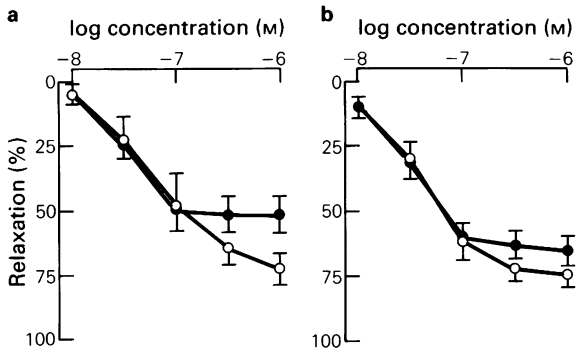

Figure 3 Relaxation by vasoactive intestinal peptide (VIP, ○, $n = 5$) and helodermin (●, $n = 6$) of femoral arteries precontracted with 3 μM phenylephrine (PhE, a) or 3–10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, b). Relaxation is expressed as a percentage of the contraction elicited by PhE or $PGF_{2\alpha}$.

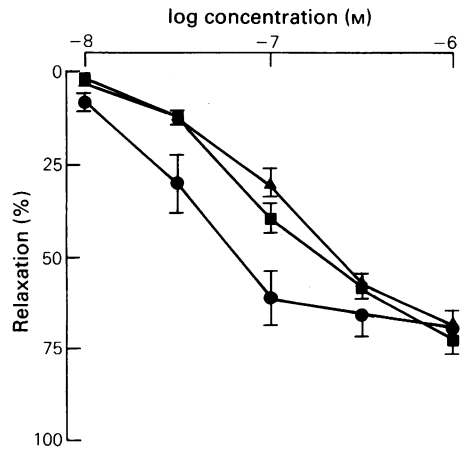

Figure 4 Relaxation by vasoactive intestinal peptide (●), helospectin I (■) and helospectin II (▲) of femoral arteries precontracted with phenylephrine 3 μM. For details, see Figure 3.

VIP in PhE-contracted arteries, but very similar to that evoked by VIP in $PGF_{2\alpha}$-contracted vessels. Helospectin I and II relaxed PhE-contracted vessels to the same extent as VIP, but with a lower potency (Figure 4, Table 2).

Addition of VIP 1 μM to preparations maximally relaxed by helodermin 1 μM ($n = 12$) or *vice versa* ($n = 6$) did not produce a further relaxation (data not shown).

Similarly, addition of VIP 1 μM to preparations exposed to 1 μM of each of the helospectins ($n = 9$–13) or *vice versa* ($n = 2$) did not produce a further relaxation (data not shown).

Discussion

The results show that helodermin, helospectin I and helospectin II, reduce blood pressure in the rat much like VIP, but that helodermin is less effective than VIP in the low dose-range. VIP had a longer duration of action than the other three peptides. Whether this reflects different susceptibility to the proteolytic activity of rat plasma, or different affinity for a common receptor remains to be established. In contrast, helodermin produces a more long-lasting increase in canine femoral blood flow than VIP (Naruse *et al.*, 1986).

In accordance with the vasodepressor effect, all four peptides were found to relax precontracted femoral arteries, although the helospectins were less potent than VIP and helodermin. However, the maximum relaxation induced by the four peptides was of similar magnitude. Further addition of any of the other peptides did not produce a further relaxation.

Helodermin and the helospectins display structural homology with VIP in 15 residues (Table 1). The findings suggest that VIP, helodermin and the helospectins have similar vascular effects. In rat liver membranes, helodermin is known to bind with high affinity to VIP receptors (Robberecht *et al.*, 1984). It is therefore not inconceivable that helodermin and VIP act on a common receptor type in the vascular bed. The helospectins may also act by the same mechanism.

The physiological relevance of the vascular effects of helodermin and helospectins is unclear. The existence of helodermin-like peptides in mammals, notably in the adrenal medulla (Bjartell *et al.*, 1989) may suggest that they could play a role in cardiovascular regulation, provided that the mammalian homologue of lizard helodermin has the same bioactivity profile.

We are grateful to Professor Claus Rerup for statistical advice.

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Central inhibition by γ-aminobutyric acid and muscimol of the release of vasopressin and oxytocin by an osmotic stimulus in the rat

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1 In water-loaded rats under ethanol anaesthesia, the injection of 2–4 μl 1.54 M NaCl solution (hypertonic saline: HS) into a lateral cerebral ventricle (i.c.v.) produced an antidiuretic and a pressor response, together with increased urinary excretion of vasopressin and 'oxytocin-like radioimmunoreactivity' (OLRI). In lactating rats HS also produced a milk-ejection response which was shown to be due to the release of oxytocin.

2 The injection of 20–40 μg γ-aminobutyric acid (GABA) or 40–80 ng muscimol i.c.v. 2 min before HS inhibited the antidiuretic, pressor and milk-ejection responses and reduced the urinary excretion of vasopressin and OLRI.

3 The pressor response to HS was abolished by a ganglion blocking agent but it was not reduced by a vasopressin antagonist. After the antagonist, the antidiuretic response to HS was abolished and the pressor response was accompanied by a diuresis both of which were blocked by muscimol.

4 The threshold dose of HS for an antidiuretic response was 4–8 times higher on injection into the cisterna magna (i.cist.) than when injected i.c.v. GABA, i.v. or i.cist, did not inhibit the response to HS i.c.v.

5 The results confirm other evidence that, in the rat, in contrast some other species, an osmotic stimulus causes release of both vasopressin and oxytocin. This release is blocked by GABA and muscimol. These drugs and HS act at a site reached not from the subarachnoid space but from the cerebral ventricles, probably the hypothalamus. The pressor response to HS under the experimental conditions used is due entirely to central sympathetic stimulation and this effect, as well as the release of vasopressin and oxytocin, is blocked by muscimol.

Introduction

There is increasing evidence for a role of γ-aminobutyric acid (GABA) as an inhibitory transmitter in the neural control of release of vasopressin. Vasopressin and oxytocin are synthesized within the neurones of the supra-optic and paraventricular nuclei (SON and PVN) in the anterior hypothalamus and they are released into the circulation from the nerve endings in the neurohypophysis. GABA reduces the firing frequency of the vasopressin-secreting neurones when applied microionophoretically to the SON in rats (Bioulac *et al.*, 1978; Arnauld *et al.*, 1983) and inhibits the spontaneous (Sladek & Armstrong, 1983) or stimulated (Hillhouse & Milton, 1988) release of vasopressin from the nerve endings in the isolated hypothalamo-neurohypophysial system. GABA inhibits the release of vasopressin in response to hypovolaemia (Knepel *et al.*, 1980). The release of vasopressin by a reduction in blood volume or pressure involves an excitatory input from chemoreceptors to the nucleus of the tractus solitarius, from which there is an excitatory projection to the SON and PVN through synapses on the ventral surface of the medulla (Bisset & Chowdrey, 1988). GABA has an inhibitory action at these synapses (Feldberg & Rocha E Silva, 1978; 1981; Sved *et al.*, 1982) and mediates baroreceptor-activated inhibition of the vasopressin-secreting cells in the SON (Jhamandas & Renaud, 1986; Kasai *et al.*, 1987). GABA blocks the release of vasopressin by carbachol, when both drugs are injected into a lateral cerebral ventricle but not into the cisterna magna: this suggests an action of GABA at the level of the hypothalamus (Bisset & Chowdrey, 1980; Chowdrey & Bisset, 1988).

GABA has now been implicated in the osmotic control of vasopressin release. Brennan and his co-workers (Brennan & Haywood, 1983; 1985; Brennan *et al.*, 1984; Haywood & Brennan, 1985) found that the injection of hypertonic CSF into a lateral cerebral ventricle (i.c.v.) in the rat produced a pressor response which was due partly to the release of vasopressin and partly to central stimulation of the sympathetic nervous system. After elimination of the sympathetic component by a ganglion blocking agent, the residual rise in blood pressure was blocked by a selective V_1 vasopressin antagonist and by hypophysectomy. The vasopressin component of the response was blocked by i.c.v. GABA or the GABA agonist, muscimol. The increase in the plasma vasopressin concentration caused by hypertonic saline was also reduced by GABA or muscimol (Iovino *et al.*, 1983; Brennan *et al.*, 1984). In bilaterally nephrectomised rats in which an injection of hypertonic saline produces a pressor response attributable to the release of vasopressin (Hatzinikolaou *et al.*, 1981), blood pressure was lowered by i.v. injection of GABA or of nipecotic acid, an inhibitor of GABA uptake (Brennan & Haywood, 1985).

A pressor response is an insensitive indicator of vasopressin release: it requires about 40 times as much vasopressin as an antidiuretic response (Bisset & Lewis, 1962). Also the use of a ganglion blocking agent to eliminate the effect of sympathetic stimulation by hypertonic saline (HS) introduces a complication, since the hypotension which it produces causes a reflex release of vasopressin (Kanjanapothi, 1975; Bisset & Chowdrey, 1984) and enhances the pressor response to circulating vasopressin. The aim of the present investigation was to

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obtain confirmatory evidence for an inhibitory role of GABA by testing its effect on the antidiuretic response to HS and the accompanying increase in the urinary excretion of vasopressin. This was the method which was used in our previous work with carbachol (Chowdrey & Bisset, 1988) but the present study incorporates improved peptide extraction and assay procedures. Moreover, in some species, for example man, the cat and the goat HS causes a preferential or independent release of vasopressin, but most of the available evidence suggests that in the rat both hormones are released (for review see Bisset & Chowdrey, 1988). We therefore tested the possibility that GABA might inhibit the release of oxytocin, as well as vasopressin, by measuring milk-ejection responses to HS in the lactating rat and the urinary excretion of 'oxytocin-like radioimmunoreactivity' (OLRI). The pressor response to HS in a number of species including the cat (Chiu & Sawyer, 1974; Bisset et al., 1989), dog (Zucker et al., 1974; Takishita & Ferrario, 1983) and goat (Andersson et al., 1972) has been attributed almost entirely to central sympathetic stimulation with no detectable contribution from a release of vasopressin. The nature of the pressor response in the rat has been further investigated in our experiments.

Methods

The experiments were carried out on Sprague-Dawley rats weighing 250–320 g. A rat was given, by stomach tube, 5 ml per 100 g body weight 12% ethanol in water, followed 30 min later by 3 ml per 100 g body weight tap water, warmed to body temperature. If necessary ether was given by inhalation to produce surgical anaesthesia. Cannulae were inserted in the trachea, an external jugular vein, a femoral artery, the bladder, a lateral cerebral ventricle and, in some experiments, the cisterna magna. The rat was placed on a balance and the fluid load maintained constant at 8% body weight by means of a feedback system which delivered an infusion into the external jugular vein of a solution containing 0.18 g NaCl, 2.2 g glucose and 1–3 ml of ethanol in 100 ml distilled water. The concentration of the ethanol was adjusted to maintain anaesthesia. Blood pressure was recorded from the femoral artery and urine flow from the bladder cannula with a drop counter set to a 1 min time base. Full experimental details have been published previously (Bisset & Chowdrey, 1984). In lactating rats, milk-ejection pressure was recorded from a cannulated teat duct (Bisset et al., 1967).

Collection of urine samples

In the experiments included in Table 1, 5 ml samples of urine were collected immediately after the i.c.v. injection of hypertonic saline (HS). The antidiuretic response (ADR) was calculated as the percentage reduction in urine flow from the expression: [100(A − B)]/A, where A is the control rate of flow immediately before the injection and B the mean rate of flow during the period in which the 5 ml urine sample was collected. Control 5 ml samples were also collected. The 5 ml samples were acidified by adding 0.5 ml 1 M formic acid and kept at 4°C until extracted.

Extraction and assay of urine samples

In our previous work with carbachol (Chowdrey & Bisset, 1988) vasopressin was extracted from urine with an ion-exchange resin and estimated in the extracts by bioassay. In the present investigation, we used an improved method of extraction for both vasopressin and oxytocin and radioimmunoassay (RIA). Urine samples were extracted by the method of La Rochelle et al. (1980), with the modifications described by Williams et al. (1985) for extracting vasopressin and oxytocin from plasma. The following were injected in sequence from 5 ml syringes through octadecasilyl silica columns (C18, Sep-Paks: Waters Associates, Northwich): 3 ml 95% methanol/5% 0.1 M formic acid; 3 ml 0.1 M formic acid; the acidified 5 ml urine sample; 3 ml 0.1 M formic acid and 3 ml 95% methanol/5% 0.1 M formic acid. The final eluate was collected and evaporated to dryness at room temperature in a vacuum centrifuge (Speed Vac concentrator, Uniscience, London). The residue was taken up in 0.5–1 ml RIA buffer (100 mM, Tris-hydroxymethyl-methylamine [Tris] (BDH, U.K.) and 3 mg ml^{-1} bovine serum albumin adjusted to pH 7.4 with HCl) to constitute the final extract.

Vasopressin was estimated in 25–100 μl of the extracts by radioimmunoassay according to the method of Horn et al. (1985), with the exception that A8 anti-arginine vasopressin (AVP) serum was used in this study. The A8 anti-AVP serum was used at a final dilution of 1:400,000. At this dilution the cross-reaction of the A8 anti-AVP serum with oxytocin was 0.1%, lysine vasopressin 10% and DDAVP 0.01% (Tannahill et al., 1988). Dilutions of the 1st International Standard for AVP (National Institute for Biological Standards and Control, London), were used as working standards and synthetic AVP (400 iu mg^{-1}; Batch No. 770110, Ferring AB, Malmo, Sweden) was used for iodination with ^{125}I. Bound hormone was separated from free hormone by precipitation with 2 volumes of 18% polyethylene glycol (PEG) in 0.1 M Tris buffer pH 8.5 containing 1.5 mg ml^{-1} bovine gamma globulin and 0.02% Triton X100. Serial dilutions of the extracts inhibited binding of the tracer in parallel with the Standard. The inter- and intra-assay coefficients of variation were 14% ($n = 5$) and 6% ($n = 10$) respectively and the lower limit of sensitivity ranged from 1 to 3 pg per tube.

In a number of extracts vasopressin was estimated also by bioassay of antidiuretic activity in the water-loaded rat, as described above but without cannulation of a lateral ventricle. Figure 1 shows a satisfactory correlation between the estimates by bioassay and RIA ($r = 0.97$; $P < 0.001$). All the estimates quoted in the Results section were obtained by RIA.

Oxytocin-like radioimmunoreactivity (OLRI) was estimated in the extracts, as described above for the AVP RIA, with an $R3_5$ anti-oxytocin serum at a final dilution of 1:225,000 (Robinson, 1980). Cross-reaction with AVP was <0.004%. Dilutions of the 4th International Standard for oxytocin (NIBSC, London) were used as working standards and oxytocin (440 iu mg^{-1}; Ferring AB) was used for iodination with ^{125}I. Bound hormone was separated from free hormone by precipitation with PEG. The inter- and intra-assay coefficients of variation were 15% ($n = 4$) and 5% ($n = 6$) respectively. The lower limit of sensitivity ranged from 2 to 5 pg per tube. In a number of extracts which had been treated with 100 μg

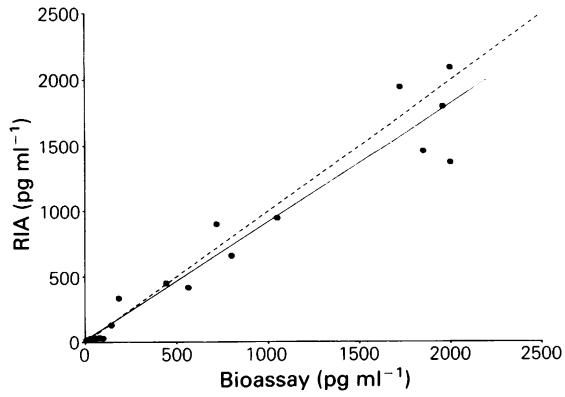

Figure 1 Correlation between estimations of vasopressin in the same urine extracts by bioassay and radioimmunoassay (RIA). The solid line represents the regression calculated from the experimental data and the dotted line indicates a perfect correlation.

trypsin to inactivate vasopressin (Bisset *et al.*, 1973), oxytocin was estimated also by bioassay of milk-ejecting activity in a lactating rat (Bisset *et al.*, 1967).

To test the efficiency of the extraction procedure, 10 ml samples of urine were collected from water-loaded rats during high rates of urine flow and divided into two 5 ml portions. Known amounts of vasopressin or oxytocin were added to one portion, the other serving as a control. Vasopressin and OLRI were estimated in extracts of the samples by RIA. The net recovery of added vasopressin after deduction of the activity in the control sample was $83 \pm 3.9\%$ ($n = 15$). The net OLRI was equivalent to $103 \pm 4.4\%$ ($n = 12$) of the added oxytocin. To measure the urinary excretion of vasopressin and oxytocin, 5 ml samples of urine were collected after i.v. injection of 500–3000 pg of the two hormones, either separately or simultaneously. After deduction of the endogenous hormone in control samples, the net amount of vasopressin excreted was $14.0 \pm 0.63\%$ ($n = 23$) of the injected dose and the net OLRI was equivalent to $4.95 \pm 0.66\%$ ($n = 15$) of the amount of oxytocin injected. When the hormones were injected together neither significantly affected the percentage excretion of the other.

Drugs

Drugs used were GABA, muscimol, hexamethonium bromide (Sigma), and the synthetic vasopressin antagonist (D-$[CH_2]_5$D-Tyr [Et] valyl arginine vasopressin (VAVP) (Sawyer *et al.*, 1981), which was a generous gift from Dr Manning of the Department of Biochemistry, Medical College of Ohio, U.S.A.

Statistics

Results are expressed as mean ± s.e.mean. Significance of differences between means was determined by Mann-Whitney U test. Correlations were determined by linear regression.

Results

The injection of hypertonic saline (HS) i.c.v. in the water-loaded rat under ethanol anaesthesia produced a pronounced antidiuretic response (ADR) and a small pressor response, both of which were reversibly inhibited by GABA and muscimol. A typical experiment is illustrated in Figure 2. The basal rate of urine flow was 150 μl min^{-1}. The injection of 4 μl 1.54 M NaCl solution (hypertonic saline: HS) produced a slight increase of urine flow for the first 2 min after the injection, followed by an ADR which reached a maximum in 15 min, with progressive recovery to the control flow about 50 min after the injection. The pressor response consisted of a rise in blood pressure of 10 mmHg which lasted for only 4 min. After the i.c.v. injection of 40 ng muscimol 2 min before HS, both the ADR and the pressor response to HS were abolished with full recovery in 15 min. Larger doses of muscimol produced an inhibition of longer duration. After 160 ng, which itself caused a slight fall in blood pressure and decrease of urine flow, the ADR and the pressor response to HS were still blocked 15 min later, with partial recovery at 30 min and fully recovery at 50 min. With 640 ng muscimol the fall in blood pressure was accompanied by a 50% reduction of urine flow. When the flow had recovered after 30 min the ADR and the pressor response to HS were abolished with a progressive, but only partial, recovery at 90, 105 and 120 min. The tracing shows commensurate changes in blood pressure and urine flow during recovery from inhibition by muscimol.

Figure 3 shows that the profile of the ADR to HS is similar to that produced in i.v. injection of vasopressin. However, whereas HS caused a rise in blood pressure of 15 mmHg, 500 pg of vasopressin, which produced a comparable ADR,

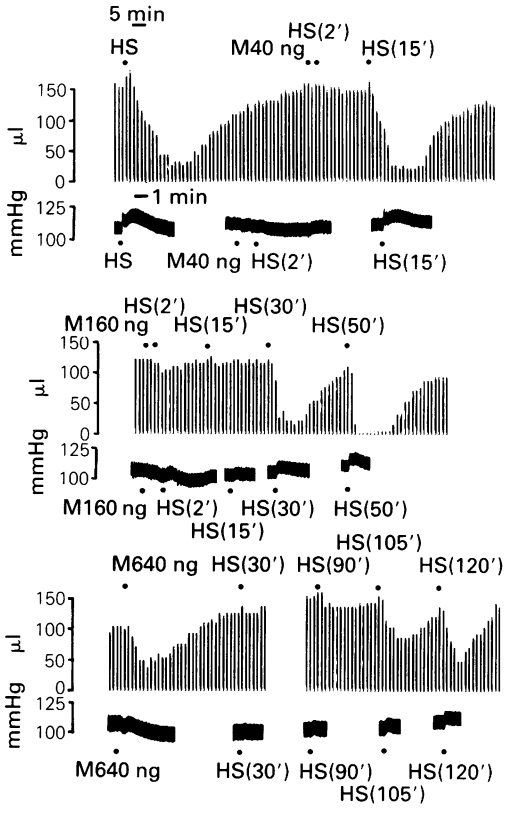

Figure 2 The effect of muscimol (M) on the antidiuretic and pressor responses to hypertonic saline (HS; 4 μl 1.54 M NaCl solution) in the water-loaded rat under ethanol anaesthesia. In each part, upper trace shows urine flow (each vertical line representing μl in 1 min) and lower trace, arterial blood pressure. The numbers in parentheses after HS represent min after the preceding injection of muscimol.

caused no rise and 1000 pg, which produced a much larger ADR, caused a barely perceptible rise. After injection of the specific vasopressin antagonist, D-$[CH_2]_5$D-Tyr [Et] VAVP, which acts on both V_1 amd V_2 receptors (Sawyer *et al.*, 1981), both the ADR and the pressor response to 1000 pg of vasopressin were abolished. A second injection of HS then produced a rise in blood pressure similar to that after the first injection, but the ADR was replaced by a pronounced diuretic response. This diuretic response and the accompanying pressor response were reversibly inhibited by an i.c.v. injection of 40 ng muscimol. Similar results were obtained in five other rats with doses of 4 μl 1.54 M NaCl solution (HS) and 40–160 ng muscimol. A measurable decrease in the pressor response to HS after the vasopressin antagonist was obtained in only one of these five rats and this decrease was of doubtful significance, since in this rat the pressor response to repeated injections of HS exhibited tachyphylaxis. In five rats the pressor response to 4 μl 1.54 or 3.08 M NaCl, before or after the administration of the vasopressin antagonist, was abolished by an i.v. injection of 3–10 mg hexamethonium.

In lactating, water-loaded rats in which milk-ejection pressure was recorded simultaneously with urine flow, HS i.c.v. produced both a milk-ejection response (MER and an antidiuretic response (ADR). This is illustrated in Figure 4. The graded MERs to control injections of 0.5 and 1.0 mu (0.8 and 1.6 ng) oxytocin consisted of a single peak and were accompanied by a slight diuresis. The injection of HS produced a sustained MER with 2 major peaks, each having an amplitude similar to that observed with the smaller dose of oxytocin, and a large ADR. The ADR was closely matched by 1000 pg vasopressin i.v. but this did not result in milk ejection. Similar results were obtained in 3 other rats. In the experiment of Figure 4, muscimol 80 ng i.c.v. injected 2 min before HS, abolished the MER and almost completely inhibited the ADR.

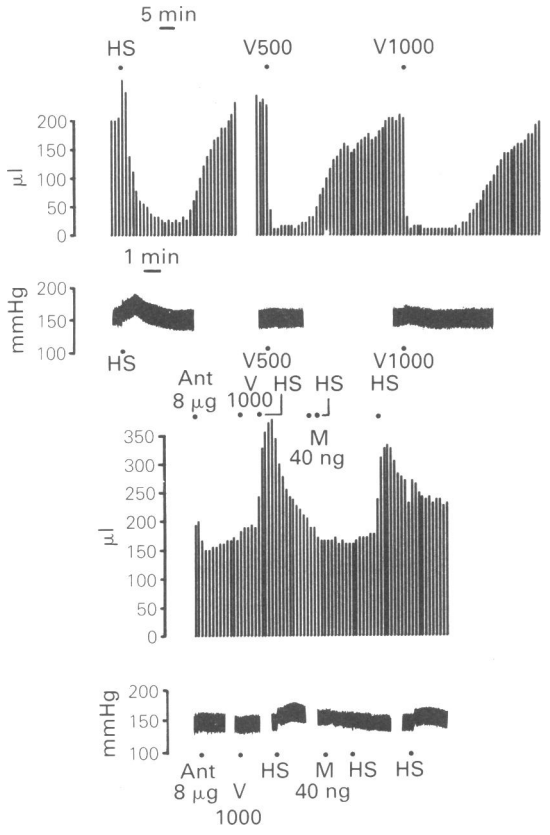

Figure 3 Comparison of the antidiuretic and pressor responses to hypertonic saline (HS; 4 µl, 1.54 M NaCl solution) and vasopressin (V: doses in pg) and the effects of a vasopressin antagonist, D-[CH_2]$_5$D-Tyr [Et] VAVP (Ant) and muscimol (M) in a water-loaded rat under ethanol anaesthesia. Urine flow and blood pressure recorded as for Figure 2.

After recovery from muscimol, the ADR and the MER to HS were blocked by the vasopressin antagonist, D-[CH_2]$_5$D-Tyr [Et] VAVP. This antagonist has potent anti-oxytocic activity *in vivo* (Manning *et al.*, 1987; 1989) and was shown (Figure 4) to block also the milk ejection response to 1 or 2 mu (1.6 to 3.2 ng) oxytocin. This finding, together with the demonstration that vasopressin makes no contribution to the MER to HS, leads to the conclusion that this response is mediated by release of oxytocin. Inhibition of the ADR to HS by the antagonist confirms the evidence from Figure 3 that this response is mediated by release of vasopressin.

Table 1 shows the results of experiments on 7 rats in which the urinary excretion of vasopressin and oxytocin-like radioimmunoreactivity (OLRI) was measured after i.c.v. injections of 1 to 4 µl HS and of 4 µl HS 2 min after 10 to 40 µg GABA. Since any inhibitory effect of GABA on the response to HS was completely reversible, injections could be given repeatedly in the same rat and the results of all the injections in the 7 rats have been pooled in the table. The effect of 1 µl HS was negligible. Both 2 and 4 µl HS produced an antidiuretic response (ADR) and a significant ($P = 0.01$) increase in the urinary excretion of vasopressin and OLRI. There was no significant difference between the ADRs and the excretion of vasopressin for the two doses but 4 µl produced a significantly greater ($P < 0.01$) excretion of OLRI. GABA 10 µg caused a slight inhibition of the response to 4 µl HS. Both 20 and 40 µg GABA caused a significant reduction in the ADR to 4 µl HS and in the urinary excretion of vasopressin and OLRI. There was no significant difference between the effects of these two doses of GABA. The excretion of OLRI after 40 µg GABA and 4 µl HS was not significantly different from the control, but the excretions of OLRI after 20 µg GABA and of vasopressin after both 20 and 40 µg were significantly higher than in the control, indicating incomplete inhibition of the response to HS. Two injections of 2 µl HS 2 min after 40 ng muscimol and one injection of 4 µl HS 2 min after 80 ng muscimol were also tested in these experiments. None of the injections of HS produced an ADR; the mean excretion of vasopressin for the three injections was 40 pg and that of OLRI 47 pg. Muscimol appeared, therefore, to cause total inhibition of the response to HS, as illustrated in Figures 2, 3 and 4, and was about 1000 times more potent than GABA.

As shown in Figure 5 the ADR to HS alone or HS after GABA or muscimol was closely correlated with the amount of vasopressin excreted in the urine ($r = 0.97$; $P < 0.001$). The intercept of the regression line corresponding with zero ADR is approximately 25 pg. This is close to the actual amount of vasopressin (33 pg) which was detected in control samples of

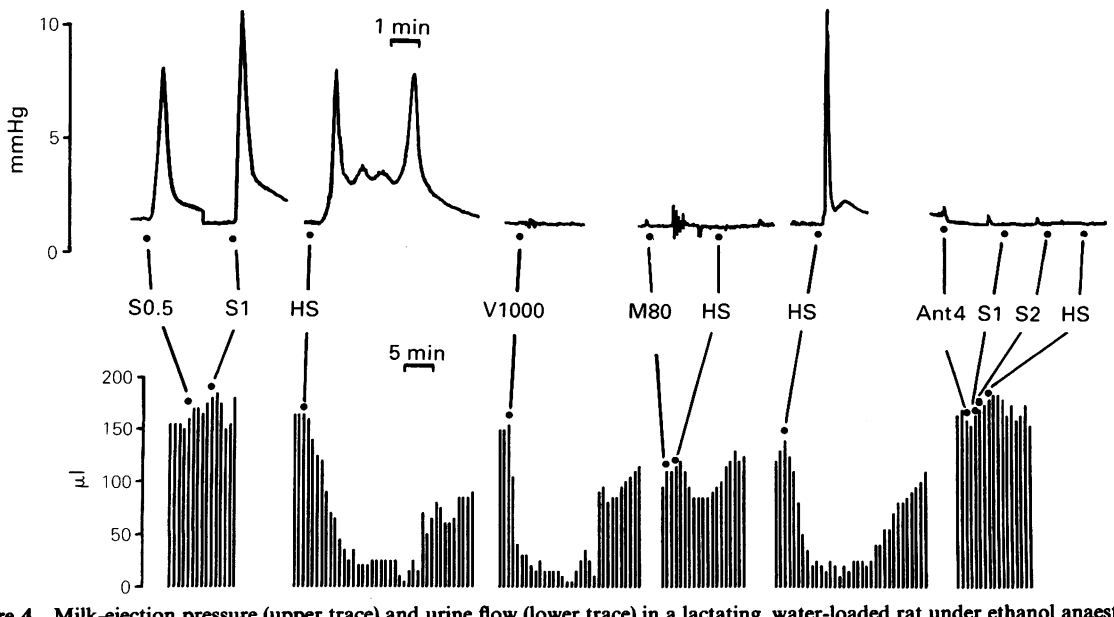

Figure 4 Milk-ejection pressure (upper trace) and urine flow (lower trace) in a lactating, water-loaded rat under ethanol anaesthesia. S: oxytocin (Syntocinon) mu. HS: 4 µl 1.54 M NaCl solution. V: vasopressin (pg). M: muscimol (ng). Ant: D-[CH_2]$_5$ D-Tyr [Et] VAVP (µg) (a dose of 4 µg had been given 15 min previously). S, V and Ant injected i.v.; HS and M i.c.v.

Table 1 Antidiuretic response (ADR) and excretion of vasopressin (Vas) and 'oxytocin-like radioimmunoreactivity' (OLRI) during collection of 5 ml urine after i.c.v. injection of 1.54 M NaCl solution (hypertonic saline: HS) alone or following GABA (G)

Drugs	n	*ADR* (%)	*Total amount* (pg) *in 5 ml urine*	
			Vas	*OLRI*
C	9	0	33 ± 3.2	36 ± 12
HS 1 μl	2	3	45	64
HS 2 μl	5	42 ± 2.4	230 ± 31	260 ± 59
HS 4 μl	14	38 ± 2.3	312 ± 28	498 ± 40
G 10 μg + HS 4 μl	2	27	169	313
G 20 μg + HS 4 μl	5	23 ± 5.9*	104 ± 22***	172 ± 61**
G 40 μg + HS 4 μl	5	10 ± 4.8***	51 ± 7.4***	118 ± 46***

Data shown are means ± s.e.mean. n = total number of injections in 7 rats. C = control collection of 5 ml urine. G was injected 2 min before HS. For calculation of ADR, see Methods. Significance of difference from HS 4 μl alone, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

urine and suggests that the secretion of vasopressin under our experimental conditions is not totally suppressed by the water load and ethanol anaesthesia, and is consistent with the finding that the vasopressin antagonist caused some increase in the basal rate of urine flow.

An experiment was carried out to determine whether the OLRI could be correlated with the excretion of biologically active oxytocin in the urine. Seven 5 ml control samples of urine, and seven 5 ml test samples after i.v. injection of 4 μl HS, were collected from 2 rats in each of which control and test samples were alternated. The seven control samples and the seven test samples were pooled and extracted. The two final extracts, each derived from 35 ml urine, were divided into 2 portions, one of which was treated with trypsin to inactivate vasopressin. The extracts were assayed for milk-ejecting activity (MEA) and antidiuretic activity (ADA); OLRI was also estimated. Results are expressed in terms of the activity in 5 ml of the original pooled urine samples. The ADA, which was assayed in the untreated extracts and represents vasopressin, increased from 19 pg in 5 ml control urine to 705 pg in 5 ml test urine; trypsin caused >90% inactivation of both of the urine extracts and of standard vasopressin. The MEA, assayed in the trypsin-treated extracts, increased from 38 to 540 pg. In the milk-ejection assay, the intrinsic MEA of standard vasopressin was equivalent to 9% of its antidiuretic activity. It was calculated that, in the trypsin-treated samples, vasopressin could not have contributed more than 2 pg to the MEA in 5 ml control urine or 12 pg to the MEA in 5 ml test urine. The MEA in these samples, therefore, represents almost entirely oxytocin. The estimates of OLRI in 5 ml urine amounted to 72 pg for the control and 839 pg for the test. This experiment showed that the i.c.v. injection of HS in the rat resulted in an increase in the urinary excretion of biologically active oxytocin, which was equivalent to about 60% of the OLRI measured in the same urine samples.

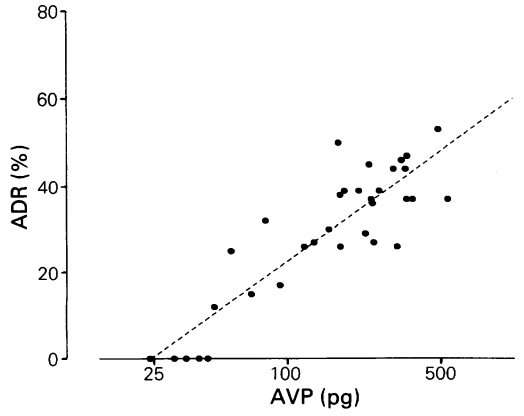

Figure 5 Correlation between antidiuretic responses (ADRs) to hypertonic saline (2–4 μl 1.5 M NaCl solution) administered either alone or after GABA and muscimol and the amounts of vasopressin (measured as arginine vasopressin, AVP) excreted in the urine. For calculation of ADR, see Methods.

In 4 rats the injection of 4 μl 3.08 M NaCl solution i.cist. produced a rise in blood pressure of 18–40 mmHg but no ADR, or only a slight ADR less than that produced by 1–2 μl 1.54 M NaCl solution injected i.c.v. in the same rat. In 4 experiments the i.cist. injection of 100 μg GABA caused no inhibition of the ADR or pressor response to 2–4 μl 1.54 M NaCl solution i.c.v., although the same or a smaller dose of GABA was effective i.c.v.

In 3 rats 4–16 μg muscimol i.v. 2 min before 2–4 μl 1.54 M NaCl (HS) i.c.v. produced no effect on urine flow or blood pressure and did not inhibit the ADR to HS, although this response was blocked in the same rat by 40 ng muscimol i.c.v. GABA injected i.v. in doses of 80 μg and above caused a fall in blood pressure and antidiuresis, but in 1 experiment 200 μg i.v. 2 min before HS caused no fall in blood pressure and did not inhibit the ADR to 1 μl 1.54 M NaCl i.c.v., although this was blocked by 20 μg i.c.v.

Discussion

The octadecasilyl method for extracting vasopressin from plasma (La Rochelle *et al.*, 1980) was highly satisfactory for extracting vasopressin and oxytocin from urine. Consistently high recoveries of both hormones were obtained. Several criteria established the identity of the substance measured in urine extracts by the RIA for vasopressin; the RIA correlated well with bioassay; the profiles of the antidiuretic responses to extracts in the bioassay were similar to those for i.v. injections of vasopressin and there was no significant deviation from parallelism between their log dose-response curves: the antidiuretic activity of the extracts was abolished by trypsin and by the vasopressin antagonist D-$[CH_2]_5$ D-Tyr [Et] VAVP The ADR to HS was correlated with the amount of vasopressin excreted in the urine. When vasopressin and oxytocin were injected i.v. the amount of OLRI in urine extracts amounted to 5% of the injected dose of oxytocin compared with 14% excretion of vasopressin. These values do not differ significantly from previous estimates by bioassay (Kanjanapothi, 1975) of the percentage excretion of vasopressin (13.1 ± 0.75; $n = 14$) and oxytocin (3.1 ± 0.76; $n = 6$). This suggests that oxytocin is more extensively metabolised than vasopressin and that OLRI measures largely intact, or biologically active, oxytocin. In the experiment in which MEA and OLRI were measured in the same urine extracts after treatment with trypsin to inactivate vasopressin. OLRI exceeded MEA by 55–90%, but the increase in the test sample collected after administration of HS compared with a control sample was

practically the same (14 fold for MEA and 12 fold for OLRI). The increase in OLRI after HS was not simply an artefact arising from concentration of the urine during the antidiuresis produced by the concomitant release of vasopressin. This was shown by one experiment in which the OLRI in urine increased from a control value of 38 pg to 919 pg after i.c.v. injection of HS producing an ADR of 33%, but to only 47 pg after an i.v. injection of vasopressin which caused a larger ADR of 43%. It may be assumed, therefore, that an increase in OLRI in urine does reflect the release of oxytocin from the neurohypophysis. Estimation of hormones in urine rather than plasma provides an indication of the total release over a period commensurate with the ADR to HS. The volume of urine collected, 5 ml, is adequate to allow substantial or complete recovery from the ADR.

The i.c.v. injection of HS produced both an ADR and a MER with increased urinary excretion of vasopressin, OLRI and, in one experiment, biologically active oxytocin. This provides further evidence that, in the rat, an osmotic stimulus causes a simultaneous release of vasopressin and oxytocin which may be related to their synergistic effect on sodium excretion (Balment *et al.*, 1986). In our experiments it was found that an i.v. injection of vasopressin, which mimicked the antidiuretic response to HS, caused little or no rise in blood pressure. Also, the pressor response to HS was abolished by a ganglion blocking agent and not significantly reduced by the vasopressin antagonist. The pressor response to HS appeared, therefore, to be due entirely to sympathetic stimulation.

The injection of 20–40 μg GABA or 40–80 ng muscimol 2 min before 2–4 μl 1.54 M NaCl solution reduced or abolished the antidiuretic, pressor and milk-ejection responses to HS and significantly reduced the urinary excretion of both vasopressin and OLRI. These results confirm the conclusion (Haywood & Brennan, 1985) that GABA and muscimol block the release of vasopressin by the injection of HS in the rat. The doses of GABA and muscimol used in our experiments and those of Haywood & Brennan (1985) are similar. Our results show that oxytocin release is also blocked by GABA and muscimol as well as the pressor response which, under our conditions, is due to central sympathetic stimulation.

The sites of action of HS and GABA in our experiments are uncertain. The finding that HS was 4–8 times more potent by i.c.v. than i.cist. injection suggests that it acts predominantly not from the subarachnoid space but through the walls of the cerebral ventricles. The most likely site is the hypothalamus. GABA was ineffective in inhibiting the antidiuretic or pressor response to HS when injected i.v. or i.cist. This finding excludes an action of GABA at the nerve terminals in the neurohypophysis, which is outside the blood-brain barrier, and the ventral surface of the medulla which would be accessible from cisterna magna. Both HS and GABA appear, therefore, to act at the level of the hypothalamus. Similar conclusions were reached for the site of action of GABA in inhibiting the release of vasopressin by carbachol (Chowdrey & Bisset, 1988). HS releases vasopressin by stimulating osmoreceptors, but their location is controversial. Some contend that the SON and PVN themselves function as osmoreceptors and others that these nuclei receive an afferent neural input from extraneous osmoreceptors, which are believed to be located in the rat in the organum vasculosum laminae terminalis (for review, see Bisset & Chowdrey, 1988). GABA could inhibit the release of vasopressin and oxytocin by HS either by acting postsynaptically on the neurosecretory cells in the SON and PVN, or presynaptically by blocking the release of an excitatory transmitter from afferent nerve terminals.

Since GABA i.cist. did not inhibit the pressor response to HS i.c.v., at least part of this response appears to be mediated at a site of action reached from the ventricles, again possibly the hypothalamus. This is consistent with the finding in the cat, that HS produced a pressor response when injected into a lateral ventricle after cannulation of the cerebral aqueduct to prevent access to the subarachnoid space (Feldberg & Wei, 1979).

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Inhibitory action of α-human atrial natriuretic peptide on noradrenaline-induced synthesis of myo-inositol 1,4,5-trisphosphate in the smooth muscle cells of rabbit aorta

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1 Interactions between the synthesis of myo-inositol 1,4,5-trisphosphate (IP_3) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the smooth muscle cells of the rabbit aorta were investigated.

2 In the presence or absence of vascular endothelium, noradrenaline (NA; 5 μM) consistently reduced the amount of phosphatidylinositol 4,5-bisphosphate (PI-P_2) and increased both phosphatidic acid (PA) and IP_3.

3 In the presence or absence of endothelium, acetylcholine (ACh; 100 μM but not 5 μM) slightly increased the amount of IP_3, but exposure to ACh (100 μM) 4 min after application of NA did not modify NA-induced synthesis of IP_3.

4 ACh (100 μM) markedly enhanced the synthesis of cyclic GMP in the presence of endothelium but not in the endothelium-denuded tissues.

5 Prazosin (5 μM) but not dibutyryl cyclic GMP (db-cyclic GMP; 100 μM) blocked the hydrolysis of PI-P_2 induced by 5 μM NA. Synthesis of IP_3 induced by NA, as estimated with [^{3}H]-inositol was not modified by application of 100 μM db-cyclic AMP or db-cyclic GMP.

6 α-Human atrial natriuretic peptide (α-hANP; 0.1 μM) increased cyclic GMP in the presence or absence of endothelium. α-hANP (0.1 μM) consistently inhibited the hydrolysis of PI-P_2 induced by 5 μM NA.

7 The results indicate that synthesis of IP_3 is inhibited neither by the synthesis of cyclic GMP in the cytosol nor by cyclic GMP itself. However, synthesis of IP_3 through hydrolysis of PI-P_2 may be inhibited by an interaction between some steps in the IP_3 synthetic process and by the activation of the α-hANP-guanylate cyclase process at the sarcolemma.

Introduction

In smooth muscle tissues including vascular beds, activation of the α_1-adrenoceptor generates myo-inositol 1,4,5-trisphosphate (IP_3) which in turn releases Ca from intracellular stores. As a consequence, contraction occurs. The Ca released by IP_3 also activates Ca-dependent K currents (Suematsu *et al.*, 1984; Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986; Iino, 1987; Ohya *et al.*, 1988), as found in many other cell types (Berridge & Irvine, 1984; Berridge, 1986; Abdel-Latif, 1986). In addition, a co-product of IP_3, myo-1,2-diacylglycerol (DG) activates protein kinase C in the presence of Ca with phosphatidylserine (Nishizuka, 1986). This activation inhibits both the synthesis of IP_3 and the contraction induced by acetylcholine (ACh) in the porcine coronary artery (Itoh *et al.*, 1988).

Synthesis of IP_3 is also regulated by other second messengers such as guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP) in a variety of cells (Takai *et al.*, 1984; Nishizuka, 1986; Abdel-Latif, 1986). In platelets, Takai *et al.* (1981, 1982) and Nakashima *et al.* (1986) showed that sodium nitroprusside and 8-bromocyclic GMP inhibit the synthesis of IP_3 induced by thrombin, and that dibutyryl cyclic AMP (db-cyclic AMP) shows a stronger inhibitory action than 8-bromocyclic GMP. Furthermore, cyclic AMP also inhibited phosphatidylinositol 4,5-bisphosphate (PI-P_2) hydrolysis. Nakashima *et al.* (1986) concluded that inhibition of PI-P_2 hydrolysis by cyclic GMP, via activation of cyclic GMP-dependent protein kinase (G-kinase), might indicate a negative feedback control mechanism on the synthesis of IP_3 in platelets. In rat aorta, Rapoport (1986) demonstrated that cyclic GMP inhibited noradrenaline (NA)-induced contractions through inhibition of PI-P_2 hydrolysis, a process independent of the mechanism by which cyclic GMP elevation occurred.

In vascular smooth muscle tissues, the synthesis of cyclic GMP is induced by various factors, such as endogenous humoural stimulation (endothelium-derived relaxing factor (EDRF) or α-human atrial natriuretic peptide (α-hANP)), and the application of nitro-compounds (Rapoport & Murad, 1983; Rapoport *et al.*, 1983; Ignarro & Kadowitz, 1985). Furthermore, cyclic GMP is synthesized in two distinct sites in smooth muscle tissues, namely via guanylate cyclase in the sarcolemma and that in the cytosol. The former is stimulated by α-hANP and the latter by nitro-compounds or EDRF (Rapoport & Murad, 1983; Waldman *et al.*, 1984; Winquist *et al.*, 1984). Furthermore, the latter but not the former can be inhibited by methylene blue or haemoglobin (Holzmann, 1982; Ignarro *et al.*, 1985; Martin *et al.*, 1985).

The present experiments were designed to clarify interactions between cyclic GMP and IP_3 synthesis in the smooth muscle cells of rabbit aorta. NA was used to activate the hydrolysis of PI-P_2, while EDRF released from endothelial cells by ACh and α-hANP were used to stimulate cyclic GMP synthesis via cytosolic and sarcolemmal guanylate cyclase, respectively.

Methods

Materials

Male albino rabbits (1.8–2.2 kg) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.v.) and exsanguinated. The abdominal aortae were removed and placed in a dissecting chamber filled with Krebs solution which had the following composition (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5,

Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^-U134.0, glucose 11.5. The solution was gassed with 97% O_2–3% CO_2 and the pH was kept at 7.3–7.4. After removal of connective tissue with fine scissors under a binocular microscope, the tissue was cut into strips (8–10 mm in length and 4–5 mm in width). Endothelium-denuded tissues were prepared by rubbing the intimal surface with a cotton ball moistened with Krebs solution.

Assays of phosphatidyl inositol 4,5-bisphosphate and phosphatidic acid

The amounts of PI-P_2 and phosphatidic acid (PA) in the muscle cells of rabbit aorta (either endothelium intact or denuded tissues) were measured before and during application of various stimulants. Muscle strips were labelled in a phosphate-free Krebs solution containing 40 μCi ml^{-1} ^{32}Pi (specific radioactivity, 30–40 Ci mmol^{-1}: Japan Atomic Energy Institute) at 37°C for 2 h. The strips were then washed three times with Krebs solution without ^{32}Pi, and were incubated with stimulants for various periods. The reaction was halted by adding a solvent containing chloroform, methanol and concentrated HCl (100:200:2, v/v), and the strips were homogenized in a glass homogenizer. Crude extracts in the solvent were chromatographed on Silica Gel 60 plates (Merk), according to the method introduced by Billah & Lapetina (1982); the plates were then autoradiographed with Fuji X-ray film for 12–15 h. The fraction corresponding to PI-P_2 and PA on the plates was cut out, and counted for radioactivity in a liquid scintillation counter. The amount of protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Assays of inositol 1,4,5-trisphosphate and cyclic GMP

Muscle strips (endothelium intact or denuded tissues) were allowed to equilibrate in flasks containing Krebs solution. The strips were exposed to the agents for various times and homogenized in 6% trichloroacetic acid in a glass homogenizer. The homogenate were centrifuged and the supernatant fractions were treated with ether three times, and assayed for IP_3 and cyclic GMP by radioimmunoassay kits from Amersham International plc. and Yamasa shoyu Co., respectively.

Drugs

Noradrenaline-HCl (NA), N^6, 2'-o-dibutyryl adenosine 3':5'-cyclic monophosphate (db-cyclic AMP) and N^2, 2'-o-dibutyryl guanosine 3':5'-cyclic monophosphate (db-cyclic GMP) were obtained from Sigma, acetylcholine-HCl (ACh) from Wako Pure Chem., prazosin HCl from Pfizer-Taito Pharmac. Co. and α-human atrial natriuretic peptide (α-hANP) from Peptide Instit. Co. Osaka.

Statistics

The experimental values are expressed as the mean ± s.e. mean (number of observations) and the statistical significance was determined by use of Student's *t* test.

Results

Effects of ACh on the hydrolysis of PI-P_2 and synthesis of PA induced by NA

Figure 1 shows the effects of 5 μM NA and 100 μM ACh on the hydrolysis of PI-P_2 and the synthesis of PA in the presence and absence of endothelial cells. The amounts of PA and PI-P_2 measured before application of NA were 3983 ± 209 c.p.m. mg^{-1} protein (n = 12) and 15291 ± 415 c.p.m. mg^{-1} protein (n = 12), respectively in the presence of endothelium and 3983 ± 264 c.p.m. mg^{-1} protein (n = 15) and 12451 ± 454 c.p.m. mg^{-1} protein (n = 15) respectively in the endothelium denuded tissues. These individual values were normalized as 100%. With application of 5 μM NA, the amount of PA was increased and that of PI-P_2 decreased, in a time-dependent manner. These changes reached a maximum at about 8 min (3.4 times and 0.6 times the control in the presence of endothelial cells and 3.2 times and 0.6 times the control in the absence of endothelial cells, respectively, $P < 0.01$, n = 12). A relatively low concentration of ACh (5 μM) did not modify the amounts of PI-P_2 and PA (1.1 times and 0.98 times the control in the presence of endothelial cells, $P > 0.05$, n = 6). However, in the presence of a high concentration of ACh (100 μM), the amount of PA was increased (after 8 min, 1.7 times and 1.6 times the control in the presence and absence of endothelium, respectively, $P < 0.05$, n = 6) and that of PI-P_2 was decreased (0.85 times and 0.80 times the control in the presence and absence of endothelium, respectively, $P < 0.05$, n = 6). These changes were much less than those observed on application of 5 μM NA, in the presence or absence of endothelial cells. When 100 μM ACh was applied 4 min after application of 5 μM NA, the amount of PI-P_2 and PA remained the same as observed on application of NA alone (Figure 1). These results indicate that in the rabbit aorta, both NA and ACh are capable of hydrolysing PI-P_2, but that the potency and efficacy of ACh were much less than those of NA.

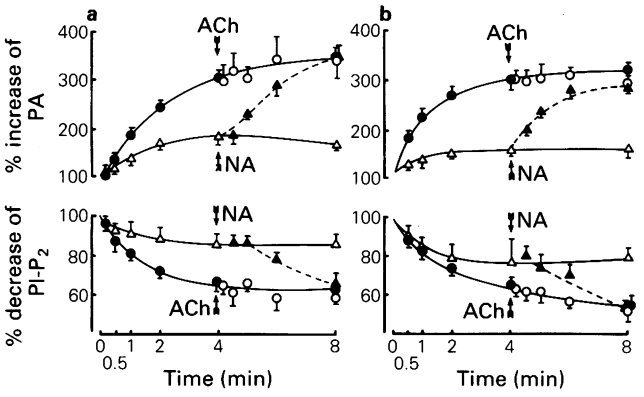

Figure 1 Effects of noradrenaline (NA) and acetylcholine (ACh) on the synthesis of phosphatidic acid (PA) and the amount of phosphatidylinositol 4,5-bisphosphate (PI-P_2) in smooth muscle cells of the rabbit aorta, in the presence (a) or absence (b) of endothelium. The amount of PA or PI-P_2 was measured at various times up to 8 min after application of NA (5 μM) or ACh (100 μM). The values are expressed as the mean (n = 6–15) and vertical lines show s.e.mean. (●) NA (5 μM); (○) 4 min after application of NA (5 μM), ACh (100 μM) was added. (△) ACh (100 μM) and (▲) 4 min after application of ACh (100 μM), NA (5 μM) was applied.

Effects of cyclic GMP and db-cyclic GMP on the hydrolysis of PI-P_2

In relation to the effects of ACh on the NA-induced hydrolysis of PI-P_2, the amount of cyclic GMP synthesis induced by 100 μM ACh was measured in the intact and endothelium denuded tissues. As shown in Figure 2, 100 μM ACh markedly increased the amount of cyclic GMP in intact tissues 30 s after application and the increase was sustained for over 4 min (after 2 min, 4.3 times the control, n = 9). In endothelium-denuded tissues, ACh slightly increased the amount of cyclic GMP 30 s after application of ACh, an effect which was less than that observed in the intact tissues (after 30 s, 1.3 times and after 2 min, 1.5 times the control, n = 8, $P > 0.01$).

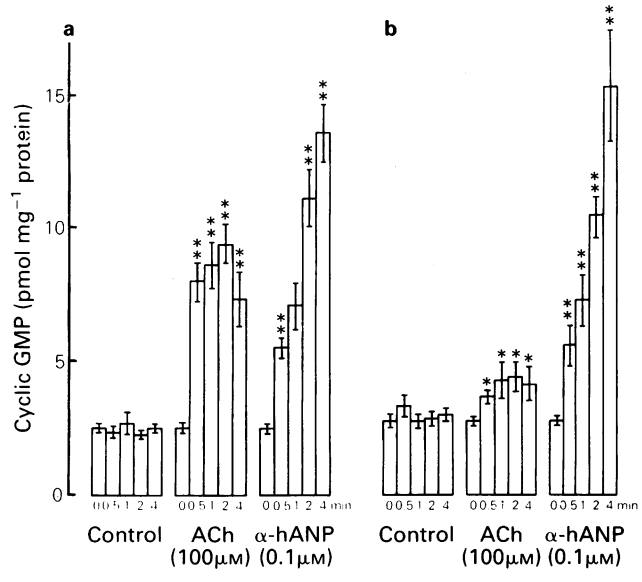

Figure 2 Effects of acetylcholine (ACh) or α-human atrial natriuretic peptide (α-hANP) on the synthesis of cyclic GMP at various exposure times in smooth muscle cells of the rabbit aorta in the presence (a) or absence (b) of endothelium. The amount of cyclic GMP is expressed as the absolute value (the mean value with vertical bars showing s.e.mean, $n = 8$–12). *Indicates $P < 0.05$ and **$P < 0.01$.

Figure 3 shows the effects of prazosin and db-cyclic GMP on the hydrolysis of PI-P_2 induced by 5 μM NA in the presence and absence of endothelial cells. The amounts of PA and PI-P_2 in intact tissues before application were 4429 ± 374 c.p.m. mg^{-1} protein ($n = 7$) and 13875 ± 1234 c.p.m. mg^{-1} protein ($n = 7$), respectively and those in endothelium-denuded tissues were 4723 ± 379 c.p.m. mg^{-1} protein ($n = 8$) and 11943 ± 699 c.p.m. mg^{-1} protein ($n = 8$), respectively. These values, obtained from intact and endothelium-denuded tissues, were normalized as 100%. Prazosin (5 μM) applied 5 min before and during application of 5 μM NA did not modify the amount of PA and PI-P_2. Furthermore, with application of 100 μM db-cyclic GMP 5 min before and during application of NA, or 2 min following application of NA, the amounts of PI-P_2 and PA were not affected (Figure 3a and b).

The effects of db-cyclic GMP and db-cyclic AMP on the synthesis of IP_3 induced by 5 μM NA were observed (Figure 4). The basal value of IP_3 before application of NA was 0.687 ± 0.091 nmol mg^{-1} protein ($n = 10$) and this value was normalized as 100%. The amount of IP_3 reached a peak at 2 min after application of 5 μM NA, and then declined to a steady level in the presence of NA. With application of db-cyclic AMP (100 μM) or db-cyclic GMP (100 μM) 5 min before and during application of NA, the synthesis of IP_3 remained the same as observed on application of NA alone. Since LiCl was not included in the solution, the reduction in the amount of IP_3 after 2 min may be due to de-phosphorylation of IP_3 by inositol 1,4,5-trisphosphate 5-phosphatase, or to synthesis of inositol 1,3,4,5-tetrakisphosphate (IP_4) by activation of inositol 1,4,5-trisphosphate 3-kinase present in the cytosol (Irvine & Moor, 1987; Yamaguchi *et al.*, 1987). The amounts of PI-P_2 and PA 3 min after application of NA did not change (Figure 3) indicating that there was no reduction of synthesis of IP_3.

The effects of α-hANP on hydrolysis of PI-P_2 induced by NA

It is known that nitro-compounds (nitroglycerin, isosorbide dinitrate, nitroprusside and others) and EDRF increase the amount of cyclic GMP in the cytosol, whereas α-hANP increases the amount of cyclic GMP in the sarcolemma. The synthesis of cyclic GMP by the former process but not the latter is inhibited by methylene blue or haemoglobin (Rapoport & Murad, 1984; Ignarro *et al.*, 1985). To determine the effects of cyclic GMP in the sarcolemma on the hydrolysis of PI-P_2 induced by NA, the effects of α-hANP were observed. As shown in Figure 2, the effects of α-hANP on synthesis of cyclic GMP were compared in the presence and absence of endothelial cells. α-hANP (0.1 μM) markedly increased cyclic GMP in the presence and absence of endothelium to the same

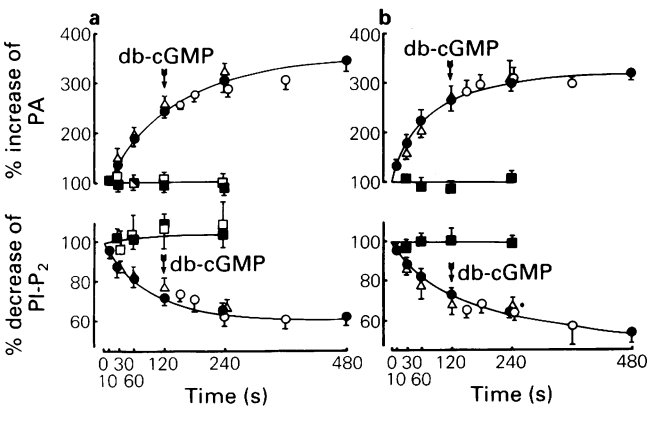

Figure 3 Effects of prazosin or dibutyryl cyclic GMP (db-cyclic GMP) on the synthesis of phosphatidic acid (PA) and amount of phosphatidylinositol 4,5-bisphosphate (PI-P_2) in smooth muscle cells of the rabbit aorta in the presence (a) or absence (b) of endothelium. Prazosin (5 μM) or db-cyclic GMP (100 μM) was applied 5 min before exposure to noradrenaline (NA), or db-cyclic GMP was applied 2 min after application of NA. The values are expressed as the mean and vertical lines show s.e.mean. ($n = 7$–10). (■) Control, (●) 5 μM NA, (□) NA after pretreatment with prazosin (5 μM), (△) NA after pretreatment with db-cyclic GMP (100 μM) and (○) application of 100 μM db-cyclic GMP 2 min after NA.

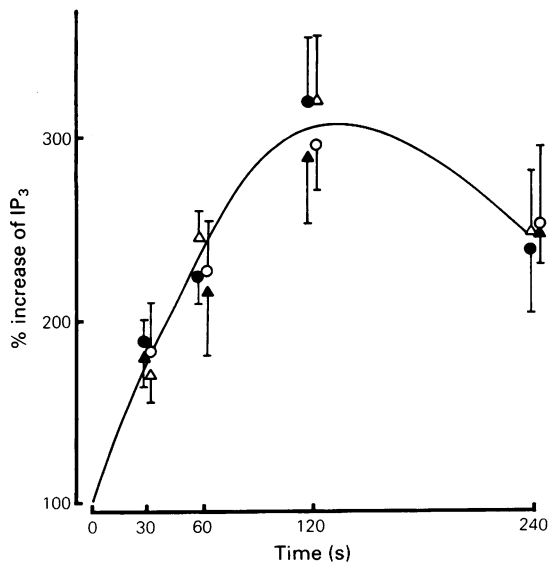

Figure 4 Effects of cyclic AMP or cyclic GMP on the synthesis of myo-inositol 1,4,5-trisphosphate (IP_3) at various exposure times in smooth muscle cells of the rabbit aorta. The endothelium was removed. The values are expressed as the mean and vertical lines show s.e.mean ($n = 6$–8). (●) Noradrenaline (NA, 5 μM), (○) application of 100 μM db-cyclic AMP 5 min before and during application of NA and application of 0.1 mM (▲) or 1 mM (△) db-cyclic GMP 5 min before and during application of NA.

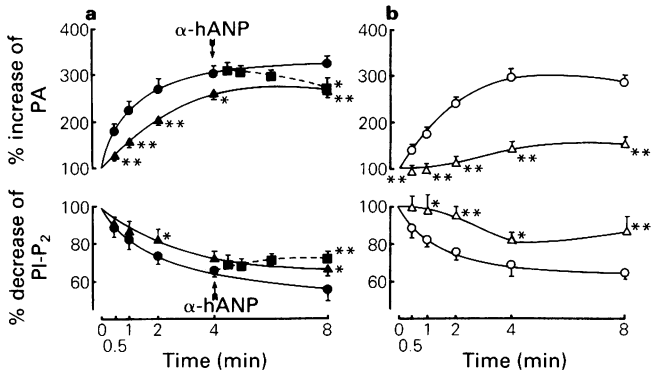

Figure 5 The effects of 0.1 μM α-human atrial natriuretic peptide (α-hANP) on phosphatidic acid (PA) and phosphatidydylinositol 4,5-bisphosphate (PI-P_2) induced by noradrenaline (NA, 5 μM; solid symbols (a) or 0.5 μM; open symbols (b)) in smooth muscle cells of the rabbit aorta. The endothelium had been removed. α-hANP was applied 5 min before and during application of NA or 4 min after application of NA. The values are expressed as the mean and vertical lines show s.e.mean, $n = 8$–12. (●, ○) NA (5 and 0.5 μM); (▲, △) application of 0.1 μM α-hANP 5 min before and during application of 5 and 0.5 μM NA and (■) application of 0.1 μM α-hANP 4 min after application of NA.

extent (after 4 min, 5.4 times the control and 5.5 times the control, $n = 10$, respectively) and this increase was greater than that observed on application of ACh (release of EDRF) in the presence of endothelial cells (after 4 min, 2.8 times the control, $n = 8$).

Figure 5 shows the effects of α-hANP on the synthesis of PA and hydrolysis of PI-P_2 in endothelium-denuded muscle tissues. The basal amounts of PA and PI-P_2 were 3180 ± 134 c.p.m. mg^{-1} ($n = 12$) and 10619 ± 353 c.p.m. mg^{-1} ($n = 12$) and these values were normalized as 100%. α-hANP (0.1 μM) itself did not modify the hydrolysis of PI-P_2. When 0.1 μM α-hANP was applied 5 min before and during application of NA (5 μM in Figure 5a and 0.5 μM in b) or 4 min after application of NA (5 μM, a and b), an increase in the amount of PA occurred to a lesser extent than that observed after application of NA alone (8 min after application of NA, 3.4 times the control and in the presence of α-hANP, 2.6 times the control, $P < 0.05$, $n = 8$). The reverse was observed on the amount of PI-P_2. As shown in Figure 5b, hydrolysis of PI-P_2 induced by 0.5 μM NA was markedly reduced by application of α-hANP (0.1 μM; 1.6 times the control, $P < 0.05$, $n = 8$). Increased concentrations of α-hANP (from 0.01 μM to 0.1 μM) inhibited hydrolysis of PI-P_2 induced by 0.5 μM NA, in a concentration-dependent manner (PA measured after application of NA, 1.52 times the control; in the presence of 0.01 and 0.1 μM α-hANP were 1.20 times, $P < 0.05$, and 1.0 times $P < 0.01$, the control, respectively, compared with that observed with NA alone, $n = 8$: PI-P_2 measured on application of NA, 0.79 times the control; in the presence of 0.01 and 0.1 μM α-hANP were 0.9 times, $P < 0.05$, and 0.99 times the control, $P < 0.01$, $n = 7$, compared with that observed with NA alone).

Discussion

Rapoport (1986) found that in the rat aorta, agents which elevate cyclic GMP inhibit agonist-induced hydrolysis of PI-P_2. This suggests that in intact vascular tissues, sodium nitroprusside, ACh, atriopeptin II, or 8-bromocyclic GMP inhibit NA-induced contractions by initial activation of cytosolic (soluble) or particulate guanylate cyclase, with secondary reduction of IP_3 synthesis (Rapoport & Murad, 1983; Waldman *et al.*, 1984; Winquist *et al.*, 1984; Tremblay *et al.*, 1985). This mechanism is consistent with that proposed by Takai *et al.* (1984) that cyclic GMP inhibits PI-P_2 breakdown in platelets through activation of G-kinase.

The present results showed that both ACh and α-hANP increased the intracellular concentration of cyclic GMP. However, the hydrolysis of PI-P_2 induced by NA was unaffected by ACh in the presence or absence of endothelial cells. Furthermore, neither db-cyclic GMP nor db-cyclic AMP had any effect on the breakdown of PI-P_2 induced by NA. In contrast, α-hANP significantly inhibited PI-P_2 hydrolysis. Since α-hANP acts directly on particulate guanylate cyclase, inhibition of PI-P_2 hydrolysis is dependent on the mechanism by which cyclic GMP elevation occurs.

The present conclusions differ from those of Rapoport (1986), which were based on studies in rat aorta. In this earlier study, slightly different procedures were adopted for measuring PI-P_2 hydrolysis under increased cyclic GMP conditions. Atriopeptin II, sodium nitroprusside or ACh were applied for 5 min or 8-bromocyclic GMP for 15 min. Subsequently tissues were exposed to 0.3 μM NA for 30 min to stimulate the hydrolysis of PI-P_2. However, in the present experiments, IP_3 synthesis induced by NA was measured within 8 min to limit any α_1-adrenoceptor desensitization (Hashimoto *et al.*, 1986). In addition, species differences may contribute to the differences between the present results and those of Rapoport (1986). In rat aorta, NA-induced contractions are markedly inhibited by Ca antagonists, or in Ca-free solutions, whereas in rabbit aorta, Ca antagonists do not inhibit NA-induced contractions, which persist in Ca-free solution (Ljung & Kjellstedt, 1987).

In the smooth muscle cells of the rabbit aorta, NA markedly and ACh slightly increased the amount of IP_3 as estimated from production of PA. This response differed from that observed in the porcine coronary artery, where ACh but not NA markedly enhanced the synthesis of IP_3 (Hashimoto *et al.*, 1986). ACh markedly enhanced the synthesis of cyclic GMP in intact tissues but only slightly increased it in the endothelium-denuded tissues. Presumably the slight increase in the cyclic GMP in the endothelium-denuded tissue may be due to incomplete ablation of endothelial cells from the tissue rather than direct action of ACh for synthesis of cyclic GMP in smooth muscle cells. Furthermore, following application of methylene blue (1 μM), synthesis of cyclic GMP by ACh in the presence of endothelium was blocked (the present authors, unpublished observations).

The particulate fraction of many cells contains a large molecular weight GTP binding protein which has been classified into several subtypes, based mainly on α-subunit differences, together with several small molecular weight GTP binding proteins located mainly in the cytosol (Bokoch *et al.*, 1984; Sternweis & Robishaw, 1984; Okajima *et al.*, 1985; Kikuchi *et al.*, 1988; Yamamoto *et al.*, 1988). The GTP binding protein is an essential component in the synthesis of IP_3 but the characteristics required for phospholipase C catalysis in vascular smooth muscle are not yet clear. Furthermore, Chinkers *et al.* (1989) have shown that the ANP receptor is guanylate cyclase itself and that this protein is a member of a new class of mammalian sarcolemmal receptors which contains an extracellular ligand binding domain and an intracellular guanylate cyclase catalytic domain.

It may be too early to postulate from the present experiments, but if the α-hANP-guanylate cyclase complex formed in the sarcolemma inhibits the action of α_1-adrenoceptor-GTP binding protein-phospholipase C complex which forms IP_3, the active complex formation of α-hANP guanylate cyclase (Chinkers *et al.*, 1989) for synthesis of cyclic GMP may interact. However, cyclic GMP-dependent protein kinase activated by cyclic GMP may not directly act on the above step for synthesis of IP_3, because exogeneously applied db-cyclic GMP and cyclic GMP synthesized in the cytosol had no effect on hydrolysis of PI-P_2. In addition, it is also postulated that if α-hANP acts as a vasodilator in vascular beds, it may result from an increase in the cyclic GMP concentration and, in part, to inhibition of the synthesis of IP_3.

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The influence of thyroid states upon responses of the rat aorta to catecholamines

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1 Mechanical responses to various stimulants and the distribution of adrenoceptor subtypes were investigated in smooth muscle cells of the rat aorta in eu- (control), hyper- and hypo-thyroid states.

2 Concentration-response relationships for K showed that the KCl EC_{50} value was slightly higher (28.4 mM K) in hypothyroid than in euthyroid and hyperthyroid states (22.5 mM and 22.8 mM K, respectively). The order of maximum amplitudes of KCl contraction was control > hypothyroid > hyperthyroid.

3 The EC_{50} values for the noradrenaline (NA)-induced contraction for eu-, hypo- and hyper-thyroid states were 7.4, 12.5 and 5.5 nM, respectively. The density of α_1-adrenoceptors was increased to 1.4 times the control in the hypothyroid state and reduced to 0.26 times the control in the hyperthyroid state.

4 The EC_{50} values for the phenylephrine-induced contraction in eu-, hypo- and hyper-thyroid states were 43.4, 247 and 41.5 nM, respectively, and those for clonidine 104, 1360 and 56.5 nM, respectively. The IC_{50} values for prazosin against 0.1 μM NA-induced contractions in eu-, hypo- and hyper-thyroid states were 5.8, 3.5 and 0.7 nM, respectively, and those for yohimbine 8.9, 5.4 and 4.1 μM, respectively.

5 The IC_{50} values for isoprenaline against phenylephrine-induced contractions were 63 nM and 2.4 μM in hyper- and eu-thyroid states, respectively, and the corresponding IC_{25} values for eu- and hypo-thyroid states were 72 and 423 nM, respectively. The density of β-adrenoceptors was increased to 3.91 times the control in the hyperthyroid and reduced to 0.68 times the control in the hypothyroid states. The amount of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) synthesized by isoprenaline increased in parallel with the density of β-adrenoceptors in the three thyroid states.

6 NA consistently increased the amount of inositol 1,4,5-trisphosphate (IP_3) within 30 s in the three thyroid states. In the hypothyroid state, the amount of IP_3 was slightly increased and in the hyperthyroid condition, it occurred to a lesser extent than in the euthyroid state.

7 The results indicate that in hypothyroidism, the density of α_1-adrenoceptors is increased and that of β-adrenoceptors reduced in vascular beds. The latter but not the former is accompanied by corresponding mechanical responses. In hyperthyroidism, the density of α_1-adrenoceptors is decreased and β-adrenoceptors increased with parallel changes in mechanical responses.

Introduction

In the hyperthyroid state, there is general agreement that the mechanical and electrical responses of cardiac muscle tissues induced by β-adrenoceptor stimulation are enhanced and those induced by α-adrenoceptor stimulation are markedly reduced. In contrast, responses to α-adrenoceptor stimulation are markedly enhanced in the hypothyroid state (Nakashima *et al.*, 1971; Rosenqvist & Boreus, 1972; Young and McNeill, 1974; Kunos, 1977; Bilezikian & Loeb, 1983; Threatte *et al.*, 1983; Ishac & Pennefather, 1983; Gross & Lues, 1985; Fox *et al.*, 1985).

However, no consensus has been reached concerning the density of α-adrenoceptors in either hyperthyroidism (unchanged: Ciaraldi & Marinetti, 1978; decreased: Ciaraldi & Marinetti, 1977; Sharma & Banerjee, 1978; Williams & Lefkowitz, 1979; McConnaughey *et al.*, 1979) or hypothyroidism (increased: Sharma & Banerjee, 1978; Kunos *et al.*, 1980; Chang & Kunos, 1981; unchanged: Ciaraldi & Marinetti, 1978; decreased: Ciaraldi & Marinetti, 1977; 1978; McConnaughey *et al.*, 1979; Noguchi & Whitsett, 1983). Several studies have also revealed that the number of β-adrenoceptors is altered in different thyroid states. In hyperthyroidism, the number of β-adrenoceptors and the responses to β-agonists are increased (Williams *et al.*, 1977; Tse *et al.*, 1980; Scarpace & Abrass, 1981; Stiles & Lefkowitz, 1981; Krawietz *et al.*, 1982; Bilezikian & Loeb, 1983; O'Donnell & Wanstall, 1986; O'Donnell *et al.*, 1987) or unchanged (Banerjee & Kung, 1977). In hypothyroid animals, the β-adrenoceptor population in cardiac muscles decreases (Banerjee & Kung, 1977; Chang & Kunos, 1981; Ciaraldi & Marinetti, 1977; McConnaughey *et al.*, 1979; Kunos *et al.*, 1980; Tse *et al.*, 1980; Stiles & Lefkowitz, 1981; Krawietz *et al.*, 1982; Noguchi & Whitsett, 1983).

In vascular smooth muscle tissues, the effects of thyroid hormones on adrenoceptors are also controversial. In rat vascular beds, it was shown that the density of smooth muscle α_1-adrenoceptors is not altered by any thyroid state (Fox *et al.*, 1985). However, others have found that in the rat pulmonary artery and aorta, responses to β_1- but not to β_2-adrenoceptor stimulation are enhanced in the hyperthyroid state (O'Donnell & Wanstall, 1986; O'Donnell *et al.*, 1987). In the hypothyroid rat aorta, Rahmani *et al.* (1987) demonstrated that the α_1-adrenoceptor-mediated response was significantly reduced and shifted to the right, and that α_2-adrenoceptor responses were blocked. In contrast, responses to the α_1- and α_2-adrenoceptors were not altered in the hyperthyroid state. These results confirmed previous observations (MacMillan & Rand, 1962; Rosenqvist & Boreus, 1972), but were in contrast to the enhanced α-adrenoceptor-mediated responses to NA obtained in rat aorta in the hypothyroid but not in the hyperthyroid state (Field *et al.*, 1973).

In vascular smooth muscle tissues, no systematic investigation has been carried out on the density of adrenoceptor distribution and its relationship to receptor stimulation in different thyroid states. The present experiments were thus designed to clarify responses of the smooth muscle of rat aorta in relation to the distribution of the catecholamine receptors

in eu-, hyper- and hypo-thyroid states. The possible actions of thyroid hormones on smooth muscle tissues are also discussed.

Methods

Materials

Male Wistar King A (WKA) rats weighing 225–350 g (8–12 weeks old) were used. The animals were caged separately and given rat purina chow and drinking water *ad libitum*. Hypothyroidism was induced by total thyroidectomy and to prevent the occurrence of hypoparathyroidism, parathyroid glands were autotransplanted. One or two parathyroid glands were identifed under the binocular microscope, carefully dissected out from the thyroid lobes and re-implanted into the hyoid muscle. The hypothyroid animals were killed not less than 3 weeks after the thyroidectomy.

Hyperthyroidism was induced by subcutaneous injections of 0.75 mg kg^{-1} L-thyroxine sodium in alkaline saline solution (0.001 N NaOH in 0.9% NaCl) daily for 7 days (Threatte *et al.*, 1983). The animals were killed at least 20 h after the last injection. The control rats (eu-thyroid status) were either sham-operated or injected with alkaline saline for the same period of time. Since the thyroxine-treated animals were sensitive to overdoses and vulnerable to spontaneous death during the treatment period, especially when the room temperature exceeded 25°C, the room temperature was maintained at 25°C.

Assessment of thyroid states

Body weight was measured at the commencement of treatment and the average daily change in body weight was compared with controls. Colonic temperature was measured under anesthesia (sodium pentobarbitone 6.5 mg 100 g^{-1} i.p.) at an ambient temperature of 25 ± 2°C by a thermistor probe (Nihon Kohden model NGA 111, type 219), which was inserted 6 cm beyond the anus for 1 min. Subsequently animals were exsanguinated and then blood and tissues were collected. Heart weights were measured in all animals as was the weight of the thyroid in eu- and hyper-thyroid rats. Heart and thyroid weights were expressed relative to body weight (mg 100 g^{-1} body weight). A blood sample taken from each rat on the day of experiment was centrifuged at 3000 r.p.m. for 10 min, and the serum was separated immediately. Serum Ca estimation was carried out for the hypothyroid rats by use of a commercially available calcium C-test (Wako kits). The rest of the serum samples were stored at −20°C. Total serum thyroxine (T_4) and tri-iodothyronine (T_3) levels of each rat were determined by radioimmunoassay with a commercially available conventional double antibody technique (provided by Eiken Chem. Co. Ltd.) and newly developed solid phase antibody-coated tubes (provided by Mallinkrodt Diagnostica, Daiichi Radioisotopes, Japan).

Measurements of isometric contraction

The isolated aorta was cleaned of surrounding connective tissue and a 2 mm wide helical strip was cut with scissors from a 6 mm length of upper thoracic aorta. The endothelium was gently removed with a cotton ball. The tissue was suspended in an organ bath and perfused with warmed Krebs solution (35–37°C), by a thermistor-controlled perfusion pump. The tissue was suspended for 1 h without stretch, and then 50–60 mg tension was applied for a further 1 h. Isometric tension was recorded by a force transducer (Nihon Kohden FD pick up, TB-621), and the measurements were started when the amplitude of contraction showed a constant value in response to repetitive application of a solution containing 39 mM KCl. To obtain concentration-response relationships, agonists and antagonists were applied cumulatively.

Krebs solution of the following composition was used (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl 134, glucose 11.5. The solution was bubbled with 97% O_2 and 3% CO_2, and the pH of the solution was maintained at about 7.3.

Cyclic AMP and inositol 1,4,5-trisphosphate assays

Rat aorta devoid of connective tissue and endothelium was cut into 5 segments and incubated at 37°C in Krebs solution for 1 h. Tissues were stimulated with 1 μM isoprenaline for 0–5 min and the reaction was halted by quickly immersing the muscle strip in liquid N_2. The samples were homogenized in cold trichloroacetic acid (0.3 mol l^{-1}) at 4°C and the concentration of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and protein content were measured by a radioimmunoassay kit (Yamasa Shoyu, Tokyo, Japan) and the method of Lowry *et al.* (1951), respectively.

In the case of inositol 1,4,5-trisphosphate (IP_3) measurements, tissues were stimulated with 50 μM NA for 30 s to 3 min, and homogenized in cold trichloroacetic acid (0.75 mol l^{-1}). The amount of IP_3 was measured by a radioimmunoassay kit (Amersham, Japan, plc., code TRK, 1000).

Radioligand binding assay

β-Adrenoceptor radioligand binding assay Aortae from 6–7 rats devoid of connective tissue and endothelium were minced with scissors in 50 mM tris-maleate buffer (pH 7.2) at 4°C, and homogenized with a polytron homogenizer (Type NS 500; Nihon Seimitsu Kogyo, Japan) for 20 s at half-maximal speed. The above procedure was repeated twice and the homogenate was then centrifuged at 10,000 *g* for 10 min and the supernatant was centrifuged again at 100,000 *g* for 1 h at 4°C. The pellet was re-suspended in buffer and protein determination was performed as described by Lowry *et al.* (1951). [^{125}I]-iodocyanopindolol ([^{125}I]-CYP; 2200 Ci $mmol^{-1}$) was incubated with 20 μg of membrane protein in triplicate in a final volume of 0.5 ml at 25°C for 1 h. The suspension was filtered through a GF/F glass fibre filter (Whatman 25) under reduced pressure. The filters were washed with 10 ml of 15 mM Tris-maleate buffer at pH 7.2, dried and counted in a gamma counter. Non-specific binding of [^{125}I]-CYP was determined as binding remaining following treatment with 50 μM isoprenaline.

α_1-Adrenoceptor radioligand binding assay α_1-Adrenoceptor binding experiments were carried out with [^{125}I]-iodo-4-hydroxy phenyl-ethyl-aminomethyl-tetralone ([^{125}I]-HEAT; 2200 Ci $mmol^{-1}$), as described by Jones *et al.* (1987), using a crude membrane fraction of 3–4 aortae in 25 mM glycylglycine buffer (pH 7.6). Briefly, the procedure was the same as for the [^{125}I]-CYP binding assay; 20 μg of crude membrane was incubated for 40 min, filtered through glass fibre filters and radioactivity counted. The determination of non-specific binding of [^{125}I]-HEAT was made by treatment with 100 μM NA.

Drugs

The following drugs were used: L-thyroxine, (−)-noradrenaline (NA), and isoprenaline hydrochloride were obtained from Sigma Chem. Co.; phenylephrine, prazosin were obtained from Taito-Pfizer Pharmac. Co., and yohimbine and clonidine from Tokyo Kasei Pharmac. Co. [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) and β-[^{125}I]-iodo-4-hydroxy phenyl-ethyl-aminomethyl-tetralone ([^{125}I]-HEAT) were obtained from NEN Research Products. Triiodo-thyronine (T_3) solid phase antibody coated kits (T_3-SPAC) were kindly donated by Daiichi Radioisotopes, Japan.

Statistics

The experimental values are expressed as the mean ± s.e. Statistical significance was determined by use of Student's *t* test.

Results

Table 1 summarizes the measurements of various parameters (see Methods) following altered thyroid states in rats. In the hypothyroid animals, significant reductions in weight gain, colonic temperature, heart/body weight ratio and serum T_4 and T_3 levels were observed. However, there was no significant change in the serum Ca level to suggest hypoparathyroidism. In contrast, significant increases in colonic temperature, heart/body weight ratio, serum T_4 and T_3 levels were observed in the hyperthyroid rat. In addition, there were significant reductions in the weight of the thyroid gland and in the amount of weight gain. In the hyperthyroid rats, the serum Ca level remained the same as that observed in the controls.

K- and NA-induced contractions observed in three different thyroid states

Figure 1 shows the concentration-response (contraction) relationships observed on application of various concentrations of KCl and NA to aortae from eu-, hyper- and hypothyroid rats. In the hypothyroid state, the KCl concentration-response relationship was shifted to the right and the maximum amplitude of contraction evoked by 118 mM KCl was inhibited compared with that observed in euthyroid rats. In hyperthyroid aortae, the maximum amplitude of the KCl-induced contraction (118 mM) was markedly inhibited, but the 11.7 mM KCl-induced contraction was not modified in comparison to the control (Table 2).

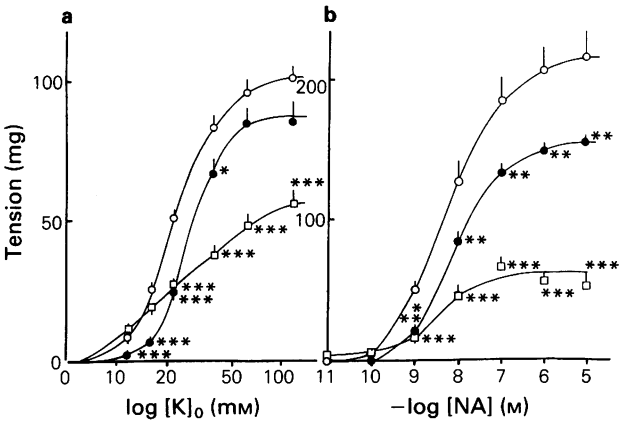

Figure 1 Concentration-response relationships for (a) KCl- and (b) noradrenaline (NA)-induced contractions of rat aorta in eu-(control; ○), hypo-(●) and hyper-thyroid states (□). Vertical lines denote s.e.mean; in (a) $n = 6$–10 and (b) $n = 15$–17. Asterisks indicate responses significantly different from the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Similar experiments were carried out with NA as the agonist. As shown in Figure 1b, the minimum concentration of NA required to produce a contraction was approximately the same in the three states (>0.1 nM). However, the contractions evoked at any given concentration of NA in aortae in the euthyroid rats were consistently larger than those observed in the hyper- and hypo-thyroid states, and the maximum amplitude of the NA (10 μM)- induced contraction evoked in tissues from the hyperthyroid rat was much smaller than that observed from eu- and hypothyroid rats. The EC_{50} values for NA in eu-, hypo- and hyper-thyroid conditions are listed in Table 2.

Table 1 Various somatic parameters measured following altered thyroid states in rats

Variable	*Treatment group* *Euthyroid (control)*	*Hypothyroid*	*Hyperthyroid*
% decrease of wt. gain over control animals	a	40.3 ± 1.7* (79)	87.8 ± 7.2* (90)
Colonic temp. in °C	38.2 ± 0.1 (45)	36.7 ± 0.1* (47)	39.7 ± 0.1* (63)
Heart/body weight (mg 100 g^{-1} body wt.)	256 ± 5.6 (48)	204 ± 2.1* (74)	403 ± 5.1* (90)
Thyroid/body weight (mg 100 g^{-1} body wt.)	4.81 ± 0.11 (54)	0	3.35 ± 0.07* (44)
Serum thyroxine (T_4) (μg dl^{-1})	3.16 ± 0.29 (58)	0.44 ± 0.04* (79)	23.7 ± 1.62* (90)
Serum triiodothyronine (T_3) (ng dl^{-1})	71.8 ± 2.95 (58)	35.2 ± 2.46* (79)	403 ± 25.7* (90)
Serum Ca^{2+} (mg dl^{-1})	8.65 ± 0.11 (32)	8.57 ± 0.09^{NS} (39)	8.68 ± 0.24^{NS} (26)

Results are expressed as mean ± s.e. (number of observations).
* Significantly different from euthyroid group by Student's *t* test, $P < 0.001$.
NS – not significant.
a, weight gains of alkaline saline injected and sham-operated rats during the treatment period were 28.7 ± 2.5 and 18.3 ± 1.5 g week^{-1}, respectively.

Table 2 The EC_{50} values measured for high K-, noradrenaline-, phenylephrine- and clonidine-induced contractions and the IC_{50} values for prazosin- and yohimbine-induced inhibition of the noradrenaline (0.1 μM)-induced contraction measured from smooth muscle tissues of the rat aorta in eu-, hypo- and hyper-thyroid states

	Control	*Hypothyroid*	*Hyperthyroid*
High-K	22.5 ± 0.8 mM ($n = 8$)	28.4 ± 0.7 mM** ($n = 10$)	22.8 ± 1.5 mM ($n = 9$)
Noradrenaline	7.4 ± 1.2 nM ($n = 15$)	12.5 ± 1.7 nM* ($n = 17$)	5.5 ± 1.0 nM ($n = 17$)
Phenylephrine	43.4 ± 3.1 nM ($n = 15$)	247 ± 58 nM** ($n = 12$)	41.5 ± 3.7 nM ($n = 11$)
Clonidine	104 ± 12.3 nM ($n = 14$)	1360 ± 251 nM*** ($n = 14$)	56.5 ± 18.6 nM** ($n = 9$)
Prazosin	5.8 ± 0.7 nM ($n = 25$)	3.45 ± 0.45 nM** ($n = 9$)	0.66 ± 0.2 nM*** ($n = 8$)
Yohimbine	8.9 ± 0.2 μM ($n = 12$)	5.44 ± 0.83 μM*** ($n = 8$)	4.07 ± 0.34 μM*** ($n = 8$)

The EC_{50} and IC_{50} values were obtained from concentration-response curves in each tissue. Data are mean ± s.e. (n = number of observations).
* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Effects of α-adrenoceptor agonists and antagonists

To clarify further the response of smooth muscle tissues to activation of α-adrenoceptors, the effects of phenylephrine and clonidine were studied (Figure 2a and b). The minimum concentration of phenylephrine required to produce a contraction was 1 nM, and the maximum amplitude of contraction evoked by phenylephrine was larger in the control than the hypo- and hyper-thyroid rats. The minimum concentration of clonidine required to evoke a contraction was about 10 nM and the maximum amplitudes of contraction evoked by 10 μM clonidine were much smaller than those evoked by 10 μM phenylephrine or NA. The EC_{50} values for phenylephrine and clonidine in the eu-, hypo- and hyper-thyroid states are given in Table 2.

To investigate further the action of thyroid hormone on responses to α-adrenoceptor subtypes, the effects of prazosin and yohimbine on NA-induced contractions were investigated (Figure 3a and b). Prazosin and yohimbine each inhibited the NA (0.1 μM)-induced contraction in the three different thyroid states. Yohimbine was less potent than prazosin (Table 2). The inhibition of the standard NA-induced contraction by yohimbine occurred at concentrations above 1 μM and even at a concentration of 10 μM, yohimbine never completely blocked the contraction in any of the three thyroid states (Figure 3b and Table 2).

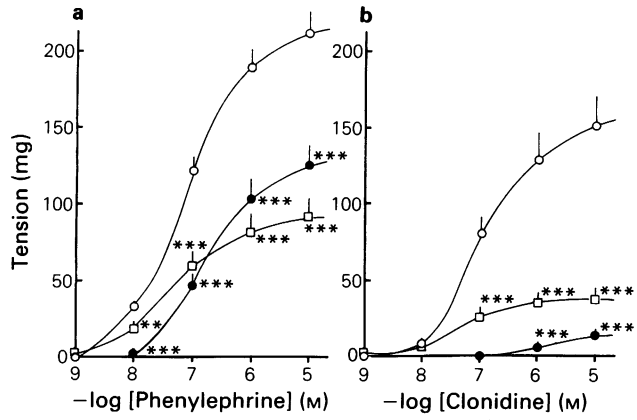

Figure 2 Concentration-response relationships for (a) phenylephrine- and (b) clonidine-induced contractions in rat aorta in eu- (○; $n = 15$ in (a) and (14) in (b)), hypo-(●; $n = 12$ in (a) and 14 in (b)) and hyper- (□; $n = 11$ in (a) and 9 in (b))-thyroid states. Vertical lines show s.e.mean $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, responses significantly different from control.

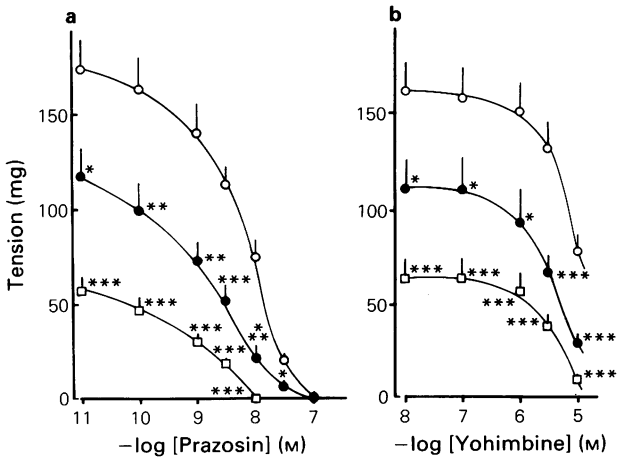

Figure 3 Concentration-dependent inhibition by prazosin (a) and yohimbine (b) of the noradrenaline (NA, 0.1 μM)-induced contraction in rat aorta in eu- (○; $n = 25$ in (a) and 12 in (b)), hypo- (●; $n = 9$ in (a) and 8 in (b)) and hyper- (□; $n = 8$ in (a) and (b)) thyroid states. Vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, responses significantly different from control.

The effects of the β-adrenoceptor agonist, isoprenaline, on the phenylephrine-induced contraction

To evaluate the role of β-adrenoceptors in the three different thyroid states, the effects of isoprenaline were studied in tissues precontracted by phenylephrine (1 μM) (Figure 4). In the euthyroid state, 1 nM isoprenaline did not relax the phenylephrine (1 μM)-induced contraction and more than 1 μM isoprenaline was required to produce 50% relaxation of the contracted tissue. A potent relaxation was induced by isoprenaline in tissues excised from the hyperthyroid rats (1 μM isoprenaline induced 0.09 ± 0.02 times the maximum amplitude of phenylephrine-induced contractions), whereas in the hypothyroid state, 1 μM isoprenaline relaxed the tissue to 0.66 ± 0.02 times the maximum amplitude of contraction observed. The IC_{50} values for isoprenaline against the phenylephrine-induced contraction were 2.4 ± 0.4 μM and 63.2 ± 5.9 nM ($n = 17$) in the eu- and hyper-thyroid states, respectively, and the IC_{25} values in eu- and hypo-thyroid states were 72.4 ± 19.5 and 423 ± 94.4 nM ($n = 16$–17), respectively.

The effects of thyroid states on [^{125}I]-CYP and [^{125}I]-HEAT binding

To investigate β-adrenoceptor distribution on smooth muscle cells of the rat aorta in eu-, hyper- and hypo-thyroid states, [^{125}I]-CYP was used. β-Adrenoceptor density was estimated from a Scatchard analysis of the saturation isotherm specific for [^{125}I]-CYP binding in the three thyroid states (Figure 5a). The total binding of [^{125}I]-CYP to the fragmented membrane increased in a time-dependent manner and reached a steady level at 45 min. Therefore the maximum binding (B_{max}) was measured after 1 h exposure to [^{125}I]-CYP. The specific binding of [^{125}I]-CYP was obtained by subtraction of non-specific binding from total binding (Methods). The B_{max} values in the eu-, hypo- and hyper-thyroid states are given in Table 3. The equilibrium dissociation constants, K_D, for the specific

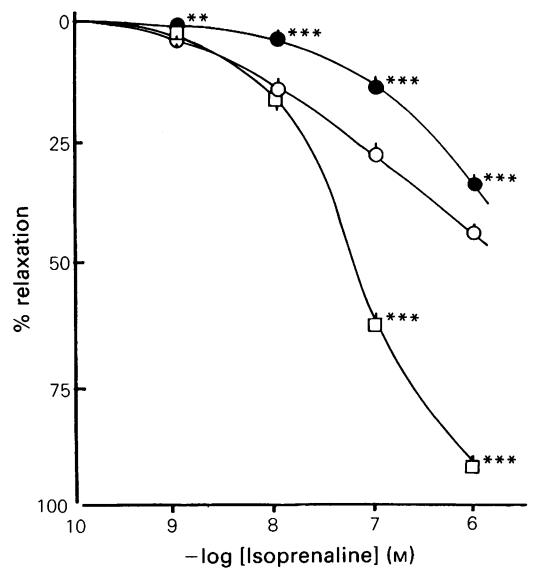

Figure 4 The concentration-response relationship of the isoprenaline-induced relaxation in rat aorta precontracted by phenylephrine (1 μM) in eu- (○; $n = 17$), hypo- (●; $n = 16$) and hyper- (□; $n = 17$) thyroid states. The contractions evoked by phenylephrine in each of the three different thyroid states were normalized as 1 and complete relaxation of precontracted tissues was normalized as 100%, respectively. Vertical lines show s.e.mean. $**P < 0.01$, $***P < 0.001$, responses significantly different from control.

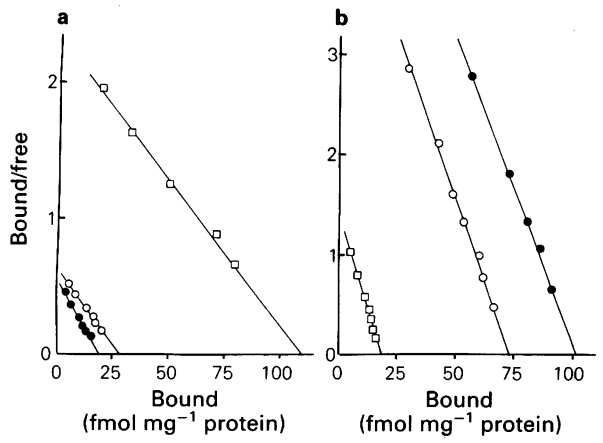

Figure 5 Scatchard analysis of saturation isotherms of β-adrenoceptor specific [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) binding (a) and α_1-adrenoceptor specific [^{125}I]-iodo-4-hydroxyphenyl-ethyl-amonimethyl-tetralone ([^{125}I]-HEAT) binding (b) to membrane fractions of smooth muscle cells of the rat aorta in eu-(○), hypo-(●) and hyper-thyroid states (□). Numbers of observations are 5–6 (34–45 animals) in (a) and 7–10 (15–20 animals) in (b).

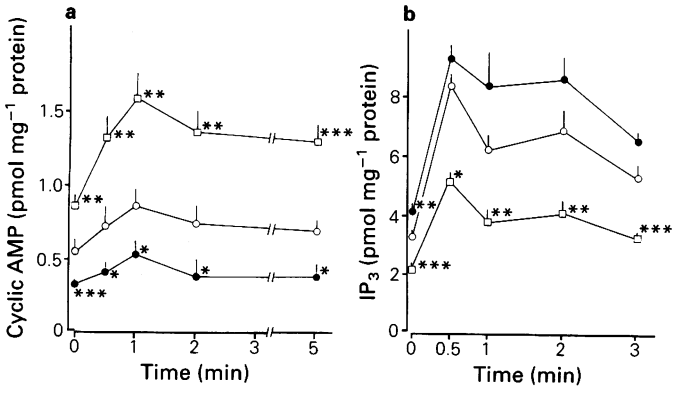

Figure 6 Time-dependent synthesis of cyclic AMP (a) and inositol 1,4,5-trisphosphate (IP_3) (b), following application of 1 μM isoprenaline or 50 μM noradrenaline, respectively in eu- (○), hypo- (●) and hyper (□)-thyroid states. Individual points indicate the mean (n = 7–9); vertical lines show s.e. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control.

binding of [^{125}I]-CYP in the above three different thyroid states were similar (Figure 5a and Table 3). Therefore, hyperthyroidism caused a 3.91 times increase and hypothyroidism caused a 0.68 times reduction in the density of specific [^{125}I]-CYP binding sites, without any change in the binding affinity in the rat aorta.

The effects of thyroid states on the specific binding of [^{125}I]-HEAT to the α_1-adrenoceptor were determined by a Scatchard analysis, using the same procedure as for the [^{125}I]-CYP binding and the results are summarised in Figure 5b and Table 3. In hyperthyroidism, the density of β-adrenoceptors on smooth muscle cell membranes was increased but that of α-adrenoceptors decreased, while the reverse sequence in receptor density was observed in hypothyroidism. However, the K_D values derived from application of radioligands were not modified in the three different thyroid states.

Effect of thyroid state on the synthesis of cyclic AMP induced by isoprenaline

Figure 6a shows the effects of 1 μM isoprenaline on the synthesis of cyclic AMP in smooth muscle cells in the three different thyroid states. The basal cyclic AMP level of smooth muscle tissues obtained from the hyperthyroid rat was increased (0.86 ± 0.07 pmol mg^{-1} protein, $n = 8$) in comparison to that from euthyroid rats (0.56 ± 0.08 pmol mg^{-1} protein, $n = 8$), while that obtained from the hypothyroid rat was decreased (0.33 ± 0.02 pmol mg^{-1} protein, $n = 7$). Following application of 1 μM isoprenaline, the amount of cyclic AMP was markedly increased within 1 min to 1.56, 1.60 and 1.84 times in the eu-, hypo- and hyper-thyroid rats, respectively and then gradually reduced with time. In the hypothyroid rat, the amount of cyclic AMP measured 5 min after application of isoprenaline was reduced to almost the same level as that observed before the application, while in the control (euthyroid) rat, the amount of cyclic AMP returned to a level just above the basal level and in the hyperthyroid rat, the amount of cyclic AMP remained at a higher level than the control (Figure 6a).

Effect of thyroid state on the synthesis of IP_3

Figure 6b shows the effects of 50 μM NA on the synthesis of IP_3 in smooth muscle cells of the eu-, hyper- and hypothyroid rats. The basal level of IP_3 in the tissue was increased in the hypothyroid rats (4.14 ± 0.54 pmol mg^{-1} protein, $n = 7$) and decreased in the hyperthyroid rats (2.22 ± 0.39 pmol mg^{-1} protein, $n = 7$) when compared with the euthyroid rats (3.33 ± 0.40 pmol mg^{-1} protein, $n = 7$). In all three thyroid conditions, the amount of IP_3 was markedly increased within 30 s following NA stimulation in a similar manner and gradually decreased with time over a period of 3 min. When the maximum amount of synthesized IP_3, measured at 30 s after application of NA, was compared to the basal levels, the increases were 2.54, 2.31, and 2.24 times basal ($n = 7$ each) in eu-, hyper- and hypo-thyroid animals, respectively. Thus, the hyperthyroid state was associated with a relatively decreased level of IP_3, whereas in the hypothyroid state, the amount of IP_3 measured following agonist stimulation was not significantly different from the controls, although the basal value was high.

Discussion

In cardiac and vascular smooth muscle tissues, figures for the density of adrenoceptor distribution in different thyroid states are controversial as described in the Introduction. Recently, Limas & Limas (1987) showed that the density of β-adrenoceptors in rat cardiac muscle increased progressively

Table 3 [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) and [^{125}I]-iodo-4-hydroxyphenyl-ethyl-amonimethyl-tetralone ([^{125}I]-HEAT) binding to smooth muscle membranes of the rat aorta in eu-, hypo- and hyper-thyroid states

[^{125}I]-CYP	*Control*	*Hypothyroid*	*Hyperthyroid*
B_{max} (fmol mg^{-1} protein)	28.3 ± 3.1 (n = 5)	19.3 ± 1.5* (n = 6)	110.6 ± 9.2*** (n = 6)
K_D (pM)	45.3 ± 3.3 (n = 5)	33.9 ± 2.9 (n = 6)	47.3 ± 3 (n = 6)
[^{125}I]-HEAT			
B_{max} (fmol mg^{-1} protein)	72.9 ± 1.7 (n = 7)	101.6 ± 3.3*** (n = 8)	18.6 ± 1.9*** (n = 10)
K_D (pM)	15.1 ± 0.66 (n = 7)	16.5 ± 0.38 (n = 8)	13.4 ± 0.8 (n = 10)

The maximum binding (B_{max}) and dissociation constant (K_D) are presented.
Results are expressed as mean ± s.e. (n = number of observations). Asterisks denote significantly different from control animals determined by Student's t test (*P < 0.05, ***P < 0.001).

during daily injections of L-thyroxine. In contrast the number of α_1-adrenoceptors declined rapidly and following cessation of thyroxine administration, the receptor number returned rapidly to normal values within 2 days. These results may indicate that the biological activity of thyroxine in the rat is a critical factor in determining the density and responsiveness of the cardiac adrenoceptor.

Rahmani *et al.* (1987) found that in aortae from hypothyroid rats, the α_1-adrenoceptor-mediated response was significantly decreased, the maximum mechanical response was reduced and the concentration-response relationship shifted to the right, compared with the euthyroid aortae, whereas the α_2-adrenoceptor-mediated response was completely inhibited. In contrast, hyperthyroidism did not alter the α_1- and α_2-adrenoceptor responses. These observations did not confirm the previous findings of Rosenqvist & Boreus (1972) and Field *et al.* (1973).

In the present experiments, the somatic condition of rats was significantly modified following thyroidectomy or injections of thyroxine (Table 1). In the hypothyroid state, responses of the α_1-adrenoceptor to NA or phenylephrine were markedly inhibited and those of the α_2-adrenoceptor to NA or clonidine almost ceased. The density of α_1-adrenoceptors in the hypothyroid rat was increased, as estimated from the B_{max} value of [^{125}I]-HEAT binding experiments, without any significant change in the K_D value. Furthermore, the basal level of IP_3 synthesis was enhanced. However, in the hypothyroid state, the increased number of the α_1-adrenoceptors did not directly correlate with the α_1-adrenoceptor-induced mechanical responses of the smooth muscle.

In the rat pulmonary artery, O'Donnell *et al.* (1987) observed a selective decrease in the responses mediated by the β_1-adrenoceptor in hypothyroidism. In the present study, thyroxine increased the density of β-adrenoceptors and the associated mechanical responses. The reverse was observed in the hypothyroid aorta, as estimated from the maximum relaxation of the tissue, the IC_{50}, the B_{max} and synthesis of cyclic AMP. However, the K_D values remained the same in all three thyroid states. These results are thus consistent with those previously observed on the β-adrenoceptor in cardiac and other smooth muscle tissues.

In the hypothyroid rat, an increased number of α_1-adrenoceptors was accompanied by an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-P_2) and the resulting synthesis of IP_3 under resting conditions. However, following agonist stimulation, time-dependent IP_3 synthesis was not significantly different, when compared with the control animals, whilst mechanical responses mediated by α_1-adrenoceptor agonists were reduced. These discrepancies in the synthesis of IP_3 and the associated mechanical response may be explained by alterations in the functions of the sarcoplasmic reticulum and myosin ATPase. Suko (1973) showed that Ca uptake and the hydrolysis of Ca-dependent myosin ATPase in cardiac muscles were markedly reduced in the hypothyroid state, presumably due to a deficiency of protein synthesis in this condition (Rovetto *et al.*, 1972; Yazaki & Raben, 1975).

In the hyperthyroid rat, a decreased α-adrenoceptor density together with a decreased basal level of IP_3, but a normal pattern of stimulated IP_3 synthesis, was observed. This was accompanied by an increased density of β-adrenoceptors, resulting in an enhanced production of cyclic AMP. These factors may combine to reduce the overall mechanical response. Cyclic AMP is known to accelerate Ca uptake into the sarcoplasmic reticulum and partly to inhibit the Ca-calmodulin-myosin light chain kinase (MLCK) complex through phosphorylation of MLCK by cyclic AMP-dependent protein kinase (Conti & Adelstein, 1981; Kuriyama *et al.*, 1982). In smooth muscle tissues, thyroid hormones have also been shown to have an inhibitory action on Ca-calmodulin binding, and this may also induce a reduction in myosin phosphorylation through inhibition of the Ca-calmodulin-MLCK complex (Hagiwara *et al.*, 1989). These results suggest that in the rat aorta, thyroid hormones negatively regulate the synthesis of the α-adrenoceptor but positively regulate the synthesis of the β-adrenoceptor.

The thyroid gland has multiple actions on cardiac and smooth muscle tissues, e.g. in cardiac ventricular but not atrial muscles, thyroid hormones regulate the actin-activated ATPase and isoenzyme patterns (Banerjee, 1983), and they also accelerate the accumulation of Ca into the sarcoplasmic reticulum through activation of Ca-ATPase (Suko, 1973). These actions may be related to an increase in cyclic AMP concentration (Suematsu *et al.*, 1985) by increasing the number of β-adrenoceptors. Furthermore, thyroid hormones also decrease the density of Ca channels (Hawthorn *et al.*, 1988). Moreover, additional multiple actions of thyroid hormones on cellular processes and components, including protein synthesis through nuclear transcription, mitochondrial activation and plasma membrane effects are well documented (Sterling, 1979; Oppenheimer, 1979; 1985).

In conclusion, thyroid hormones regulate cell functions including the distribution of α- and β-adrenoceptors in rat aorta. Thyroid hormones accelerate the production of the β-adrenoceptor with concomitant synthesis of cyclic AMP, while these hormones suppress the production of the α-adrenoceptor and thereby the synthesis of IP_3, without affecting the cellular process involved in their synthesis. A deficiency of thyroid hormones reverses the above sequence, again without affecting the enzymatic reactions that synthesize cyclic AMP or IP_3. An investigation of the molecular mechanisms involved in the action of thyroid hormones on the coding system in the nucleus is urgently needed.

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Effect of quinine on the release of catecholamines from bovine cultured chromaffin cells

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1 The effects of quinine on catecholamine release from cultured bovine chromaffin cells were studied.

2 Quinine (25–400 μM) produced a dose-related inhibition of catecholamine release in response to depolarizing concentrations (12.5–50 mM) of K^+.

3 The inhibition of the secretory response to high K^+ produced by quinine decreased with the increase in the extracellular concentration of Ca^{2+}.

4 Stimulation of cultured chromaffin cells with 50 mM K^+ produced a significant increase in Ca^{2+} influx. In the presence of 100 μM quinine a 54% inhibition of the K^+-induced Ca^{2+} influx was observed.

5 Quinine treatment of chromaffin cell cultures produced a small but significant decrease in membrane resting potential and a less pronounced depolarization in response to 50 mM K^+.

6 The results suggest that the inhibition of the K^+-evoked release of catecholamines produced by quinine is at least partly due to a decrease in Ca^{2+} influx. Ca^{2+} influx is lower because quinine reduces the sensitivity of the membrane potential to changes in extracellular K^+ but direct effects of quinine on Ca^{2+} channels cannot be excluded.

Introduction

Quinine and its stereoisomer, quinidine, belong to a category of compounds known for their antiarrhythmic action on cardiac cells. It was originally reported to be the specific blocker of the Ca^{2+}-activated K^+ conductance in red blood cells (Armando-Hardy *et al.*, 1975; Lew & Ferreira, 1978). However, recent patch clamp studies on secretory cells have shown that quinine is far from being a specific blocker but is in fact a very inefficient flickery blocker of Ca^{2+}-activated K^+ channels (Findlay *et al.*, 1985; Glavinović & Trifaró, 1988). On the other hand, quinine and quinidine appear to affect delayed rectifier currents in *Aplysia* (Hermann & Gorman, 1984) and inward rectifier currents in insulin secreting cells (Findlay *et al.*, 1985) as well as Na^+ conductances in frog auricular fibres (Ducouret, 1976). Quinine and quinidine also affect Ca^{2+} conductances in frog heart (Ducouret, 1976), mammalian heart muscles (Nawrath, 1981) and in molluscan neurones (Plant & Standen, 1981). It is therefore to be expected that quinine alters the catecholamine release induced by high potassium, a condition responsible for an increased Ca^{2+} influx. Ca^{2+} plays a key role in the secretory process of the chromaffin cell and in other systems that store their secretory products in subcellular granules (Trifaró, 1977; 1982). In addition, the increase in intracellular Ca^{2+} brought about by cell stimulation activates Ca^{2+}-dependent K^+ channels leading to hyperpolarization and inhibition of further Ca^{2+} influx. Furthermore, any change in the potassium conductance ought to alter the sensitivity of the membrane potential to changes in extracellular potassium. In these studies, we have examined whether and if so how, quinine affects the catecholamine release from cultured chromaffin cells. A preliminary account of this study has appeared elsewhere (Glavinović *et al.*, 1985).

Methods

Isolation of chromaffin cells

Bovine adrenal glands obtained from a slaughterhouse were freed from their cortices and perfused *in vitro* for 10 min (37°C) with Ca^{2+}-free Mg^{2+}-free Locke solution as described previously (Trifaró *et al.*, 1967). Perfusion was continued for 60 min with fresh solution to which 0.05% collagenase (Sigma Chemical Co., St. Louis, MO, U.S.A.) has been added and chromaffin cells were isolated as described previously (Trifaró *et al.*, 1978). The cell preparation thus obtained was layered on a Percoll (Pharmacia) density gradient and chromaffin cells were isolated by centrifugation as described by Trifaró & Lee (1980).

Cell cultures

Chromaffin cells isolated as described above were plated either on 35 mm falcon dishes (release studies) or glass coverslips (electrophysiology studies) previously coated with collagen in Dulbeco modified Eagle's medium (DMEM, Gibco) containing 10% foetal calf serum, ascorbic acid (0.1 mM), glucose (1 mg ml^{-1}), HEPES buffer (3.6 μg ml^{-1}) and the following antibiotics: penicillin (10 μg ml^{-1}), streptomycin (10 μg ml^{-1}), tetracycline (5 μg ml^{-1}), gentamicin (10 μg ml^{-1}) and micostatin (25 units ml^{-1}). The culture medium also contained 5-fluorodioxyuridine 10^{-5} M and cytosine arabinoside 10^{-5} M to prevent fibroblast proliferation by inhibiting cell division. The culture dishes were incubated at 37°C in a humidified incubator (National) and in air + CO_2 (95:5%) atmosphere. The culture medium was changed every 3 days and cultures were also inspected at the same time by phase contrast optics. The cultured cells were used for experiments within 10 days of plating. For further details about chromaffin cell culture systems, see Trifaró & Lee (1980).

Catecholamine release studies

Isotope incubations Thirty-five mm diameter culture dishes containing 7–14 day old chromaffin cell cultures were incubated for 5 min at room temperature with 1 ml/culture dish of amino acid-antibiotic-free DMEM containing [^{3}H]-noradrenaline 10^{-7} M (specific activity: 1 μCi 0.1 mmol^{-1}). After this labelling period, each dish was incubated with 6 changes of 1 ml of Locke solution over a 60 min period before commencing with the actual release. For a more thorough description of this procedure, see Kenigsberg & Trifaró (1980). Work from our laboratory has demonstrated that [^{3}H]-noradrena-

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line, taken up by cultured chromaffin cells, is homogeneously distributed within the cellular amine stores, and upon stimulation, is released concomitantly and in parallel with endogenous amines (Trifaró & Bourne, 1981). Therefore, release of [^{3}H]-noradrenaline from prelabelled chromaffin cells was measured by simply assaying for radioactivity in the incubation medium. The media employed in these release studies were of the following composition (mM): (a) regular Locke solution, NaCl 152, $CaCl_2$ 2.2, $MgCl_2$ 1.2, KCl 2.5, K_2HPO_4 2.15, KH_2PO_4 0.85 and dextrose 10; (b) high K^+ Locke solution contained different amounts of K^+ (5.6–50 mM) (in this solution, NaCl was reduced by an equivalent amount) and (c) regular Locke solution containing quinine (25–400 μM). All solutions were equilibrated with 5% CO_2, and the final pH of each solution was 7.2. Catecholamine release from chromaffin cells was monitored during periods of rest (in the absence of secretagogue) and during periods of stimulation with high K^+. The incubation medium was collected every 3 min and the [^{3}H]-noradrenaline in the media measured in a liquid scintillation spectrometer (Beckman), 15 ml of Aquasol (New England Nuclear) being used per vial as a scintillation cocktail. Statistical significance between the difference of the means was tested by Student's t test.

Calcium flux and uptake studies

Cultured chromaffin cells were washed with amino acid-free, antibiotic-free DMEM for 10 min followed by two 5 min washes with calcium-free regular Locke solution in the absence or in the presence of 200 μM quinine. The cells were subsequently incubated with radiolabelled Ca^{2+} (^{45}Ca = 5 μCi/dish) in 2.2 mM Ca^{2+} Locke solution containing 50 mM K^+ in the absence or in the presence of quinine. The cells were then washed with regular buffered Locke solution. Each wash interval was kept constant at 5 min. The washes were collected and radioactivity within, assayed in vials containing 15 ml of Aquasol in a liquid scintillation spectrometer (Beckman). Once the washing was completed, the cells were collected by scraping them from the dishes with a rubber policeman in 2 ml of H_2O. One ml aliquots of cell scrappings were assayed for radioactivity as described above. Cellular Ca^{2+} uptake was calculated by the peeling method (Aguirre *et al.*, 1977; Kenigsberg & Trifaró, 1980; Kenigsberg *et al.*, 1982). Extracellular Ca^{2+} is removed in the first few washes, while in the later washes intracellular Ca^{2+} that have been taken up by the cells appear in the wash (see Kenigsberg & Trifaró, 1980 and Bourne & Trifaró, 1982 for details). The actual amount of Ca^{2+} that has been accumulated by the cells at the end of the labelling period can be obtained by extrapolating to zero time from the values obtained from the last 6 washes (25–50 min) (Kenigsberg & Trifaró, 1980).

Electrophysiology

The whole-cell patch clamp method (Hamill *et al.*, 1981) was used to study the changes in membrane potential due to quinine and/or high K^+ exposure. Patch pipettes were prepared of Kimax 51 glass and were heat polished. The pipettes had resistances between 2 and 7 MΩ when filled with intracellular solution which consisted of (mM): KCl-KOH 160, HEPES 10, $MgCl_2$ 1, EGTA and $CaCl_2$ 5 at pH 7.2, giving a free Ca^{2+} concentration of 0.01 μM. Bath solutions were (mM): NaCl 154, KCl 2.5, $CaCl_2$ 2.2, $MgCl_2$ 1.2, K_2HPO_4 2.15, KH_2PO_4 0.85 and dextrose 10. Since the chamber volume was < 1 ml, complete change in the bath solution occurs in less than 1 min. All solutions were adjusted to a final pH of 7.2. Experiments were performed at room temperature (20–23°C).

Chemicals

(−)-[ring-2,5,6^{3}H]-noradrenaline and ^{45}Ca were purchased from New England Nuclear, Boston, Mass, U.S.A. Quinine monohydrochloride dihydrate was purchased from Aldrich, Milwaukee, Wisconsin, U.S.A.

Results

Effect of different concentrations of quinine on catecholamine release induced by a depolarizing concentration of potassium

Chromaffin cells labelled as described in the Methods were stimulated for 3 min with 50 mM K^+ in the absence or presence of increasing concentrations (25–400 μM) of quinine. Quinine was added to the medium 18 min before the high K^+ stimulation. Stimulation of cultured chromaffin cells by high K^+ induced a release equivalent to 6.1% of the total catecholamine content (Figure 1). In the presence of quinine, there was a dose-dependent inhibition of the catecholamine release in response to K^+ depolarization. Quinine 25 μM produced a small but significant inhibition of the response to K^+ stimulation (Figure 1). Maximal inhibition (70%) was obtained with quinine 200 μM.

Effects of quinine on the secretory response of chromaffin cells to different extracellular concentrations of potassium

Cultured chromaffin cells were divided into three groups and each group was stimulated with a different K^+ concentration (12.5, 25 and 50 mM) in the absence or presence of quinine 200 μM (Figure 2). When cells were tested in the presence of quinine, the drug was added to the incubation media 18 min before K^+ stimulation. Stimulation of chromaffin cells with either 25 or 50 mM K^+ produced a highly significant secretory response. K^+ 12.5 mM produced a small secretory response. As expected, the increment in catecholamine output was linearly related to the concentration of K^+ in the extracellular medium (Figure 3). In presence of 200 μM quinine in the medium, there was a significant inhibition of K^+-induced catecholamine release for all concentrations of K^+ tested (Figures 2 and 3).

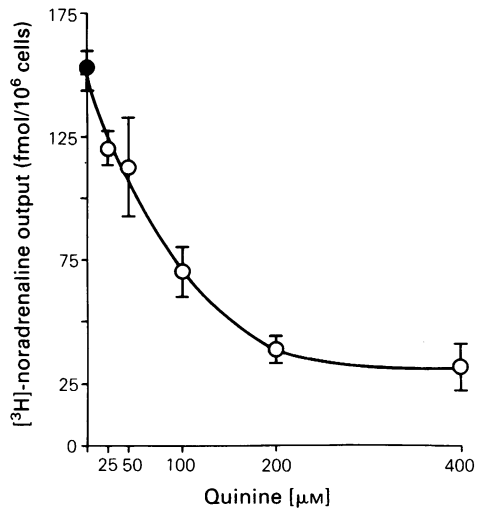

Figure 1 Inhibition of K^+-evoked release of [^{3}H]-noradrenaline from bovine cultured chromaffin cells by quinine. Seven day old cultures were labelled with 10^{-7} M [^{3}H]-noradrenaline for 5 min and were subsequently washed for 60 min (see Methods for details). The cells were stimulated with 50 mM KCl Locke solution in the absence (●) or presence of increasing concentrations of quinine (○). K^+-induced noradrenaline output was determined by subtracting basal release values from those obtained during three consecutive 3 min periods of stimulation with K^+. Each value represents the mean (with s.e. indicated by vertical bars) of 4 separate culture dishes.

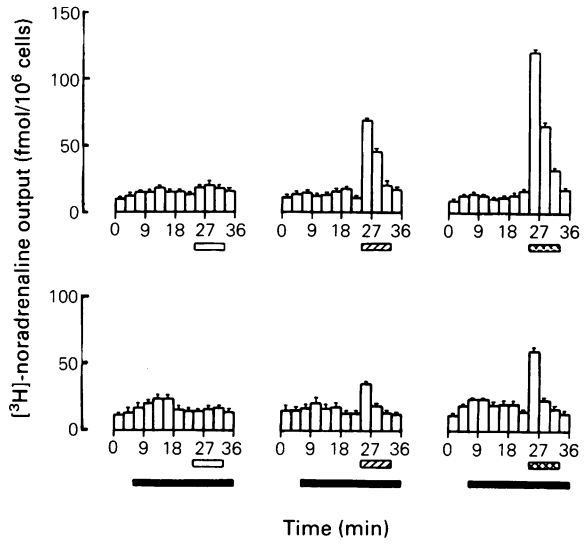

Figure 2 Effect of quinine on the K^+-induced release of [^{3}H]-noradrenaline from bovine cultured chromaffin cells. Cell cultures were labelled and washed as indicated in the legend to Figure 1. Quinine (200 μM, indicated by solid bar) was introduced into the medium 18 min prior to and during the period within which [^{3}H]-noradrenaline output was monitored in response to three different extracellular K^+ concentrations: open bar, 12.5 mM K^+; hatched bar, 25 mM K^+; cross-hatched bar, 50 mM K^+. Each column represents the mean of 4 different culture dishes; s.e. shown by vertical bars.

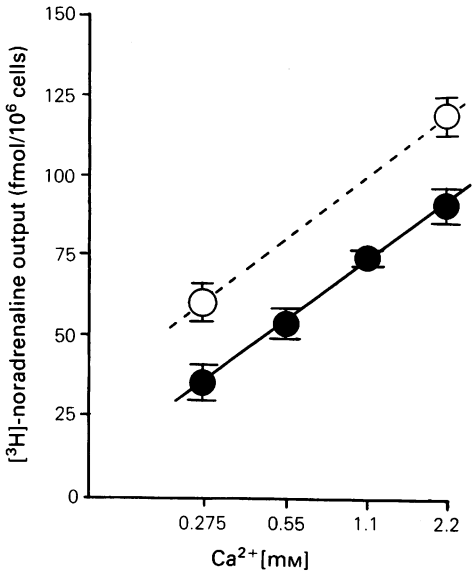

Figure 4 Effect of extracellular concentration of Ca^{2+} on quinine inhibition of K^+-evoked [^{3}H]-noradrenaline release. Bovine chromaffin cell cultures were labelled and washed as indicated in the legend to Figure 1. [^{3}H]-noradrenaline output was stimulated by KCl 50 mM in the absence (○) or presence of quinine 100 μM (●) at the concentration of extracellular Ca^{2+} indicated on the abscissa scale. [^{3}H]-noradrenaline output was determined by subtracting basal release values from those obtained during three consecutive 3 min periods of stimulation with high K^+. Each value represents the mean of 4 separate culture dishes; s.e. indicated by vertical bars.

Inhibitory effects of quinine on potassium-induced catecholamine release at different extracellular concentrations of calcium

Chromaffin cells were stimulated for 3 min with 50 mM K^+ in the presence of different extracellular Ca^{2+} concentrations and the effects of quinine (100 μM) were tested under these experimental conditions (Figure 4). Stimulation of chromaffin cells with 50 mM K^+ produced a secretory response which increased with the increase in the extracellular concentration of calcium (0.275–2.2 mM) reaching a maximum at the extracellular Ca^{2+} concentration of 2.2 mM (Figure 4). The inhibition of the secretory response to high K^+ produced by 100 μM quinine decreased with the increase in the extracellular concentration of Ca^{2+} (Figure 4).

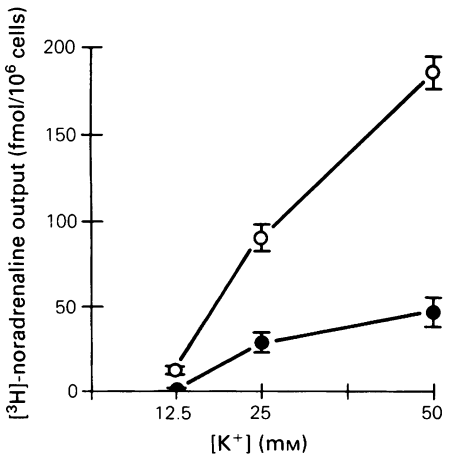

Figure 3 Effects of the extracellular concentration of K^+ on the inhibition produced by quinine. Chromaffin cell cultures were labelled, washed and stimulated as described in the legend to Figure 1. [^{3}H]-noradrenaline release was measured in cells stimulated by increasing concentrations of extracellular potassium in the presence (●) or absence (○) of quinine 200 μM. Each point represents the mean of 4 separate culture dishes; s.e. shown by vertical bars.

Effect of quinine on the depolarization-induced Ca^{2+} uptake and on the Ca^{2+} efflux from cultured chromaffin cells

It has been shown that there is an increase in ^{45}Ca influx into either the perfused adrenal medulla or cultured chromaffin cells (Bourne & Trifaró, 1982) in response to K^+ depolarization and that this increase was completly blocked in the presence of Ca^{2+} channel blocker such as nickel. In the latter experiments as well as in the experiments described here, cellular uptake was calculated by the peeling method (Kenigsberg & Trifaró, 1980; Bourne & Trifaró, 1982). The amount of ^{45}Ca taken up by the cells at the end of labelling period (control vs. stimulation) can be obtained by extrapolating to zero time from the last 6 values (25–50 min) obtained from the efflux (washout) curves (Figure 5). Stimulation of chromaffin cells with 50 mM K^+ for 5 min produced 218% ($n = 4$) increase in Ca^{2+} influx into chromaffin cells (1.67 ± 0.07 nmol $Ca^{2+}/10^6$ cells during 5 min and 5.31 ± 0.09 nmol/10^6 cells during 5 min for controls and high K^+ respectively). Quinine alone did not modify the Ca^{2+} influx under resting conditions (1.66 ± 0.07 nmol $Ca^{2+}/10^6$ cells during 5 min); neither was calcium influx affected when 100 μM quinine was added at the time of K^+ stimulation. However, when quinine was added to the culture dishes 10 min before K^+ stimulation, there was a 54% (3.27 ± 0.06 nmol $Ca^{2+}/10^6$ cells during 5 min, $n = 4$) inhibition of the K^+-evoked Ca^{2+} influx (Figure 5).

Effect of quinine on the membrane resting potential

The resting membrane potentials were stable in individual cells under resting conditions, but ranged from one cell to

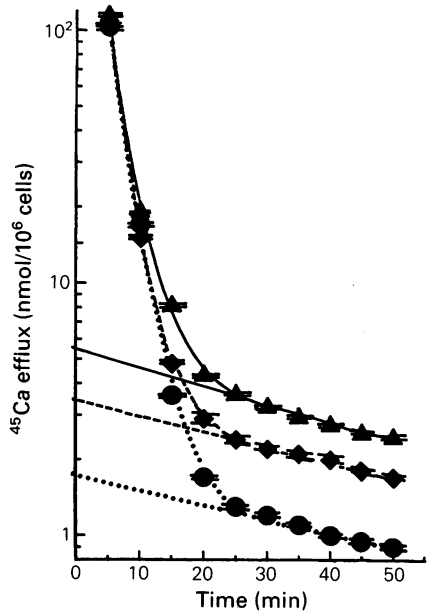

Figure 5 Effects of quinine on ^{45}Ca uptake by and on ^{45}Ca efflux from bovine cultured chromaffin cells during K^+ stimulation. Three groups of 4 dishes each (10^6 cells/dish) were incubated for 10 min in regular Locke solution in the absence or in the presence of quinine (100 μM). This was followed by incubation with radiolabelled calcium (5 μCi ^{45}Ca/dish) in regular Locke solution (●) or in high K^+ Locke solution with (◆) andwithout (▲) quinine for 5 min. Subsequent to labelling, the cells were washed with regular Locke solution for 50 min. Wash intervals were kept constant at 5 min. The washes were collected and ^{45}Ca content within determined as described in Methods. With the values obtained, desaturation curves were plotted (-·-·-, - - -, ——) and the uptake of ^{45}Ca into chromaffin cells was calculated by extrapolating to zero time (ordinate scale) the last 6 values (25–50 min) obtained from the efflux (washout) curves (Kenigsberg & Trifaró, 1980; Bourne & Trifaró, 1982). Each point represents the mean from 4 different culture dishes; s.e. indicated by bars (within size of symbols).

another from −75 to −95 mV (mean = 82 ± 5 mV; n = 11). When quinine was added, the resting membrane potentials decreased (depolarized) (Figure 6) by 2 to 11 mV (8 ± 3 mV; n = 7) in good agreement with previous findings in other systems (see Discussion). Change to high K^+ (50 mM) Locke solution led, as expected, to membrane depolarization (56 ± 7 mV; n = 11). Depolarization was less pronounced (50 ± 6 mV; same 11 cells) if the change to high K^+ occurred in the presence of quinine 200 μM. The difference in membrane depolarization was statistically significant ($P < 0.02$). This suggests that in high K^+ Locke solution, quinine does not depolarize membrane potential as much as in normal Locke solution, or that in fact, it may hyperpolarize the membrane. This was tested directly. Quinine (200 μM) in high K^+ (50 mM)

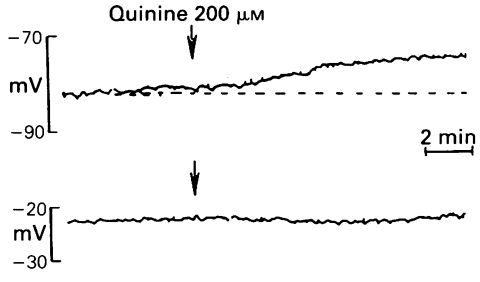

Figure 6 Addition of quinine (200 μM) leads to membrane depolarization if extracellular K^+ concentration is low (5.5 mM; upper trace) but not if extracellular K^+ concentration is high (50 mM; lower trace) in cultured chromaffin cell.

Locke solution did not appear to have any effect on membrane potential (in two cells, membranes were depolarized by 1–2 mV and in another two hyperpolarized by 1–3 mV).

Discussion

The present study clearly shows that quinine markedly, and in a concentration-dependent manner, reduced the catecholamine release induced by a high extracellular K^+. Spontaneous release, on the other hand, was increased ($P < 0.001$, n = 14). Lower resting membrane potential induced by quinine, reported previously in cat spinal motorneurones (Puil & Krnjević, 1976), in *Helix* neurones (Walden & Speckman, 1981), in guinea-pig hippocampal pyramidal neurones (Yoshida *et al.*, 1986) and in pancreatic beta cells (Atwater *et al.*, 1979), can explain greater spontaneous release.

It is possible that quinine reduces the release of catecholamine induced by high K^+ by directly affecting the release process. Nevertheless, this study clearly shows that the reduction must be at least partially due to lower calcium influx. It may be entirely so, although the reduction of the calcium influx by quinine is less pronounced than the reduction of catecholamine release because the relationship between the entry of calcium and release is very nonlinear (Katz & Miledi, 1969).

The calcium influx might have been reduced because quinine affects calcium channels directly. This would not be surprising as several previous reports have shown that quinidine, an isomer of quinine, blocks Ca^{2+} current in frog heart (Ducouret, 1976), mammalian heart muscle fibres (Nawrath, 1981) and in molluscan pacemaker neurones (Hermann & Gorman, 1984), but not in neuroblastoma cells (Fishman & Spector, 1981).

Furthermore, it has been shown that quinine inhibits in a concentration-related fashion, the contractions of smooth muscle induced by both K^+ and acetylcholine and that these inhibitory effects of quinine were readily reversed by increasing the extracellular concentrations of Ca^{2+} (Savage & Akinlalu, 1985). These changes could also have occurred because of direct effects of quinine on calcium channels. Alternatively, calcium influx could have been reduced because quinine blocks K^+ channels.

Earlier reports suggested that quinine is a specific blocker of Ca^{2+}-activated K^+ conductance in red blood cells (Armando-Hardy *et al.*, 1975; Lew & Ferreira, 1978) but that is now known not to be the case (Findlay *et al.*, 1985; Iwatsuki & Petersen, 1985). Quinine is, even at high concentrations (>1 mM), a rather inefficient blocker of Ca^{2+}-activated large conductance K^+ channels (Glavinovic & Trifaró, 1988). In addition, quinine and quinidine are now known to block inward rectifier channels in insulin secreting cells (Findlay *et al.*, 1985) as well as delayed rectifier currents in *Aplysia* (Hermann & Gorman, 1984). Furthermore, quinine appears to block the K^+ transport across the membrane (Christensen & Zeuthen, 1987; Glavinovic & Trifaró, 1988). We did not examine what K^+ channels are affected, but both the lower resting membrane potentials and the diminished sensitivity of the membrane to changes in extracellular K^+ produced by addition of quinine could be explained by a reduction of K^+ permeability by quinine.

Finally, it can be argued that quinine reduces the catecholamine release by blocking the voltage-dependent sodium channels. This, however, can explain lower release only when release is induced by relatively small elevations of extracellular K^+, since in such a case slight depolarization probably induces calcium entry by evoking sodium spikes. When release is induced by high extracellular K^+, greater depolarization directly activates calcium channels while inactivating sodium channels.

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Release of a neutrophil-derived vascoconstrictor agent which augments platelet-induced contractions of blood vessels *in vitro*

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1 The effects of neutrophil-derived products on vascular tone *in vitro* were examined by adding purified rabbit neutrophils to siliconized organ baths containing rings of rabbit thoracic aorta.

2 Neutrophil-derived products induced a concentration-dependent contraction of the blood vessels generating 3.9 ± 0.2 g of tension at a cell concentration of 2×10^6 ml^{-1}. This contractile response was not dependent on an intact endothelium and was not ameliorated by treatment of the neutrophils with inhibitors of lipoxygenase or cyclo-oxygenase enzymes, by free radical scavengers or by the use of end organ antagonists to angiotensin II, desArg^9-bradykinin, histamine, catecholamines or acetylcholine.

3 Neutrophils stimulated with phorbol myristate acetate, formyl methionyl-leucyl-phenylalanine or calcium ionophore A23187 released a contractile factor into the supernatant which produced qualitatively similar contractions compared to those elicited by incubation with intact unstimulated neutrophils.

4 Ten to twenty times more platelets were required to evoke equivalent contractions to those observed with neutrophils. However, neutrophil supernates significantly augmented platelet-mediated contractions ($P < 0.001$).

5 Contractions elicited by neutrophils and supernates derived from activated neutrophils were partially antagonized by 5-hydroxytryptamine (5-HT) receptor antagonists, methysergide and ketanserin. However, the inhibition of exogenous 5-HT-induced contractions on rabbit aorta and rat stomach strips by both antagonists was greater than the inhibition of contractions produced by neutrophils and neutrophil-derived supernates.

6 Extraction of the biologically active material from supernatants of activated neutrophils into acetone, but not chloroform-methanol or ethyl acetate, suggests the contractile factor may be a protein/peptide. Partial purification on a Sephadex G100 column yields a contractile factor with a molecular weight of less than 4,000 daltons.

7 This factor may augment vascular tone, either directly or by interactions with platelets, in pathophysiological states associated with neutrophil activation and accumulation.

Introduction

Neutrophils adhere to the vascular endothelium and accumulate at discrete sites within the circulation in many pathological states including acute inflammatory disorders, myocardial infarction, respiratory distress syndrome, hypertension and endotoxin or haemorrhagic shock (Brigham & Meyrick, 1984; Chobanian *et al.*, 1984; Schmid-Schonbein & Engler, 1986; Mullane, 1988). The interaction of neutrophils and blood vessels may be important in the regulation of vascular function since activated neutrophils release a variety of factors that are capable of influencing vascular tone.

The range of neutrophil-derived factors possessing vasoactive properties include reactive oxygen species, lipid metabolites and proteolytic enzymes. Activated neutrophils generate oxygen-derived metabolites, superoxide anion and hydrogen peroxide (H_2O_2) (Babior, 1984) which may indirectly affect vascular tone by inactivating endothelial-derived relaxing factor (EDRF) (Gryglewski *et al.*, 1986) and by diminishing prostacyclin release (Whorton *et al.*, 1985), respectively. Conversely, H_2O_2 itself may evoke vascular relaxation due to stimulation of guanylate cyclase (Burke & Wolin, 1987). Lipid-derived mediators, platelet-activating factor and 15-hydroperoxyeicosatetraenoic acid provoke endothelial-dependent relaxations of isolated blood vessels (Kamitani *et al.*, 1984; Thomas & Ramwell, 1986). Cathepsin G, a proteolytic enzyme released from neutrophil azurophilic granules, cleaves angiotensinogen to release angiotensin II, which contracts vascular smooth muscle (Wintroub *et al.*, 1984). However, it is not clear whether such factors are released from neutrophils interacting with blood vessels. In addition, some neutrophil-derived mediators including platelet activating factor (PAF), hypochlorous acid, cathepsin G and elastase can augment platelet activation and the release of mediators from platelets (see Mullane 1989, for review). The addition of platelets to organ baths containing rings of blood vessels provokes a contraction, attributed to 5-hydroxytryptamine (5-HT) and thromboxane A_2 (TxA_2; Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986).

The present study was designed to investigate the functional significance of neutrophil/blood vessel interactions and to elucidate the role of various classes of neutrophil-derived factors on vascular tone. We show that neutrophils elicit a concentration-dependent contraction of isolated rabbit aortic rings, which apparently is not mediated by oxygen radicals, arachidonic acid metabolites, catecholamines, acetylcholine, angiotensin II or desArg^9-bradykinin. We also demonstrate that activated neutrophils release a factor into the supernate which contracts vascular rings *in vitro* and potentiates platelet-induced contractions. A thermostable, low molecular weight material of non-lipidic nature which elicits a contractile response in rabbit aortic rings is preliminarily identified.

Methods

Male New Zealand white rabbits (3–3.5 kg) were anaesthetized by an intramuscular injection of ketamine HCl (Ketoset, 50 mg kg^{-1}) and xylazine (Rompum, 8 mg kg^{-1}).

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Assay of vascular smooth muscle contractile activity

The rabbit thoracic aorta was gently removed and placed into ice cold Krebs-bicarbonate buffer (pH 7.4) and cut into 2 mm wide rings. The composition of the Krebs-bicarbonate solution was ($g l^{-1}$): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.280, KH_2PO_2 0.162, $MgSO_4 \cdot 7H_2O$ 0.294, $NaHCO_3$ 2.1 and dextrose 2.0. Aortic rings were mounted in 5 ml silconized water-jacketed organ baths maintained at 37°C and continuously gassed with 95% O_2:5% CO_2. Tissues were equilibrated under 2 g of resting tension for 2 h. The solution in the organ baths was changed at 15 min intervals and basal tension was adjusted as required during the equilibration period. Two grams of basal tension was optimal for concentration-response curves to both phenylephrine and cell extracts. Changes in tension were measured with Grass model FTO3C force transducers coupled to a Hewlett-Packard 775BA chart recorder.

Isotonic contractions of rat fundic strips were used as a bioassay for 5-HT-mediated contractions (Vane, 1957). The fundic portion of the stomach was removed, cut into a strip 4 to 5 cm long, a thread attached to each end, and mounted with a 1.5 g preload in a water-jacketed organ bath maintained at 37°C. The strip was superfused with Krebs-bicarbonate buffer (pH 7.5) containing indomethacin (1 μg ml^{-1}) and scopolamine (1 $\mu g ml^{-1}$) at a flow rate of 4 $ml min^{-1}$. Contractions were monitored via Harvard transducer coupled to a Soltec 1244 recorder.

Preparation of rabbit neutrophils

Anaesthetized rabbits were exsanguinated via a cannula inserted into the abdominal aorta. Blood was collected into two 50 ml syringes each containing 5 ml of sodium citrate (3.15, w/v). The citrated blood was mixed (1.9 v/v) with 6% dextran (mol. wt. 500,000) and erythrocytes were allowed to sediment for 2 h at room temperature. The supernate was centrifuged (180 g) for 5 min and the cell pellet subjected to hypotonic lysis of residual erythrocytes. Leukocytes were washed with 0.9% saline, layered onto a ficoll-hypaque density gradient (Histopaque 1077, Sigma) and centrifuged at 200 g for 20 min. The neutrophil-rich pellet in which 90 to 95% of the cells were neutrophils, was suspended in Hank's buffered saline at a concentration of 1×10^7 to 2×10^7 cells ml^{-1}. Cell suspensions contained $1.75 \pm 0.56 \times 10^7$ neutrophils ml^{-1} with a platelet contamination of $2.5 \pm 0.45 \times 10^7$ platelets ml^{-1} ($n = 4$). Examination of the cell suspension by light microscopy revealed greater than 99% viability as assessed by Trypan blue exclusion.

Preparation of neutrophil-derived contractile material

Aliquots of neutrophil suspensions (10^6–10^7 cells) were placed in conical Eppendorf 1 ml microfuge tubes and incubated with phorbol myristate acetate (PMA, 0.016 and 1.6 μM), formyl methionyl-leucyl-phenylalanine (FMLP, 1 μM) or calcium ionophore A23187 (1.9 μM) for 15 min at room temperature followed by centrifugation for 60 s in an Eppendorf microfuge. Cell supernates were then added to organ baths and changes in tension measured. In other experiments, 200 μl supernate prepared from 10^7 neutrophils stimulated with A23187 was added to the aortic rings before the addition of platelets (5×10^5–1×10^8 ml^{-1}). Protein concentration of cell supernates was measured by the method of Bradford (1976), with bovine serum albumin used as a standard. For purification of the contractile factor, supernates from agonist-stimulated neutrophils (5×10^7–8×10^7) were slowly added to ice cold acetone (1:10 v/v) and stirred for 1 h. Acetone precipitable proteins were filtered, washed with ether, dried under N_2 and reconstituted in Tris buffer (0.1 M, pH 7.4).

The crude neutrophil extract was then applied to a Sephadex G-100 gel filtration column for estimation of molecular weight. The column was equilibrated with 50 mM Tris-HCl/200 mM NaCl buffer (pH 7.4 at 4°C at a flow rate at 1 $ml min^{-1}$). Fractions were collected every 30 s, and the protein concentration monitored spectrophotometrically at 280 nm u.v. Blue dextran was used for determining the void volume of the column and the following protein standards (2 mg each) were used for calibration of molecular weight: bovine serum albumin (mol. wt. 67,000), chymotrypsin (25,000) and ribonuclease A (13,700). At each step of the isolation (acetonic extract, G-100 eluate) activity was verified by bioassay on rabbit aortic rings.

Preparation of washed rabbit platelets

Citrated whole blood was centrifuged at 1000 g for 15 min to obtain platelet-rich plasma. The plasma was then subjected to hypotonic lysis to remove residual erythrocytes. The erythrocyte-free plasma was centrifuged at 1500 g for 1 h, resultant pellets combined, resuspended in 50 ml saline-EDTA, pH 7.4 and centrifuged at 5000 g for 10 min. The pellet was then re-centrifuged at 200 g for 5 min. The low-speed supernate, free of contaminating elements, was then centrifuged at 4000 g for 20 min to obtain a pure platelet pellet. The platelet pellet was re-suspended in saline (pH 7.4) at a final concentration of 5×10^8–8×10^8 platelets ml^{-1} ($n = 8$), which were free of leukocyte contamination.

Experimental protocol

Cumulative concentration-response curves to phenylephrine (10^{-9}–10^{-6} M) were obtained before the addition of neutrophils to assess individual tissue responsiveness. Accordingly, after each experiment, phenylephrine concentration-response curves were repeated and results expressed as a percentage of the maximal phenylephrine response. The maximum tension developed at 10^{-6} M phenylephrine was 5.24 ± 0.12 g ($n = 20$).

After initial phenylephrine concentration-response curves, tissues were washed and neutrophils (1×10^5–2×10^6 ml^{-1}) or platelets (5×10^5–1×10^8 ml^{-1}) were added in a cumulative fashion to the organ baths. Increasing amounts of blood elements were added only after the maximal response was obtained to the preceding concentration.

In studies with receptor antagonists of various vasoactive mediators, tissues were pretreated 15 min before neutrophil addition. The antagonists and the concentrations (μM) used were as follows: saralasin (1.0), ^{8}Leu-desArg-bradykinin (1.0), pyrilamine (0.3), propranolol (2.0), phenoxybenzamine (0.4), scopolamine (0.4), methysergide (0.1) and ketanserin (0.1). In some experiments, neutrophils and/or tissues were pretreated with the cyclo-oxygenase inhibitor, indomethacin (3 μM) or lipoxygenase inhibitors, BW755C (3-amino-1[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline, 50 μM) or REV-5901 (1-pentyl-3-(2-quinolinylmethoxy)benzene methanol 5 μM), or with scavengers of oxygen derived free radicals, superoxide dismutase and catalase (both at 60 u ml^{-1}).

In experiments assaying the contractile activity of material released from activated neutrophils, supernates from vehicle-treated cells and the direct effects of the neutrophil stimulating agents (PMA, calcium ionophore and FMLP prepared in Hanks buffered saline) served as controls.

Statistical analysis

Results are expressed as mean $\pm$s.e. The data were analyzed by a two-way analysis of variance. If statistical differences were noted, multiple comparisons were analyzed by a Bonferroni test. EC_{50} values and maximal responses were evaluated by Student's t test for unpaired data.

Results

Neutrophil-induced contractions of rabbit aorta

The cumulative addition of neutrophils (1×10^5–2×10^6 cells ml^{-1}) to the organ baths elicited concentration-dependent iso-

metric contractions of rabbit thoracic aortic rings (Figure 1a). The initiation of the contractile response occurred within 60 s after the addition of neutrophils and was sustained while the neutrophils were present in the organ bath (>30 min). Upon washout, tension declined back to baseline values. Figure 1b depicts the absolute changes in tone, expressed in g. The same data, expressed as percentage of maximal contraction to a standard agonist (phenylephrine 1 μM) permitted comparisons of neutrophil-derived contractions to those obtained for another vasoactive agent (Figure 1c). The addition of 2×10^6 cells ml^{-1} achieved 80% of the maximal response to PE. The subsequent addition of neutrophil stimulants, FMLP or A23187, did not increase further the neutrophil-mediated responses.

Contamination of neutrophil preparations with platelets

Ficoll-hypaque prepared neutrophils were contaminated with platelets, where platelet:neutrophil ratios varied between 1.4:1 to 2.2:1. Because activated platelets release the vasoconstrictor mediators TxA_2 and 5-HT and the addition of platelet suspensions to organ baths containing rings of canine coronary arteries elicit a contraction (Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986) it was possible that the contractions observed with the neutrophil preparations could be attributed to the contaminating platelets. The cumulative addition of platelets (5×10^5–1×10^8 ml^{-1}) to the suspended aortic rings evoked a concentration-dependent contraction (Figure 2). The EC_{50} was $8 \pm 0.9 \times 10^6$ platelets ml^{-1} which was ~10 fold higher than the concentration of neutrophils required to elicit a similar response ($P < 0.01$, ANOVA), and ~8 fold higher than the number of platelets contaminating the neutrophil suspensions. Moreover, the magnitude and time-course of neutrophil-induced contractions were not influenced by the presence or absence of an intact endothelium, in contrast to the responses observed upon adding platelets (Cohen *et al.*, 1983; Houston *et al.*, 1986). For example, when neutrophils were added to the organ baths containing endothelial intact and denuded vessels, no significant differences in tone were observed (0.3 ± 0.06 vs. 0.5 ± 0.15, 1.0 ± 0.13 vs. 1.3 ± 0.21, 2.4 ± 0.58 vs. 2.4 ± 0.53 g of tension developed in intact and denuded vessels, respectively at 10^5, 2×10^5 and 10^6 neutrophils ml^{-1} added to the organ baths, $n = 4$). Finally, pretreatment of rabbit neutrophils (5×10^6) with

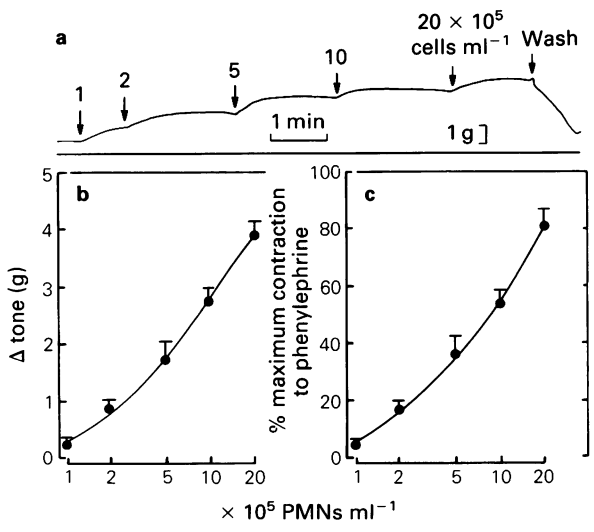

Figure 1 Neutrophil-induced concentration-dependent contractions of rabbit thoracic aortic rings: (a) depicts a typical trace of a cumulative concentration-response curve performed by the addition of purified rabbit neutrophils to organ baths containing arterial rings. The data generated are then expressed as (b) g of tension, or (c) percentage of maximal response to phenylephrine (10^{-6} M, $n = 20$).

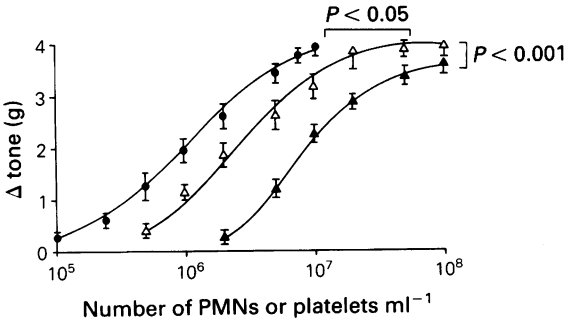

Figure 2 Platelet-induced concentration-dependent contractions of rabbit thoracic aortic rings in the absence (▲) and in the presence (△) of neutrophil supernate (200 μl). For comparison, the concentration-response curve to neutrophils (●) is included ($n = 36$). Each data point represents tension development in response to the platelet suspension alone ($n = 30$) or in the presence of neutrophil supernate ($n = 16$).

indomethacin (3 μM for 15 min) did not impair neutrophil-mediated contractions, yielding 113% of the control response compared to vehicle-treated cells run in parallel incubates ($n = 2$). This observation argues against a role for TxA_2 (or other vasoconstrictor prostanoids) in the contractile response.

Treatment of neutrophils with inhibitors of arachidonic acid metabolism and free radical scavengers

Pretreatment of rabbit neutrophils (5×10^6) for 15 min with inhibitors of arachidonic acid metabolism, BW 755C (50 μM) or REV-5901 (5 μM), did not diminish contractions provoked by 5×10^6 cells, with 100 and 103% of the control response being obtained, respectively ($n = 2$). Furthermore, pretreatment of cells (5×10^6) with scavengers of O_2-derived mediators, superoxide dismutase and catalase (60 u ml^{-1} of each) did not attenuate the neutrophil-induced contractions with treated cells producing 102% of the control response (mean values from two experiments).

Treatment of blood vessels with pharmacological antagonists

Incubation of vessels for 15 min with an antagonist mixture consisting of pyrilamine, propranolol, phenoxybenzamine and scopolamine blocked the contractile response induced by the appropriate agonists added at a concentration of 0.5 μM. However, the antagonists did not attenuate the contractile response to neutrophils added to the organ baths (Figure 3). In addition, treatment of vessels with saralasin and ^{8}Leu-desArg-bradykinin did not impair neutrophil-mediated contractions (i.e. 10^6 cells ml^{-1} added to the organ baths generated 1.2, 1.2 and 1.3 g of tension for control-, saralasin- and ^{8}Leu-desArg-bradykinin-treated vessels, respectively) whereas the responses to angiotensin II and desArg^9-bradykinin (both at 1 μM) were completely abolished. Indomethacin or superoxide dismutase and catalase treatment of aortic rings also did not reduce the cell mediated constrictor responses to 5×10^6 cells ml^{-1} with 4.7, 4.7 and 4.4 g of tension generated for control, indomethacin or superoxide dismutase and catalase-treated vessels (mean values from two experiments each).

In contrast, the 5-HT antagonist, methysergide, attenuated the contractions induced by neutrophils, displacing the cell concentration-response curve to the right with an increase in the EC_{50} values from $5.2 \pm 0.19 \times 10^5$ to $1.04 \pm 0.15 \times 10^6$ neutrophils ml^{-1} ($P < 0.025$, $n = 6$, Figure 4). Similar results were obtained with the 5-HT_2-receptor selective antagonist, ketanserin, which increased the EC_{50} value to $1.01 \pm 0.07 \times 10^6$ cells ml^{-1} ($P < 0.005$, $n = 6$). This represents displacement of the cell concentration-response curves

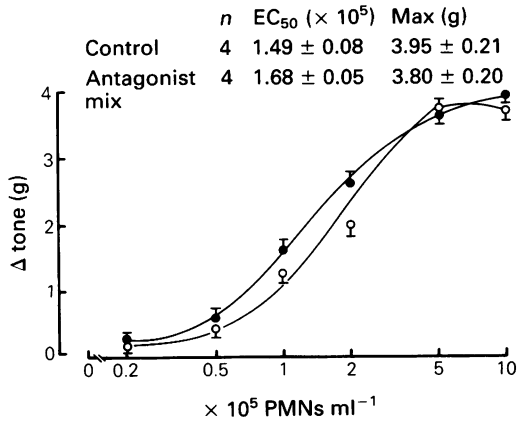

Figure 3 Lack of effect of pyrilamine, phenoxybenzamine, propanolol and scopolamine (○) on neutrophil-mediated contractions of rabbit aortic rings (●, control). The antagonist mix was added to the organ baths 15 min prior to the cumulative addition of neutrophil-enhanced suspensions.

of 2 and 1.9 fold to the right with methysergide and ketanserin, respectively. These results suggest that a portion of the vascoconstrictor activity can be attributed to 5-HT.

Biological activity of supernates from activated neutrophils

To assess if neutrophils can release a mediator that induces contraction of rabbit aortic rings, neutrophils were activated by incubation with an agonist for 15 min, the incubate rapidly centrifuged (1000 *g*, 1 min) and the cell supernates added to the organ baths. Treatment of neutrophils (5×10^6) with three different agonists; PMA (0.016 and 1.6 μM), calcium ionophore A 23187 (1.9 μM) or FMLP (1 μM) provoked the release of a vasoactive substance into supernates, which was not found in vehicle-treated neutrophil supernates (Table 1). Moreover, the direct addition of the neutrophil agonists themselves to the organ baths did not alter vascular tone. In addition, FMLP (1 μM) treatment of neutrophils (5×10^6) evoked the time-dependent release of vasoconstrictor activity into the supernatant (0.8, 1.3 and 2.3 g of tension at 2, 10 and 30 min incubations of neutrophils, $n = 2$). The contractions of the rabbit aorta produced by the cell supernates were not attenuated by the antagonist mixture, but were partially antagonized by ketanserin (Figure 5). This antagonism was characterized by a reduction in the maximal contraction with no change in the EC_{50} value. In contrast, the contractions provoked by 5-HT (5×10^{-7} to 7.5×10^{-6} M) were displaced to the right in parallel by ketanserin and methysergide, with significant

Table 1 Activation of neutrophils (5×10^6) by different agonists evokes the release of a vasoactive material into the supernate

Agonist	*T* (g)	% *phenylephrine response*
Cells (5×10^6) alone (10)	3.3 ± 0.2	57 ± 7.0
Control supernate (5)	0.1 ± 0.1	2 ± 0.5
Phorbol myristate acetate		
0.016 μM (3)	0.4 ± 0.1	13 ± 6.0
1.6 μM (6)	1.6 ± 0.2	25 ± 2.8
Calcium ionophore		
1.9 μM (4)	0.9 ± 0.4	20 ± 3.0
Formyl methionyl-leucyl-phenylalanine 1 μM (4)	0.8 ± 0.2	22 ± 2.0

Values represent mean ± s.e., the number of observations in parentheses.

increases in the EC_{50} values, without diminutions in the maximal response (Figure 6). Moreover, a comparison of equi-constrictor concentrations of 5-HT (3×10^{-6} M) and the neutrophil-derived material (100 μl) were compared in the absence and presence of ketanserin (10^{-7} M). 5-HT produced 2.1 ± 0.3 g of tension which was reduced 93% by ketanserin to

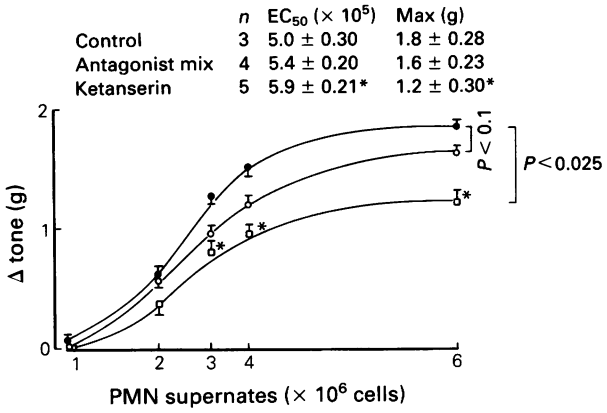

Figure 5 Aortic contractions elicited by supernates from activated neutrophils in the absence (●) and presence of pyrilamine, phenoxybenzamine, propranolol and scopolamine (○) or ketanserin (□). PMNs (1×10^6 to 6×10^6) were incubated with calcium ionophore A23187 (1.9 μM) for 15 min at room temperature, rapidly centrifuged and supernates added to the organ baths in a cumulative fashion. *$P < 0.05$.

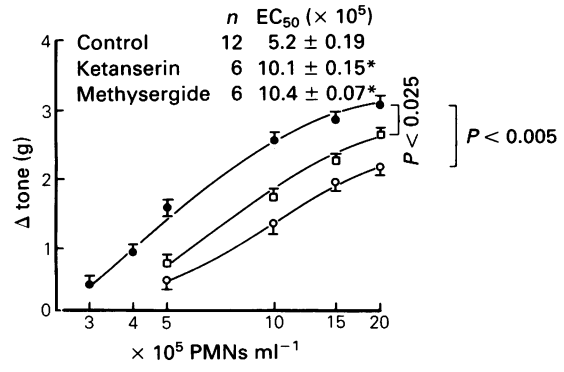

Figure 4 Neutrophil-induced contractions of rabbit thoracic aortic rings in the absence (●) and the presence of ketanserin (□) or methysergide (○). Both 5-hydroxytryptamine receptor antagonists significantly reduced neutrophil-mediated contractions at all concentrations of cells added (ANOVA). *$P < 0.05$ for EC_{50} values (unpaired *t* test).

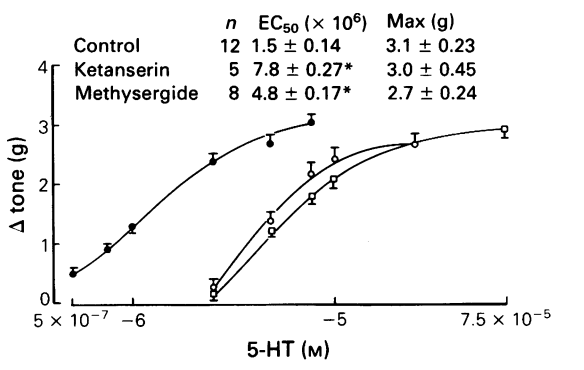

Figure 6 Cumulative dose-response curves to 5-hydroxytryptamine (5-HT) in rabbit aortic rings in the absence (●) and presence of ketanserin (□) or methysergide (○). In the presence of both receptor antagonists, significant differences were noted at all concentrations of 5-HT added. *$P < 0.05$.

Table 2 Comparison of extraction procedures used to isolate the contractile material from neutrophil supernates

	T (g)	% *phenylephrine response*
Ethyl acetate – organic	0	0
– polar	2.0	54
Chloroform : methanol – organic	0.5	7
– polar	1.5	22
Acetone ppt	3.8	61

0.14 ± 0.06 g tension ($n = 7$). In contrast, the neutrophil supernate elicited 2.13 ± 0.31 g of tension by itself, which declined 33% to 1.43 ± 0.37 g of tension with ketanserin ($n = 8$). Similar results were obtained on the rat stomach strip treated with methysergide where the dose ratios for 5-HT and the neutrophil-derived material (the concentrations of agonists required to induce a response of similar magnitude) in the presence and absence of methysergide were approximately 5,000 for 5-HT and 300 for the neutrophil factor. These results imply that a portion of the contractions elicited by cell supernates can also be attributed to 5-HT, as was observed with the neutrophils themselves.

Neutrophil supernates augment platelet-induced contractions

To determine if neutrophil supernates influenced the contractions provoked by washed platelets, 200 μl of supernate prepared from 10^7 neutrophils stimulated with A23187 (1.9 μM) was added to 2 ml organ baths containing aortic rings, followed by the cumulative addition of platelets. The supernate alone increased vascular tone by 0.24 ± 0.05 g ($n = 16$) and potentiated platelet-induced contractions. The concentration of platelets required to elicit 50% maximum response (EC_{50}) was decreased from 8.0 ± 0.9 × 10^6 ml^{-1} to 2.8 ± 0.7 × 10^6 ml^{-1} by the neutrophil supernate ($P < 0.001$; $n = 16$; Figure 2). This EC_{50} value is close to that of neutrophils alone (1.0 ± 0.35 × 10^6 ml^{-1}, $n = 36$), although the difference is still statistically significant ($P < 0.05$). Other volumes of cell supernate were not examined. In contrast the addition of A23187 (0.2 μM) alone did not influence the contractile activity of added platelets.

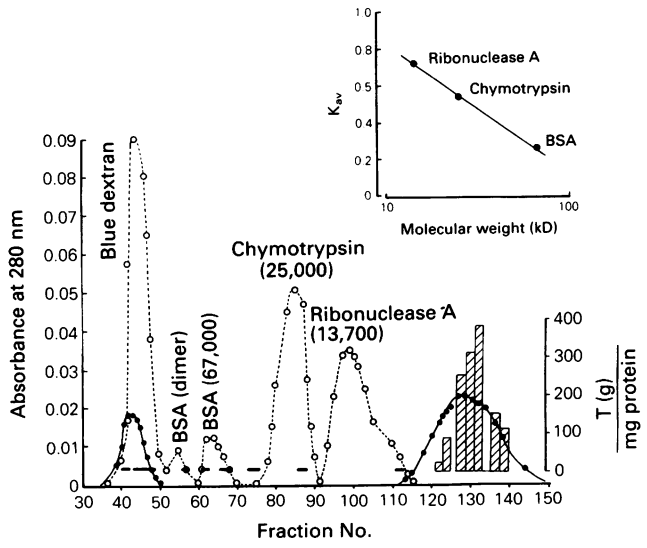

Figure 7 Partial purification of the neutrophil-derived vasoconstrictor material by Sephadex G-100 chromatography. Neutrophil supernates were extracted with cold acetone (1:10) and applied (2 mg protein) to the G-100 column. Peaks of u.v. absorbance from neutrophil supernates (●), molecular weight standards (○). Hatched columns represent column fractions assayed for vasoconstrictor activity in rabbit aortic rings. The biologically active material was eluted between fractions 120 and 140.

Extraction, purification and bioassay of the neutrophil-derived contractile factor

The supernates of neutrophils (1 × 10^7 to 3 × 10^7) activated with PMA (0.016 μM) or A23187 (1.9 μM) were first subjected to lipid extraction with ethyl acetate or chloroform : methanol (2 : 1). Minimal biological activity was observed in the organic phase with most of the activity remaining in the polar phase (Table 2). Conversely, supernatant proteins from activated neutrophils precipitated with cold acetone and reconstituted in Tris buffer (0.1 M, pH 7.4), contained a factor which enhanced vascular tone, suggesting that the mediator is protein-like or is bound to a protein. Boiling of the acetone extract from the supernatants of 5 × 10^7 PMA-activated neutrophils for 15 min resulted in a 22% reduction in biological activity.

Subsequent Sephadex G-100 purification of 2 mg of semi-purified protein (after acetone extraction) yielded 2 peaks of u.v. absorbance at 280 nm (Figure 7). The first peak coelutes with the void volume and is devoid of vasoconstrictor activity. The second peak elutes after the low molecular weight standard, and contains all the vasoconstrictor activity. Consequently, the molecular weight is approximated at less than 4 kD.

Discussion

The present study demonstrates that the addition of rabbit neutrophils to organ baths containing rings of rabbit thoracic aorta provokes a concentration-dependent contraction. The increase in vascular tone is apparently not secondary to neutrophil-mediated tissue damage because rinsing the organ baths with fresh buffer returns tone to basal levels, and concentration-response curves established to phenylephrine before and after the addition of neutrophils do not differ significantly. The neutrophil-mediated contraction of vascular rings is not mediated by eicosanoids or oxygen metabolites derived from neutrophils because neither inhibitors of cyclooxygenase and lipoxygenase enzymes, nor superoxide dismutase and catalase, attenuate the vasconstriction. Moreover, receptor antagonists to acetylcholine, catecholamines, histamine, angiotensin II and des-Arg^9-kinins are without effect. These observations indicate that the vasoconstriction cannot be attributed to products such as 15-HPETE or angiotensin II formed by neutrophils (Thomas & Ramwell, 1986; Wintroub *et al.*, 1984).

The preparation of neutrophils by ficoll-hypaque procedures results in significant platelet contamination. It is well recognized that platelets can provoke vasoconstriction by the formation of TxA_2 and the liberation of 5-HT (Ellis *et al.*, 1976; DeClerck & David, 1981). Consequently, the possibility that the vasoconstriction attributed to the neutrophils was in reality due to contaminating platelets was examined. However, there are a number of inconsistencies with this proposal. First the response was concentration-dependent to neutrophils and independent of the number of contaminating platelets. The number of platelets required to elicit contractions of a similar magnitude were 8 to 10 fold higher than those found in the neutrophil preparations. Moreover, endothelial denudation of the aortic rings did not modify the contractions to the cell suspensions, in contrast to the platelet-mediated response (Houston *et al.*, 1986). Stimulation of neutrophils by FMLP, which has no effect on platelets (Issekutz *et al.*, 1983), evokes the release of vasoconstrictor material. Taken together, these observations do not support a role for platelets as the principal source of mediators of the aortic contractions.

It can be argued that the addition of neutrophils to siliconized organ baths enables the cells to interact with components of the blood vessel or surfaces (silica, metal rings) which they may not encounter *in vivo*. However, useful insights into mechanisms by which platelets affect vascular tone have been provided by similar experimental procedures (Cohen *et al.*, 1983; Houston *et al.*, 1985; 1986). Moreover, this approach permits the observation of responses to labile substances as well as synergistic interactions between different mediators, which may not be reproduced by the exogenous addition of an individual mediator. We assume that the neutrophils are being activated upon addition to the organ baths because the subsequent addition of a stimulus such as FMLP does not augment the response. To investigate further whether neutrophil activation is required for the vascular contractions, the cells were stimulated *in vitro* and the supernates added to the baths. Irrespective of the stimulus used (PMA, calcium ionophore or FMLP), the neutrophil supernates evoked contractions of the aortic rings. However, it should be noted that the contraction elicited by the cell supernates rarely exceeded 50% of that induced by addition of the cells themselves. This may imply that either the physical presence of the neutrophil is required for full expression of the response via direct cell-cell interactions, or that a labile mediator also contributes to the contraction.

Contractions induced by neutrophils or neutrophil-derived supernates are partially antagonized by ketanserin or methysergide, thereby implicating 5-HT as one component of the response. However, there are a number of reasons for concluding that the contractions cannot be attributed solely to 5-HT. In particular, a comparison of the antagonism of 5-HT and neutrophils or neutrophil-derived supernates by ketanserin or methysergide reveals a greater antagonism of the amine than the neutrophil-mediated responses. Endothelial denudation does not modify neutrophil-mediated contractions, whereas it augments those to 5-HT (Cocks & Angus, 1983) and to platelets (Houston *et al.*, 1986). Platelets are the primary source of 5-HT, and, as stated, substantially more platelets are required to elicit similar contractions to those evoked by neutrophils. However, platelet-derived 5-HT may augment the contractions elicited by the neutrophil-derived mediator, as reported for a variety of other vasoconstrictor agents (VanNeuten *et al.*, 1982; Mullane *et al.*, 1982), and could account for a diminution but not abolition of the response to neutrophils.

Another interesting observation is that the neutrophil-derived supernate enhances platelet-induced contractions. Indeed, in the presence of the material from neutrophils, the response to platelets becomes similar to that elicited by the neutrophil suspensions contaminated with platelets (Figure 2). This interaction appears to be synergistic, because stimulating platelets with A23187 after their addition to the organ baths does not increase further the platelet-induced contraction. A variety of interactions between neutrophils and platelets have been described, where neutrophils or neutrophil-derived mediators promote platelet sequestration and activation (see Mullane, 1989, for review). Consequently the presence of both neutrophils and platelets may be required for full expression of the vasoconstrictor activity.

Extraction of the active principle from neutrophil supernates by cold acetone, but not organic solvents, indicates the mediator is not a lipid, and, therefore, unlikely to be PAF. Because the active principle also co-purifies with other proteins released into the supernates, that is the specific activity of the material increases as the proteins are purified (Figure 7), it may imply that the material is either a protein/peptide, bound to a protein, or co-localized with proteins, perhaps within the granules of the neutrophil.

A review of the literature reveals preliminary reports suggesting neutrophils may influence vascular smooth muscle function (Gillespie *et al.*, 1986; Nishida *et al.*, 1986; Carpenter *et al.*, 1987; Mullane & Sessa, 1988; Rimele *et al.*, 1988). In isolated hearts of rabbits perfused with an artificial buffer containing neutrophils, activation of the cells with PMA produced an increase in coronary perfusion pressure, indicative of coronary vasoconstriction which was ameliorated by free radical scavengers (Gillespie *et al.*, 1986). However, in the isolated heart preparation, coronary perfusion pressure is critically dependent upon left ventricular diastolic pressure, which was also increased by the activated neutrophils. Consequently, it is not clear if the neutrophils enhanced coronary vascular resistance by vasoconstriction or secondary to impaired contractility, which may be due to vascular plugging, perivascular oedema or a direct effect on contractility. In isolated lungs of rats perfused with a neutrophil-containing buffer, the addition of PMA increased pulmonary arterial pressure and vascular permeability (Carpenter *et al.*, 1987). In this preparation the combination of superoxide dismutase and catalase attenuated the vascular permeability changes without reducing the pulmonary arterial pressure, indicating that the increase in pulmonary vascular resistance was not secondary to oedema, and may imply that activated neutrophils evoked pulmonary vasoconstriction. Isolation and identification of specific neutrophil-derived mediators will provide direct evidence to help establish a role for neutrophils in modulating vascular smooth muscle tone.

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Assessment of imiloxan as a selective α_{2B}-adrenoceptor antagonist

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1 The α_2-adrenoceptor binding sites of rabbit spleen and rat kidney, labelled with [^{3}H]-rauwolscine, were characterized using a range of subtype selective ligands.

2 In rabbit spleen, the α-$_2$-adrenoceptor binding sites displayed high affinity for oxymetazoline and WB 4101 and low affinity for prazosin and chlorpromazine suggesting the presence of an α_{2A} subtype.

3 There was evidence for heterogeneity of the α_2-adrenoceptor binding sites present in rabbit spleen. The results obtained with oxymetazoline and WB 4101 indicated that at least 75% of the [^{3}H]-rauwolscine binding sites in this preparation displayed a pharmacology consistent with the presence of an α_{2A} subtype.

4 In rat kidney, the α_2-adrenoceptor binding sites displayed high affinity for prazosin and chlorpromazine and low affinity for oxymetazoline and WB 4101 suggesting the presence of an α_{2B} subtype.

5 The inclusion of guanylylimidodiphosphate (Gpp(NH)p, 0.1 mM) did not modify the pharmacology of the α_2-adrenoceptor binding sites present in the two preparations. Furthermore, when the two membrane preparations were combined, the resultant pharmacology was still consistent with the presence of two receptors that retained the characteristics of the α_{2A} and α_{2B} subtypes.

6 Imiloxan was identified as a selective α_{2B} ligand while benoxathian displayed a high degree of selectivity for the α_{2A}-adrenoceptor binding site. The selectivity of imiloxan for the α_{2B}-adrenoceptor binding site, coupled with its specificity for α_2-adrenoceptors, should make it a valuable tool in the classification of α_2-adrenoceptor subtypes.

Introduction

Direct binding studies conducted in several laboratories have now provided evidence for α_2-adrenoceptor binding site heterogeneity (Bylund, 1981; 1985; Cheung *et al.*, 1982; Summers *et al.*, 1983; Neylon & Summers, 1985). On the basis of these receptor binding studies, α_2-adrenoceptors have now been proposed to comprise α_{2A}- and α_{2B}-adrenoceptor subtypes. α_{2A}-Adrenoceptors display relatively low affinity for prazosin and high affinity for oxymetazoline while α_{2B}-receptors display the converse selectivity for these two ligands (Bylund, 1985).

While functional evidence in support of this concept has been limited (Turner *et al.*, 1984), the recent demonstration by Regan *et al.* (1988) that two α_2-adrenoceptor genes exist and that the gene products display differential sensitivity to prazosin and oxymetazoline lends considerable credence to the concept of α_2-adrenoceptor subtypes.

With the renewed interest in α_2-adrenoceptor subtypes (Bylund, 1988), the aims of the present study were two fold. Firstly, to obtain a receptor binding assay for both the α_{2A}- and α_{2B}-adrenoceptor subtypes and, secondly, to determine the α_2-adrenoceptor subtype selectivity of the α_2-adrenoceptor antagonist, imiloxan (RS 21361; Michel & Whiting, 1981). To this end we have characterized the α_2-adrenoceptors present in two tissues which appear to express homogeneous populations of the α_{2A}- and α_{2B}-adrenoceptor subtypes. The rat kidney α_2-adrenoceptor displays high affinity for prazosin relative to other α_2-adrenoceptor binding sites (Neylon & Summers, 1985) and may therefore correspond to the α_{2B}-adrenoceptor. In rabbit spleen, prazosin displays low affinity for α_2-adrenoceptor binding sites (Dickinson *et al.*, 1986) and these receptors may therefore correspond to the α_{2A}-adrenoceptor.

Methods

Membrane preparation

All membrane preparation procedures were conducted at 4°C and used ice-cold buffers. Rat kidney and rabbit spleen were obtained from Pel-Freez (AK). Tissues were homogenized for 30 s in 3 volumes (w/v) of 50 mM Tris, 5 mM EDTA homogenizing buffer (pH 7.4 at 4°C) by a Waring Blender at maximum setting. The homogenates obtained were further homogenized by a Polytron P10 tissue disrupter (setting 10; two 10 s bursts) and filtered through a double layer of cheesecloth. The homogenates were centrifuged at 500 *g* for 10 min and the supernatants reserved. The pellets were resuspended in 2 volumes of homogenizing buffer by the Polytron P10 tissue disrupter and centrifuged at 500*g* for 10 min. The supernatants from this step were combined with the original supernatants and centrifuged for 12 min at 43,500*g*. The crude membrane pellets obtained were washed by resuspending in homogenizing buffer and centrifuging at 43,500*g* for 12 min. The pellets from this step were washed twice, in a similar manner, but with ice-cold 50 mM Tris, 0.5 mM EDTA assay buffer (pH 7.4 at 4°C). The final pellets were resuspended in assay buffer (pH 7.4 at 25°C) and stored under liquid nitrogen until required.

Ligand binding assays

Membranes, [^{3}H]-rauwolscine and competing drugs were incubated in a final volume of 0.25 ml of assay buffer (pH 7.4 at 25°C) for 45 min at 25°C. In competition studies, inhibition curves were obtained by incubating a fixed concentration of radioligand (1–2 nM) and membrane (about 0.20 mg protein per tube) with 10 or 22 concentrations of the competing compound. All experiments were performed in duplicate. In saturation studies, 9 concentrations of [^{3}H]-rauwolscine ranging from 0.1 to 40 nM were used. At each concentration of radioligand, total binding was determined in triplicate and non-specific binding was determined in duplicate. In all experiments non-specific binding was defined with 10 μM phentolamine. Incubations were terminated by vacuum filtration over 0.1% PEI pretreated glass fibre filters by a Brandel cell harvester. The filters were washed for 10 s with ice-cold 0.1 M NaCl. Individual filters were placed in scintillation vials and 4 ml of Aquasol (NEN, Boston, MA) scintillation cocktail was added. The vials were shaken at room temperature for 1 h and radioactivity retained on the filters was determined by liquid scintillation spectrometry.

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Data analysis

Competition and saturation binding data were analysed as described previously by iterative curve fitting techniques (Michel & Whiting, 1988). IC_{50} values were converted to K_i values with the Cheng-Prusoff approximation (1973). In some cases, binding data from competition experiments were fitted to both one-site and two-site models. The one-site and two-site models were compared with the F value defined by the following equation:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}$$

where SS_1 is the residual sum of squares for the single site, SS_2 is the residual sum of squares for the two-site model, df_1 is the degrees of freedom for the single site model and df_2 the degrees of freedom for the two-site model (Munson & Rodbard, 1980). A two-site fit was assumed to be significantly better than a one-site fit if the calculated F value had a $P < 0.05$.

Materials

[^{3}H]-rauwolscine (specific activity 82 Ci mmol^{-1}) was obtained from New England Nuclear. Chlorpromazine, guanylylimidodiphosphate (Gpp(NH)p), noradrenaline and oxymetazoline were obtained from Sigma chemical company, as were all chemicals and reagents used. Phentolamine was obtained from Ciba-Geigy. Prazosin was obtained from Pfizer. Benoxathian and WB 4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane) were obtained from Research Biochemical Inc. Idazoxan, imiloxan, UK 14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) and SFK 104078 (6-chloro-9-[(3-methyl-2-butenyl)oxyl-3-methyl-1H-2,3,4,5-tetrahydro-3]-benzazepine) were synthesized by Dr R. Clark, IOC, Syntex.

Results

Saturation studies

In saturation studies [^{3}H]-rauwolscine labelled homogeneous populations of binding sites in the two preparations examined. The K_D for the radioligand was marginally lower in the rabbit spleen ($K_D = 4.6 \pm 0.8$ nM; $n = 4$) than in the rat kidney ($K_D = 2.2 \pm 0.5$ nM; $n = 4$). B_{max} values were 104 ± 20 and 124 ± 9 fmol mg^{-1} protein in rat kidney and rabbit spleen, respectively. Saturation curves for the two tissues were shown in a previous study (Michel *et al.*, 1989).

Competition studies

The results of competition studies performed with a range of α-adrenoceptor antagonists are presented in Table 1. Several of the compounds examined were able to differentiate between the α_2-adrenoceptors identified in rat kidney and rabbit spleen. Of these, chlorpromazine, imiloxan (Figure 1a) and prazosin (Figure 1b) displayed greater than 10 fold higher affinity for the rat kidney than for rabbit spleen α_2- adrenoceptors ($P < 0.001$, t test), while benoxathian, UK 14304, oxymetazoline (Figure 1a) and WB 4101 (Figure 1b) displayed greater than 3 fold higher affinity for the rabbit spleen than for the rat kidney α_2-adrenoceptors ($P < 0.05$, t test).

Idazoxan and rauwolscine displayed a small (less than a 3 fold) but significant difference in affinity between rat kidney and rabbit spleen α_2-adrenoceptors ($P < 0.05$, t test), while phentolamine and SKF 104078 were not selective.

In rabbit spleen the Hill coefficients for several compounds were significantly less than unity and for both oxymetazoline and WB 4101, the data were better described by assuming the presence of two populations of binding sites (Table 2). Approximately 77% of the sites displayed high affinity for both oxymetazoline and WB 4101. The affinity of oxymetazoline for the remaining sites was similar to its affinity for rat kidney α_2-adrenoceptors. However, for WB 4101 the low affinity value in the rabbit spleen was 3.3 fold lower than the potency of this ligand in rat kidney.

Table 1 Binding parameters of adrenoceptor antagonists at the α_2-adrenoceptor of rat kidney and rabbit spleen

Ligand	*Rabbit spleen* (α_{2A}) pKi/nH	*Rat kidney* (α_{2B}) pKi/nH	*Selectivity ratio*
Benoxathian	7.65 (0.10)	6.37 (0.01)	0.05
	0.88 (0.07)	1.12 (0.05)	
Chlorpromazine	6.05 (0.02)	7.27 (0.04)	16
	1.00 (0.07)	1.05 (0.04)	
Idazoxan	7.12 (0.02)	7.29 (0.02)	1.5
	0.96 (0.11)	0.83 (0.06)	
Imiloxan	5.52 (0.03)	7.26 (0.03)	55
	0.92 (0.05)	0.97 (0.02)	
Noradrenaline	6.37 (0.10)	6.98 (0.03)	4
	0.81 (0.05)	0.78 (0.04)	
Oxymetazoline	8.11 (0.04)	6.60 (0.02)	0.03 (0.01)
	0.74 (0.04)	0.71 (0.02)	
Phentolamine	7.78 (0.06)	7.97 (0.09)	1.5
	0.92 (0.04)	1.02 (0.04)	
Prazosin	5.33 (0.08)	7.22 (0.07)	78
	0.93 (0.08)	0.96 (0.04)	
Rauwolscine	8.06 (0.10)	8.46 (0.10)	2.5
	0.92 (0.11)	0.86 (0.07)	
SKF 104078	7.00 (0.04)	6.96 (0.05)	0.91
	1.03 (0.06)	1.22 (0.03)	
UK 14304	7.46 (0.08)	6.61 (0.01)	0.14
	0.76 (0.04)	0.86 (0.07)	
WB 4101	8.17 (0.09)	7.46 (0.04)	0.19 (0.06)
	0.67 (0.04)	1.02 (0.06)	

The radioligand used was [^{3}H]-rauwolscine (1–2 nM). The values shown are the mean ($\pm$s.e.mean; $n = 4$–9) for the Hill coefficient (nH) and the negative logarithm of the K_i (pK_i). The selectivity ratio shown represents the ratio between the K_i values at the rabbit spleen and rat kidney. The selectivity ratios shown in parentheses for oxymetazoline and WB 4101 represent the ratio between the affinity of these ligands for site 1 in rabbit spleen (Table 2) and their affinity at rat kidney.

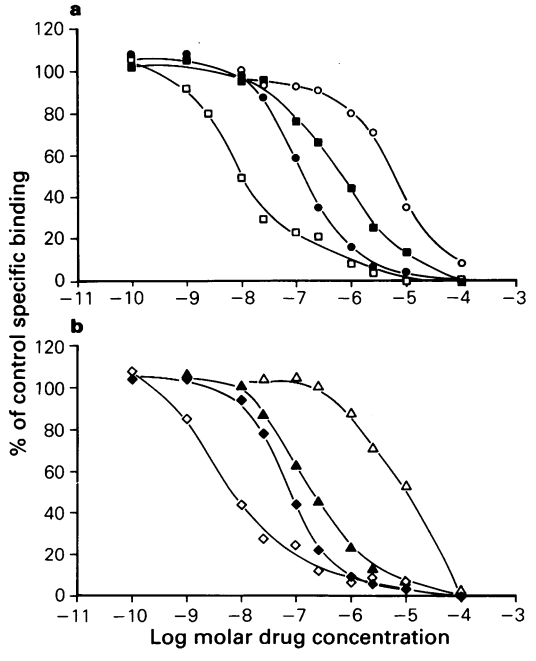

Figure 1 (a) Displacement of [^{3}H]-rauwolscine binding from rabbit spleen (open symbols) or from rat kidney (closed symbols) by either imiloxan (○, ●) or by oxymetazoline (□, ■). (b) Displacement of [^{3}H]-rauwolscine binding from rabbit spleen (open symbols) or from rat kidney (closed symbols) by either prazosin (△, ▲) or by WB 4101 (◇, ◆). In both cases membranes, [^{3}H]-rauwolscine (1–2 nM), and various concentrations of the competitors were incubated for 45 min at 25°C before bound and free ligand were separated by vacuum filtration. The data are from a single representative experiment. In rabbit spleen total binding was 1306 d.p.m. and NSB was 320 d.p.m. In rat kidney total binding was 1112 d.p.m. and NSB was 220 d.p.m.

Table 2 Two-site analysis of the interaction of WB 4101 and oxymetazoline with the α_2-adrenoceptor of rabbit spleen

	Site 1		Site 2	
Compound	pK_i	%	pK_i	%
Oxymetazoline	8.59 (0.06)	79 (5)	6.48 (0.23)	21 (5)
WB 4101	8.69 (0.08)	75 (6)	6.94 (0.17)	25 (6)

The data presented in Table 1 were analysed by assuming the presence of two populations of [^{3}H]-rauwolscine binding sites. The two-site fit was assumed to be significantly better than the single site fit if the determined F value had a $P < 0.05$ (see Methods section). The values shown represent the mean (±s.e.mean, n = 7–8) of the pK_i value or the proportion of [^{3}H]-rauwolscine binding sites displaying the indicated affinity for each ligand.

Combination studies

To determine if some factor present in, or property of the respective membrane preparations was affecting the apparent antagonist affinity estimates in the two tissues, the affinities of both prazosin and imiloxan were determined in assays where rat kidney and rabbit spleen membranes were combined in a 1:1 ratio.

With this approach, both compounds produced shallow inhibition curves that could be best analysed by assuming the existence of two populations of binding sites present in an approximately 1:1 ratio (Table 3; Figure 2). The high and low affinity estimates obtained from this analysis were similar to affinity estimates obtained at the rat kidney and rabbit spleen α_2-adrenoceptors, respectively.

Table 3 Two-site analysis of the interaction of imiloxan and prazosin with the α-adrenoceptor present in rat kidney, rabbit spleen and a 1:1 mixture of rat kidney and rabbit spleen membranes

	Rat kidney	*Rabbit spleen*	*Rat kidney plus rabbit spleen*			
			Site 1		*Site 2*	
Compound	pK_i	pK_i	pK_i	%	pK_i	%
Imiloxan	7.26 (0.03)	5.52 (0.03)	7.14 (0.18)	51 (10)	5.40 (0.13)	49 (10)
Prazosin	7.22 (0.07)	5.33 (0.08)	7.13 (0.20)	46 (3)	5.57 (0.07)	54 (3)

Values represent the mean (±s.e.mean; n = 3) pK_i value or the proportion of [^{3}H]-rauwolscine binding sites displaying the indicated affinity for each ligand. Competition curves to imiloxan and prazosin were determined in a membrane preparation that was comprised of a 1:1 mixture of the rat kidney and rabbit spleen membranes. The data were best described by a model assuming the presence of two populations of binding sites in this preparation. The pK_i values for imiloxan and prazosin in rat kidney or rabbit spleen were from Table 1.

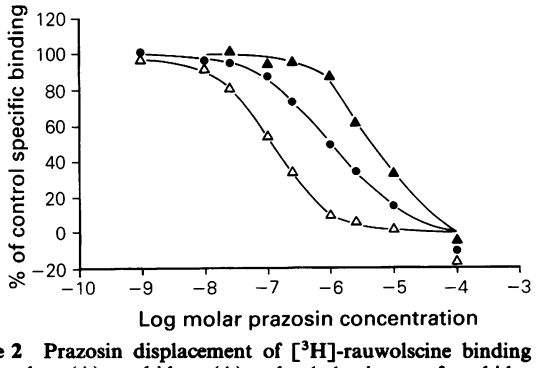

Figure 2 Prazosin displacement of [^{3}H]-rauwolscine binding from rabbit spleen (▲), rat kidney (△) and a 1:1 mixture of rat kidney and rabbit spleen membranes (●). In all cases, membranes, [^{3}H]-rauwolscine (1–2 nM), and various concentrations of the competitors were incubated for 45 min at 25°C before bound and free ligand were separated by vacuum filtration. The data are from a single representative experiment. In rabbit spleen total binding was 1426 d.p.m. and NSB was 380 d.p.m. In rat kidney total binding was 1480 d.p.m. and NSB was 420 d.p.m. In the combination study total binding was 1500 d.p.m. and NSB was 408 d.p.m.

Table 4 Effect of guanylylimidodiphosphate (Gpp(NH)p) on the interaction of ligands with the α_2-adrenoceptors of rat kidney and rabbit spleen

	Rabbit spleen (α_{2A}) pK_i		*Rat kidney* (α_{2B}) pK_i		*Selectivity ratio**	
Ligand	−	+	−	+	−	+
Noradrenaline	6.53 (0.15)	6.23 (0.14)	6.99 (0.05)	6.87 (0.05)	3.3 (1.1)	4.7 (1.1)
Oxymetazoline	8.12 (0.05)	8.14 (0.07)	6.61 (0.02)	6.52 (0.03)	0.03 (0.01)	0.03 (0.01)
WB 4101	8.25 (0.07)	8.24 (0.09)	7.48 (0.02)	7.48 (0.04)	0.17 (0.03)	0.19 (0.04)

The values represent the mean (±s.e.mean, n = 3) for the pK_i value or the selectivity ratio obtained either in the presence (+) or absence (−) of 100 μM Gpp(NH)p. In the absence of Gpp(NH)p, the Hill slope (nH) values for noradrenaline, oxymetazoline and WB 4101 were 0.80 (0.08), 0.74 (0.09) and 0.74 (0.03) respectively, in rabbit spleen, and 0.83 (0.07), 0.71 (0.04) and 1.02 (0.11) respectively in rat kidney. Addition of Gpp(NH)p did not significantly change the slope values.
* Selectivity ratio is the antilog of the difference between the pK_i value obtained in the rat kidney and the pK_i values obtained in the rabbit spleen either in the presence (+) or absence (−) of 100 μM Gpp(NH)p.

Effects of guanyl nucleotides

To examine the possible contribution of affinity states to the differences in ligand affinities seen at the α_2-adrenoceptors of the two tissues, assays in both rat kidney and rabbit spleen were conducted in the presence and absence of the non-hydrolysable GTP analogue, Gpp(NH)p (0.1 mM). As can be seen from Table 4, neither the affinity estimates nor selectivity ratios of noradrenaline, oxymetazoline or WB 4101 were affected by this assay addition.

Discussion

The major aim of the present study was to establish a receptor binding assay for the α_{2A}- and α_{2B}-adrenoceptor subtypes. To this end the rabbit spleen and rat kidney were selected, based upon data generated with prazosin in previous studies. The results obtained support the contention that these two tissues contain predominantly α_{2A}- and α_{2B}-adrenoceptors, respectively, as defined by Bylund (1988). Thus, the relatively high affinity of chlorpromazine and prazosin and the low affinity of oxymetazoline for rat kidney α_2-adrenoceptors indicated that this preparation contained mainly α_{2B}-adrenoceptors, while the converse selectivity of these compounds for rabbit spleen α_2-adrenoceptors suggested that rabbit spleen contained mainly α_{2A}-adrenoceptors.

Indeed, there was good agreement between the 78 and 16 fold selectivity of prazosin and chlorpromazine, respectively, for rat kidney α_2-adrenoceptors compared with rabbit spleen (Table 1), and the 69 and 18 fold selectivity of these compounds for the α_{2B} compared with the α_{2A}-adrenoceptor sites described by Bylund *et al.* (1988). Furthermore, oxymetazoline and WB 4101 were, respectively, 100 and 17 fold selective for rabbit spleen compared with rat kidney in the present study and have been shown to display, respectively, an 83 and 7 fold higher affinity for the α_{2A}-adrenoceptor compared with the α_{2B} sites (Bylund *et al.*, 1988).

Although these data further substantiate the classification of α_2-adrenoceptors into α_{2A}- and α_{2B}-adrenoceptor subtypes, it is possible that the differences in affinity estimates obtained in the two membrane preparations resulted from factors other than receptor heterogeneity. This, however, seemed unlikely

since in rat cortex and human platelet, Kawahara & Bylund (1985) have conducted extensive studies to rule out the role of differential metabolism of the receptors, the presence of soluble factors and membrane environment. Furthermore, in the present study when rat kidney and rabbit spleen membrane preparations were combined, biphasic inhibition curves were obtained for both imiloxan and prazosin. These curves could be resolved into both high and low affinity components and the affinities of prazosin and imiloxan for these two sites were similar to the affinity estimates at the rat kidney and rabbit spleen, respectively. This observation would suggest that differential metabolism of the ligand was not occurring.

The differences in the affinity estimates in rabbit spleen and rat kidney for agonist ligands such as oxymetazoline did not appear to be due to the presence of different proportions of high and low affinity states of the α_2-adrenoceptors, since Gpp(NH)p, a non-hydrolysable GTP analogue, did not modify either the potency or the apparent selectivity of noradrenaline, oxymetazoline or WB 4101 in the absence of Mg^{2+}. This would suggest that the ability of oxymetazoline and WB 4101 to differentiate between α_2-adrenoceptors in the two tissues was independent of affinity states.

On the basis of the present studies, then, it would appear that rabbit spleen α_2-adrenoceptors are predominantly of the α_{2A} subtype while rat kidney α_2-adrenoceptors correspond to the α_{2B} subtype. It should be noted that in rabbit spleen nH values for several compounds were low and indicated possible heterogeneity of the receptors labelled by [^{3}H]-rauwolscine. This was especially true for WB 4101 and oxymetazoline where 75–79% of the sites displayed high affinity for the competing ligand.

The major population of sites presumably represented the α_{2A}-adrenoceptor, since the selectivity of WB 4101 (17 fold) and oxymetazoline (100 fold) for these sites over the rat kidney α_{2B}-adrenoceptors was similar to the 7.5 and 83 fold selectivity of WB 4101 and oxymetazoline, respectively, for the α_{2A}- as opposed to α_{2B}-adrenoceptor (Bylund *et al.*, 1988).

The nature of the second site in rabbit spleen was uncertain. For oxymetazoline and WB 4101, the affinity for the low affinity site was possibly consistent with the presence of an α_{2B}-adrenoceptor. Although, it should be noted that for WB 4101, the low affinity K_i was 3.3 fold lower than the affinity of this compound at the α_{2B}-adrenoceptor of rat kidney. Furthermore, the α_{2B}-selective agents, chlorpromazine and prazosin, could not detect heterogeneity of binding in rabbit spleen even when more detailed 22 point competition curves were constructed (data not shown). With respect to the nature of the second site in rabbit spleen, it is relevant to note that in addition to the two α_2-adrenoceptor subtypes that have been identified to date, several other sites can be labelled by α_2-adrenoceptor ligands. Both high and low affinity binding of [^{3}H]-rauwolscine and [^{3}H]-yohimbine in rat cortex have been demonstrated by several groups (Diop *et al.*, 1983; Michel & Whiting, 1984; Boyajian & Leslie, 1987; Broadhurst *et al.*, 1988), while Bylund (1988) has proposed the existence of a third subtype of α_2-adrenoceptor, the α_{2C}-adrenoceptor. In addition, imidazolines can bind to an imidazoline binding site (Coupry *et al.*, 1987). Whether the second site in rabbit spleen corresponds to any of these other potential sites requires further study.

Several additional agents were also able to differentiate between the two receptor subtypes in rabbit spleen and rat kidney. Benoxathian (Melchiorre *et al.*, 1984), a close analogue of WB 4101, was selective for the rabbit spleen α_{2A}- adrenoceptor, while imiloxan possessed higher affinity for rat kidney α_{2B}- than for rabbit spleen α_{2A}-adrenoceptors.

The observation that imiloxan displayed a high degree of selectivity for the α_{2B}-adrenoceptor should make this compound a useful tool in the study of α_2-adrenoceptor subtypes. This is important at present since the other available subtype selective ligands such as prazosin, chlorpromazine and WB 4101 possess high affinity for other receptor systems. In contrast, imiloxan displays low affinity ($pK_i < 5$) for a wide range of receptors including dopamine, α_1-adrenoceptor, β-adrenoceptor, muscarinic and histamine receptors (A.D. Michel, unpublished observations).

In previous functional studies on the α_2-adrenoceptor in rat and rabbit vas deferens, it is interesting to note that imiloxan displayed high affinity for rat α_2-adrenoceptors but low affinity for rabbit α_2-adrenoceptors (Lattimer & Rhodes, 1985). While it is tempting to speculate that the receptors in rabbit and rat vas deferens correspond to the α_{2A}- and α_{2B}- adrenoceptors identified in the present study, it should be noted that idazoxan was marginally selective in the present study but displayed a 100 fold higher affinity for α_2-adrenoceptors of the rat vas deferens than for those present in rabbit vas deferens (Lattimer & Rhodes, 1985).

With respect to the relationship of the present binding results to functional studies it was interesting to note that SKF 104078, which, in functional studies, has been described as a selective postsynaptic α_2-adrenoceptor antagonist (Ruffolo *et al.*, 1988), was non-selective between the α_{2A}- and α_{2B}-adrenoceptors in the present study. This may indicate, as suggested by Regan *et al.* (1988), that a third subtype of α_2-adrenoceptor exists at which SKF 104078 displays low affinity and which would be the subtype functioning to mediate α_2-adrenoceptor autofeedback at sympathetic nerve terminals.

In conclusion, in the present study the α_2-adrenoceptor present in rabbit spleen has been shown to display a pharmacology consistent with the presence of an α_{2A}-adrenoceptor, while rat kidney α_2-adrenoceptors appeared to be of the α_{2B}-adrenoceptor subtype. Imiloxan displayed a 55 fold higher affinity for α_{2B}- than for α_{2A}-adrenoceptors and therefore represents a useful tool for the further characterization of α_2-adrenoceptor subtypes.

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Release of endothelium-derived relaxing factor from pig cultured aortic endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by a phorbol ester

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1 Cultured aortic endothelial cells of the pig respond to the endothelium-derived relaxing factor (EDRF) they release with an increase in cyclic GMP content. This response is inhibited by haemoglobin or by L-N^G-monomethyl-arginine (L-NMMA), and has been used to investigate the effects of phorbol esters on EDRF release.

2 Pretreatment with phorbol-12,13-dibutyrate (PDB) but not the inactive 4α-phorbol-12,13,-didecanoate (PDD), inhibited increases in cyclic GMP induced by substance P (10^{-8} M) in a time and concentration-dependent manner. PDB did not affect basal cyclic GMP levels.

3 PDB (3×10^{-7} M), but not PDD (3×10^{-7} M), also inhibited ATP (10^{-5} M)-induced increases in cyclic GMP, but did not affect those induced by bradykinin (10^{-7} M).

4 Increases in cyclic GMP induced by low (10^{-7} M) but not high (10^{-6} M) concentrations of the calcium ionophore A23187 were inhibited by PDB (3×10^{-7} M). This inhibitory effect was due to enhanced destruction of EDRF by superoxide anions rather than inhibition of EDRF release, as the inhibition was abolished in the presence of superoxide dismutase (SOD, 30 u ml^{-1}) and catalase (CAT, 100 u ml^{-1}).

5 SOD and CAT did not affect the inhibitory action of PDB on substance P or ATP-induced increases in cyclic GMP.

6 Increases in endothelial cell cyclic GMP content induced by sodium nitroprusside (10^{-5} M) were unaffected by PDB pretreatment.

7 The inhibitory effects of PDB are probably a result of an action of protein kinase C on the steps between receptor occupation and phospholipase C activation.

Introduction

Endothelium-derived relaxing factor (EDRF) has been the subject of intense investigation since it was first described by Furchgott & Zawadzki (1980). Recently attention has focussed on the factors controlling the release of EDRF. Extracellular calcium and ATP have been shown to be necessary for EDRF release (Singer & Peach, 1982; Edwards *et al.*, 1985), and an inhibitory role has been suggested for the intracellular messenger guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Busse *et al.* (1988) have shown that atrial natriuretic peptide (ANP) reduces EDRF release from cultured cells, and this has been confirmed in intact blood vessels by Hogan *et al.* (1989). ANP, like EDRF and the nitrovasodilators, increases cyclic GMP in cultured endothelial cells (Leitman & Murad, 1986; Martin *et al.*, 1988), and it has been suggested that the elevated cyclic GMP levels are responsible for the inhibition of EDRF release (Busse *et al.*, 1988; Hogan *et al.*, 1989). Support for this view comes from the observation that 8-bromo-cyclic GMP inhibits EDRF release from rabbit aorta, and this led to the suggestion that EDRF, by elevating endothelial cell cyclic GMP levels, would inhibit its own release through a negative feedback mechanism (Evans *et al.*, 1988). It has been shown that cyclic GMP inhibits phosphatidyl inositol hydrolysis in vascular smooth muscle (Lewis *et al.*, 1988), and cyclic GMP may inhibit EDRF release by a similar mechanism in endothelium.

Another intracellular messenger has been implicated in the control of EDRF release by the demonstration that phorbol esters can inhibit EDRF release from intact rings of guinea-pig pulmonary artery (Weinheimer *et al.*, 1986) or rabbit aorta (Lewis & Henderson, 1987) and from bovine cultured aortic endothelial cells in a bioassay system (de Nucci *et al.*, 1988). Each of these studies used smooth muscle relaxation as a measure of EDRF release. Tumour promoting phorbol esters mimic the stimulating action of diacylglycerol (DAG) on protein kinase C (Castagna *et al.*, 1982). The study of Lewis & Henderson (1987) was necessarily limited to short exposure times and lower concentrations of phorbol esters, because of their complicating smooth muscle constrictor effects. The study of de Nucci *et al.* (1988) used only a single concentration of phorbol ester, and did not examine the time course of the effects of the phorbol ester. The study of Weinheimer *et al.* (1986) used a range of phorbol ester concentrations, but again did not examine the time course of the effects of the phorbol ester.

Martin *et al.* (1988) demonstrated that cultured aortic endothelial cells of the pig respond to the EDRF they produce with an increase in cyclic GMP levels. This technique has been used in this study to investigate the time course and concentration-dependence of the effects of phorbol esters on agonist-stimulated EDRF release from pig cultured aortic endothelium. The specific inhibitor of EDRF production L-N^G-monomethyl arginine (L-NMMA) has also been used to confirm that the elevation of cyclic GMP in the endothelial cells is due to release of EDRF.

A preliminary account of some of these findings has been published (Smith, 1989a,b).

Methods

Endothelial cell culture

Pig aortae, obtained from a local abattoir, were flushed immediately after removal from the animal (approx 10–15 min after death) with sterile saline containing 200 u ml^{-1} benzylpenicillin and 200 μg ml^{-1} streptomycin. One end of the aorta

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was tied off, and the other end cannulated with a 60 ml syringe containing the same saline. The lumen of the aorta was then filled with saline from the syringe, and the vessel transported to the laboratory, where the endothelial cells were isolated essentially as described by Gordon & Martin (1983). Briefly, the intercostal arteries were ligated, the lumen filled with 0.2% collagenase (type II, Sigma) and the vessel incubated at 37°C for 20 min. The endothelial cells were then harvested into 40 ml of Medium E199 supplemented with 10% foetal bovine serum, 10% newborn bovine serum, glutamine (6 mM), benzyl penicillin (200 u ml^{-1}), streptomycin (200 $\mu g\,ml^{-1}$) and kanamycin (100 $\mu g\,ml^{-1}$). The cells were then seeded into three plates each containing 6 wells (9.6 cm^2). Culture medium was replaced the following day and subsequently every other day, and the cells used once they became confluent, usually within 5–7 days of isolation.

Experimental protocol

The tissue culture medium was aspirated and the endothelial cells washed with 2 × 2 ml of Krebs solution of the following composition (mM): NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The cells were then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. Drug concentrations and exposure times were selected on the basis of previous work by other authors (Martin *et al.*, 1988), and from previous experience with the drugs in intact ring preparations from various species. Drugs were added at the concentrations and times indicated in Results, and at the appropriate time the Krebs solution was rapidly aspirated and the cells immediately extracted with 0.5 ml of ice cold 6% trichloroacetic acid (TCA). The cells were scraped from the well and together with the TCA, aspirated into plastic tubes. Each well was extracted with a second 0.5 ml of ice-cold 6% TCA. The combined extracts were centrifuged at 13,000 r.p.m. for 2 min and the pellet and supernatant separated. The DNA content of the cell pellet was measured by the fluorimetric method of Kissane & Robins (1958). The supernatant was neutralised to approx pH 6 by vortexing for 90 s with 2 ml of 0.5 M tri-n-octylamine in freon (1,1,2 trichlorotrifluoroethane). The cyclic GMP content of the aqueous upper layer was measured by radioimmunoassay with a commercially available kit (New England Nuclear). The cyclic GMP content of each well was expressed as fmol μg^{-1} DNA.

Drugs

Adenosine triphosphate (ATP), calcium ionophore A23187, catalase (bovine liver), haemoglobin (bovine type I), phorbol-12,13-dibutyrate (PDB), 4α-phorbol-12,13-didecanoate (PDD), sodium nitroprusside (SNP), substance P and superoxide dismutase (horseradish) were obtained from Sigma Chemical Co. L-NMMA (L-N^G-monomethyl arginine) was obtained from Ultrafine Chemicals (Manchester). All drugs were dissolved in distilled water except for A23187, which was dissolved in dimethyl sulphoxide (DMSO), and PDB and PDD which were dissolved in ethanol. The final concentrations of these solvents in the medium bathing the cells were 0.002% and 0.02% DMSO for 10^{-7} M and 10^{-6} M A23187 respectively, and 0.003%, 0.03% or 0.3% ethanol for 3×10^{-8} M, 3×10^{-7} M or 3×10^{-6} M PDB or PDD respectively.

Haemoglobin solutions were reduced to the ferrous form with dithionite as described by Martin *et al.* (1986). Sera, culture medium, glutamine and kanamycin were obtained from Flow Laboratories, benzyl penicillin (crystapen) from Glaxo and streptomycin sulphate from Evans Medical Ltd.

Statistical analysis

The resting endothelial cell content of cyclic GMP was found to vary from batch to batch, hence in all experiments each 6 well plate served as its own internal control. For the time course experiments a large number of batches of cells were needed, and it was necessary to normalise the cyclic GMP levels by expressing the cyclic GMP content of each experimental well on a plate as a percentage of the cyclic GMP content of the control well on the same plate. Mean cyclic GMP levels were compared by two way analysis of variance between time points and drug pretreatment. Significant differences between means and the fixed value of 100% were detected by paired comparison, by use of the residual mean square from the analysis of variance and the *t* statistic. In all other experiments, absolute values of cyclic GMP were used, and results are expressed as arithmetic mean ± s.e.mean. Data were analysed by one-way analysis of variance followed by Student Newman-Keuls (SNK) multiple range test to identify significant differences at the $P < 0.05$ level.

Results

Phorbol esters and substance P

Substance P (10^{-8} M, 1.5 min) induced a 17.5 fold increase in the cyclic GMP content of the cultured endothelial cells, which was significantly reduced to a 10.7 fold increase by pretreating the cells for 2 min with phorbol-12,13-dibutyrate (PDB, 10^{-7} M), but was unaffected (21.6 fold increase) by 2 min pretreatment with the inactive 4α-phorbol-12,13-didecanoate (PDD, 10^{-7} M) (Figure 1a). Pretreatment of the endothelial cells

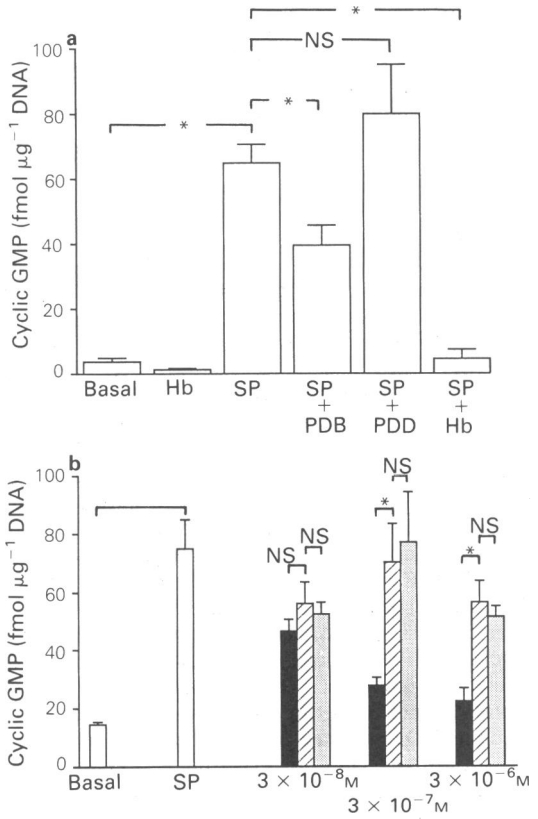

Figure 1 (a) Effects of 2 min pretreatment with phorbol-12,13-dibutyrate (PDB, 10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 10^{-7} M), or 20 min pretreatment with haemoglobin (Hb, 10^{-5} M) on substance P (SP, 10^{-8} M for 1.5 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Results are expressed as the mean of 5 or 6 observations. (b) Effects of 5 min pretreatment with various concentrations of PDB (solid columns), PDD (hatched columns) or the appropriate solvent control (ethanol, EtOH; stippled columns) on SP (10^{-8} M for 1.5 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Concentrations of phorbol esters are indicated under each group of columns. The concentrations of EtOH are 0.003%, 0.03% abd 0.3% for 3×10^{-8} M, 3×10^{-7} M and 3×10^{-6} M phorbols respectively. Results are expressed as the mean of 4 to 8 observations. Vertical bars indicate s.e.mean, NS = not significant, $*P < 0.05$.

with the specific EDRF inhibitor haemoglobin (10^{-5} M) for 20 min before the addition of substance P significantly inhibited the substance P-induced increase in endothelial cell cyclic GMP content (Figure 1a).

To provide confirmation that the increases in cell cyclic GMP content were due to EDRF release, cells were incubated with the competitive inhibitor of EDRF synthesis, L-N^G-monomethyl arginine (L-NMMA, 5×10^{-5} M) (Palmer *et al.*, 1988), for 15 min before the addition of substance P. This concentration of L-NMMA inhibited the substance P-induced increase in cyclic GMP by 55% (Table 1).

As 10^{-7} M PDB for 2 min only produced 41% inhibition of the response, the higher concentration of 3×10^{-7} M was used for all subsequent experiments with the phorbol esters. The time course of the effects was investigated in a series of experiments using different pretreatment times (Figure 2). Pretreatment with PDD (3×10^{-7} M) produced a statistically significant ($P < 0.05$) initial inhibition of the substance P (10^{-8} M)-induced increases in cyclic GMP at the 30 s time point, but no inhibition at later time points. The reason for this effect of PDD at 30 s is not known. Increasing pretreatment time with the active PDB (3×10^{-7} M) produced a progressive inhibition of the response to substance P (10^{-8} M) which became significant at 1 min pretreatment ($P < 0.01$ at 1 and 2 min, $P < 0.001$ at 3, 5 and 10 min cf. 100%), and after 3 min prevented significant elevation of cyclic GMP above basal levels by substance P (10^{-8} M). Five minutes was chosen as the pretreatment time for all subsequent experiments.

Preatreatment for 5 min with 3×10^{-7} M PDB or PDD, or the equivalent concentration of the solvent, ethanol (0.03%), had no effect on basal levels of cyclic GMP in the cells (Table 2).

The effects of 5 min pretreatment with PDB on substance P-induced increases in cyclic GMP levels were concentration-dependent (Figure 1b). When the response to substance P in the presence of PDB was compared with the response in the presence of the same concentration of PDD (after subtraction of basal levels), there was no inhibition of the substance P-induced increase with 3×10^{-8} M PDB, 76.4% inhibition with 3×10^{-7} M PDB ($P < 0.05$) and 81.3% inhibition with 3×10^{-6} M PDB ($P < 0.01$). The response in the presence of PDD is the most appropriate control, as both PDD and ethanol are present. At each concentration of phorbol ester used, the effect of the solvent (ethanol) alone was tested. There were no significant differences between the substance P-induced increases in cyclic GMP in the presence of any of the concentrations of ethanol or PDD in ethanol, hence only solvent plus PDD was examined in all subsequent experiments.

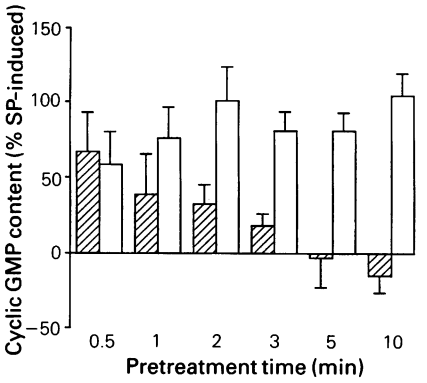

Figure 2 Time course of the effects of phorbol ester pretreatment on substance P (SP)-induced increases in pig aortic endothelial cell cyclic GMP content. The SP (10^{-8} M, 1.5 min)-induced increases in the cyclic GMP content of the cells are normalised to 100%. Zero cyclic GMP content is equivalent to basal levels. The pairs of columns show the effects on the SP-induced increase of pretreatment with phorbol-12, 13-dibutyrate (PDB, 3×10^{-7} M, hatched columns) or 4α-phorbol-12, 13-didecanoate (PDD, 3×10^{-7} M, open columns) for the times indicated under each pair of columns. Results are expressed as the mean of 7 or 8 observations, vertical bars indicate s.e.mean.

The inhibitory effect of PDB on the substance P-induced increases in endothelial cell cyclic GMP levels was also examined in the presence of superoxide dismutase (SOD, 30 u ml^{-1}) and catalase (CAT, 100 u ml^{-1}). Substance P (10^{-8} M, 1.5 min) produced a 5 fold increase in cyclic GMP content of the cells (from 13.1 ± 1.1 to 66.1 ± 12.5 fmol cyclic GMP μg^{-1} DNA, $n = 9$) in the presence of SOD and CAT, which was significantly ($P < 0.001$) reduced to 2.7 fold by 5 min PDB (3×10^{-7} M) pretreatment (to 35.8 ± 3.7 fmol cyclic GMP μg^{-1} DNA, $n = 9$), but unaffected (5.4 fold) by 5 min PDD (3×10^{-7} M) pretreatment (70.1 ± 6.1 fmol cyclic GMP μg^{-1} DNA, $n = 9$). The effect of substance P (with SOD and CAT) was completely inhibited by 20 min haemoglobin (10^{-5} M) pretreatment (4.4 ± 1.2 fmol cyclic GMP μg^{-1} DNA, $n = 9$). The 5 fold increase in cyclic GMP produced by substance P (10^{-8} M) in this experiment appears at first to be considerably less than the 17.5 fold increase produced by substance P (10^{-8} M) in the absence of SOD and CAT (Figure 1). However, this is almost entirely due to the higher resting levels of cyclic GMP in this experiment in the presence of SOD and CAT (13.1 ± 1.1 vs 3.7 ± 1.1 in Figure 1), as the stimulated values in the presence and absence of SOD and CAT are similar (66.1 ± 12.5 and 64.7 ± 5.9 respectively). It is not clear whether these higher resting levels in the presence of SOD and

Table 1 Effect of L-N^G-monomethyl arginine pretreatment on substance P-induced increases in pig aortic endothelial cell cyclic GMP content

Group	*Stimulus*	*Preatreatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (control)	None	18 ± 1.8	8	
2	SP, 10^{-8} M	None	40.6 ± 2.6	8	$P < 0.001$ cf group 1
3	SP, 10^{-8} M	L-NMMA, 5×10^{-5} M	28.1 ± 3.1	9	$P < 0.05$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. The other two groups were exposed to substance P (SP, 10^{-8} M) for 1.5 min, with or without 15 min pretreatment with 5×10^{-5} M L-N^G-mono methyl arginine (L-NMMA).

Table 2 Effect of phorbol esters on the basal levels of cyclic GMP in pig aortic endothelial cells

Group	*Treatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None	18 ± 1.7	8	No significant
2	PDB, 3×10^{-7} M	20 ± 6.2	9	differences between
3	PDD, 3×10^{-7} M	26.5 ± 5.5	9	any of the groups
4	Ethanol, 0.03%	15.9 ± 2.7	9	

Group 1 shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. Groups 2, 3 and 4 were respectively exposed to phorbol-12,13-dibutyrate (PDB), 4α-phorbol-12,13-didecanoate (PDD) or the equivalent concentration of the solvent ethanol (0.03% final concentration) for 5 min.

CAT are due to enhanced effects of basally released EDRF (because its half-life is prolonged), or variation between batches of cells, as resting levels were found to vary between batches.

Phorbol esters and ATP

PDB produced a similar inhibition of the responses of the endothelial cells to adenosine 5'-triphosphate (ATP; Figure 3). ATP (10^{-5} M, 3 min) induced a 3.2 fold increase in endothelial cell cyclic GMP content that was prevented by PDB pretreatment (3×10^{-7} M, 5 min, $P < 0.05$), but was unaffected by PDD pretreatment (2.2 fold increase; 3×10^{-7} M, 5 min, $P > 0.1$). Haemoglobin pretreatment (10^{-5} M, 20 min) also prevented the ATP-induced increase in cyclic GMP ($P < 0.05$). Similar results were seen in the presence of SOD (30 u ml^{-2}) and CAT (100 u ml^{-1}) where ATP (10^{-5} M, 3 min) induced a 2.8 fold increase in cyclic GMP (from 19.4 ± 2.3 to 55.1 ± 3.4 fmol μg^{-1} DNA, $n = 6$) which was significantly ($P < 0.05$) reduced to a 1.5 fold increase (28.3 ± 5.8 fmol μg^{-1} DNA, $n = 6$) by PDB pretreatment (3×10^{-7} M, 5 min), but unaffected (3 fold increase, 58.5 ± 10.1 fmol μg^{-1} DNA, $n = 5$, $P > 0.7$) by PDD pretreatment (3×10^{-7} M, 5 min). The ATP-induced increase in cyclic GMP (with SOD and CAT) was prevented by haemoglobin pretreatment (10^{-5} M, 20 min, 4.5 ± 0.8 fmol μg^{-1} DNA, $n = 5$, $P < 0.001$).

Phorbol esters and bradykinin

The experiments with bradykinin were only done in the presence of SOD and CAT. Bradykinin (BK, 10^{-7} M, 1.5 min) induced a significant ($P < 0.001$) 3.4 fold increase in endothelial cell cyclic GMP content that was not affected by pretreatment with PDB (3.1 fold increase) or PDD (3 fold increase) (Table 3). However, the BK-induced rise in cyclic GMP was significantly ($P < 0.001$) reduced to a 1.5 fold increase by 20 min pretreatment with 10^{-6} M haemoglobin.

Phorbol esters and sodium nitroprusside

Sodium nitroprusside (10^{-5} M, 3 min) induced a 6.4 fold increase in endothelial cell cyclic GMP content, that was prevented by 20 min haemoglobin (10^{-5} M) pretreatment (Table 4). The response to sodium nitroprusside was not inhibited by 5 min pretreatment with 3×10^{-7} M PDB or PDD (Table 4).

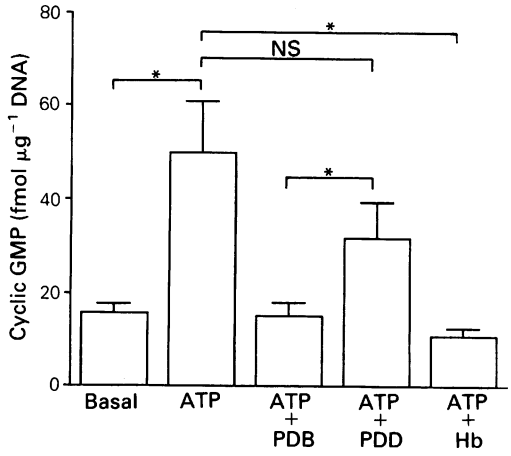

Figure 3 Effects of 5 min pretreatment with phorbol-12,13-dibutyrate (PDB, 3×10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 3×10^{-7} M), or 20 min pretreatment with Hb (10^{-5} M) on adenosine trisphosphate (ATP, 10^{-5} M for 3 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Results are expressed as the mean of 6 observations, vertical bars indicate s.e.mean, NS = not significant, $^*P < 0.05$.

Phorbol esters and calcium ionophore

A23187 (calcium ionophore, 10^{-7} M, 3 min) induced a 6.3 fold increase in endothelial cell cyclic GMP content that was significantly ($P < 0.05$) reduced to 4.2 fold by PDB pretreatment (3×10^{-7} M, 5 min), but unaffected (6.3 fold increase) by PDD pretreatment (3×10^{-7} M, 5 min; Figure 4). The response to 10^{-7} M A23187 was completely inhibited by 20 min haemoglobin pretreatment (10^{-5} M, $P < 0.001$). The response to the higher concentration of 10^{-6} M A23187 (3 min) was much greater, producing a 13.1 fold increase in endothelial cell cyclic GMP content that was unaffected by 5 min PDB (3×10^{-7} M,

Table 3 Effect of phorbol esters or haneoglobin pretreatment on bradykinin-induced increases in pig aortic endothelial cell cyclic GMP content in the presence of superoxide dismutase and catalase

Group	*Stimulus*	*Pretreatment* (30 u ml^{-1} SOD + 100 u ml^{-1} CAT in all groups)	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (control)	None	41.3 ± 3.2	5	
2	BK, 10^{-7} M	None	141.7 ± 6.6	5	$P < 0.001$ cf group 1
3	BK, 10^{-7} M	PDB, 3×10^{-7} M	129.5 ± 6.6	5	NS cf group 4
4	BK, 10^{-7} M	PDD, 3×10^{-7} M	123.1 ± 9.5	5	NS cf group 2
5	BK, 10^{-7} M	Hb, 10^{-6} M	61.3 ± 4.8	5	$P < 0.001$ cf group 2

Group 1 (control) show mean ± s.e.mean resting levels of cyclic GMP in endothelial cells treated with SOD (30 u ml^{-1}) and CAT (100 u ml^{-1}) for 6.5 min. All other groups received SOD and CAT 5 min before the addition of bradykinin (BK). Hb was added 20 min before BK, and PDB or PDD added 5 min before BK. Groups 2 to 5 were exposed to BK (10^{-7} M) for 1.5 min following the various pretreatments indicated. NS = not significant.

Table 4 Effects of phorbol esters or haemoglobin pretreatment on sodium nitroprusside-induced increases in pig aortic endothelial cell cyclic GMP content

Group	*Stimulus*	*Pretreatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (Controls)	None	15.6 ± 1.6	12	
2	SNP, 10^{-5} M	None	100.4 ± 9.2	6	$P < 0.001$ cf group 1
3	SNP, 10^{-5} M	PDB, 3×10^{-7} M	86 ± 10.7	6	NS cf group 4
4	SNP, 10^{-5} M	PDD, 3×10^{-7} M	78.9 ± 3.2	6	NS cf group 2
5	SNP, 10^{-5} M	Hb, 10^{-5} M	10.1 ± 1.1	6	$P < 0.001$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. The other groups were exposed to sodium nitroprusside (SNP, 10^{-5} M) for 3 min, with or without 5 min pretreatment with PDB or PDD, or 20 min pretreatment with Hb. Abbreviations as in Table 3.

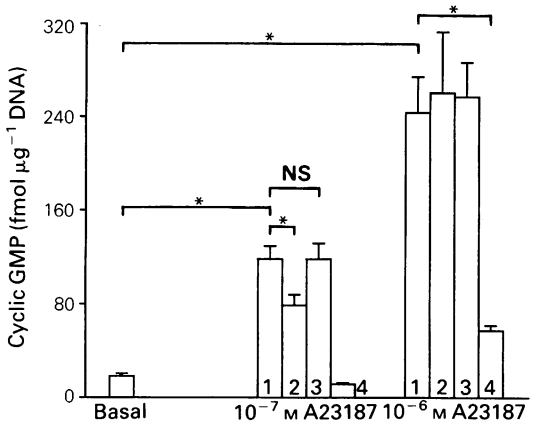

Figure 4 Effects of 5 min pretreatment with phorbol-12,13-dibutyrate (PDB, 3×10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 3×10^{-7} M), or 20 min pretreatment with Hb (10^{-5} M) on calcium ionophore (A23187, 10^{-7} or 10^{-6} M for 3 min)-induced increases in cyclic GMP content. In each group of 4 columns, (1) represents A23187 treated cells, (2) A23187 + PDB, (3) A23187 + PDD, (4) A23187 + Hb. Concentrations of A23187 are shown under each group of columns. Results are expressed as the mean of 6 or 12 observations, vertical bars indicate s.e.mean, NS = not significant, $*P < 0.05$.

14 fold increase) or PDD (3×10^{-7} M, 13.8 fold increase) pretreatment. The response to 10^{-6} M A23187 was significantly reduced ($P < 0.01$) to a 3 fold increase by 20 min pretreatment with haemoglobin (10^{-5} M) (Figure 4).

In the presence of SOD (30 u ml^{-1}), 5 min pretreatment with 3×10^{-7} M PDB no longer inhibited the response to 10^{-7} M A23187 (3 min; 1.7 fold increase in cyclic GMP) but tended to increase the response (2.2 fold increase in cyclic GMP), although the effect just failed to reach significance (Table 5). Pretreatment with PDD (3×10^{-7} M, 5 min) was without effect (1.9 fold increase in cyclic GMP), and 20 min pretreatment with haemoglobin (10^{-5} M) completely inhibited ($P < 0.01$) the response to 10^{-7} M A23187 (Table 5).

In the presence of SOD (30 u ml^{-1}) plus CAT (100 u ml^{-1}), A23187 (10^{-7} M, 3 min) produced a 1.7 fold increase in endothelial cell cyclic GMP that was unaffected by 5 min pretreatment with 3×10^{-7} M PDB (1.8 fold increase) or PDD (1.7 fold increase). In the presence of SOD and CAT the response to 10^{-7} M A23187 was completely inhibited ($P < 0.01$) by 20 min haemoglobin (10^{-5} M) pretreatment. Thus the addition of CAT had no significant additional effect to that of SOD alone.

Discussion

Agonist-induced elevation of porcine cultured endothelial cell cyclic GMP levels has previously been shown to be due to EDRF released from those cells stimulating soluble guanylate cyclase (Martin *et al.*, 1988). The inhibition by L-NMMA of substance P-induced increases in endothelial cell cyclic GMP content obtained in this study provides further evidence that the increase in cyclic GMP is due to EDRF release. This technique has been used in the present study to investigate the effects of phorbol esters on EDRF release.

Figure 1 shows that 10^{-7} M PDB but not the inactive 4α-PDD inhibited the elevation of cyclic GMP levels in pig cultured aortic endothelial cell, induced by substance P. These results in cultured endothelial cells from a different species (pig rather than rabbit) are in agreement with the observations of Lewis & Henderson (1987) that PDB inhibits substance P-stimulated EDRF release from intact rings of rabbit aorta. The present study extends these observations, showing that the inhibitory effects of PDB are both time and concentration-dependent. These results also agree with the observations of de Nucci *et al.* (1988) in bovine cultured aortic endothelial cells.

The mechanism by which phorbol esters, through stimulation of protein kinase C, inhibit receptor-mediated release of EDRF has yet to be established. Ryan *et al.* (1988) have shown that phorbol myristate acetate inhibits agonist-induced increases in endothelial cell free intracellular calcium. Brock & Capasso (1988) have further shown that a number of activators of protein kinase C inhibited agonist-induced inositol 1,4,5-triphosphate (IP_3) formation and increases in free intracellular calcium in endothelial cells. The inhibitory effects of phorbol esters probably result from an action of protein kinase C on the steps linking receptor occupation to phospholipase C activation. However, other possible actions of phorbol esters need to be excluded.

Endothelial cells have recently been shown to generate and secrete superoxide anions (Rosen & Freeman, 1984), which are known to inactivate EDRF (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). Matsubara & Ziff (1986) have demonstrated that the phorbol ester, phorbol myristate acetate, increases superoxide anion production by endothelial cells. Hence increased superoxide anion production and destruction of EDRF may be a potential mechanism for the inhibitory effects of PDB shown in Figures 1 and 2. However, PDB was still able to exert its inhibitory effects in the presence of SOD and CAT, and PDB did not inhibit bradykinin-induced EDRF release, hence it is unlikely that PDB inhibits substance P-induced EDRF release by enhancing superoxide anion production. The concentration of SOD used (30 u ml^{-1}) would be sufficient to remove phorbol ester-induced superoxide production, as the same concentration of SOD was able to reverse the synergistic production of superoxide by phorbol and ionophore (Figure 4). The inhibitory effects of PDB on substance P-induced EDRF release are also unlikely to be due to antagonism of substance P at its receptors, as PDB similarly inhibited ATP-induced EDRF release in the presence or absence of SOD and CAT.

SOD, CAT and indeed haemoglobin, are all large molecules which are unable to enter the cell, and can only interact with superoxide anions, hydrogen peroxide and nitric oxide respectively, when they are in the extracellular space. However, this

Table 5 Effect of phorbol esters or haemoglobin pretreatment on A23187-induced increases in pig aortic endothelial cell cyclic GMP content in the presence of superoxide dismutase (SOD)

Group	*Stimulus*	*Pretreatment* (30 u ml^{-1} SOD in all groups)	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (Control)	None	27 ± 6.7	6	
2	A23187, 10^{-7} M	None	46.7 ± 4.6	6	$P < 0.05$ cf group 1
3	A23187, 10^{-7} M	PDB, 3×10^{-7} M	60.4 ± 6.8	6	NS cf group 4
4	A23187, 10^{-7} M	PDD, 3×10^{-7} M	51.1 ± 5.7	6	NS cf group 2
5	A23187, 10^{-7} M	Hb, 10^{-5} M	10.9 ± 2.2	6	$P < 0.001$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in endothelial cells treated with SOD (30 u ml^{-1}) for 8 min. All other groups received SOD 5 min before the addition of A23187. Hb (10^{-5} M) was added 20 min before A23187, and PDB or PDD added 5 min before A23187. Groups 2 to 5 were exposed to A23187 (10^{-7} M) for 3 min following the various pretreatments indicated. Abbreviations as in Table 3.

does not mean that they are unable to affect the concentrations of these agents inside the cell. Haemoglobin for example will avidly bind extracellular nitric oxide, producing a steep concentration gradient out of the cell, increasing the net rate of diffusion of nitric oxide out of the cell, and hence effectively lowering the intracellular nitric oxide concentration. Whether or not superoxide anions cross endothelial cell membranes, and if so how, is at present unclear. However, it is clear from bioassay experiments that addition of extracellular SOD prolongs the half-life of released EDRF (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986).

Protein kinase C has been shown to phosphorylate rat brain soluble guanylate cyclase *in vitro* (Zwiller *et al.*, 1985). However, the inhibitory effects of PDB in this study cannot be explained by an action on soluble guanylate cyclase (which is stimulated by EDRF), as the elevation of endothelial cell cyclic GMP levels by sodium nitroprusside was unaffected by PDB pretreatment. Sodium nitroprusside generates nitric oxide spontaneously in solution (Feelisch & Noack, 1987) and thus stimulates soluble guanylate cyclase directly (Braughler *et al.*, 1979). EDRF and sodium nitroprusside will stimulate the same soluble guanylate cyclase, as the active principle in both cases is nitric oxide, a freely lipid permeable gas capable of reaching all parts of the cell.

The calcium ionophore, A23187, is thought to act by directly translocating calcium ions across the cell membrane. Its effects are not therefore subject to the same intracellular regulatory mechanisms as receptor-mediated agonists. However, in the absence of SOD or CAT, PDB pretreatment inhibited the increase in cyclic GMP levels produced by 10^{-7} M A23187, but not that produced by 10^{-6} M A23187. Phorbol esters and calcium ionophore have been shown to act synergistically to increase superoxide anion production by endothelial cells (Matsubara & Ziff, 1986). It is possible therefore that at the lower concentration (10^{-7} M) of calcium ionophore the enhanced superoxide anion production results in more rapid EDRF destruction, less elevation of cyclic GMP and an apparent inhibition of release. With 10^{-6} M A23187, very large increases in cyclic GMP were seen (Figure 4), indicating a large release of EDRF, which was not affected by PDB pretreatment. This suggests that the amount of EDRF released is more than sufficient to saturate the soluble guanylate cyclase, and any enhanced destruction of EDRF by increased superoxide anion production is not apparent.

Evidence to support this interpretation comes from the experiments with A23187 in the presence of SOD and SOD plus CAT. In the presence of SOD only, PDB pretreatment no longer inhibited 10^{-7} M A23187-induced elevation of cyclic GMP levels, but if anything tended to increase the response, although this was not significant when compared to the response in the presence of PDD. Care must be exercised in interpreting these results as a lack of inhibitory effect of PDB on A23187-induced EDRF release, as the end product of SOD-mediated superoxide anion dismutation is hydrogen peroxide, which can stimulate soluble guanylate cyclase either directly (White *et al.*, 1986) or indirectly by reacting with superoxide anions to produce hydroxyl radicals which in turn stimulate guanylate cyclase (Murad *et al.*, 1978). The apparent reversal of PDB-induced inhibition by SOD alone could therefore be due to elevation of cyclic GMP levels by hydrogen peroxide and/or hydroxyl radical. Similar results were obtained when the experiment was repeated in the presence of SOD and CAT so that any hydrogen peroxide formed by SOD would be converted to water by CAT. Pretreatment with PDB in the presence of SOD and CAT had no effect on 10^{-7} M A23187-induced elevation of cyclic GMP levels. Failure of PDB to inhibit A23187-induced EDRF release in rabbit aorta was similarly shown by Lewis & Henderson (1987). It is apparent that A23187-induced cyclic GMP production is less in the presence of SOD or SOD and CAT than in their absence (cf Table 5 and Figure 4). This may well reflect protein binding of the ionophore, as A23187 is known to bind to albumin: indeed binding of A23187 to albumin has been used as a technique for rapid removal and reversal of the effects of A23187 (Sarkadi *et al.*, 1976).

The lack of an inhibitory effect of PDB on BK-induced EDRF release was an unexpected result, and implies that BK has a different pathway of cellular activation from substance P and ATP. These experiments were only performed in the presence of SOD and CAT, but there is no obvious reason why a different result would be obtained in the absence of SOD and CAT. Not all agonists release EDRF through the same mechanism. For example pertussis toxin, which inactivates a G_i-protein involved in receptor-response coupling (Murayama & Ui, 1983), inhibits endothelium-dependent relaxation of pig coronary arteries produced by the α_2-agonist UK 14304 (Pfizer, Sandwich, Kent), 5-hydroxytryptamine and thrombin, but not those produced by bradykinin and ADP (Flavahan *et al.*, 1989).

Evidence that phospholipase C can participate in the pathways leading to EDRF release has been provided by a number of studies. For example, de Nucci *et al.* (1988) found that addition of purified phospholipase C to bovine cultured aortic endothelial cells released both EDRF and prostacyclin. Bradykinin is known to stimulate phosphatidyl inositol hydrolysis in cultures of both bovine (Derian & Moskowitz, 1986) and porcine (Hong & Deykin, 1982; Lambert *et al.*, 1986) aortic endothelial cells, and ATP has been shown to increase the formation of inositol-1,4,5-trisphosphate in bovine cultured aortic endothelial cells (Pirotton *et al.*, 1987). Substance P stimulates phosphatidyl inositol hydrolysis in smooth muscle (Watson & Downes, 1983; Yousufzai *et al.*, 1986) and paratoid acinar cells (Aub & Putney, 1984), and is likely to act in a similar manner in endothelial cells. Thus, if all three agonists act by increasing phosphatidyl inositol hydrolysis (proven for bradykinin and ATP, assumed for substance P), but PDB only inhibits EDRF release induced by substance P and ATP, one must question whether the mechanism of action of PDB acting via protein kinase C, is through inhibition of the phospholipase C enzyme *per se*.

It has been demonstrated that phorbol esters induce the phosphorylation of a number of receptor types, e.g. insulin (Jacobs *et al.*, 1983; Takayama *et al.*, 1984), α_1-adrenoceptor (Leeb-Lundberg *et al.*, 1985) and β-adrenoceptor (Sibley *et al.*, 1984; Kelleher *et al.*, 1984), and that in some of these systems this is associated with a reduction in agonist binding affinity and in response (see for example Leeb-Lundberg *et al.*, 1985). Protein kinase C has also been shown to phosphorylate a G_i protein *in vitro* (Katada *et al.*, 1985). It is possible that different agonists utilise different G proteins to couple receptors to phospholipase C, and that protein kinase C only affects some G proteins. There is therefore a number of possible sites where phorbol ester-stimulated protein kinase C could act to inhibit agonist-induced EDRF release. Brock & Capasso (1988) suggested a similar mechanism to explain inhibition of thrombin- and histamine-induced increases in IP_3 formation and intracellular calcium by various activators of protein kinase C. The exact mechanism by which protein kinase C acts requires further investigation.

In conclusion, PDB but not the inactive 4α-PDD inhibit increases in pig aortic endothelial cell cyclic GMP content induced by substance P and ATP, but not by bradykinin, sodium nitroprusside and, in the presence of SOD and CAT, the calcium ionophore A23187. The inhibitory effects of PDB probably result from an action of protein kinase C on the steps leading from receptor occupation to phospholipase C activation.

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Alterations of responsiveness to adrenoceptor agonists and calcium of non-infarcted hypertrophied muscles from rats with chronic myocardial infarction

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1 The responsiveness of hypertrophied right ventricular muscle from rats with chronic myocardial infarction (MI) was examined. Myocardial infarction was produced by ligating the left coronary artery. The positive inotropic effects of α- and β-adrenoceptor agonists and calcium on the isolated non-ischaemic muscles were determined at one and four weeks after ligation, and were compared with those in sham-operated (SO) rats.

2 The contractile force of electrically-driven right ventricular papillary muscles was measured isometrically. We also determined the change of beating rate of the right atrium in response to a β-adrenoceptor agonist.

3 Basal contractile force increased at four weeks after ligation.

4 The pEC_{50} values of the α_1-adrenoceptor agonist, methoxamine, and calcium increased at four weeks after ligation, but the pEC_{50} values of the β-adrenoceptor agonist, dobutamine did not change.

5 Maximal contractile force did not change with these agonists at either one and four weeks after ligation.

6 Tissues from rats four weeks after MI exhibited slight increases in the B_{max} of α- but not β-receptors, without any changes of affinity.

7 The increases in the sensitivity to an α_1-adrenoceptor agonist and calcium may be compensatory mechanisms of non-infarcted ventricular tissue which has undergone hypertrophy in chronic MI.

Introduction

The gradual morphological change of the non-ischaemic cardiac muscle after myocardial infarction in rats has been described by many investigators (Anversa *et al.*, 1980a,b; 1984; 1986; Fishbein *et al.*, 1978; Rubin *et al.*, 1983). The necrotic myocardial tissue is completely replaced by connective tissue within 21 days and changes in the morphology of the right ventricle begin from three to twelve weeks (Fishbein *et al.*, 1978). In guinea-pigs with myocardial infarction, the responsiveness to β-adrenoceptor stimulation of non-ischaemic ventricular muscle has been shown to decrease three days after ligation of the coronary artery, and to return to normal seven days after ligation (Baumann *et al.*, 1981). However, the responsiveness of non-infarcted muscles over a longer period of time has not been investigated. In this study, we determined the responsiveness to drugs of hypertrophied ventricular muscles at one and four weeks after ligation of the coronary artery in rats.

Methods

Operative procedure

Myocardial infarction (MI) was induced in male Wistar rats (200–220 g) by a method similar to that described previously (Johns *et al.*, 1954; Selye *et al.*, 1960; Fishbein *et al.*, 1978). Briefly, the rats were anaesthetized with ether, followed by pentobarbitone (35 mg kg^{-1}, i.p.), intubated and ventilated by a positive pressure respirator. The heart was exposed through an incision between the fourth to fifth, or fifth to sixth ribs on the left side. The left coronary artery was then ligated 1–2 mm from its origin with 5-0 nylon. The chest was closed, and the rats were allowed to recover. There was a 20–30% mortality rate within the first 24 h following this procedure. The sham-operated (SO) rats were treated similarly without the procedure of coronary ligation. Surviving rats were maintained under the same conditions in a 12 h light/dark cycle and given standard chow and water *ad libitum*.

Contractions of right ventricular papillary muscles

At one and four weeks after the left coronary artery ligation or sham-operation, rats were killed by a blow on the head. The hearts were rapidly removed and papillary muscles of the right ventricle excised. The isolated preparations were suspended in 10 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 120, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 1.4. To determine the sensitivity to exogenous Ca^{2+}, Ca^{2+}-free perfusate was prepared and Ca^{2+} 0.2 to 5 mM was added in a cumulative manner. The solution was maintained at a temperature of 32.0 ± 0.2°C and bubbled with a gas mixture of 95% O_2–5% CO_2. Preparations were stimulated at 100 min^{-1} through an electrode by a square wave pulse of 3 ms duration and a voltage of approximately twice threshold, delivered by a stimulator (Nihon Koden SE-1101). Papillary muscles were stretched by a resting tension of about 1.0 g, and tension development was recorded via force-displacement transducers (Nihon Koden, TB-612T, TB-652T) on a thermal array recorder (Nihon Koden, WS-641G). After a stabilization period of one hour, cumulative concentration-response curves were determined (Hashimoto *et al.*, 1978). In preliminary experiments, resting tension-active tension development curves for muscles from one and four week old MI and SO rats were determined (Figure 1). The basal contractile forces were maximal with a tension of 1.0 g in one and four week old MI and SO rats. We measured the diameter of the centre of the papillary muscles under tension after all experimental procedures had been completed. Cross-sectional area of individual papillary muscles was calculated as a circle. The tension development was represented as mg mm^{-2}.

Beating rate of right atrium

At one week and four weeks after ligation or sham-operation, rats were killed and the hearts were rapidly removed. Right

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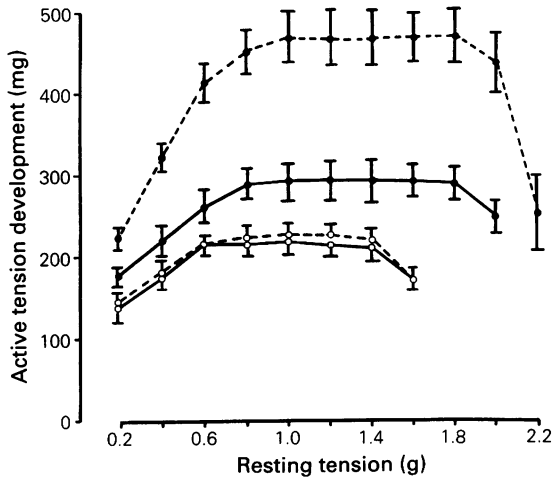

Figure 1 The active tension development of the isolated right ventricular papillary muscles with various resting tensions in one and four week old myocardial infarcted (MI) or sham-operated (SO) rats. (○——○) One week SO rats ($n = 6$); (●——●) one week MI rats ($n = 6$); (○---○) four week SO rats ($n = 6$); (●---●) four week MI rats ($n = 6$). Each point represents the mean and vertical lines show s.e.mean in this and subsequent figures.

atria were dissected and suspended in 10 ml organ baths containing Krebs-Henseleit solution. The solution was maintained at 32.0 ± 0.2°C and bubbled with 95% O_2–5% CO_2. The spontaneously beating atria were connected to force-displacement transducers and contractions were recorded. The beating rate was counted with a tachometer (Nihon Koden, AT-601G). The resting tension on the preparations was set to 0.5 g. Concentration-response curves were determined cumulatively.

α- and β-adrenoceptor binding assays

Four weeks after the left coronary artery ligation or sham-operation, rats were killed by a sharp blow on the head. The hearts were rapidly removed from the rats, placed in ice-cold buffer and the right ventricular free walls were dissected. Due to the large amount of protein required for receptor binding assays and Scatchard analysis, segments from two to five hearts were combined for the preparation of the membrane fraction. The right ventricular free walls were minced with a pair of scissors and homogenized in 10 to 20 times volume (w/v) of ice-cold buffer (0.25 M sucrose, 5 mM Tris/HCl, 1 mM $MgCl_2$, pH 7.5: buffer A) by a Brickman Polytron PT-10 at setting 6 for 20, followed by 10 strokes of motor-driven teflon-glass homogenizer.

These homogenates were centrifuged at 1000 *g* for 10 min at 4°C. The pellet was removed, centrifuged at 30,000 *g* for 20 min at 4°C. The membrane pellet was washed in 20 times volume of the original wet weight of ice-cold buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.5: buffer B) and recentrifuged at 30,000 *g* for 20 min at 4°C. The final pellet was resuspended in 20 times volume (for the [^{3}H]-prazosin and [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding assay) of original wet weight of ice-cold buffer B.

α_1- and β-adrenoceptor binding was measured by a method similar to that described previously (Asano *et al.*, 1983).

For binding to α_1-adrenoceptors, membrane preparations (250 μl) were incubated, with shaking, for 20 min at 30°C with [^{3}H]-prazosin (0.1–2.0 nM) in a total volume of 550 μl of buffer A containing 0.1 mg ml^{-1} bovine serum albumin. The incubations, which were performed in duplicate, were terminated by the addition of 4 ml ice-cold buffer B, followed by rapid filtration through Whatman GF/C glass fibre filter disks. The filter disks were washed three times with 4 ml of ice-cold buffer B, and the radioactivity trapped on the filter disks was measured in an Aloka scintillation spectrometer (LSC-671) with a counting efficiency of 34%. Non-specific binding was defined as non-displacable binding in the presence of 10 μM phentolamine and the specific binding was defined as the difference between total and non-specific binding.

The binding assay with [^{3}H]-DHA was carried out in the same way as that for [^{3}H]-prazosin, using [^{3}H]-DHA (1.0–20 nM) instead of [^{3}H]-prazosin, and non-specific binding of [^{3}H]-DHA was defined in the presence of 10 μM propranolol.

The protein concentration was measured by the method of Lowry *et al.* (1951). We analysed the data with the aid of a weighted non-linear square curve fitting programme, 'LIGAND' (Munson & Rodbard, 1980).

Drugs

The drugs used were as follows: dobutamine hydrochloride (Shionogi Chem.), methoxamine hydrochloride (Tanabe Chem.), [^{3}H]-prazosin (80.9 Ci mM^{-1}), [^{3}H]-DHA (54.8 Ci mM^{-1}) were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.), bovine serum albumin (Sigma), phentolamine hydrochloride (Chiba-Geigy), propranolol (Sigma).

Statistical analysis

The results are expressed as mean ± s.e.mean. Statistical analysis of the data was performed by Student's *t* test and differences were considered significant only if $P < 0.05$.

Results

Heart weights and right ventricular weights

Table 1 shows the body weight and heart weight of MI and SO rats. The body weights of four week old MI and SO rats

Table 1 Body, heart and ventricular weight ratios in rats with myocardial infarction (MI) and sham-operated (SO) rats

	1 week		*4 weeks*	
	SO	*MI*	*SO*	*MI*
n	6	7	9	8
BW (g)	236 ± 4	204 ± 6**	278 ± 4	253 ± 10
HW (mg)	742 ± 10	797 ± 18*	827 ± 20	1139 ± 65**
RV (mg)	117 ± 2	134 ± 4**	137 ± 3	247 ± 17**
RA (mg)	34.1 ± 2.1	41.2 ± 3.1*	38.3 ± 2.6	69.5 ± 4.9*
SC (mg)	—	90 ± 8	—	86 ± 18
HW/BW (mg g^{-1})	3.12 ± 0.04	3.91 ± 0.10**	2.98 ± 0.06	4.40 ± 0.20**
RV/BW (mg g^{-1})	0.50 ± 0.02	0.65 ± 0.03**	0.50 ± 0.01	0.98 ± 0.06**
SC/HW (%)	—	11.3 ± 0.5	—	7.6 ± 0.4††

Results are expressed as mean ± s.e.mean. BW = body weight, HW = heart weight, RV = right ventricular weight, RA = right atrial weight, SC = scar weight.
*$P < 0.05$, **$P < 0.01$ (MI vs SO).
††$P < 0.01$ (1 week vs 4 weeks).

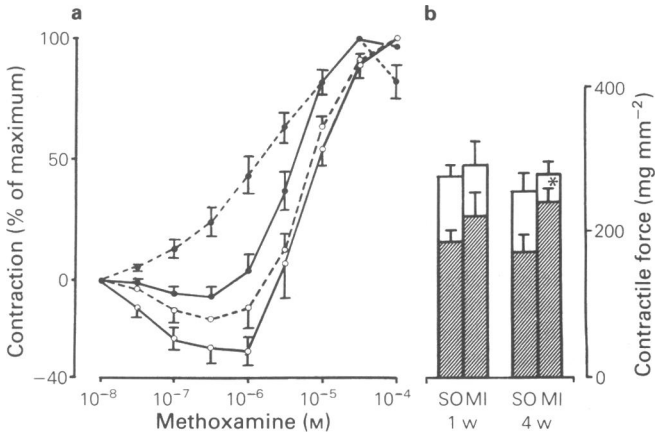

Figure 2 The positive inotropic effect of methoxamine on the isolated right ventricular papillary muscles of one and four week old myocardial infarcted (MI) or sham-operated (SO) rats. (a) Concentration-response curves, (○——○) one week SO rats ($n = 6$), (●——●) one week MI rats ($n = 7$), (○---○) four week SO rats ($n = 7$). (●---●) four week MI rats ($n = 6$). (b) The contractile force per cross-sectional area with methoxamine. Hatched columns, basal contractile force. Hatched + open columns, maximal contractile force with methoxamine. 1 w, one week old rats; 4 w, four week old rats. *$P < 0.05$ vs SO rats.

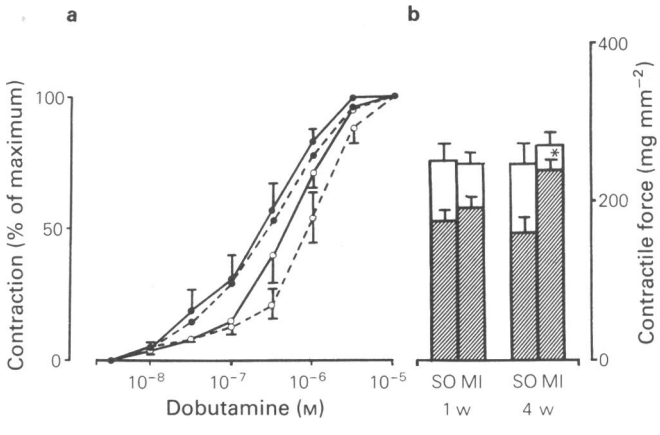

Figure 3 The positive inotropic effect of dobutamine on the isolated right ventricular papillary muscles of one and four week old myocardial infarcted (MI) or sham-operated (SO) rats. (a) Concentration-response curves, (○——○) one week SO rats ($n = 6$), (●——●) one week MI rats ($n = 6$), (○---○) four week SO rats ($n = 6$), (●---●) four week MI rats ($n = 7$). (b) The contractile force with dobutamine. Hatched columns, basal contractile force. Hatched + open columns, maximal contractile force with dobutamine. 1 w, one week old rats; 4 w, four week old rats. *$P < 0.05$ vs SO rats.

were not significantly different. However, the body weights of one week old MI rats were significantly less than those of the SO rats. The heart weights and right ventricular weights in both one and four week old MI rats were significantly greater than those of SO rats. Thus, the ratios of whole heart weight/body weight and of right ventricular free wall weight/body weight in one and four week old MI rats significantly increased in comparison with the SO rats. The right ventricular weight to body weight ratios increased 32% in one week old MI rats and 100% in four week old MI rats. The ratios of the scar weight/heart weight were 11.3 ± 0.5% in one week old and 7.6 ± 0.4% in four week old rats.

Positive inotropic effect of adrenoceptor agonists on right ventricular papillary muscles

Figure 2a shows the concentration-response curves of contractile force in response to an α_1-agonist, methoxamine. In one week old SO rats, low concentrations of methoxamine produced a slight negative inotropic effect, but in other SO or MI rats these responses were not observed. The pEC_{50} values for methoxamine of tissues from one week old MI and SO rats were identical, but those from four week old MI and SO rats were significantly different (Table 2). Figure 2(b) shows the values of the basal and the maximal contractile force per cross-sectional area of right ventricular papillary muscles with methoxamine. In four week old rats, the basal contractile force per area was greater in MI rats. The maximal contractile force per area with methoxamine was not different in MI rats and SO rats.

In preliminary experiments, the pEC_{50} of dobutamine in the papillary muscle was 6.30 ± 0.12 ($n = 6$). The pEC_{50} of dobutamine with 10^{-6} M propranolol was 5.36 ± 0.15 ($n = 6$, $P < 0.01$). The pEC_{50} of dobutamine with 3×10^{-6} M phentolamine was 6.22 ± 0.17 ($n = 6$). Thus it can be regarded as a β-adrenoceptor agonist in papillary muscle of rats. Figure 3a shows the concentration-response curves of contractile responses to dobutamine. The values of pEC_{50} of one or four week old MI rats were not significantly different from those of SO rats (Table 2). Figure 3b shows the values of the basal and the maximal contractile force per cross-sectional area with dobutamine. In four week old rats, the basal contractile force was larger in MI than SO rats. The maximal contractile force with dobutamine was not different in MI and SO rats.

Positive inotropic effects to calcium on right ventricular papillary muscles

Figure 4a shows the concentration-response curves for contractile force in response to exogenous calcium. The sensitivity of right ventricular muscle to calcium significantly increased in one and four week old MI rats (Table 2). Figure 4b shows the absolute increases in the contractile force of right ventricular papillary muscles with calcium. The maximal contractile

Table 2 pEC_{50} (negative logarithm of EC_{50}) of methoxamine, dobutamine and calcium in the papillary muscles of rats with myocardial infarction (MI) and sham-operated (SO) rats

	1 week		*4 weeks*	
	SO	*MI*	*SO*	*MI*
Methoxamine	5.09 ± 0.11 ($n = 6$)	5.34 ± 0.08 ($n = 7$)	5.09 ± 0.04 ($n = 7$)	5.62 ± 0.04* ($n = 6$)
Dobutamine	6.26 ± 0.11 ($n = 6$)	6.60 ± 0.16 ($n = 6$)	6.19 ± 0.10 ($n = 6$)	6.54 ± 0.17 ($n = 7$)
Calcium (mM)	3.10 ± 0.06 ($n = 6$)	3.38 ± 0.10* ($n = 7$)	2.98 ± 0.05 ($n = 6$)	3.33 ± 0.09** ($n = 6$)

Results are expressed as mean ± s.e.mean.
*$P < 0.05$, **$P < 0.1$ (MI vs SO).

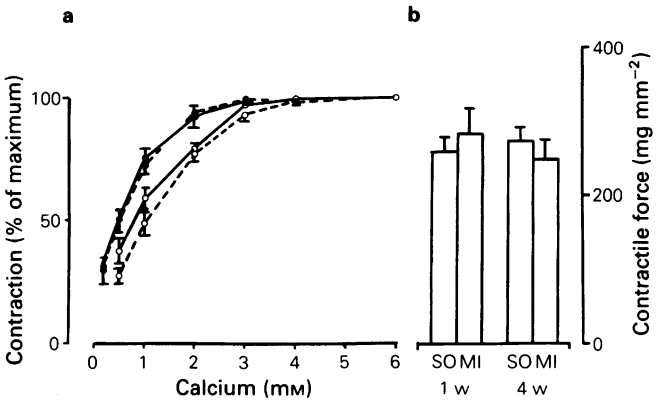

Figure 4 The positive inotropic effect of calcium on the isolated right ventricular papillary muscles of one and four week old myocardial infarcted (MI) or sham-operated (SO) rats. (a) Concentration-response curves, (○——○) one week SO rats ($n = 6$), (●——●) one week MI rats ($n = 7$), (○---○) four week SO rats ($n = 6$), (●---●) four week MI rats ($n = 6$). (b) The maximal contractile force with calcium. 1 w, one week old rats; 4 w, four week old rats.

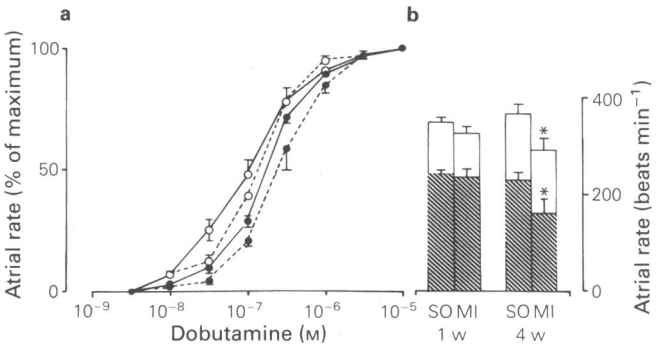

Figure 5 The positive chronotropic effect of dobutamine on the isolated right atrial muscles of one and four week old myocardial infarcted (MI) or sham-operated (SO) rats. (a) Concentration-response curves for the effect of dobutamine on atrial rate; (○——○) one week SO rats ($n = 7$), (●——●) one week MI rats ($n = 6$), (○---○) four week SO rats ($n = 6$), (●---●) four week MI rats ($n = 6$). (b) The absolute beating rate of the atria with dobutamine. Hatched columns, basal rate; hatched + open columns, maximal atrial rate with dobutamine. 1 w, one week old rats; 4 w, four week old rats. *$P < 0.05$ vs SO rats.

forces per area with calcium were not different in one and four week old MI rats.

Positive chronotropic effect of a β-agonist in right atrium

Figure 5a shows the concentration-response curves for the beating rate of right atria to the β-agonist, dobutamine. The values of pEC_{50} in one week old MI and SO rats were identical (6.70 ± 0.02 ($n = 6$), 6.93 ± 0.11 ($n = 7$)), and those of four week old MI and SO rats were also identical (6.55 ± 0.17 ($n = 6$), 6.38 ± 0.10 ($n = 6$)). Figure 5b shows the absolute beating rate of right atrium with dobutamine. The basal rate in four week old MI rats (162 ± 29) was significantly decreased compared with SO rats (232 ± 22). The maximal atrial rate with dobutamine in four week old MI rats (294 ± 25 beats min^{-1}) was also significantly decreased as compared with SO rats (365 ± 31 beats min^{-1}). The maximal increases in the atrial rate with dobutamine were not different between MI and SO rats.

Ligand binding at α- and β-adrenoceptors

The B_{max} of [^{3}H]-prazosin binding in four week old MI rats was slightly but significantly increased, but the K_D did not change (Table 3). The B_{max} and K_D of [^{3}H]-DHA binding in four week old MI rats were not significantly different from those of SO rats.

Table 3 Binding of [^{3}H]-prazosin and [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) to right ventricle from sham-operated (SO) rats and rats with myocardial infarction (MI) at four weeks

	B_{max} (fmol mg^{-1} protein)	K_D (nM)
[^{3}H]-prazosin		
SO	67.4 ± 2.4 ($n = 6$)	0.25 ± 0.02 ($n = 6$)
MI	77.6 ± 1.8** ($n = 6$)	0.26 ± 0.01 ($n = 6$)
[^{3}H]-DHA		
SO	42.9 ± 3.6 ($n = 6$)	1.49 ± 0.28 ($n = 6$)
MI	51.4 ± 3.8 ($n = 6$)	1.26 ± 0.21 ($n = 6$)

Results are expressed as mean ± s.e.mean.
**$P < 0.01$ (MI vs SO).

Discussion

In a previous study with guinea-pigs with myocardial infarction, the responsiveness to β-receptor stimulation of residual non-ischaemic ventricular papillary muscles decreased three days after ligation of the coronary artery and returned to normal by seven days after ligation (Baumann *et al.*, 1981). These alterations of responsiveness were due to a down-regulation of adrenoceptors caused by the increased level of plasma catecholamines. However, in chronic MI the alterations of the responsiveness of non-ischaemic muscle have not been examined. In the present study, we determined the responsiveness of hypertrophied muscles at one and four weeks after MI.

The hypertrophy of non-ischaemic areas in MI rats occurs during the early days after ligation and the necrotic myocardial tissue is completely replaced by connective tissue within 21 days (Fishbein *et al.*, 1978). According to previous studies, the increases in weight due to right ventricular hypertrophy in rats were 5–65% at 21 days after occlusion, and about 30–79% at 30 to 40 days after occlusion (Anversa *et al.*, 1984; 1986). It varied according to the infarct size (Pfeffer *et al.*, 1979). The right ventricular hypertrophy (as assessed by increase in tissue weight) in our studies was 32% and 100% at one and four weeks, respectively, which was larger than those found in previous studies, probably because of differences in infarct size. The marked increase in the basal contractile force in MI rats may be partially caused by hypertrophy, and the increased contractile force may contribute to the compensatory mechanisms in MI.

The sensitivity to the α-adrenoceptor agonist increased at four weeks after ligation. The α-adrenoceptor density slightly but significantly increased in MI rats at this time, and this may partially contribute to the increased sensitivity to the α-adrenoceptor agonist.

The sensitivity to the β-adrenoceptor agonist did not change in right papillary muscles or atrial preparations of MI rats. Maximal contractile force with methoxamine or dobutamine in one and four week old MI rats was not different from SO rats. However, basal contractile force in four week old MI rats was larger than that in four week old SO rats. Calcium (2 mM) produced a larger contractile force in MI rats than in SO rats, and this resulted in the increased basal contractile force in four week old MI rats. The basal atrial rate (beats min^{-1}) was significantly decreased in right atria from 4 week old MI rats; this may be due to some deterioration of pacemaker cells during hypertrophy of the right atrium, but this needs further clarification.

Clozel et al. (1987) found that at three weeks following MI, a slight increase in β-adrenoceptor density occurred, but no change in the EC_{50} of isoprenaline, in right papillary muscle from rats. In our study, the pEC_{50} of dobutamine did not significantly change, which is consistent with the result of Clozel et al. (1987). The B_{max} of β-adrenoceptors in MI rats did not significantly change in our study.

An alteration of cardiac α_1-receptors has been observed in other models of cardiac hypertrophy, such as in spontaneously hypertensive rats (Hanna et al., 1986) and in rats with aortic constriction (Karliner et al., 1980). In hereditary cardiomyopathy of the Syrian hamster, responsiveness to stimulation of α- but not β-adrenoceptors was increased (Bohm et al., 1986). It seems that an increase in cardiac α_1-adrenoceptors in cardiac hypertrophy is a common phenomenon.

The sensitivity to calcium significantly increased in four week old MI rats, which led to the increased level of basal contractile force in this group. Thus, the increased sensitivity to calcium is considered to be one of the compensatory mechanisms in MI.

Anversa et al. (1985) found that the sensitivity to calcium of the left ventricle was significantly decreased after MI in working hearts. The difference between the results of Anversa et al. and ours may lie in the different muscles studied, i.e., the residual muscles included the infarcted area of the left ventricle in their study, but in our study only the intact papillary muscles of the right ventricle were used.

In conclusion, increases in the sensitivity to an α_1-adrenoceptor agonist and calcium occur in four week old MI rats; these may be compensatory mechanisms of the non-infarcted hypertrophied ventricle in chronic MI.

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Effects of antiarrhythmic agents classified as class III group on ischaemia-induced myocardial damage in canine hearts

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1 The cardioprotective effects of antiarrhythmic agents classified as class III, amiodarone, sotalol and E-4031, were investigated in anaesthetized dogs.

2 The left anterior descending coronary artery was occluded for 2 h.

3 Heart mitochondria were prepared from both the ischaemic and non-ischaemic areas, and their function was estimated polarographically.

4 Activities of the lysosmal enzymes, N-acetyl-β-glucosaminidase and β-glucuronidase, were measured in each fraction.

5 Two hour occlusion induced ventricular arrhythmias, and amiodarone, sotalol and E-4031 greatly suppressed the development of arrhythmias.

6 Amiodarone, sotalol and E-4031 significantly protected mitochondria against ischaemia, and prevented ischaemia-induced leakage of lysosomal enzymes.

7 Antiarrhythmic agents classified as class III show cardioprotective effects, which might participate in their antiarrhythmic effect.

Introduction

Of the estimated episodes of acute myocardial infarction, about 50% will cause a patient to die before hospitalization. Patients who die during hospitalization usually succumb to either arrhythmias or pump failure. The introduction and widespread employment of coronary care units designed for continuous electrocardiographic monitoring of patients with acute myocardial infarction have resulted in reduced mortality (Singh *et al.*, 1980). Recent advances in cardiology have shown that substantial quantities of ischaemic myocardium, destined to undergo necrosis, can be salvaged by treatment with several drugs such as β-blockers, nitrates, calcium antagonists and hyaluronidase (Maroko *et al.*, 1972; Smith *et al.*, 1973; Opie *et al.*, 1975, Sugiyama *et al.*, 1983; Nagai *et al.*, 1984). The feasibility of protection of ischaemic myocardium has been demonstrated experimentally by many different techniques in various laboratories and in many different species.

The sudden onset of ischaemia induced by occlusion of the coronary artery is associated with a series of metabolic, functional and ultrastructural changes. The dependence of myocardium on aerobic, in other words, mitochondrial metabolism, is reflected in the fact that about 30% of the volume of a normal left ventricular myocyte is occupied by mitochondria (Page & McCallister, 1973). A persistent decrease in tissue adenosine triphosphate level, based on mitochondrial dysfunction, leads to cellular dysfunction. In addition, lysosomes contain phospholipases and proteinases and leakage of lysosomal enzymes might play an important role in irreversible cellular damage (Hearse, 1979). Indeed, Kennet & Weglicki (1978) showed that in ischaemic myocardium lysosomal enzyme activities were decreased around the 20000 *g* fraction, and these increased in the supernatant of homogenized myocardium. Hence, the estimation of mitochondrial function and of lysosomal enzyme release is a useful biochemical marker to assess myocardial damage following coronary occlusion.

Since an iodinated benzofuran derivative, amiodarone, was introduced, effects of antiarrhythmic drugs of class III on myocardial ischaemia have attracted much interest (Marcus *et al.*, 1981). It is well known that amiodarone has a mild non-competitive sympathetic antagonistic effect and significant coronary and peripheral vasodilator actions (Mason, 1987). Sotalol is a β-adrenoceptor blocking drug with class III activity (Strauss *et al.*, 1970), It was shown that sotalol prevented ventricular arrhythmias in dogs with coronary artery occlusion (Cobbe *et al.*, 1983). Nevertheless, effects of antiarrhythmic agents classified as class III on ischaemia-induced myocardial damage have been little studied. In the present study, we examined effects of amiodarone, sotalol, and a newly developed class III agent, E-4031 (Sawada *et al.*, 1988), on mitochondrial function and stability of the lysosomal membrane after coronary artery occlusion.

Methods

Animal preparations

Thirty three mongrel dogs of either sex weighing between 8–15 kg were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}) given intraperitoneally. After endotracheal intubation, each animal was mechanically ventilated with a Harvard type ventilator. Lead II of the electrocardiogram was monitored continuously by a VC-640G oscillographic recorder and recorded simultaneously by an ICR-7200 ambulatory electrocardiogram recorder throughout the experiment. The right femoral artery and left femoral vein were cannulated for measurement of arterial blood pressure and for infusion of the drug and physiological saline, respectively.

The chest was opened at the 4th or 5th intercostal space and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free of fat and connective tissue immediately distal to the first diagonal branch. A silk ligature was placed around the LAD for acute occlusion.

Experimental design

The dogs were divided into 4 groups. Twenty-five minutes before LAD occlusion, physiological saline (25 ml) was administered for 10 min to the control group ($n = 12$). For the amiodarone group ($n = 7$), 25 min before LAD occlusion, amiodarone (3 mg kg^{-1}) dissolved in 5 ml distilled water was administered for 10 min. For the sotalol group ($n = 7$), 25 min before LAD occlusion, sotalol (1 mg kg^{-1}) dissolved in 5 ml of physiological saline was administered for 10 min. For the E-4031 group ($n = 7$), 15 min before LAD occlusion, 2.5 μg kg^{-1} min^{-1} E-4031 (1-(2-(6-methyl-2-pyridyl)ethyl)-4-(4-

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Table 1 Total number of ventricular premature beats, arrhythmia ratio, and total duration time of ventricular tachycardia in all groups

Group	*Total number of ventricular premature beats*	*Arrhythmia ratio (%)*	*Total duration time of ventricular tachycardia*
Control	416.4 ± 275.8	2.57 ± 1.85	34.5 ± 47.8
Amiodarone	11.3 ± 11.2**	0.08 ± 0.07**	0.0 ± 0.0*
Sotalol	87.6 ± 73.1**	0.51 ± 0.39**	0.8 ± 1.4*
E-4031	26.1 ± 29.1**	0.19 ± 0.45**	0.7 ± 1.9*

*$P < 0.05$, **$P < 0.01$ versus control group.

methylsulphonyl aminobenzoyl)piperidine) was infused throughout the experiment. Two hours after occlusion of LAD. Evans' blue dye was infused via the femoral vein, and then the heart was excised The ischaemic area of the left ventricle was identified as a non-staining zone and the non-ischaemic area as a blue staining zone.

Analysis of arrhythmias

With the ambulatory electrocardiogram recorder, the number of ventricular premature beats during a 2h period of occlusion was recorded and analysed by auto analyzer. To express the severity of arrhythmias, the total number of ventricular premature beats was counted and the arrhythmia ratio, defined as the number of ventricular premature beats divided by the total heart beats, was determined. The total duration time of ventricular tachycardia, which was defined as more than 3 successive ventricular premature beats, was also calculated.

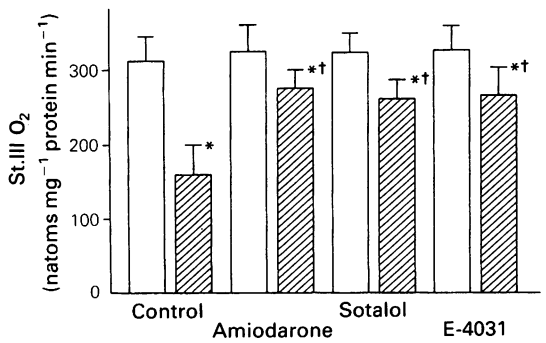

Figure 1 The rate of oxygen consumption in state III (St. III O_2) in heart mitochondria from the ischaemic (hatched columns) and non-ischaemic areas (open columns) of each group. Two hour occlusion induced a decrease in St. III O_2. Amiodarone, sotalol and E-4031 lessened the decrease. Each column shows the mean and vertical bars indicate s.d. *$P < 0.01$ versus the non-ischaemic area of the corresponding group. †$P < 0.01$, versus the ischaemic area of the control group.

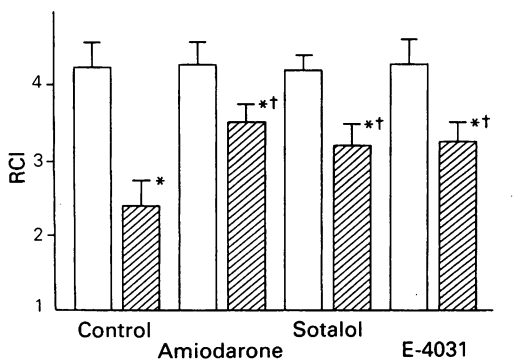

Figure 2 The respiratory control index (RCI) in heart mitochondria from the ischaemic (hatched columns) and non-ischaemic (open columns) areas of each group. Beneficial effects of amiodarone, sotalol and E-4031 were observed. Each column represents the mean and vertical bars show s.d. *$P < 0.01$, versus the non-ischaemic area of the corresponding group. †$P < 0.01$, versus the ischaemic area of the control group.

Measurement of mitochondrial function

Heart mitochondria from the ischaemic and non-ischaemic areas of each group were prepared as we have described previously (Sugiyama *et al.*, 1980), and the rate of oxygen consumption in state III (St. III O_2) and the respiratory control index (RCI) were measured immediately after the preparation, by an oxygen electrode and closed cell. The incubation medium contained 0.3 mol l^{-1} mannitol, 10 mmol l^{-1} KCl, 10 mmol l^{-1} potassium phosphate, pH 7.4, 2.5 mmol l^{-1} $MgCl_2$ and 0.25 mmol l^{-1} ethylendiaminetetraacetic acid (EDTA) in a total of 0.7 ml. Respiration was initiated by addition of 0.03 mmol l^{-1} of mitochondrial suspension (10 mg protein ml^{-1}), and then 0.03 ml of succinate (0.2 mol l^{-1}) as a substrate and 0.03 ml adenosine diphosphate (10 mmol l^{-1}) were added subsequently.

Assays of lysosomal enzymes

According to the methods of Kennett & Weglicki (1978) and Ruth *et al.* (1978), the endocardial tissue from the ischaemic and non-ischaemic areas of each group was immersed in the extraction medium (0.25 mol l^{-1} sucrose, 0.6 mol l^{-1} KCl, 1 mmol l^{-1} adenosine triphosphate, 1 mmol l^{-1} EDTA, and 1 mmol l^{-1} $MgCl_2$, buffered with imidazole at pH 7.2). The tissue was then homogenized with a Teflon pestle tissue grinder. Pellets were obtained by differential centrifugation; the duration in min at the various g-forces were; 1,000 g for 10 min; 2,500 g for 10 min; 9,000 g for 10 min; 20,000 g for 30 min; 140,000 g for 60 min. They were resuspended in 0.25 mol l^{-1} sucrose containing 10 mmol l^{-1} imidazole, buffered at pH 7.2. All pellets and the final supernatant were assayed for total activity of N-acetyl-β-glucosaminidase (NAG) and β-glucuronidase (β-gluc) by the methods of Noto *et al.* (1983) and Kato *et al.* (1960).

Statistics

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

Results

Mortality

Of the 33 dogs that underwent coronary artery occlusion, 5 out of 12 dogs in the control group, died within 2h of occlusion (4: ventricular fibrillation, 1: pump failure), and were excluded from the subsequent analysis. No dogs died in either the amiodarone, sotalol or E-4031 groups. The mortality in the control group was thus higher than that in the other 3 groups ($P < 0.05$). Data for the remaining 28 dogs formed the basis of the results presented below.

Arrhythmias analysis

Table 1 shows the total number of ventricular premature beats, arrhythmia ratio, and the total duration time of ven-

Table 2 Total activities of N-acetyl-β-glucosaminidase (nmol g^{-1} wet weight min^{-1}) in myocardial fractions of each group

Fraction	*1,000 g*	*2,500 g*	*9,000 g*	*20,000 g*	*140,000 g*	*Supernatant*
Non-ischaemic area						
Control	114.6 ± 29.8	19.9 ± 5.1	22.4 ± 3.6	21.5 ± 3.6	23.3 ± 2.7	91.1 ± 16.9
Amiodarone	112.7 ± 18.7	22.2 ± 4.5	20.2 ± 4.2	23.6 ± 4.8	23.3 ± 4.5	84.3 ± 15.6
Sotalol	106.4 ± 20.9	20.9 ± 3.6	22.0 ± 3.0	20.4 ± 3.2	22.4 ± 3.3	85.2 ± 19.3
E-4031	117.0 ± 30.1	21.1 ± 5.3	23.7 ± 2.3	21.5 ± 4.7	20.4 ± 3.2	89.5 ± 8.8
Ischaemic area						
Control	93.2 ± 34.0	13.8 ± 6.6	13.8 ± 5.4**	10.6 ± 2.0**	11.7 ± 3.2**	117.3 ± 14.7**
Amiodarone	113.4 ± 25.7	21.6 ± 3.1†	21.5 ± 6.8†	19.7 ± 5.5††	19.5 ± 4.8††	93.2 ± 18.9†
Sotalol	99.9 ± 27.7	19.6 ± 3.4	21.7 ± 4.4†	20.6 ± 4.3††	20.5 ± 2.5††	93.0 ± 14.7†
E-4031	104.0 ± 28.3	20.6 ± 5.2†	21.3 ± 4.4†	17.5 ± 3.2††	17.9 ± 3.3††	94.3 ± 15.2†

Data shown are means ± s.d.
** $P < 0.01$ versus the non-ischaemic area in the corresponding group.
† $P < 0.05$, †† $P < 0.01$ versus the ischaemic area in the control group.

tricular tachycardia in each group. In the control group, the total number of ventricular premature beats and arrhythmia ratio were significantly larger than in the drug-treated groups. Furthermore, the total duration time of ventricular tachycardia in the control group was significantly longer than that of the drug-treated groups.

Mitochondrial function

Figure 1 shows St. III O_2 of mitochondria prepared from ischaemic and non-ischaemic areas in each group. In the control group, St. III O_2 from the ischaemic area (161 ± 38 natoms mg^{-1} protein min^{-1}) was decreased significantly compared with that from the non-ischaemic area (313 ± 31). In the amiodarone, sotalol and E-4031 groups, St. III O_2 from the ischaemic area (275 ± 25, 260 ± 26, 264 ± 39, respectively) were lower than that of the non-ischaemic area (respective values of 325 ± 30, 323 ± 24, 326 ± 31). However, this decrease was significantly less than that of the ischaemic area in the control group. Figure 2 shows the RCI in each group. Administration of amiodarone, sotalol and E-4031 also lessened ischaemia-induced decrease in the RCI.

Lysosomal enzyme activity

Table 2 shows the changes in total activities of NAG in all particulate and supernatant fractions. In the control group, a significant loss of NAG activity occurred in the post-2,500 *g* particulate fractions of the ischaemic area, and a significant increase was observed in the supernatant fraction of the ischaemic area compared with those of the non-ischaemic area. However, in the amiodarone, sotalol and E-4031 groups, the decrease in NAG activity of the post-2,500 g particulate fractions and the increase of the supernatant fraction of the ischaemic area were significantly inhibited compared with those of the control group. The changes in total activity of β-gluc in all particulate and supernatant fractions are shown in Table 3. Amiodarone, sotalol and E-4031 also prevented significantly the leakage of β-gluc.

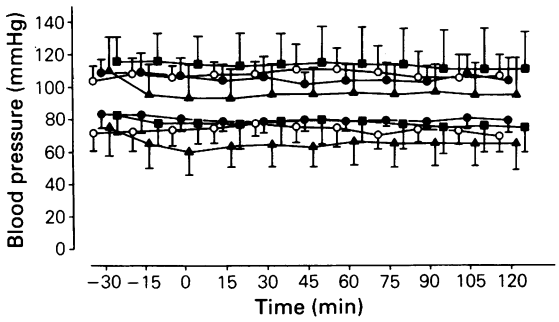

Figure 3 Time course of changes in systolic and diastolic blood pressure in each group: (○) control group, (●) amiodarone, (▲) sotalol and (■) E-4031-treated groups. Blood pressure was not significantly different between the groups. Each symbol represents the mean and vertical lines show s.d.

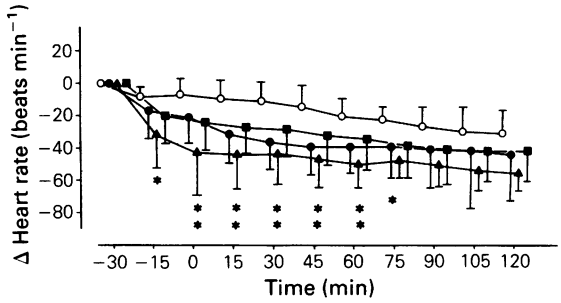

Figure 4 Time course of changes in heart rate in each group. No significant changes in heart rate were observed in the control (○) and E-4031 (■) groups. In the amiodarone group (●), 30 and 45 min after the coronary occlusion, a significant decrease in heart rate was observed. Sotalol (▲) decreased heart rate significantly until 75 min after left anterior descending (LAD) coronary artery occlusion. *$P < 0.05$, **$P < 0.01$. Each symbol represents mean and vertical lines show s.d.

Table 3 Total activities of β-glucuronidase (μg g^{-1} wet weight h^{-1}) in myocardial fractions of each group

Fraction	*1,000 g*	*2,500 g*	*9,000 g*	*20,000 g*	*140,000 g*	*Supernatant*
Non-ischaemic area						
Control	83.9 ± 28.9	15.1 ± 5.3	20.0 ± 3.2	20.3 ± 2.9	23.0 ± 3.3	71.3 ± 17.2
Amiodarone	78.5 ± 14.3	17.7 ± 4.3	20.5 ± 3.5	21.2 ± 3.4	22.2 ± 5.2	77.0 ± 28.2
Sotalol	81.8 ± 16.3	19.7 ± 5.1	19.9 ± 2.3	20.0 ± 3.1	20.9 ± 3.3	72.1 ± 10.8
E-4031	81.0 ± 10.3	18.2 ± 2.8	20.1 ± 3.1	20.5 ± 3.3	22.7 ± 3.1	77.0 ± 14.7
Ischaemic area						
Control	76.1 ± 14.1	8.1 ± 4.8*	12.4 ± 2.8**	10.2 ± 2.0**	10.9 ± 3.3**	108.9 ± 15.5**
Amiodarone	80.5 ± 14.7	16.6 ± 3.2††	19.3 ± 3.7††	20.5 ± 5.3††	18.2 ± 2.4††	80.9 ± 18.3††
Sotalol	77.8 ± 16.7	15.8 ± 4.7††	17.4 ± 3.4†	20.1 ± 2.3††	19.9 ± 4.1††	80.2 ± 14.0††
E-4031	75.6 ± 10.5	18.5 ± 4.0††	18.7 ± 3.7††	18.5 ± 5.4††	18.9 ± 3.8††	79.1 ± 15.8††

Data shown are means ± s.d.
* $P < 0.05$, ** $P < 0.01$ versus the non-ischaemic area in the corresponding group.
† $P < 0.05$, †† $P < 0.01$ versus the ischaemic area in the control group.

Time course of changes in blood pressure and heart rate

Figure 3 shows the time course of changes in arterial blood pressure. No significant changes in blood pressure were seen between the control and drug treated groups, though in the sotalol group, slight but insignificant decreases in blood pressure were observed. Figure 4 shows changes in heart rate, i.e., the difference between heart rate preinjection (physiological saline or drugs) and that after administration of drugs or saline. No significant changes in heart rate were observed in the control and E-4031 groups. In the amiodarone group, 30 and 45 min after LAD occlusion, a significant decrease in heart rate was observed. Administration of sotalol decreased heart rate significantly until 75 min after LAD occlusion.

Discussion

The general principles of treatment of acute ischaemia are to increase oxygen supply, to decrease oxygen demand, to increase anaerobic metabolism, and to prevent direct cell damage (Kirk & Jennings, 1982). Theoretically, energy transduction in ischaemic tissue might be increased by stimulating anaerobic glycolysis. However, activity of phosphofructokinase, a key enzyme of anaerobic glycolysis, is inhibited due to acidosis in association with ischaemia. Accordingly, anaerobic metabolism cannot contribute to energy production. It is well known that the best way to increase oxygen supply is reperfusion to the ischaemic myocardium. Recent advances in thrombolysis, coronary artery bypass grafting and percutaneous transluminal coronary angioplasty have produced favourable results. However, duration of ischaemia remains a determining factor for the recovery of ischaemic tissue. The most efficient clinical means to decrease oxygen demand is to induce cardiac hypothermias. This is applied to open heart surgery. β-Blockers are also known to decrease oxygen demand. The effectiveness of β-blockers on acute myocardial infarction is widely accepted. Other preventive methods of cellular damage have also been proposed; administration of allopurinol, coenzyme Q_{10}, steroids etc. (Shatney *et al.*, 1976; Nagai *et al.*, 1985). These drugs depend on the identification of the mechanisms responsible for cellular injury associated with ischaemia. With these surgical and/or pharmacological interventions, it is of primary important to prevent or, at least, to delay the onset of irreversible injury until reperfusion.

It is becoming increasingly clear that class III compounds are likely to emerge as the most significant ones for the control of cardiac arrhythmias (Beckers & Kulbertus, 1987). In the present study, we used three class III drugs: amiodarone, sotalol and E-4031. In untreated animals, occlusion of the coronary artery caused ventricular arrhythmias. On the other hand, a significant decrease in the number of ventricular arrhythmias was observed in each drug-treated group. Moreover, indices of mitochondrial function from the ischaemic area were decreased significantly compared with those from the non-ischaemic area in the control group. The leakage of lysosomal enzymes, i.e., instability of lysosomal membranes, was observed in the ischaemic myocardium in the control group, whereas, mitochondrial function from the ischaemic area in the drug-treated groups was improved significantly compared with that in the control group. Nokin *et al.* (1987) showed a protective effect of amiodarone against ischaemia-induced mitochondrial dysfunction. In the present study, stability of lysosomal membranes was also demonstrated by administration of amiodarone, sotalol or E-4031. These results suggest that class III agents show cardioprotective properties as well as antiarrhythmic effects. The acute effect of amiodarone might not be ascribed to its class III action (Wellens *et al.*, 1984). Hieda *et al.* (1988) showed that propranolol maintained mitochondrial function and prevented the leakage of lysosomal enzymes during coronary occlusion. Sugiyama *et al.* (1988) demonstrated that SUN 1165, a sodium channel inhibitor shows a similar cardioprotective effect to propranolol. Hanaki *et al.* (1989) also found that other sodium channel blockers, classified as group I antiarrhythmic agents, show cardioprotective effects. It is suggested that amiodarone has a potent sodium channel blocking action (Mason *et al.*, 1984). Hence, we cannot rule out the possibility that the protective effects of amiodarone and sotalol might be based on their respective sodium channel blocking action or β-blocking action, irrespective of their class III action. Class I antiarrhythmic drugs, as well as propranolol, have a membrane stabilizing effect (Courtney, 1988). Rhodes & Herbette (1988) suggested that this effect might depend on structural similarity to the lipophilic site in a membrane bilayer. Amiodarone, sotalol and E-4031 might have a similar effect on biomembranes, which might be linked to their cardioprotective effect.

According to the classification of Vaughan Williams (1979), antiarrhythmic compounds are categorized by their effects on (I) the fast sodium current, (II) the sympathetic activity of the heart, (III) the repolarization current, and (IV) the slow inward current. The classifications might be useful for assessment of various types of antiarrhythmic drugs. However, they are controversial in the treatment of arrhythmias associated with ischaemia, because the classification mainly depends on the effect on action potentials (Harrison, 1985). It has been found that counterpulsation reduces ventricular arrhythmias (Hanson *et al.*, 1980). The severity of ventricular arrhythmias has been shown to be related to the extent of myocardial necrosis (Cox *et al.*, 1976). Moreover, cardioprotective drugs, which are not classified as antiarrhythmic drugs such as coenzyme Q_{10} and phosphocreatine, improve lethal arrhythmias (Nagai *et al.*, 1985; Ruda *et al.*, 1988). Accordingly, it is deduced that not only electrophysiological derangements but also biochemical derangements have been implicated in the genesis of arrhythmias related to acute ischaemia. Muller *et al.* (1986) emphasized the relationship between the antiarrhythmic effect of β-blockers and their cardioprotective effect. The cardioprotective effect of antiarrhythmic agents classified as class III observed here might also participate in their antiarrhythmic effect.

In conclusion, we would like to emphasize that amiodarone, sotalol and E-4031 show a cardioprotective as well as an antiarrhythmic effect in ischaemic heart.

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Mechanism of the potentiation of neurally-induced bronchoconstriction by gallamine in the guinea-pig

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1 Electrical stimulation of the cervical vagi (15 Hz, 0.2 ms, 3 s, 7–15 V) produced a slight bronchoconstriction in the anaesthetized guinea-pig. This effect was fully abolished by atropine, while gallamine (0.1–10 μmol kg^{-1}) produced a dose-dependent increase up to ten fold.

2 Gallamine-induced potentiation of neurally-mediated bronchoconstriction was not inhibited by depletion of sensory neuropeptides with capsaicin or by pretreatment with pyrilamine. In propranolol-pretreated guinea-pigs the potentiation induced by gallamine 3 and 10 μmol kg^{-1} was inhibited by 40 and 46%, respectively.

3 Physostigmine (0.5 mg kg^{-1}) produced a very slight and slowly developing bronchoconstriction in the anaesthetized guinea-pig, which was also potentiated dose-dependently by gallamine (0.1–10 μmol kg^{-1}).

4 Gallamine (10 μmol kg^{-1}) potentiated the bronchial anaphylactic response induced by aerosol challenge with ovalbumin in actively sensitized guinea-pigs.

5 These results suggest that neither sensory neuropeptides nor histamine are involved in the gallamine-induced potentiation of neurally-mediated bronchoconstriction, while inhibition of the sympathetic nervous system may play a minor role. They are in general agreement with the hypothesis that gallamine antagonizes acetylcholine selectively at prejunctional muscarinic receptors in the guinea-pig airways, thus increasing its release from parasympathetic nerve terminals. These autoreceptors appear to be operant during anaphylactic bronchoconstriction.

Introduction

The role of the parasympathetic nervous system as the dominant neural bronchoconstrictor mechanism in all animals, including man, is soundly established (see Nadel, 1980 and Barnes, 1986 for reviews). The efferent supply to the airways is derived from ganglia located in the airway wall via short parasympathetic postganglionic fibres, the terminals of which are situated near the smooth muscle at sites extending from trachea to terminal bronchioles. Electrical stimulation of the vagus nerve causes bronchoconstriction in animals, a response that is potentiated by cholinesterase inhibitors, blocked by atropine and most likely depends on contraction of airway smooth muscle (Nadel, 1980; Barnes, 1986). Efferent cholinergic stimulation causes release of acetylcholine (ACh) from postganglionic vagal fibres. ACh activates muscarinic receptors on airway smooth muscle and thus stimulates the breakdown of membrane phosphoinositides, resulting in the release of calcium ions from intracellular stores and in smooth muscle contraction (Nadel, 1980; Barnes, 1986).

Recent studies suggest the existence of prejunctional muscarinic receptors located on airway parasympathetic nerve terminals of guinea-pigs (Fryer & Maclagan, 1984), cats (Blaber *et al.*, 1985) and man (Minette *et al.*, 1987) and endowed with an inhibitory function on ACh release. This hypothesis is mainly based on indirect evidence: gallamine, which is known to antagonize ACh at muscarinic receptors of the heart, potentiates bronchoconstriction induced by electrical stimulation of the vagus nerve, while the cholinoceptor agonist pilocarpine inhibits this response (Fryer & Maclagan, 1984; Blaber *et al.*, 1985). These authors suggest that gallamine and pilocarpine are a selective antagonist and agonist, respectively, of prejunctional muscarinic receptors in the airways.

However, conclusive evidence for the existence of these receptors and for their selective antagonism by gallamine is not yet available. We have, therefore, examined the possibility that the potentiating action of gallamine on the bronchoconstriction may be due to additional effects of this drug, such as the inhibition of the sympathetic nervous system (possibly due to its ganglion blocking activity, Bowman & Webb, 1972) and histamine release (Wali *et al.*, 1987) or to an increased release of neuropeptides, which are known to be mobilized by vagal stimulation, under certain experimental conditions, from airway sensory neurones (Lundberg *et al.*, 1983). In order to obtain a deeper insight into the mechanism and the physiopathological relevance of the potentiating action of gallamine, we also examined the effects of this agent on the bronchoconstriction induced by endogenous ACh accumulation as a result of acetylcholinesterase inhibition and anaphylactic challenge.

Methods

Male Dunkin-Hartley guinea-pigs (320–450 g) from Rodentia (Torre Pallavicina, Bergamo, Italy) were used. They were anaesthetized with urethane, 1.8 g kg^{-1} i.m. A tracheal cannula was connected to a Rodent Ventilator (U.Basile, Comerio, Varese, Italy) calibrated at 3–4 ml and 60 strokes min^{-1}. Pulmonary inflation pressure (PIP) was recorded through a side-arm of the cannula connected to a Hewlett-Packard (HP, Waltham, MA, U.S.A.) 1280 c pressure transducer. Changes in PIP probably reflect changes of both resistance and compliance components of the lungs (Fügner, 1985). Both jugular veins were cannulated, one for administration of succinylcholine chloride (100 μg kg^{-1} as a bolus followed by an infusion of 10 μg kg^{-1} min^{-1} throughout the whole experiment) and the other for the administration of other drugs. The right carotid artery was cannulated for the measurement of blood pressure by means of a HP 1280 c transducer. Heart rate, triggered by the pulse pressure, was measured by a HP 8812 A rate computer. All signals were displayed on a HP 7758 D Recorder.

Both cervical vagal nerves were carefully dissected free and cut. Their peripheral ends were placed over a Dastre electrode

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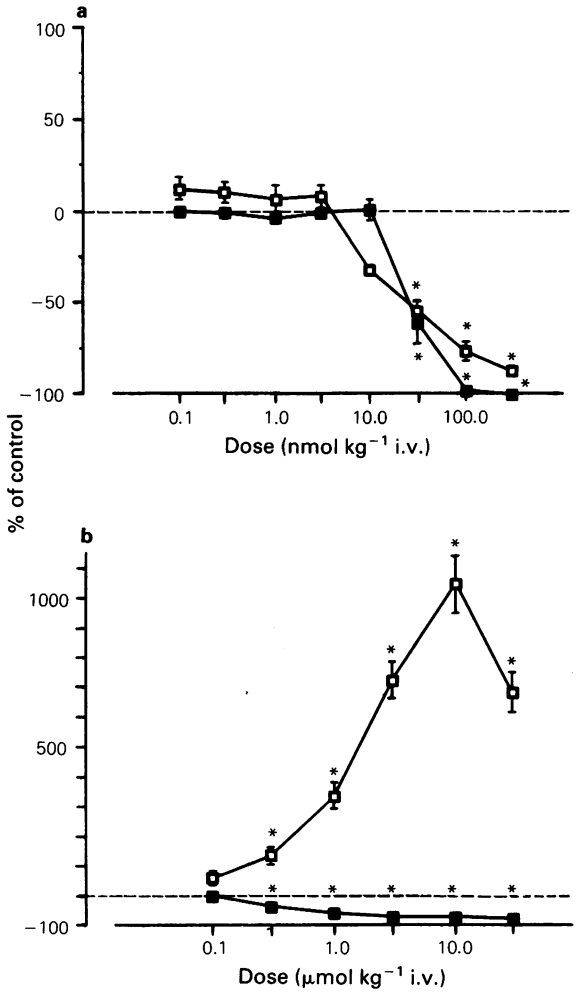

Figure 1 Effects of the intravenous administration of atropine (a) and gallamine (b) on bronchoconstriction (□) and bradycardia (■) evoked by vagal stimulation (15 Hz, 0.2 ms, 3 s, 7–15 V) in the anaesthetized guinea-pig. Each point is mean of 4–5 experiments with s.e.mean shown by vertical bars. * $P < 0.01$ (Student's unpaired t test).

(Havard Apparatus, South Natick, MA, U.S.A.) and immersed in liquid paraffin. The body temperature of the animals was maintained at 37°C with a heater control device (MARB f1/B6, Chiesina Uzzanese, Pistoia, Italy). After a stabilization period of 30 min the vagal nerves were stimulated with square waves, from a BM-ST3 stimulator (Biomedica Mangoni, Pisa, Italy), (15 Hz, 0.2 ms, 3 s, 7–15 V), at 5 min intervals. Once three similar responses (increase in PIP and decrease in heart rate) were obtained, the vehicle or the test drugs were administered 2 min before the following vagal stimulation. Each animal was used for evaluating the effects of the vehicle (repeated administrations of 1 ml kg^{-1}) or of increasing doses of a single drug at 5 min intervals. Duration of experiments never exceeded 3 h: the indicators of depth of anaesthesia were spontaneous changes in blood pressure and heart rate.

The effects of gallamine were assayed with the same protocol in the absence and in the presence of pyrilamine and propranolol and in capsaicin-desensitized guinea-pigs. Pyrilamine was administered at the dose of 0.5 mg kg^{-1} i.v., followed by a continuous infusion of 10 μg kg^{-1} min^{-1}, 13 min before the injection of the first dose of gallamine. Propranolol was given at a dose of 0.5 mg kg^{-1} i.p. immediately after the anaesthetic, followed by a continuous infusion of 10 μg kg^{-1} min^{-1}. Capsaicin desensitization was performed according to a modification of the method described by Gamse *et al.* (1981). Increasing doses of capsaicin were injected for a total of 52 mg kg^{-1} s.c., over two days. On the first day the doses, injected at 2 h intervals were: 0.3, 0.6, 1.2 and 2.4 mg kg^{-1}. On the second day they were: 2.5, 10, 15 and 20 mg kg^{-1}. Fifteen min before each capsaicin injection the animals received intraperitoneally a mixture of atropine (1.25 mg kg^{-1}), pyrilamine (2.5 mg kg^{-1}) and theophylline (10 mg kg^{-1}), plus salbutamol 0.1 mg kg^{-1} s.c. Since the first five capsaicin injections are highly irritating, pentobarbitone sodium was injected at a dose of 20 mg kg^{-1} i.p. 15 min before the first and the fifth injection and at a dose of 10–15 mg kg^{-1} i.p. 15 min before the second, third and fourth injection; 65% of the animals survived this treatment and were used 5–11 days after desensitization. In order to check the validity of this procedure, at the end of the vagal stimulation experiment each guinea-pig received capsaicin 10 μg kg^{-1} i.v., a dose that produced a maximal bronchoconstriction in normal animals but did not affect PIP in any of our desensitized guinea-pigs.

Effects of gallamine on vagally-mediated atropine-resistant bronchoconstriction

The experimental procedure was the same as described above, except for stimulation parameters, which were: 5 Hz, 5 ms, 30 s, 4–8 V, according to Grundström & Andersson (1985). The nerves were stimulated at 10 min intervals. When three similar responses were obtained, atropine, 0.28 μmol kg^{-1} min^{-1}, was infused intravenously. After three additional stimulations, vehicle or gallamine, 10 μmol kg^{-1}, was injected 2 min before the following stimulation (see Figure 3).

Effects of gallamine on physostigmine-induced bronchoconstriction

The animals were prepared as described above, but the vagi were left intact. Following a 30 min stabilization period physostigmine, 0.5 mg kg^{-1}, was injected i.v. After 5 min, vehicle (1 ml kg^{-1}) or increasing doses of gallamine were administered repeatedly at 2 min intervals (see Figure 4).

Effects of gallamine on bronchial anaphylaxis

The method described by Daffonchio *et al.* (1987) was used, except that the vagi were left intact. Briefly, animals were sensitized with ovalbumin, 50 mg i.p. together with 50 mg s.c. Sixteen days after sensitization the animals were prepared as described above. Following a 30 min stabilization period, the animals received either vehicle or gallamine, 10 μmol kg^{-1} i.v. After 2 min an aerosol of ovalbumin, generated by a Pulmosonic ultrasonic nebulizer (Devilbiss, Somerset, PA, U.S.A.) connected in series with the efferent limb of the ventilator circuit, was administered for 10 s. The concentration used was 3 mg ml^{-1}, which produced in our conditions a submaximal bronchoconstriction (microshock according to Daffonchio *et al.*, 1987).

Analysis of data

Results are expressed as mean ± s.e.mean. Responses obtained in drug-treated animals were compared with those obtained in vehicle-treated controls by means of Student's unpaired t test. A P value of 0.05 or less was taken to be significant.

Drugs

Atropine sulphate, gallamine triethiodide, physostigmine hemisulphate, pyrilamine maleate, (±)-propranolol hydrochloride, succinylcholine chloride and ovalbumin (Grade V) were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). Capsaicin and urethane were purchased from Fluka (Buchs, Switzerland) and theophylline monohydrate from C.Erba (Milan, Italy). Salbutamol sulphate was a kind gift of Glaxo (Verona, Italy).

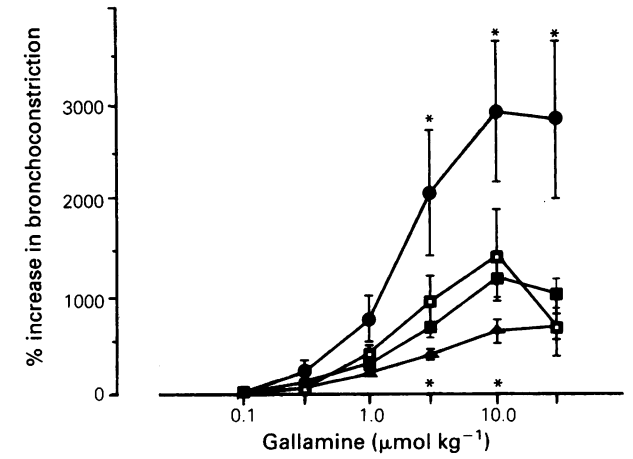

Figure 2 Effects of vehicle (■), pyrilamine (□), propranolol (▲), and capsaicin desensitization (●) on gallamine-induced potentiation of vagally-mediated bronchoconstriction (15 Hz, 0.2 ms, 3 s, 7–15 V) in anaesthetized guinea-pigs. Propranolol and pyrilamine were administered at the dose of $0.5\,mg\,kg^{-1} + 10\,\mu g\,kg^{-1}\,min^{-1}$. For capsaicin desensitization see Methods. Each point is the mean of 4–6 experiments with s.e.mean shown by vertical bars. $^{*}P < 0.05$ (Student's unpaired t test).

The drugs were diluted in 0.9% w/v NaCl solution and administered intravenously in a volume of $1\,ml\,kg^{-1}$. Capsaicin was dissolved in 10% ethanol and 10% Tween 80 in saline and given in a volume of $2\,ml\,kg^{-1}$ s.c.

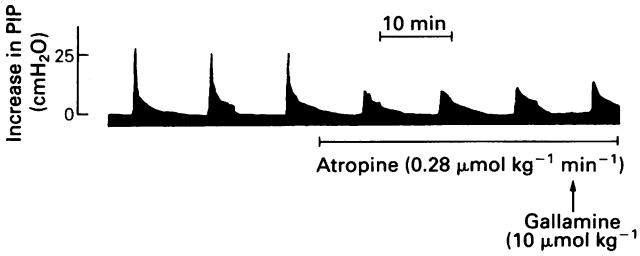

Figure 3 Effect of gallamine on the atropine-resistant portion of the response to vagal stimulation (5 Hz, 5 ms, 30 s, 4–8 V). A representative tracing of one of three similar experiments is shown. The response to vagal stimulation was slightly and non-significantly increased (32 ± 9%) by gallamine, when compared to vehicle-treated controls ($n = 3$).

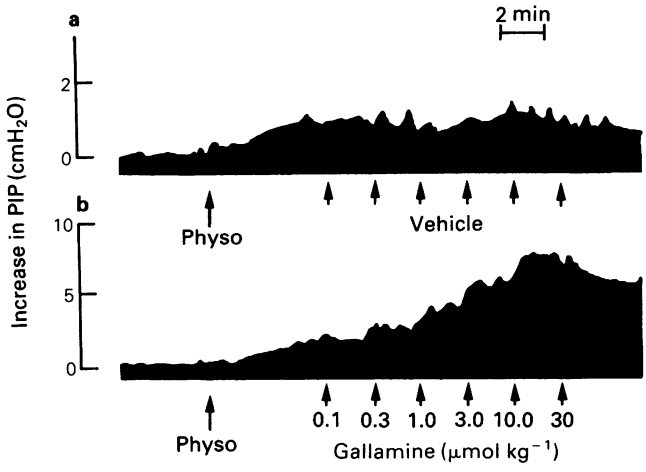

Figure 4 Representative tracings of the bronchial response to physostigmine (Physo) $0.5\,mg\,kg^{-1}$ in the anaesthetized guinea-pig. The slight and slowly developing increase of pulmonary inflation pressure (PIP) shown in the control tracing (a) is amplified dose-dependently by gallamine (b).

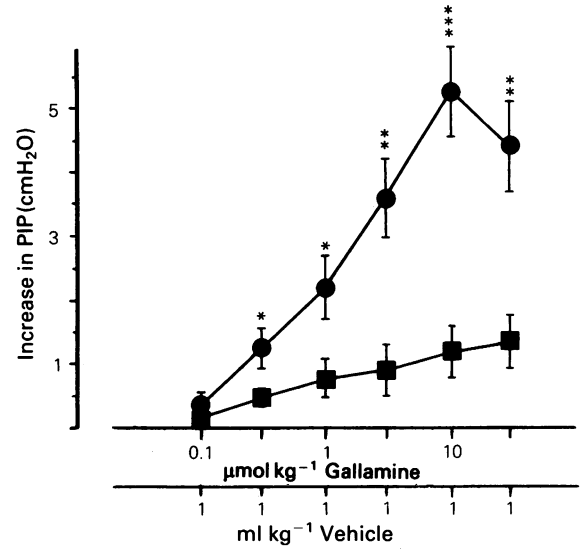

Figure 5 Potentiation by gallamine of the effect of physostigmine on pulmonary inflation pressure (PIP) of anaesthetized guinea-pigs. After the injection of physostigmine ($0.5\,mg\,kg^{-1}$ i.v.), either normal saline (■, $1\,ml\,kg^{-1}$) or gallamine (●, 0.1 to $30\,\mu mol\,kg^{-1}$) were injected repeatedly at 2 min intervals (see Figure 4). Each point is mean of 6 (control) or 4 (gallamine) animals with s.e.mean shown by vertical bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's unpaired t test).

Results

Effects of muscarinic antagonists

Vagal stimulation (15 Hz, 0.2 ms, 3 s, 7–15 V) in anaesthetized guinea-pigs rapidly induced an increase in PIP by 2.27 ± 0.16 cmH_2O (mean value ± s.e.mean, $n = 27$) and a decrease of HR by 157 ± 15 beats min^{-1} ($n = 17$). Vehicle administration for six consecutive times at 5 min intervals did not produce any change in the bronchoconstriction or bradycardia (data not shown). Atropine (10–100 $nmol\,kg^{-1}$) inhibited dose-dependently both responses with the same potency (Figure 1a). Gallamine (0.1–10 $\mu mol\,kg^{-1}$) caused a dose-related increase, up to ten fold, in vagally-mediated bronchoconstriction and a concomitant inhibition of the bradycardic response, which was abolished at the highest doses (Figure 1b).

Effects of propranolol, pyrilamine and capsaicin desensitization on gallamine-induced potentiation of bronchoconstriction

The effects of several drugs on the potentiating action of gallamine on vagally-mediated bronchoconstriction were evaluated in separate groups of animals. Capsaicin-desensitized animals were less responsive to vagal stimulation: in 3 out of 7 animals bronchoconstriction was not achieved even at 20–30 V, while the remaining guinea-pigs responded at 13–18 V with a less intense bronchoconstriction (0.87 ± 0.05 cmH_2O, $n = 4$, $P < 0.001$ vs controls). In these animals the potentiating effect of gallamine, 3–30 $\mu mol\,kg^{-1}$, was significantly higher than in normal guinea-pigs (Figure 2). This is probably a consequence of the lower basal response of desensitized animals to vagal stimulation: in fact, when the same results are plotted as absolute values rather than % increase, the potentiation induced by gallamine is similar in desensitized and in normal guinea-pigs (data not shown).

In propranolol-pretreated guinea-pigs, the dose-response curve for gallamine appeared to be reduced, the difference being significant at 3 and 10 $\mu mol\,kg^{-1}$ of gallamine, with an inhibition of 40% and 46% respectively. On the contrary,

pyrilamine did not affect the potentiation of bronchoconstriction by gallamine (Figure 2).

Effects of gallamine on atropine-resistant vagally-induced bronchoconstriction

A longer lasting vagal stimulation (5 Hz, 5 ms, 30 s, 5–8 V) induced an increase in PIP by 21.8 ± 1.1 cmH_2O ($n = 6$). This response was inhibited only partially (55 ± 7%, $n = 6$) by an infusion of atropine (0.28 $\mu mol\,kg^{-1}\,min^{-1}$ for 40 min). Gallamine at 10 $\mu mol\,kg^{-1}$, the dose that produced the maximal enhancing action with the previously described stimulation parameters (Figure 1b), provoked a slight and non-significant potentiating effect (32 ± 9%, $n = 3$) (Figure 3).

Effects of gallamine on physostigmine-induced bronchoconstriction

Physostigmine, 0.5 $mg\,kg^{-1}$, produced a slight and slowly developing increase in PIP (Figure 4 and 5). Gallamine caused a dose-dependent potentiation of this response (Figure 4 and 5), with a pattern resembling that observed in the vagal stimulation experiments.

Effects of gallamine on bronchial anaphylaxis

Figure 6 shows the effects of an aerosol administration of ovalbumin in actively sensitized guinea-pigs with intact vagi. The antigen administration, at a submaximal dose, induced an increase in PIP, which peaked at 2 min and appeared largely reversible. After the administration of gallamine (10 $\mu mol\,kg^{-1}$) the anaphylactic response was considerably enhanced and the difference from controls was significant during the period of 1 to 5 min after the administration of gallamine.

Discussion

Our results are in good agreement with those of Fryer & Maclagan (1984), showing that gallamine produces a massive potentiation of vagally-induced bronchoconstriction, while at the same time it inhibits the bradycardic response. Fryer & Maclagan (1984) interpreted these results as indicating that gallamine blocked putative prejunctional muscarinic receptors, located in the parasympathetic nerves supplying the lungs and endowed with an inhibitory function.

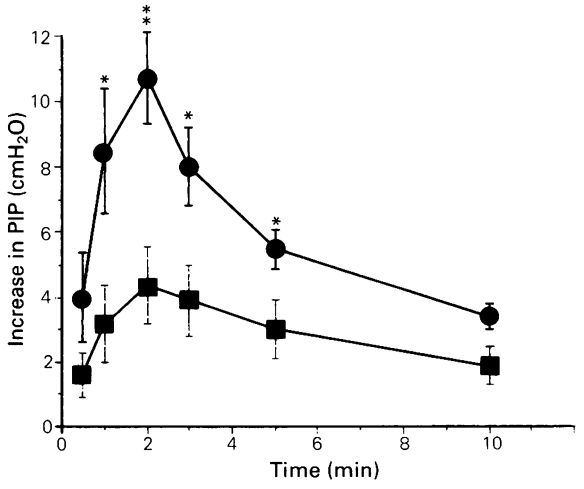

Figure 6 Potentiation by gallamine of the bronchoconstriction induced by an aerosol antigen challenge in actively sensitized anaesthetized guinea-pigs. Gallamine (●, 10 $\mu mol\,kg^{-1}$) or vehicle (■), were injected 2 min before the inhalation of ovalbumin 3 $mg\,ml^{-1}$ for 10 s. Each point is the mean of 6 animals with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.01$ (Student's unpaired t test).

However, the existence of prejunctional inhibitory muscarinic receptors can only be proven by direct measurement of ACh release, which should be enhanced by muscarinic antagonists and inhibited by muscarinic agonists. To our knowledge this evidence, available for central cholinergic neurones as well as for a number of peripheral cholinergic nerves (Kilbinger, 1984), has not yet been provided for the airway parasympathetic nerves. The potentiating action of gallamine might also be explained by its ganglion blocking activity (Bowman & Webb, 1972). This activity may inhibit the sympathetic nervous system, particularly adrenaline release from the adrenal medulla, which may lead to blockade of a compensatory bronchodilator reflex following bronchoconstriction (McCulloch *et al.*, 1967). In fact, propranolol did inhibit part of the potentiating response to gallamine. However, this inhibition never reached 50%, indicating that inhibition of sympathetic neurotransmission is not likely to be the major determinant of this response. Another possible action of gallamine that must be taken into account is histamine release. In fact, gallamine (0.1–7 μM) produces marked contractions of rat isolated ileum, which are reduced by antihistamines (Wali *et al.*, 1987). Although it is unlikely that gallamine releases large amounts of histamine from the guinea-pig airways, since it does not cause, given alone, any bronchoconstriction (data not shown), it could release subthreshold quantities, acting synergistically with ACh released during vagal stimulation. However, the lack of influence of pyrilamine on the potentiation of bronchoconstriction by gallamine shows that this is not the case.

So far we have postulated that bronchoconstriction induced by vagal stimulation is dependent exclusively on the release of ACh from airway parasympathetic nerve terminals. In fact, in our experiments bronchoconstriction was inhibited by atropine with a dose-response curve which is superimposable on that of the inhibition of vagally-induced bradycardia and both responses were suppressed by atropine 100 $nmol\,kg^{-1}$ (Figure 1a). However, in different conditions of vagal stimulation the increase in PIP is only partially inhibited by atropine and there is evidence suggesting that in this case a major portion of the response is caused by the release of substance P or other neuropeptides from antidromically stimulated C-fibre afferents (Lundberg *et al.*, 1983). Accordingly, two experimental protocols were designed in order to check the possibility that gallamine may favour the release of bronchoconstrictor neuropeptides. The effects of gallamine were assayed (a) on the vagally-induced bronchoconstriction in capsaicin-desensitized guinea-pigs, which had been depleted of sensory neuropeptides (Gamse *et al.*, 1981) and (b) on the atropine-resistant component of a more intense bronchoconstriction, elicited by use of higher parameters for vagal stimulation (Grundström & Andersson, 1985). Both results speak against an involvement of sensory neuropeptides in the potentiating action of gallamine.

Physostigmine, the classical anticholinesterase agent, produces a very slight increase in PIP in the anaesthetized guinea-pig. This effect is most probably due to accumulation of ACh at muscarinic airway smooth muscle receptors, released physiologically in the absence of electrical stimulation. Marked potentiation of physostigmine-induced bronchoconstriction by gallamine is further evidence that this atypical muscarinic antagonist works by suppressing a feedback mechanism inhibiting endogenous ACh release and fits very well with the hypothesis of the existence of prejunctional inhibitory muscarinic receptors. It should be also pointed out that the dose-response curves of the potentiation of physostigmine and vagal stimulation by gallamine are superimposable, suggesting a similar mechanism of action.

Finally, we tried to find out if this hypothetical mechanism of action of gallamine (selective antagonism of ACh at airway prejunctional muscarinic receptors) is operant during the immediate hypersensitivity reaction. For this reason we chose

the 'microshock' aerosol challenge stimulus in actively sensitized guinea-pigs (Daffonchio *et al.*, 1987), which seems to cause bronchoconstriction through the release of histamine and leukotrienes. Vagal reflex is partly responsible for the anaphylactic bronchoconstriction in guinea-pigs, provided anaesthesia is not very deep and the shock not excessively severe (Fügner, 1985), as is the case of the present experiments. Therefore the enhancing action of gallamine on the 'microshock' is likely to depend on the potentiation of this vagal reflex, through the blockade of prejunctional inhibitory muscarinic receptors in the airways.

Our analysis of the mechanism of action of gallamine in potentiating neurally-mediated bronchoconstriction is still based on indirect evidence, due to the difficulties of measuring ACh release from vagal nerves *in vivo*. In spite of this limitation, our results are in agreement with the hypothesis that at least a major part of the potentiating action of gallamine on neurally-mediated bronchoconstriction depends on the blockade of inhibitory prejunctional muscarinic receptors. In addition, it is important to underline that these autoreceptors appear able to modulate anaphylactic bronchoconstriction and therefore may play an important role in asthma.

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Somatostatin decreases the calcium inward current in guinea-pig atria

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1 The effects of somatostatin on mechanical and electrophysiological responses were studied in guinea-pig atrial muscle preparations and single cells.

2 Somatostatin ($\geqslant 10^{-8}$ M) decreased the twitch contraction in a concentration-dependent manner in electrically driven left atria and spontaneously beating right atria. However, the beating rate was not affected.

3 The negative inotropic effect of somatostatin was transient. Desensitization to this agent developed slowly during continuous exposure to the peptide.

4 In single atrial cells, somatostatin significantly shortened the action potential duration, but the resting potential and action potential amplitude were not affected.

5 Under whole cell voltage-clamp conditions, somatostatin decreased the calcium inward current without affecting the sodium and potassium currents.

6 These results suggest that somatostatin selectively acts on the calcium channel of guinea-pig atrial cells to reduce the calcium inward current, which in turn gives rise to the negative inotropic effect.

Introduction

Somatostatin is present in sympathetic and/or sensory nerves in many peripheral tissues including heart (Pradayrol *et al.*, 1980; Hökfelt *et al.*, 1980; Day *et al.*, 1985). Although the functional significance of somatostatin in the cardiovascular system is still unclear, the peptide may be implicated in a variety of cardiac disorders including arrhythmia. In a clinical trial, intravenous administration of somatostatin has been shown to restore sinus rhythm in patients with paroxysmal supraventricular tachycardia and junctional tachycardia (Greco *et al.*, 1984; Ghirlanda *et al.*, 1985). Somatostatin also has a negative inotropic effect in the human isolated atria (Franco-Cereceda *et al.*, 1987), guinea-pig atria (Quirion *et al.*, 1979; Diez *et al.*, 1985; Lin *et al.*, 1988) and dog Purkinje fibres (Lin *et al.*, 1988). Since somatostatin inhibited calcium influx in the experiment with ^{45}Ca (Diez & Tamargo, 1987), the negative inotropic and/or chronotropic effects may be related to the decrease in calcium inward current.

In the present study, we analysed the effects of somatostatin on guinea-pig cardiac cells with special reference to calcium current. The results obtained here suggest that the negative inotropic action of somatostatin may be attributable to a decrease in the calcium inward current.

Methods

Contractility measurement

Male guinea-pigs weighing 250–350 g were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.), exsanguinated from the carotid arteries and the hearts were quickly isolated. Right and left atria and papillary muscles were dissected and mounted vertically in 20 ml organ baths containing modified Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, NaH_2PO_4 0.21 and glucose 5.6, gassed with a mixture of 95% O_2 and 5% CO_2 at 30°C. Square wave pulses of 1 ms duration and suprathreshold voltage were applied at frequencies ranging from 0.1 Hz to 2 Hz in left atria and papillary muscles. In right atria, spontaneous beating was recorded. Contractile force was measured isometrically by a force displacement transducer (T7-30-240, Toyo boldwin, Japan) and recorded on a strip chart recorder (RJG-4128, Nihon Kohden, Japan). The preparations were equilibrated at a resting tension of 1 g (in atria) or 0.5 g (in papillary muscle) for 90 min before the experiments were started. Propranolol (10^{-6} M) and atropine (10^{-6} M) were added to the baths 15 min before the treatment with somatostatin in order to eliminate possible neurogenic responses.

Isolation of single atrial cells

Guinea-pig heart was isolated and mounted on the Langendorff apparatus to perfuse the coronary artery with oxygenated and pre-warmed (37°C) normal external solution (Table 1) at a pressure of 65 cmH_2O. After the heart had been cleared of blood, the perfusate was changed to Ca-free external solution containing 0.4 mg ml^{-1} collagenase (Type I, Sigma, U.S.A.) and the solution recirculated by a peristaltic pump for 25 min. Thereafter, the preparation was washed out with 100 ml of high K-low Cl solution (storage solution) and was stored in this solution at 4°C until the experiments were started.

Whole-cell clamp experiment

A piece of tissue was dissected from the collagenase-perfused left atrium mentioned above and the cells dispersed in the experimental chamber (0.5 ml in volume) filled with the storage solution. After the cells had settled on the floor of the chamber, the perfusion of normal external solution was started at a rate of 2–3 ml min^{-1}. The temperature of the perfusate in the experimental chamber was maintained at 33°C.

Patch electrodes were fabricated from glass capillaries of 1.5 mm in outer diameter by a double step microelectrode puller (PP-83, Narishige, Japan). After the tip was polished by heat, the electrode was filled with internal solution (Table 1).

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Table 1 Composition of external and internal solutions (mM)

	External solution					
	NaCl	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*Glucose*	*HEPES*
Normal	136.9	5.4	1.8	0.5	10	5
K-free	136.9	—	1.8	0.5	10	5

	Internal solution							
	K aspartate	*KCl*	*Cs aspartate*	*$CsCl_2$*	*$MgCl_2$*	*Phospho-creatine**	*ATP***	*EGTA*
K-rich	100	20	—	—	2.5	5	5	5
Cs-rich	—	—	100	20	2.5	5	5	5

The pH of the external solution was adjusted to 7.2 by NaOH. In internal solutions, pH was adjusted to 7.2 by KOH or CsOH.
* Phosphocreatine (disodium salt); **adenosine-5'-triphosphate (dipotassium salt).

Outer diameter of the electrode tip was 2–5 μm and the electrode resistance ranged between 1 and 3 MΩ. Patch clamp amplifier (S-3666, Nihon Kohden) was used to record the whole-cell membrane currents. After the electrode tip touched the cell surface, a negative pressure of 20–40 cmH_2O was applied to the interior of the electrode. Within a few seconds, a GΩ seal was established between the electrode tip and the cell surface. Then the membrane patch at the tip of the electrode was ruptured by applying further suction of -50 to $-100\,cmH_2O$. Rupture was indicated by a large increase in the capacitive current. The current signals and membrane potentials were displayed on an oscilloscope (VC-10, Nihon Kohden) and the signals were digitized by a PCM recording system (NF Circuit Design Block, RP-880) and stored on videotape for later analysis.

Solutions and drugs

The compositions of the external and internal solutions used are listed in Table 1. The composition of high K-low Cl storage solution was (mM): taurine 10, oxalic acid 14, glutamic acid 80, KCl 25, KH_2PO_4 10, N-(2-hydroxyethyl)piperazine-N'-3-propanesulphonic acid (HEPES) 10, glucose 11, ethylene glycol *bis* (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.5, pH was adjusted to 7.2 by adding KOH (Isenberg & Klöckner, 1982). Somatostatin (Peptide Institute Inc., Osaka, Japan) was dissolved in saline solution containing 2% gelatin. (±)-Propranolol hydrochloride (Nacalai Tesque Inc., Kyoto, Japan) and atropine sulphate (Wako Pure Chemical Industries Ltd., Osaka, Japan) were dissolved in distilled water.

Data analysis

Experimental values are given as mean ± s.e.mean. The effects of drugs were considered significant if $P < 0.05$ by Student's t test for unpaired data.

Results

Effects of somatostatin on mechanical responses in atrial and papillary muscle preparations

Somatostatin attentuated the twitch contraction both in electrically driven left atria (0.33 Hz) and spontaneously beating right atria (Figure 1). A decrease in twitch contraction by 10^{-6} M somatostatin was produced immediately after the application and attained the maximum in a few minutes, then contraction recovered to the control level gradually. Subsequent cumulative application of 10^{-6} M somatostatin failed to cause further reduction in twitch contraction (Figure 1). These results suggested the occurrence of a comparatively slow but apparent desensitization phenomenon in the response of somatostatin. Figure 2 shows the concentration-response curves of somatostatin, in which somatostatin was applied non-cumulatively and the maximum response at each concentration was plotted. The effects of somatostatin were concentration-dependent and no difference was observed in the potencies between left and right atria. Beating-rate of the right atria was not significantly affected by 10^{-8} M to 10^{-6} M

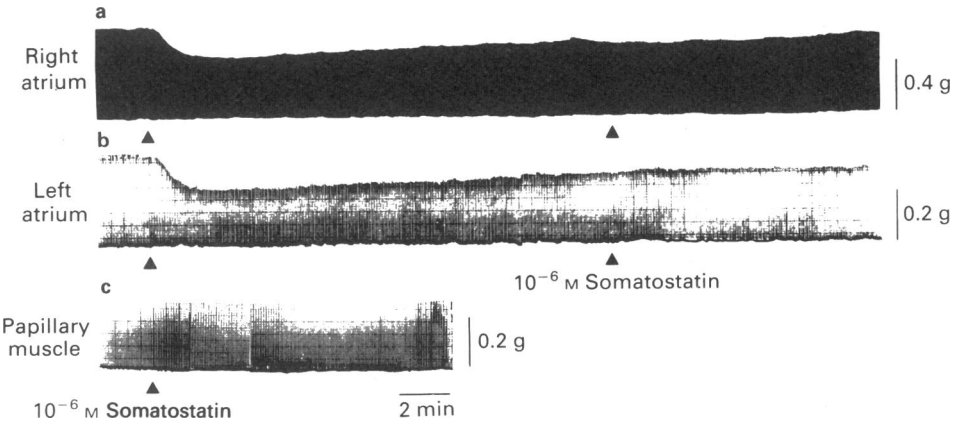

Figure 1 Representative recordings showing the negative inotropic effect of somatostatin (10^{-6} M) in isolated right atrium (a), left atrium (b) and papillary muscle (c). Right atrium beat spontaneously. Left atrium and papillary muscle were electrically driven at a frequency of 0.33 Hz. Somatostatin (10^{-6} M) was applied cumulatively in (a) and (b).

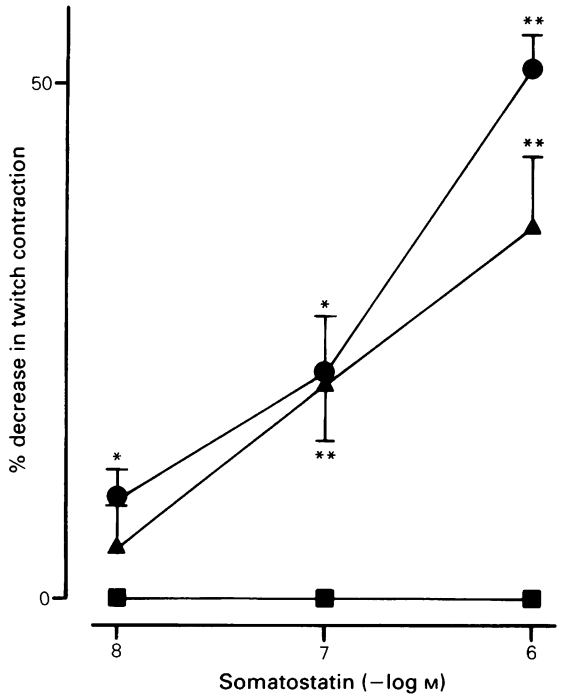

Figure 2 Concentration-inhibition curves of somatostatin in the right atria (●), left atria (▲) and papillary muscles (■). Left atria and papillary muscle were driven at a frequency of 0.33 Hz. The extent of inhibition of twitch contraction was plotted against the concentrations of somatostatin. Each value indicates the mean (n = 4–8). Vertical lines show s.e.mean. *P < 0.05, **P < 0.01 (significant difference from control).

somatostatin (131 ± 15 beats min^{-1} and 134 ± 17 beats min^{-1}, before and after application of 10^{-6} M somatostatin, respectively; n = 5).

In contrast to the atria, somatostatin (10^{-8} M–10^{-6} M) did not affect the twitch contraction in the papillary muscles stimulated at a constant rate of 0.33 Hz (Figure 1) or 1 Hz (data not shown).

Frequency-dependent effects on mechanical response in the left atria

Figure 3 shows the effect of the stimulus frequencies on the negative inotropic action of somatostatin in electrically driven left atria. Since the effect of somatostatin was transient, as mentioned above, 10^{-6} M somatostatin was applied under conditions where twitch contractions reached a steady state at each frequency. Significant reduction of twitch response by somatostatin was produced at all frequencies tested (0.1–2 Hz). However, the effects varied with stimulus frequency; the inhibition was more marked at lower frequencies. Therefore, we used a frequency of 0.33 Hz in the following experiments.

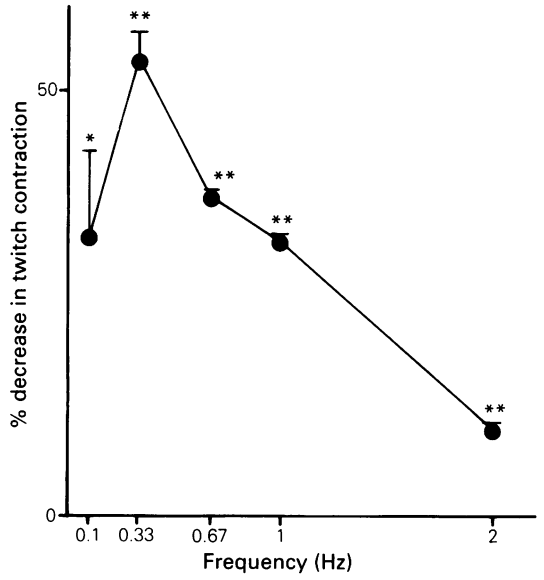

Figure 3 Effects of stimulus frequency on the negative inotropic effect of somatostatin in left atria. Each value indicates mean (n = 4–7). Vertical lines show s.e.mean. *P < 0.05, **P < 0.01 (significant difference from control).

Effects on the resting and action potentials in single atrial cells

Somatostatin at concentration of 10^{-7} M or 10^{-6} M shortened the action potential duration in the single atrial cells. Representative recordings of the effects of 10^{-7} M and 10^{-6} M somatostatin are shown in Figure 4. The cells were stimulated at a frequency of 0.33 Hz. The shortening of action potential

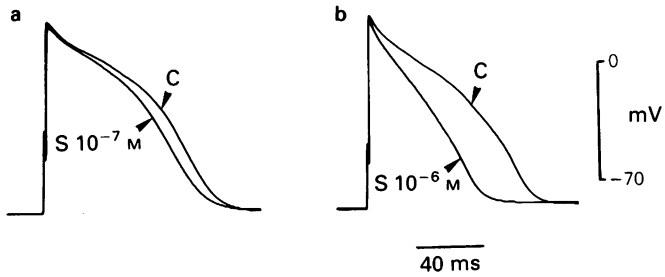

Figure 4 Effects of somatostatin on the action potentials in single left atrial cells. The cells were stimulated at a frequency of 0.33 Hz. (a) Shows action potentials before (C) and 5 min after the treatment with 10^{-7} M somatostatin(S). (b) Shows action potentials before (C) and 5 min after the treatment with 10^{-6} M somatostatin(S). Recordings from two different cells.

Table 2 Effects of somatostatin on resting and action potentials of guinea-pig single atrial myocytes

Treatment	n	*RP*	*APA*	APD_{30}	APD_{50}	APD_{90}
Control	6	100% (−75.5 ± 1.2 mV)	100% (107.7 ± 2.9 mV)	100% (67.8 ± 13.1 ms)	100% (90.7 ± 12.6 ms)	100% (109.8 ± 15.5 ms)
Somatostatin 10^{-7} M	3	98.8 ± 0.7%	100.6 ± 0.9%	80.9 ± 4.2%*	86.7 ± 5.2%	94.1 ± 6.3%
10^{-6} M	3	100.8 ± 0.2%	99.1 ± 0.5%	69.6 ± 4.0%**	72.6 ± 3.5%**	78.0 ± 2.0%**

RP, resting potential; APA, action potential amplitude; APD_{30}, APD_{50} and APD_{90}, action potential duration at 30%, 50% and 90% repolarization. n: number of cells. Values after somatostatin treatment are expressed as % of control. Absolute values in control are also shown in parentheses.
*P < 0.05, **P < 0.01 (significant difference from control).

appeared immediately after the perfusion of somatostatin was commenced and lasted for more than 5 min. The effects of somatostatin on membrane potential parameters are summarized in Table 2, where each parameter is expressed as percentage of the control value because of the large variation in absolute values between the cells. Somatostatin (10^{-7} M and 10^{-6} M) significantly shortened the action potential duration, an effect which was more prominent at 30% repolarization (APD_{30}) than at 50% repolarization (APD_{50}) or 90% repolarization (APD_{90}). The action potential amplitude and resting membrane potential were unaffected by somatostatin.

Effects of somatostatin on ionic currents in single atrial cells

Whole-cell voltage clamp experiments were carried out using single atrial cells of guinea-pig. In experiments with normal external and potassium-rich internal solutions (Table 1), the membrane potential was held at the resting potential (−70 mV in Figure 5) and the depolarizing or hyperpolarizing pulses for 300 ms to various potentials were delivered every 3 s. Somatostatin (10^{-6} M) did not affect the currents flowing at the end of the 300 ms test pulses (Figure 5). However, the inward currents at the early phase of depolarizing pulses were somewhat attenuated by somatostatin. In order to analyse the effects on the calcium inward current, we dialyzed the cells internally with 120 mM caesium (Lee & Tsien, 1982; Nishio *et al.*, 1986) and superfused externally with potassium-free solution (Table 1). Furthermore, the membrane potential was held at −50 mV to inactivate sodium channels. Thus, calcium inward current was isolated from overlapping sodium and potassium currents (Muramatsu *et al.*, 1987). Figure 6 shows

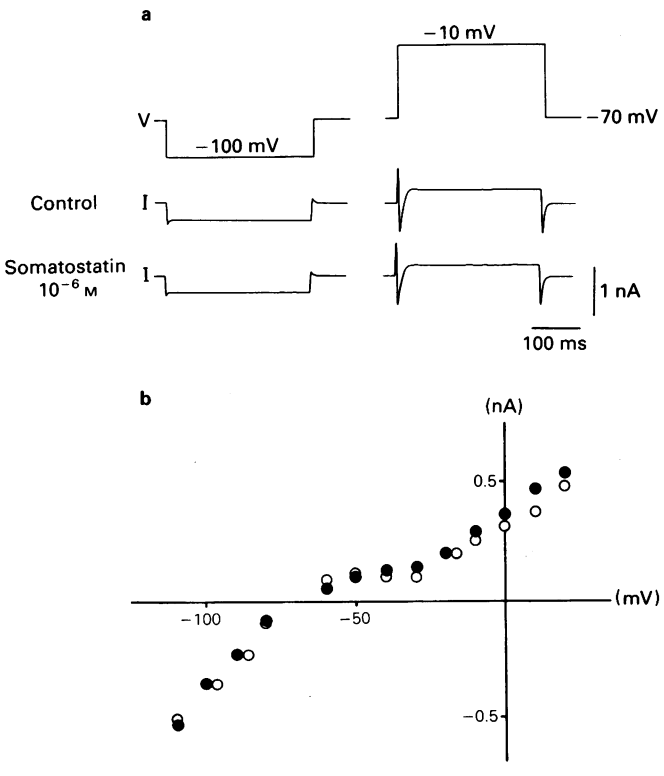

Figure 5 Effects of somatostatin on ionic currents in a single atrial cell. (a) Representative currents recorded upon clamp steps from −70 mV (holding potential) to −10 mV and −100 mV before and after application of 10^{-6} M somatostatin. The sodium currents at a depolarization to −10 mV could not be recorded exactly. (b) Current-voltage relationships. The current amplitude was measured at the end of 300 ms clamp pulses before and 5 min after application of somatostatin.

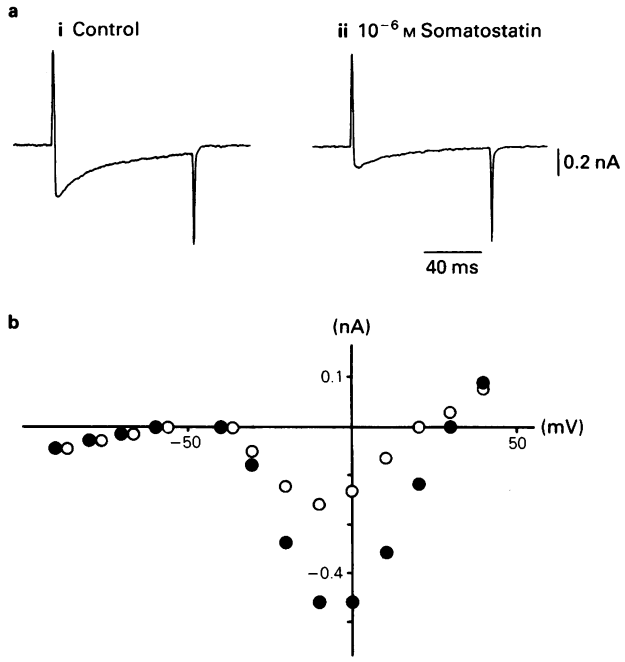

Figure 6 Effect of somatostatin on calcium currents. The atrial cell was dialysed with caesium-rich internal solution and perfused with potassium-free external solution. (a) The membrane was held at −50 mV under voltage clamp conditions and step depolarizing pulses to 0 mV were applied every 3 s. (i) Before and (ii) 5 min after application of 10^{-6} M somatostatin. (b) Current-voltage relationships before (●) and 5 min after the application of 10^{-6} M somatostatin (○).

the calcium inward currents elicited by the depolarizing test pulses to 0 mV, before and 4 min after application of somatostatin, and the current-voltage relationships, in which the current amplitude was measured at the peak of the inward current. The inward calcium currents were elicited by test pulses to membrane potentials more positive than −40 mV. The calcium inward current was significantly reduced after perfusion of 10^{-6} M somatostatin (45.1 ± 8.2% reduction in the current amplitude at 0 mV, $n = 6$).

Finally, we examined the effect of somatostatin on sodium current. The cell was perfused with normal external solution and the membrane was held at the resting potential (−75 mV). The experimental temperature was 22°C to slow down the sodium channel kinetics. Figure 7 shows representative recordings, in which the sodium current was elicited by a depolarization from −75 mV to −30 mV. Somatostatin (10^{-6} M) did not affect the sodium current ($n = 3$).

Discussion

Somatostatin produced a negative inotropic effect in guinea-pig atria and shortened the action potential duration (APD) of

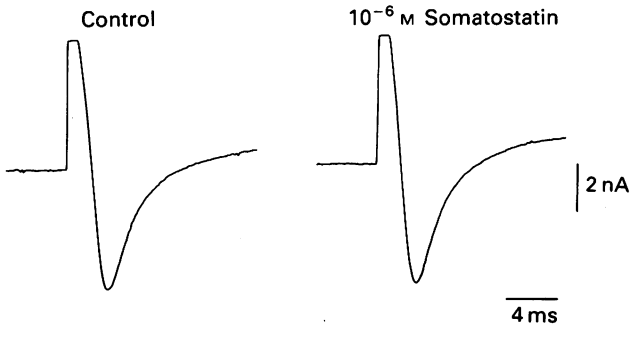

Figure 7 Effects of somatostatin (10^{-6} M) on sodium current elicited by 50 ms clamp pulses to −30 mV from a holding potential of −75 mV in a single atrial cell. Temperature 22°C.

single atrial cells. The shortening of APD was more dominant at the plateau phase rather than at the end of repolarization. Since the calcium inward current contributes to maintain the plateau phase of action potential in the atrial myocytes (Hume & Uehara, 1985), it is likely that somatostatin causes the shortening of action potential duration by inhibiting calcium inward current. In fact, somatostatin inhibited the calcium inward current in single atrial cells without affecting the potassium and sodium currents under voltage clamp conditions. It is well known that calcium inward current is responsible for excitation-contraction coupling (Reuter, 1973). Therefore, the negative inotropic effect of somatostatin seems to be accounted for by the decrease of calcium inward current.

In contrast to the atria, somatostatin failed to produce any significant effects in the ventricular muscles (Figure 1c). The reasons for such regional differences in the effects of somatostatin are not known. However, it is interesting to note that somatostatin is present in the sinotrial and atrioventricular node but not in the ventricular muscle (Day *et al.*, 1985). Such heterogeneous distribution of somatostatin suggests that the peptide may play a physiological role in the conduction system of the heart. In fact, intravenous administration of somatostatin has been shown to restore sinus rhythm in paroxysmal supraventricular tachycardia and junctional tachycardia in man (Greco *et al.*, 1984; Webb *et al.*, 1986; Ghirlanda *et al.*, 1989). The present study also indicates that the antiarrhythmic effect of somatostatin may result from an inhibition of calcium channels.

Somatostatin has been shown to act on various ionic channels in various tissues. For example, somatostatin decreases the calcium current in neuroblastoma cells (Tsunoo *et al.*, 1986) and increases an inwardly rectifying potassium current in guinea-pig submucous plexus neurones (Mihara *et al.*, 1987). The present study indicates that somatostatin inhibits calcium channel current, without affecting the other ionic channels, in guinea-pig heart and that its negative inotropic effect can be attributed to this decrease in calcium inward current.

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The β_1- and β_2-adrenoceptor affinity and β_1-blocking potency of S- and R-metoprolol

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1 The β-adrenoceptor affinity and blocking potency of the two enantiomers and the racemate of metoprolol were investigated *in vitro*, by use of a receptor-binding technique, and *in vivo* in the anaesthetized cat.

2 The enantiomeric purity of the S- and R-form was: >99.2% and >99.9%, respectively.

3 The β_1- and β_2-adrenoceptor affinity (−log equilibrium dissociation constant) of the enantiomers was determined from competition binding experiments (radioligand: [^{125}I]-(S)-pindolol) performed in membranes prepared from the guinea-pig left ventricular free wall (predominantly β_1) and soleus muscle (β_2). The β_1-adrenoceptor affinity was (means ± s.d.): 7.73 ± 0.10 and 5.00 ± 0.06 for the S- and R-form of metoprolol, respectively. The corresponding values for β_2-adrenoceptors were 6.28 ± 0.06 (S) and 4.52 ± 0.09 (R). Thus, the difference in affinity for the two enantiomers was greater on β_1- (about 500) than on β_2-adrenoceptors (about 50). The β_1-adrenoceptor selectivity of the S-form (about 30) was similar to that of the racemic metoprolol, while the R-form was almost non-selective (3 fold β_1-selective).

4 In the anaesthetized cat, the (−log) intravenous doses (μmol kg^{-1}) of S- and R-metoprolol causing a 50% reduction (ED_{50}) in the heart rate response to sympathetic nerve stimulation were determined. The doses inducing a 25% depression (DD_{25}) of the basal myocardial contractility were also estimated. For the two enantiomers, the β_1-blocking potency (−log ED_{50}) was 7.04 ± 0.16 (S) and 4.65 ± 0.16 (R). A significant cardiodepressive effect was observed at high doses (−log DD_{25}): 4.18 ± 0.20 (S) and 4.08 ± 0.10 (R).

5 It is concluded that the binding of metoprolol to β_1-adrenoceptors has a stricter steric requirement than that for the binding of this β-blocker to β_2-adrenoceptors. Furthermore, the non-specific cardiodepressive effect of metoprolol was observed at equally high doses for the two enantiomers.

Introduction

Metoprolol is a β_1-adrenoceptor selective antagonist used in the treatment of various cardiovascular disorders, including hypertension and ischaemic heart disease (Åblad *et al.*, 1980; Cruickshank & Prichard, 1987). Like most β-blockers, metoprolol is a racemic compound with two enantiomeric forms, S and R. For many β-blockers, the S-enantiomer has been shown to possess greater β-blocking potency than the corresponding R-form (Patil *et al.*, 1975; Toda *et al.*, 1978; Morris & Kaumann, 1984; Kaumann, 1987; El Tayar *et al.*, 1988; Nathansson, 1988), while other properties such as the membrane stabilizing effect is similar for the two enantiomers (Barrett & Cullum, 1968; Harry *et al.*, 1974; Badia *et al.*, 1984). In the majority of these earlier studies the enantiomeric purities of the two forms of the β-blocker were not stated. Thus, due to the possible contamination of the R-enantiomer with significant quantities of the S-form, conclusions regarding the actual β-adrenoceptor affinity of the R-form could not be drawn from these studies. One exception is a study by Kaumann and coworkers (Wächter *et al.*, 1980), demonstrating that the R-form of bupranolol (enantiomeric purity >99.9%) is about 100 times less potent as a β-blocker compared to the corresponding S-form.

Results obtained in a number of studies suggest that the steric requirements of the β_1-adrenoceptor is more stringent than that of the β_2-adrenoceptor (Lemoine & Kaumann 1983; Morris & Kaumann 1984; Walter *et al.*, 1984; Nathanson, 1988). Indeed, Nathanson (1988) demonstrated a marked potency of R-timolol (enantiometric purity >99.9%) to antagonize the β_2-adrenoceptor stimulant effect of isoprenaline in the rabbit ciliary process. In contrast to the findings on β_1-adrenoceptors in the heart, where the S-form was more potent than the R-enantiomer, the β-blocking potency of R-timolol in the iris/ciliary body of the eye was equal to that of the S-form.

The techniques for stereoselective synthesis of compounds have markedly improved in recent years. The use of optically pure precursors makes it possible to synthesize compounds with a high degree of enantiomeric purity (Lamm *et al.*, 1987). With this technique, the S- and R-forms of metropolol with an enantiomeric purity of >99.2% (S) and >99.9% (R) were recently synthesized by the Department of Organic Chemistry, AB Hässle. The aim of this study was to investigate the β_1- and β_2-adrenoceptor affinity of these enantiomers and, furthermore, to compare the β_1-blocking potency of S- and R-metoprolol with the doses producing significant cardiodepressive effects.

Methods

β-Adrenoceptor binding experiments

Male Dunkin-Hartley guinea-pigs (400–600 g) were used in the experiments. The animals were killed by a blow on the neck, and the left ventricular free wall (LV) and the soleus muscle were excised from each animal for preparation of membranes. Both this procedure and the binding assay were essentially the same as described previously (Abrahamsson & Nerme, 1987; Abrahamsson *et al.*, 1988). The membrane suspensions from LV and soleus muscle had a protein concentration of 0.29–0.50 mg ml^{-1} and 0.30–0.73 mg ml^{-1}, respectively. The incubation with [^{125}I]-(S)-pindolol, at a final concentration of 100 pM, was performed at 30°C (18 min) and always in the presence of guanosine 5'-triphosphate (Nerme *et al.*, 1985). The specific binding of [^{125}I]-(S)-pindolol, determined in the presence of 50 μM R-isoprenaline, constituted 85–90% of the total binding.

The negative logarithm of the equilibrium dissociation constant (pK_D) for [^{125}I]-(S)-pindolol was derived from saturation binding experiments. In each tissue studied, saturation

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binding experiments were performed at 30°C (18 min incubation) with 9 concentrations of the radioligand (20–440 pM) and the pK_D values obtained were (means ± s.d., $n = 5$): 9.9 ± 0.11 (LV) and 10.3 ± 0.09 (soleus muscle). These constants are in good agreement with those obtained from association/dissociation kinetic experiments (means ± s.d., $n = 2$): 10.2 ± 0.18 (LV) and 10.1 ± 0.07 (soleus muscle). Moreover, the kinetic analysis of [^{125}I]-(S)-pindolol binding confirmed that an incubation period of 18 min was sufficient when binding experiments were carried out at 30°C (means ± s.d., $n = 2$): $k_{+1} = 2.3 \pm 0.57\,nM^{-1}\,min^{-1}$, $k_{-1} = 0.16 \pm 0.008\,min^{-1}$ (LV) and $k_{+1} = 1.6 \pm 0.28\,nM^{-1}\,min^{-1}$, $k_{-1} = 0.12 \pm 0.044\,min^{-1}$ (soleus muscle).

In order to determine the pK_D values for the various forms of metoprolol, competition binding experiments were performed with 14 concentrations for each drug. For S and racemic metoprolol the concentration ranges studied were 1 nM–20 μM (LV) and 10 nM–0.2 mM (soleus muscle). The corresponding ranges for R-metoprolol were 10 nM–0.2 mM (LV) and 20 nM–0.5 mM (soleus muscle). The competition binding curves were analysed by fitting the experimental data to a non-linear function, based on the method of least squares, by means of a computer assisted program. Each curve was tested according to a mathematical equation describing a one-site or a two-site receptor interaction. The two-site model was accepted when the fitting gave an improvement in the goodness of fit value that was statistically significant ($P < 0.05$) compared to the fitting to the one-site model.

A β_2-adrenoceptor selectivity of about 3 has been obtained for [^{125}I]-(S)-pindolol (McGonigle *et al.*, 1986; Neve *et al.*, 1986; Abrahamsson & Nerme, 1987). Such a selectivity may give rise to potential problems when competition binding experiments are used to determine the β_1- and β_2-adrenoceptor affinity of a drug (McGonigle *et al.*, 1986; Neve *et al.*, 1986). Thus, in this study the β_1-adrenoceptor affinity of metoprolol was determined from the β_1-adrenoceptor population in the LV (about 80% β_1-adrenoceptors, Abrahamsson *et al.*, 1988), while the corresponding β_2-adrenoceptor affinity was determined in the soleus muscle, a tissue with an almost homogeneous β_2-adrenoceptor population (Abrahamsson *et al.*, 1988). The β_1-selectivity of the compounds was then calculated from the β_1- and β_2-adrenoceptor affinities obtained from the two tissues from each animal.

As described above, the β-adrenoceptor affinity of the radioligand was determined in each tissue preparation studied (both LV and soleus muscle). Since the guinea-pig LV contains both β_1- and β_2-adrenoceptors, the affinity of the radioligand obtained in this tissue was regarded as an approximate value of the β_1-adrenoceptor affinity of [^{125}I]-(S)-pindolol. However, we do not think that this approximation will significantly affect the calculated β_1-adrenoceptor affinity of metoprolol. In fact, recalculation of the pK_D values for metoprolol, with the 'true' β_1-adrenoceptor affinity of [^{125}I]-(S)-pindolol (mean value $pK_D = 9.7$; Neve *et al.*, 1986), gave similar values to those obtained when the affinity values of the radioligand obtained in the guinea-pig LV were used (see above). In this study, a comparison between the receptor affinities for the radioligand obtained from saturation binding experiments in the LV and soleus muscle revealed a β_2-adrenoceptor selectivity of 2.9 ± 1.10 (mean ± s.d., $n = 5$).

In vivo *experiments*

Male cats (body weight 3.2–5.2 kg) were used in the experiments. Anaesthesia was induced by sodium pentobarbitone (30 mg kg^{-1} i.v.) and maintained by i.v. infusion of 0.5 mg kg^{-1} h^{-1}. A tracheotomy was performed and the animals were ventilated mechanically with room air. The respiratory rate and tidal volume were adjusted and bicarbonate was administered to keep blood gases and blood pH within the normal range. The body temperature was kept between 37.5°C and 38.5°C by an automatic thermoregulator connected to a water-heated operating table. Catheters were inserted into the right carotid artery (for blood pressure measurement), the left jugular vein (for drug administration) and the left femoral vein for administration of pentobarbitone. After sternectomy, a tip-transducer (Millard) was passed through the apex of the heart into the left ventricular cavity. The transducer was used to measure left ventricular pressure, and the *dP/dt max* was derived from these signals. Pace electrodes were placed on the right atrial appendage. Bilateral vagotomy was performed to prevent reflex vagal effects secondary to sympathetic stimulation. The stellate ganglionic rami going to the heart were identified by test stimulation, the rami were then cut and a stimulating electrode placed on the distal part. Throughout the experiment the haemodynamic signals were recorded on a Grass Polygraph and the signals (heart rate, *dP/dt max*, mean arterial blood pressure) were analysed by a computerized system (Axenborg, 1988).

The cardiac sympathetic nerves were stimulated (supramaximal current, duration 1 ms) for 60 s and the chronotropic response was recorded. The stimulating frequency required to obtain 80% of the maximal chronotropic response was determined in each cat (mean value ± s.d.: 1.6 ± 0.78 Hz). In three groups of five cats, increasing doses of S, R and racemic metoprolol were administered i.v. over a period of 15 min. At each dose level, the nerves were stimulated at the end of the perfusion period (13–15 min) for determination of the β-blocking effect. In each cat the non-specific cardiodepressive effect of the compounds was studied. Starting at 10 μmol kg^{-1}, doses in increments of two were given until the left ventricular *dP/dt max* (determined at constant heart rate) was decreased by more than 50%.

The percentage blockade of the chronotropic response to nerve stimulation was plotted against the cumulative dose of the test compound. The negative logarithm of the dose causing 50% blockade of the chronotropic response ($-\log ED_{50}$) was calculated as the geometrical mean of ED_{50} values from the individual experiments. Similarly, the $-\log$ dose reducing the *dP/dt max* by 25% ($-\log DD_{25}$) was obtained by plotting the negative inotropic effect against the cumulative dose of the compound. A dose-ratio comparing the $-\log ED_{50}$ and the $-\log DD_{25}$ was calculated for each cat.

Drugs

S, R and racemic metoprolol were obtained from the department of Organic Chemistry, AB Hässle. The method for synthesis of the S-enantiomer of metoprolol has recently been described (Lamm *et al.*, 1987). The R-antipode used in the present work was prepared from D-mannitol via (R)-1-O-methanesulphonyl-2,3-O-isopropylidene-glycerol, following a previously published method (Danilevicz & Kemp, 1973) with one modification, the cyclic sulphite was used instead of the primary tosylate of the 1-aryloxy-2,3-propranediol. The preparation of racemic metoprolol via cyclic sulphite has been described in a recent patent (Jpn. Kokai Tokyo Koho, 1985). The enantiomeric purity of the R- and S-form of metoprolol was determined by h.p.l.c. analysis of the diastereomeric (R)-O-methyl-mandelic acid amides (Helmchen & Strubert, 1974) and was shown to be at least 99.9 and 99.2%, respectively.

[^{125}I]-(S)-pindolol was purchased from New England Nuclear. R-isoprenaline bitartrate was obtained from Sigma.

Results

β_1- *and* β_2-*adrenoceptor affinity* (in vitro)

Competition binding curves for S, R and racemic metoprolol in LV and soleus muscle are shown in Figure 1. These curves clearly show that considerably higher concentrations were needed for R-metoprolol to displace the radioligand when compared to the other compounds studied. The β_1- and β_2-adrenoceptor affinity of S, R and racemic metoprolol are summarized in Table 1. The β_1-adrenoceptor affinity of the

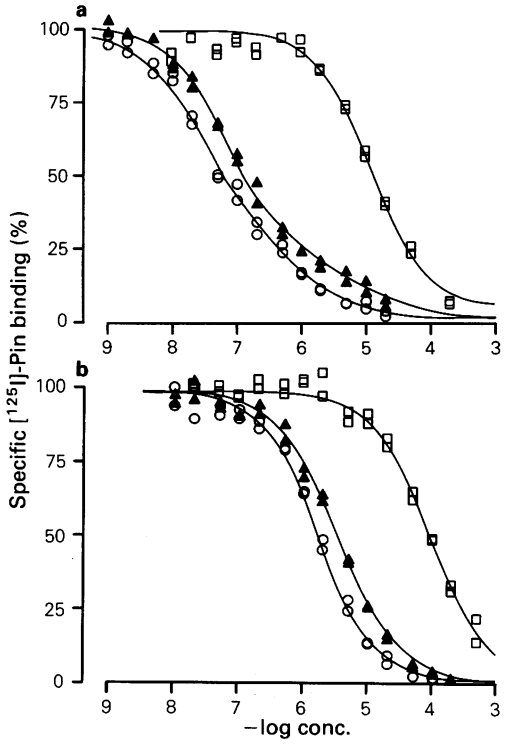

Figure 1 Inhibition of binding of [^{125}I]-(S)-pindolol ([^{125}I]-Pin) by increasing concentrations of **S** (○), **R** (□) and racemic (▲) metoprolol in membrane preparations of the guinea-pig left ventricular free wall (LV) (a) and soleus muscle (b). Shown are curves (binding was performed in duplicate) from the two tissues in one animal. In this experiment the affinity (pK_D) of S-metoprolol was 7.69 (β_1 LV), 6.16 (β_2 LV) and 6.23 (soleus). The corresponding values for racemic metoprolol were 7.49 (β_1 LV), 5.97 (β_2 LV) and 5.97 (soleus). For **R**-metoprolol the β-adrenoceptor affinity was 5.00 (LV) and 4.50 (soleus).

S-form was about 500 times greater than that of the **R**-form, while the corresponding difference for the β_2-adrenoceptors was about 50. As expected, the β-adrenoceptor affinity of the S-form was about twice as great as that of racemic metoprolol. The β_1-adrenoceptor selectivity ratio of the compounds was calculated by comparison of the β_1- and β_2-adrenoceptor affinity obtained from LV and soleus muscle in each animal. The β_1-adrenoceptor selectivity was similar for the racemic and the S-enantiomer of metoprolol, while the **R**-enantiomer was only 3 fold β_1-selective.

From the displacement curves with racemic and S-metoprolol in the LV (Figure 1), we found evidence of two subpopulations of β-adrenoceptors (mean ± s.d., $n = 5$): 74 ± 2.2% (β_1) and 26 ± 2.2% (β_2). This confirms the work on guinea-pig LV previously described (Lemoine *et al.*, 1985; Abrahamsson *et al.*, 1988). In contrast to the other compounds studied, it was not possible to identify the two subpopulations in the LV with **R**-metoprolol. The β_2-adrenoceptor affinity (pK_D) of racemic and S-metoprolol obtained from the small β_2-adrenoceptor population in the LV were ($n = 5$): 5.61 ± 0.35 (racemic) and 6.09 ± 0.39 (S). Although the variability was greater, these values from the LV are in agreement with those obtained from the soleus muscle (Table 1).

Table 1 The β_1- and β_2-adrenoceptor affinity for **S**, **R** and racemic metoprolol obtained from receptor binding studies *in vitro*

		pK_D		
Metoprolol	n	β_1	β_2	*β_1-selectivity*
Racemate	5	7.32 ± 0.13	5.94 ± 0.09	25 ± 9
S-form	5	7.73 ± 0.10	6.28 ± 0.06	29 ± 6
R-form	5	5.00 ± 0.06	4.52 ± 0.09	3 ± 0.1

Mean values ± s.d. are shown. n = number of experiments. pK_D = −log equilibrium dissociation constant.

β_1-adrenoceptor blocking potency and non-specific cardiodepressive effects (in vivo)

Before the start of the drug administration, the haemodynamic parameters monitored were similar for the three groups of animals (Table 2). In addition, the chronotropic responses to nerve stimulation were the same in the three groups (increase in beats min^{-1}): 77 ± 8 ('racemate'), 72 ± 11 ('S') and 74 ± 7 ('R'). The heart rate response to nerve stimulation was antagonized following administration of increasing doses of **S**, **R** or racemic metoprolol (Figure 2). The β-blocking potency was markedly lower for the **R**-form than for S and racemic metoprolol. Calculations of $-\log ED_{50}$ values showed that the S-form was about 250 times more potent than the **R**-enantiomer (Table 3).

Table 2 Basal haemodynamics in the anaesthetized cats before drug administration

Metoprolol	*n*	*Heart rate* (beats min^{-1})	*MABP* (mmHg)	*Left ventricular peak* dP/dt max
Racemate	5	165 ± 4	110 ± 5	2873 ± 150
S-form	5	158 ± 3	120 ± 5	3486 ± 84
R-form	5	158 ± 3	105 ± 8	3305 ± 233

Mean values ± s.d. are shown. n = number of experiments. MABP = mean arterial blood pressure. The different groups were subsequently given **S**, **R** or racemic metoprolol.

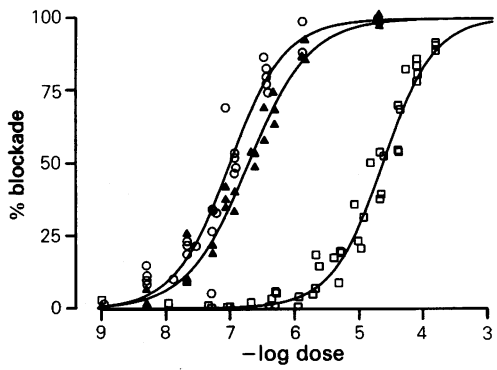

Figure 2 Blockade of the heart rate response to sympathetic nerve stimulation after intravenous administration of increasing doses of **S** (○, $n = 5$), **R** (□, $n = 5$) and racemic (▲, $n = 5$) metoprolol. Each dose of the drug was administered over a 15 min period and the cardiac nerves were stimulated at the end of the perfusion period (min 13–15) for determination of the β-blocking effect. The β-blocking effect is presented for all the doses administered to each individual cat.

Table 3 The β_1-adrenoceptor blocking potency and non-specific cardiodepressive effects in anaesthetized cats of **S**, **R** and racemic metoprolol

Metoprolol	*n*	*β_1-blockade* ($-\log ED_{50}$)	*Cardiac depression* ($-\log DD_{25}$)	*Dose-ratio* (DD_{25}/ED_{50})
Racemate	5	6.74 ± 0.13	4.30 ± 0.13	293 ± 129
S-form	5	7.04 ± 0.16	4.18 ± 0.20	779 ± 302
R-form	5	4.65 ± 0.16	4.08 ± 0.10	4 ± 2

Mean values ± s.d. are shown. n = number of experiments. ED_{50}: dose (μmol kg^{-1}) causing 50% blockade of the increased heart rate. DD_{25}: dose (μmol kg^{-1}) causing 25% reduction of cardiac contractility (at paced heart rate).

The non-specific cardiodepressive effects of the compounds were studied after administration of high doses. A significant decrease in basal cardiac contractility (*dP/dt max*) was obtained at similar concentrations for **S**, **R** and racemic metoprolol (Table 3). The ratio between the dose causing a β-blocking effect (ED_{50}) and the dose producing a 25% reduction in basal contractility (DD_{25}) was about twice as high for S-metoprolol compared to the racemic form, while the corresponding ratio for the **R**-form was markedly lower (Table 3).

Discussion

The enantiomeric purity of the two forms of metoprolol studied was very high. This is particularly essential when investigating the β-adrenoceptor affinity and β-blocking potency of the less active **R**-enantiomer. The enantiomeric purity of the **R**-form used in this study was more than 99.9%. Thus, the contamination of the **R**-form with the more active S-enantiomer of metoprolol was less than 0.1%.

An interesting observation in this study was that the affinity difference for the S- and **R**-enantiomers of metoprolol was 10 times greater on β_1- than on β_2-adrenoceptors. Moreover, the β_1-adrenoceptor selectivity of the S-form was similar to that of the racemate, while the **R**-form was only 3 fold β_1-selective. Thus, it seems as if the binding of metoprolol to include β_1-adrenoceptors has a stricter steric requirement than that for the binding of this drug to β_2-adrenoceptors.

Several other stereoisomeric pairs of β-blockers have been shown to possess a different potency with regard to β_1- and β_2-adrenoceptor blockade. Using a receptor-binding technique, Morris & Kaumann (1984) demonstrated a two fold greater difference between the β_1- and β_2-adrenoceptor affinity for the S-enantiomers of ten different β-ligands compared to that of the corresponding **R**-forms. A similar result was also obtained for the enantiomers of the β-blocker bunolol (Quast & Vollmer, 1984). Taken together, these earlier observations and the findings in this study clearly show that the steric requirements for the binding of β-blockers are different for β_1- and β_2-adrenoceptors. Nathanson (1988) recently suggested that the β_2-adrenoceptor, due to its different configuration, may provide an additional binding site for the **R**-enantiomer of a β-blocker.

In the anaesthetized cat, the S-enantiomer of metoprolol was demonstrated to be about 250 times more potent than the **R**-form in attenuating the chronotropic response to sympathetic nerve stimulation. Moreover, the S-enantiomer was about twice as potent as racemic metoprolol. The increase in heart rate produced by sympathetic nerve stimulation in this model is predominantly mediated via stimulation of cardiac β_1-adrenoceptors (Åblad *et al.*, 1974; Bryan *et al.*, 1981). Accordingly, the difference in potency for the enantiomers in the cat experiments was quite similar to the affinity difference for the S- and **R**-forms on β_1-adrenoceptors, obtained from the binding studies *in vitro*.

A non-specific cardiodepressive effect of β-blockers has been demonstrated at high concentrations (Badia *et al.*, 1984; Hellenbrecht & Enenkel, 1984). This effect, due to the membrane stabilizing properties of the β-blocker, seems to be related to the lipophilicity of the compound (Hellenbrecht *et al.*, 1973; Hellenbrecht & Enenkel, 1984). In this study, a significant decrease in basal cardiac contractility was not observed until doses $> 50\,\mu mol\,kg^{-1}$ for the three forms of metoprolol. The cardiodepressive effect of the S-form and racemate was observed at doses several hundred fold greater than those required for β_1-adrenoceptor blockade, while these effects were observed at similar (high) doses for the **R**-enantiomer.

It has been claimed that racemic drugs are impure compounds with an 'isomeric ballast' (Ariëns, 1986). For the β_1-selective blocker metoprolol it is clear from this study that the β_1-adrenoceptor blocking effect of the racemic compound, at therapeutically effective plasma concentrations (50–300 nM) (Harron *et al.*, 1981; Rydén *et al.*, 1988) resides solely in the S-enantiomer (K_D = 20 nM). The **R**-form is much less active as a β-adrenoceptor antagonist (K_D = 10,000 nM) than the S-enantiomer. Although the difference in affinity for the two enantiomers was less pronounced on β_2- than on β_1-adrenoceptors, this difference does not mean that the β_1-selectivity of racemic metoprolol is less than that of the S-enantiomer. It is also very unlikely that the **R**-form, by its membrane stabilizing properties, will cause any significant effects during therapy with clinically relevant doses of racemic metoprolol.

Today, racemic metoprolol is widely used in the treatment of various cardiovascular disorders. Like other racemic β-blockers in clinical use, metoprolol is well tolerated and the adverse effects are mild and relatively uncommon (Cruickshank & Prichard, 1987). Thus, since β-blockers constitute a group of drugs with a very high benefit/risk ratio, it may be concluded that it is not likely that an active S-enantiomer of a β-blocker will have any therapeutic advantages over the racemic form of the drug.

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The effect of diltiazem on the coronary haemodynamic and cardiac functional effects produced by intracoronary administration of endothelin-1 in the anaesthetized dog

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1 We have studied the effect of the calcium channel antagonist, diltiazem, on the coronary haemodynamic and cardiac functional responses produced by intracoronary (i.c.) administration of endothelin-1 (ET-1) in anaesthetized dogs.

2 ET-1, 1, 3 and 10 ng kg^{-1} i.c., produced dose-delated increases in coronary blood flow with no cardiac functional or systemic haemodynamic changes. ET-1, 30, 100 and 300 ng kg^{-1} i.c., produced dose-related reductions in coronary artery blood flow. The reduction in coronary blood flow was accompanied by dose-related falls in cardiac output, mean arterial pressure, $+dP/dt$ and $-dP/dt$ and increases in left ventricular end-diastolic pressure. However, there was no reflex tachycardia in response to the fall in blood pressure and at 300 ng kg^{-1}, ET-1 produced a 22% reduction in heart rate.

3 Following a series of abnormal ECG changes, four out of five dogs died of ventricular fibrillation at 13 ± 2 min after 300 ng kg^{-1} ET-1.

4 The administration of diltiazem (15 μg kg^{-1} min^{-1}, i.v.) reduced mean arterial pressure by 10% and heart rate by 15%, and increased coronary blood flow by 39%. Diltiazem did not have any significant effect on the coronary dilator response to low doses of ET-1. Although there was a general trend for diltiazem to inhibit the coronary vasoconstrictor responses to ET-1, diltiazem significantly attenuated only the reduction in coronary blood flow produced by 100 ng kg^{-1} ET-1 by 60% but not the response to 30 or 300 ng kg^{-1} ET-1. Two out of five diltiazem-treated dogs died of ventricular fibrillation with a mean time to death of 20 min following treatment with ET-1 (300 kg^{-1}).

5 ET-1 is a very potent coronary vasodilator. At slightly higher doses ET-1 is also a coronary vasoconstrictor. ET-1 also appears to have direct cardiotoxicity independent of myocardial ischaemia. The vasoconstrictor activity and direct cardiotoxicity are only weakly inhibited by diltiazem.

Introduction

It has been known for several years that the vascular endothelium can produce vasodilator substances that have profound effects on the underlying vascular smooth muscle, e.g. prostacyclin and endothelium-derived relaxing factor (Vanhoutte *et al.*, 1986). More recent studies have shown that the vascular endothelium can also produce vasoconstrictor substances. The demonstrations by Hickey *et al.* (1985), Gillespie *et al.* (1986) and O'Brien *et al.* (1987) that cultured vascular endothelial cells can produce a stable, potent vasoconstrictor substance was rapidly followed by the isolation, purification and characterization of this substance as a 21 amino acid peptide that was termed endothelin (Yanagisawa *et al.*, 1988). It is now known that there are genes for three forms of endothelin, termed ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). ET-1, the originally described endothelin and formerly called porcine/human endothelin, is expressed by endothelial cells, whereas ET-2 and ET-3, which differ from ET-1 by two and six amino acid residues, respectively, are not (Inoue *et al.*, 1989). It is possible that ET-3 is produced in neural tissue, but at the present time no tissue has been found to express ET-2 (Inoue *et al.*, 1989).

ET-1 was shown by Yanagisawa *et al.* (1988) to be a potent constrictor of the porcine coronary artery *in vitro* and to produce a long-lasting pressor effect in ganglion-blocked rats. These vasoconstrictor responses were insensitive to a wide variety of receptor antagonists but were sensitive to blockade by voltage-operated calcium channel antagonists. Thus, it was postulated that ET-1 may be acting as an agonist to activate voltage-operated calcium channels in vascular smooth muscle (Yanagisawa *et al.*, 1988). It is now known that ET-1 probably does not directly activate voltage-operated calcium channels (Chabrier *et al.*, 1989) but, similar to a wide variety of other vasoconstrictor substances, does activate phospholipase C to increase the production of inositol phosphates (Pang *et al.*, 1989; Ohlstein *et al.*, 1989) and cause the release of intracellular calcium (Van Renterghem *et al.*, 1989) and the influx of extracellular calcium through receptor-operated calcium channels (Pang *et al.*, 1989). However, it has been shown in rat cultured aortic smooth muscle cells that conditioned medium from bovine cultured aortic endothelial cells and synthetic ET-1 both cause membrane depolarization and generate verapamil-sensitive action potentials (Highsmith *et al.*, 1989). Thus, it is possible that ET-1 may produce different effects on different blood vessels, with the relative importance of the influx of extracellular calcium through voltage-operated calcium channels varying from negligible to very high.

In view of the potent, calcium channel blocker-sensitive, coronary vasoconstrictor activity of ET-1 *in vitro* and the well-documented *in vivo* coronary vasodilator activity of calcium antagonists such as diltiazem (Nagao *et al.*, 1972), we have studied the effects of ET-1 on the coronary circulation and cardiac function following direct intracoronary administration in the anaesthetized dog and the effects of diltiazem on those responses.

Methods

Anaesthetized dogs

Adult male mongrel dogs, 10–15 kg, were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.). An endotracheal tube was inserted and the animals were artificially ventilated with a positive-pressure respirator (Harvard Apparatus Co.) at a frequency of 12 cycles min^{-1} with a tidal volume of 20 ml kg^{-1}. Body temperature was measured with a rectal

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probe and maintained at 38°C with a heating pad. A femoral artery and vein were cannulated for the measurement of blood pressure with a Statham P23ID pressure transducer and the administration of supplemental anaesthesia and fluids, respectively. The left cephalic vein was cannulated for the administration of saline or diltiazem. Needle electrodes were inserted subcutaneously to monitor the lead II ECG with a Grass 7PGD ECG amplifier. Heart rate was measured by a Grass 7P4H tachograph triggered by the Q wave of the ECG.

A left thoracotomy was performed at the fifth intercostal space and the heart was suspended in a pericardial cradle. The left atrium was retracted and a segment of the left circumflex coronary artery distal to the atrial branch and proximal to any major ventricular branches was dissected free and an electromagnetic flow probe, 6 mm in circumference and connected to a CME model FM501 flowmeter (Carolina Medical Electronics), was placed on the artery to measure coronary blood flow. A snare occluder was placed around the artery distal to the flowprobe for the determination of zero flow. A 25 gauge L-shaped needle catheter was inserted into the circumflex coronary artery distal to the flowprobe for the intracoronary (i.c.) administration of ET-1. The root of the aortic arch was cleaned of adhering fat and an electromagnetic flowprobe, 35–45 mm in circumference, connected to a CME model FM501 flowmeter (Carolina Medical Electronics) was placed around it to measure pulsatile and mean aortic blood flow. The pulsatile diastolic blood flow was used to set zero flow on the flowmeter and was checked periodically throughout the experiment for baseline drift.

The right carotid artery was exposed and a 5 French Millar Mikro-Tip catheter pressure transducer (model SPC-350) was passed down the carotid artery into the left ventricle for the measurement of left ventricular pressure. The maximal rate of change of left ventricular pressure during systole ($+dP/dt$) and during diastole ($-dP/dt$) were obtained by differentiating the left ventricular pressure signal with a Grass 7P20C differentiator. Left ventricular end-diastolic pressure was measured by amplifying the left ventricular pressure signal. All parameters were recorded on a Grass Model 7E Polygraph.

Experimental protocol

Following the completion of surgery the animals were allowed to stabilize for at least 30 min before the start of the experiment. After stabilization baseline haemodynamic parameters were recorded and an intravenous infusion of either 0.9% saline or diltiazem ($15\,\mu g\,kg^{-1}\,min^{-1}$) was started at a rate of $0.1\,ml\,kg^{-1}\,min^{-1}$. Ten minutes after the start of the infusion, when the haemodynamic parameters had stabilised, the dose-response curve to ET-1 was started. Bolus doses of endothelin were given over approximately 30 s directly into the coronary artery in volumes of less than 0.5 ml and were flushed in with 1 ml 0.9% NaCl. Each dose of ET-1 was administered when the response to the previous dose had subsided and the haemodynamic parameters had returned to baseline. Measurements were taken at the peak coronary blood flow response.

All procedures were performed in accordance with the 'Guide for the care and use of laboratory animals', NIH Publication No:86-23 and were approved by the Institutional Animal Care and Use Committee.

Drugs

Stock solutions of ET-1 (Peninsula Labs) were made in distilled water and stored at 4°C. Subsequent dilutions were made in 0.9% NaCl. ET-1 has a molecular weight of 2419.8, therefore the dose-range of endothelin studied, 1 to $300\,ng\,kg^{-1}$, corresponds on a mole basis to 0.4 to $120\,pmol\,kg^{-1}$, respectively. Diltiazem hydrochloride (Sigma Chemical Co.) was dissolved in 0.9% NaCl.

Statistical analysis

All data are expressed as the mean ± s.e.mean of n observations. The statistical significance of the effect of diltiazem and saline on basal haemodynamics was determined by Student's paired t test. The statistical significance of the difference between the effects of ET-1 in saline- and diltiazem-treated dogs was determined by Student's unpaired t test.

Results

Table 1 shows that an infusion of saline had no significant effects on coronary or systemic haemodynamics. However, diltiazem ($15\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) lowered blood pressure by 10 ± 2%, heart rate by 14 ± 4%, coronary vascular resistance by 31 ± 9%, total peripheral resistance by 11 ± 3% and increased coronary blood flow by 39 ± 19% (Table 1).

At low doses ($1–10\,ng\,kg^{-1}$, i.c.) ET-1 produced a dose-related increase in coronary blood flow with no concomitant changes in cardiac function or systemic haemodynamics (Figure 1). The coronary vasodilator response produced by low doses of ET-1 was very short-lived (see Figure 1). Although this dilator response was observed in all animals studied there was a very large variation in the magnitude of this effect between animals. This effect was not due to an injection artifact since equal volumes of ET-1 vehicle did not produce any coronary vascular or cardiac functional effects. The coronary vasodilator response produced by ET-1 was observed in both saline- and diltiazem-treated animals and as a result of the wide variability of this response there was no significant difference between the two groups of animals.

Higher doses of ET-1 ($30–300\,ng\,kg^{-1}$, i.c.) produced dose-dependent reductions in coronary blood flow (Figure 2). In many cases these reductions in coronary blood flow were preceded by transient and variable increases in coronary blood flow. In contrast to the vasodilator responses seen at low doses, the constrictor responses to ET-1 were of a much longer duration, such that following $100\,ng\,kg^{-1}$ it took approximately 30 min for coronary blood flow to return to normal. The coronary vasoconstrictor response curve for ET-1 was very steep such that $30\,ng\,kg^{-1}$ reduced coronary flow by 9 ± 2% and $300\,ng\,kg^{-1}$ reduced coronary flow by 89 ± 4%. Diltiazem ($15\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) produced a small rightward shift in the dose-response curve to ET-1 such that the response to $100\,ng\,kg^{-1}$ ET-1 was significantly reduced (Figure 2). The reduction in coronary blood flow was accom-

Table 1 The effect of saline ($0.1\,ml\,kg^{-1}\,min^{-1}$, i.v.) (a) and diltiazem ($15\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) (b) on resting coronary and systemic haemodynamics

		Mean arterial pressure (mmHg)	*Heart rate* (beats min^{-1})	*Total peripheral resistance* ($mmHg\,l^{-1}\,min^{-1}$)	*Coronary blood flow* ($ml\,min^{-1}$)	*Coronary vascular resistance* ($mmHg\,ml^{-1}\,min^{-1}$)
(a)	Control	104 ± 6	150 ± 9	90.5 ± 11.3	23.0 ± 2.4	4.64 ± 0.30
	+ Saline	104 ± 6	153 ± 9	88.9 ± 12.1	24.2 ± 2.6	4.40 ± 0.29
(b)	Control	106 ± 4	144 ± 8	95.0 ± 4.5	23.0 ± 3.4	5.20 ± 0.88
	+ Diltiazem	95 ± 4*	123 ± 4*	84.6 ± 5.7*	32.8 ± 7.2	3.68 ± 0.87*

Values are expressed as the mean ± s.e.mean of 5 observations per group. *$P < 0.05$ (Student's one-tailed paired t test).

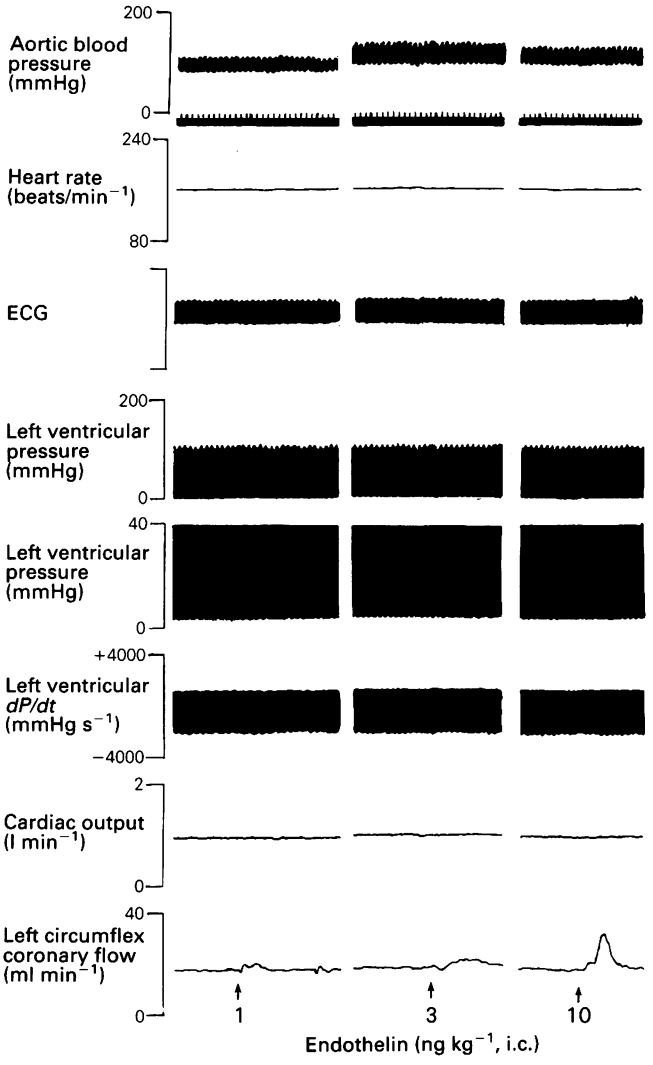

Figure 1 Original recording of the coronary vasodilator effect of low doses of endothelin 1 in the anaesthetized dog.

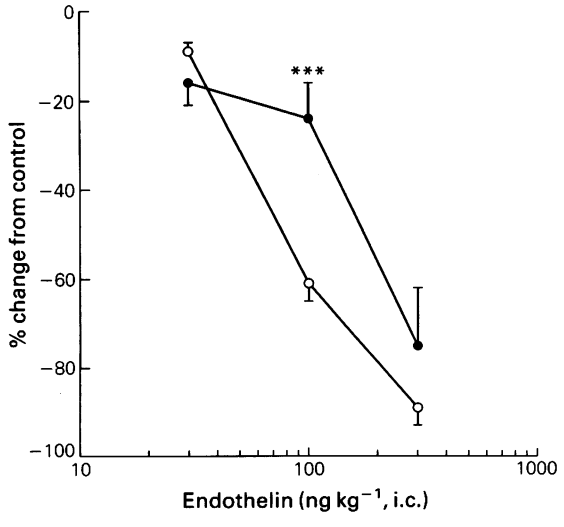

Figure 2 The effect of endothelin-1 (ET-1, 30–300 ng kg^{-1}, i.c.) on left circumflex coronary blood flow in the anaesthetized dog. (○) Responses in saline (0.1 ml kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$); (●) responses in diltiazem (15 μg kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$). Each point represents the mean percentage change from control values obtained immediately before administration of ET-1 and the vertical bars represent 1 s.e.mean. ***$P < 0.001$ (Student's unpaired t test).

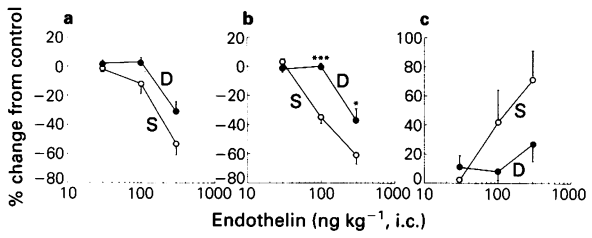

Figure 3 The effect of coronary vasoconstrictor doses of endothelin-1 (ET-1) on (a) $+dP/dt$, (b) $-dP/dt$ and (c) left ventricular end-diastolic pressure. (○, S) Responses in saline (0.1 ml kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$); (●, D) responses in diltiazem (15 μg kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$). Each point represents the mean percentage change from control values obtained immediately before administration of ET-1 and the vertical bars represent 1 s.e.mean. *$P < 0.05$, ***$P < 0.001$ (Student's unpaired t test).

panied by systemic haemodynamic changes. ET-1 produced dose-related reductions in $+dP/dt$ and $-dP/dt$ with an increase in left ventricular end-diastolic pressure (Figure 3). Cardiac output and mean arterial pressure were reduced (Figure 4). However, there was no reflex tachycardia in response to the fall in blood pressure. Indeed, 300 ng kg^{-1} ET-1 reduced heart rate by 22 ± 7% (Figure 4). Diltiazem tended to reduce the haemodynamic responses produced by intracoronary ET-1. However, these inhibitions, like the inhibition of the coronary blood flow response, were slight.

Four out of five saline-treated dogs died of ventricular fibrillation at 13 ± 2 min after receiving 300 ng kg^{-1} ET-1 following a series of ECG changes that are not normally associated with myocardial ischaemia. Figure 5 shows that within 1 min of the end of the injection of 300 ng kg^{-1} ET-1, there was a marked elevation of the ST segment and a very small prolongation of the PR interval. Two minutes later there was a marked prolongation of the PR interval and marked prolongation of the RR interval, as reflected in the bradycardia. Approximately 10 min later the animal went into ventricular flutter and at 15 min following the administration of ET-1 died of ventricular fibrillation (Figure 5). Two out of five diltiazem-treated dogs died of ventricular fibrillation with a mean time to death of 20 min following 300 ng kg^{-1} ET-1.

Discussion

This study shows that ET-1 has profound vasoconstrictor effects in the coronary circulation and effects, both direct and indirect, on myocardial function in the anaesthetized dog and that diltiazem can reduce, but not abolish, these responses to ET-1. These studies also clearly show that in addition to the well characterized vasoconstrictor response, ET-1 also produces a potent coronary vasodilator response.

Kurihara *et al.* (1989) have also shown that ET-1, in the same dose-range used in this study, produces potent coronary

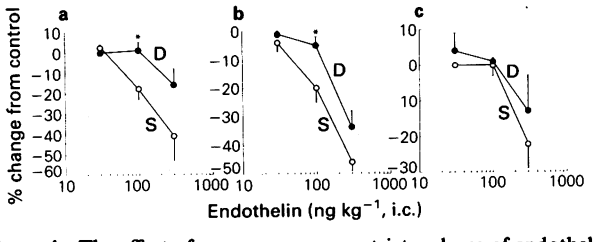

Figure 4 The effect of coronary vasoconstrictor doses of endothelin-1 (ET-1) on (a) cardiac output, (b) mean arterial pressure and (c) heart rate. (○, S) Responses in saline (0.1 ml kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$); (●, D) responses in diltiazem (15 μg kg^{-1} min^{-1}, i.v.)-treated animals ($n = 5$). Each point represents the mean percentage change from control values obtained immediately before administration of ET-1 and the vertical bars represent 1 s.e.mean. *$P < 0.05$ (Student's unpaired t test).

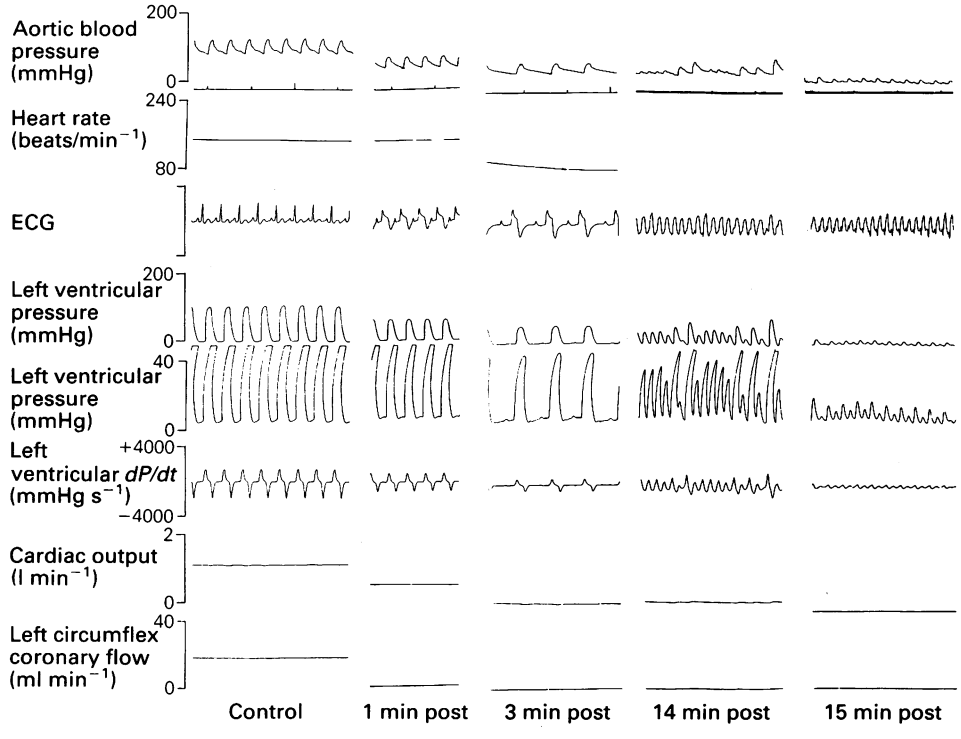

Figure 5 The effects of a high dose of endothelin-1 (300 ng kg^{-1}, i.c.) on coronary and systemic haemodynamics leading to death at 15 min.

vasoconstriction when administered directly into the coronary circulation of the anaesthetized dog. However, in contrast to our findings, Kurihara *et al.* (1989) did not observe a coronary vasodilator response to ET-1 at low doses. This discrepancy may arise from the fact that in the study of Kurihara *et al.* (1989) the coronary circulation was autoperfused with femoral arterial blood, whereas in our experiments the coronary circulation was left intact. It is noteworthy, however, that low doses of ET-1 have been shown to produce vasodilatation in other vascular beds including the renal (Lippton *et al.*, 1988), hindquarter (Wright & Fozard, 1988) and cerebral (R.N. Willette, personal communication) vascular beds. The fact that ET-1 produces direct coronary vasoconstriction, as shown in this study and by Kurihara *et al.* (1989), and that ET-1 gene expression in porcine aortic endothelial cells is induced by agents related to thrombus formation, e.g. thrombin, has led Kurihara *et al.* (1989) to suggest that ET-1 may play a role in the pathogenesis of coronary vasospasm associated with endothelial injury and subsequent thrombus formation. However, the nature of the response to physiologically or pathophysiologically released ET-1, if it does indeed occur *in vivo*, would appear to depend upon the amount of ET-1 released. If ET-1 is only released in very small amounts, then it is possible that the physiological or pathophysiological role of ET-1 is the production of vasodilatation.

Concomitant with the reductions in coronary blood flow, ET-1 produced marked cardiac functional effects. The increase in left ventricular end-diastolic pressure that was produced is commonly seen during myocardial ischaemia following coronary artery occlusion. However, the reductions in cardiac contractility, as assessed from $+dP/dt$, left ventricular diastolic relaxation, as assessed from $-dP/dt$, and cardiac output leading to a profound fall in systemic arterial pressure were much greater and more prolonged than those commonly associated with myocardial ischaemia. This suggests that endothelin may have some direct effect on myocardial function in addition to the myocardial dysfunction that would occur as a result of the ischaemia produced by the reduction in coronary blood flow. Interestingly, it has been shown that myocardium from rat, pig and man possesses binding sites for ET-1 (Davenport *et al.*, 1989), supporting the hypothesis that ET-1 can have direct actions on the myocardium. Previous *in vitro* studies have shown that ET-1 produces a positive inotropic effect in rabbit isolated papillary muscles (Watanabe *et al.*, 1989) and Langendorff perfused hearts of the rat (Baydoun *et al.*, 1989). However, it was found that the initial positive inotropic response to ET-1 observed in the rat perfused heart became a negative inotropic response upon repeated administration (Baydoun *et al.*, 1989). Thus, ET-1 appears to have both positive and negative inotropic actions *in vitro* and predominantly or exclusively negative inotropic effects *in vivo*.

The electrocardiographic changes that occurred following 300 ng kg^{-1} ET-1 were very unusual in that they were not identical to those produced merely by myocardial ischaemia. Myocardial ischaemia produced by occlusion of a coronary artery is associated with ST segment elevation and the frequent occurrence of premature ventricular contractions (PVCs) and occasionally ventricular tachycardia and fibrillation. Myocardial ischaemia *per se* does not produce prolongation of the PR interval or bradycardia similar to that seen with ET-1. Moreover, the incidence of ventricular fibrillation following coronary artery occlusion in mongrel dogs in our laboratory is low (approximately 10%, A.J. Nichols, unpublished observations), in contrast to the 80% incidence following ET-1. Thus, it would appear that ET-1 has direct effects on the electrical properties of the myocardium and its conduction system. It is interesting to note that the snake venom, sarafotoxin S6b, which has a peptide structure very similar to that of ET-1 (Takasaki *et al.*, 1988a), is believed to act as a direct cardiotoxin (Takasaki *et al.*, 1988b) and has been shown to produce ST segment elevation and severe atrioventricular block in mice (Wollberg *et al.*, 1988). Thus, if ET-1 can indeed be produced and released in large quantities from coronary arterial endothelial cells under pathophysiological conditions, then it may not only cause coronary artery vasoconstriction, but may also produce cardiac arrhythmias that may ultimately lead to ventricular fibrillation and death.

The inhibitory effect of diltiazem on the responses to ET-1 suggests that ET-1 may be acting, either directly or indirectly, *via* activation of voltage-operated calcium channels. However, the degree of inhibition produced by diltiazem was not large

despite the large dose of diltiazem which has previously been shown to inhibit myocardial calcium influx under pathological conditions (Bush *et al.*, 1982). Moreover, the evidence from *in vitro* experiments would suggest that ET-1 does not activate voltage-operated calcium channels to produce vasoconstriction (Chabrier *et al.*, 1989; Ohlstein *et al.*, 1989). However, ET-1 has been shown to depolarize vascular smooth muscle cells and generate action potentials that are sensitive to inhibition by calcium channel antagonists (Highsmith *et al.*, 1989). It is possible, therefore, that at least a small component of the response to ET-1 in the coronary vasculature may be mediated by such a process and consequently is sensitive to inhibition by diltiazem. It is also possible that diltiazem is acting as a functional antagonist to inhibit the response to ET-1 by a mechanism that is unrelated to the mechanism of action of ET-1. The infusion of diltiazem produced coronary and systemic haemodynamic changes that are commonly seen with calcium channel antagonists, e.g. vasodilatation, which suggests that intracellular calcium levels were reduced by diltiazem. Thus, the diltiazem-treated dogs had a lower vascular tone and possibly reduced intracellular calcium levels that may have attenuated the responses to ET-1.

Although the incidence of ventricular fibrillation and death was lower in the diltiazem-treated animals relative to the saline-treated animals, diltiazem did not abolish the cardiotoxicity produced by ET-1. Thus, although this dose of diltiazem has been shown to have a profound effect in reducing calcium influx and be cardioprotective in canine ischaemic/reperfused myocardium *in vivo* (Bush *et al.*, 1982) it was, at most, only partially effective at inhibiting ET-1 mediated cardiotoxicity. Thus, the mechanism of the direct cardiotoxic response to ET-1 is not likely to be mediated by calcium influx through voltage-operated calcium channels. If this were the case, it would be expected that there would be at least some evidence of a positive inotropic response, similar to that seen with the calcium channel activator Bay K 8644 (Schramm *et al.*, 1983). Thus, the mechanism of the direct cardiotoxic response to ET-1 remains unclear.

In conclusion, we have shown that ET-1, when administered directly into the left circumflex coronary artery of the anaesthetized dog, produces coronary vasodilatation at low doses and vasoconstriction at high doses. The vasoconstrictor responses are accompanied by cardiac functional responses, e.g. profound negative inotropic effects and electrocardiographic changes, that are not normally associated with myocardial ischaemia, and thus suggest a direct cardiotoxic effect of ET-1. The responses to ET-1 are weakly attenuated by diltiazem, but this may be the result of functional antagonism rather than inhibition of ET-1-mediated calcium influx through voltage-operated calcium channels.

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L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle

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1 The effects of L-N^G-monomethyl arginine (L-NMMA) and L-N^G-nitro arginine (L-NOARG) on non-adrenergic, non-cholinergic (NANC) relaxations of the mouse anococcygeus were investigated.

2 L-NMMA (10–200 μM) produced a concentration-related inhibition of the NANC response; the inhibitory effect of 50 μM L-NMMA was completely reversed by L-arginine but not D-arginine (both 100 μM).

3 L-NOARG (1–50 μM) also produced a concentration-related inhibition of the NANC response and was some 30–50 times more potent than L-NMMA; again, the effects of 10 μM L-NOARG were reversed by 100 μM L-, but not D-, arginine. By itself 100 μM L-arginine did not relax the tissue, but did cause a slight potentiation of the NANC response.

4 Sodium nitroprusside (0.01–10 μM), hydroxylamine (0.1–100 μM), sodium azide (1–100 μM) and nitric oxide (3–120 μM) all relaxed carbachol-induced tone; relaxations to submaximal concentrations of these nitrovasodilators were unaffected by either 50 μM L-NMMA or 10 μM L-NOARG.

5 L-NOARG 10 μM did not inhibit, but rather potentiated, contractions of the mouse anococcygeus due to stimulation of its sympathetic nerves.

6 The inhibitory effects of 10 μM L-NOARG on NANC relaxations were reversed by L-arginine (by 131%), L-citrulline (by 75%), L-arginine methyl ester (by 46%) and L-homoarginine (by 22%), but were unaffected by a variety of other amino acids and their derivatives (all at 100 μM).

7 The results provide strong evidence that NANC relaxations of the mouse anococcygeus are mediated by an endogenous nitrate material, probably derived from L-arginine, and confirm that L-NOARG provides a very useful and potent drug for the investigation of endogenous nitrate function.

Introduction

Stimulation of the autonomic nerves to several organs causes responses which are neither adrenergic nor cholinergic. Although identification of the responsible transmitter has been the subject of much research (Burnstock, 1986), in the majority of cases the transmitter remains obscure. One tissue in which such non-adrenergic, non-cholinergic (NANC) relaxations have been observed is the anococcygeus muscle, first described in the rat by Gillespie (1972). Although most work has been carried out using the rat anococcygeus, similar NANC responses have been demonstrated in muscles from several other species (Gillespie, 1980; Gillespie, 1987). In the mouse anococcygeus, these NANC relaxations may be mimicked by application of nitrovasodilators (Gibson & Mirzazadeh, 1989a); N-methylhydroxylamine inhibited relaxations induced by NANC field stimulation and by nitrovasodilator drugs, but did not affect responses to a variety of other, non-nitrovasodilator smooth muscle relaxants, and, conversely, M&B 22948 selectively potentiated NANC- and nitrovasodilator-induced relaxations. Further, an inhibitory substance has been extracted from the rat anococcygeus, and from the related bovine retractor penis, which mimics the NANC transmitter and which has the characteristics of a nitrovasodilator-like material (Gillespie & Martin, 1980; Bowman & Gillespie, 1982; Bowman & Drummond, 1984; Gillespie, 1987; Gillespie & Sheng, 1988). Evidence has been presented that the activity of this inhibitory substance may be due to nitric oxide (NO) derived from extracted nitrite (Martin *et al.*, 1988). These observations raise the possibility that the NANC transmitter itself may be nitrovasodilator-like, and therefore the NANC transmission in the anococcygeus may represent another example of a physiological process mediated by the release of an endogenous nitrate, like endothelium-derived relaxing factor (EDRF, Moncada *et al.*, 1989). EDRF has been identified as NO which is synthesized from L-arginine within the endothelial cell; L-N^G-monomethyl arginine (L-NMMA) has been shown to block the synthesis pathway and hence attenuate EDRF function (Moncada *et al.*, 1989). In addition, we have recently presented evidence that another guanidino-substituted arginine derivative, L-N^G-nitro arginine (L-NOARG) is more potent than L-NMMA as an inhibitor of EDRF function (Moore *et al.*, 1989) and, like L-NMMA, the inhibitory effect of L-NOARG is reversed by L-, but not D-, arginine. Consequently, if NANC relaxations of the mouse anococcygeus are indeed mediated by release of an endogenous nitrate, it might be expected that both L-NMMA and L-NOARG would produce an L-arginine-reversible inhibition of the transmission process; the object of the present study was to test this possibility. Preliminary accounts of some of this work have been presented to the British Pharmacological Society (Gibson & Mirzazadeh, 1989b; Gibson *et al.*, 1989a).

Methods

Male mice (LACA, 25–35 g) were killed by stunning and exsanguination. The paired anococcygeus muscles were dissected out and set up in series, joined by the ventral bar, in 2 ml glass organ baths containing Krebs-bicarbonate buffer (composition, mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) which was maintained at 37°C and gassed with 95% O_2:5% CO_2. A resting tension of 200–400 mg was placed on the tissues and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to a Graphtec pen recorder (WR 3101). Muscles were allowed to equilibrate for 45 min before beginning the experiment.

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Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue (Gibson & Wedmore, 1981); these were attached to a Grass S48 stimulator (0.5 ms pulse width; 70 V). To observe NANC relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and to negate contractions due to stimulation of sympathetic nerve endings. Tone was raised with 50 μM carbachol in all cases; sympathetic responses were prevented by including 1 μM phentolamine in the Krebs solution and by pre-incubating each muscle with 30 μM guanethidine for 10 min, during the 45 min equilibration period.

To record relaxations to drugs, tone was first raised with 50 μM carbachol. When carbachol-induced tone had reached a plateau (usually within 3 min), the relaxant drug was added to the organ bath. The response was calculated as the peak % reduction of carbachol-induced tone occurring within 4 min of addition of the relaxant drug; if no peak occurred within 4 min the response at 4 min was used. Both carbachol and relaxant were then washed out of the organ bath and the muscle allowed to rest for 20 min before tone was raised again.

All drugs were dissolved in distilled water. Nitric oxide solutions were prepared, and bath concentrations calculated, as described previously (Gibson & Mirzazadeh, 1989a). Drugs used during the study were: L-arginine hydrochloride (Sigma), D-arginine hydrochloride (Sigma), L-arginine methyl ester hydrochloride (Sigma), carbachol (BDH), L-citrulline (Sigma), L-cysteine (Sigma), glycocyamine (Sigma), guanethidine sulphate (Ciba), γ-guanidino-butyric acid (Sigma), L-homoarginine hydrochloride (Sigma), L-leucine (Sigma), L-lysine hydrochloride (Sigma), L-N^G-monomethyl arginine (kindly donated by Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent), nitric oxide (99%, BDH), L-N^G-nitro arginine (Sigma or Aldrich), L-ornithine hydrochloride (Sigma), phentolamine mesylate (Ciba), L-phenylalanine (Sigma), sodium azide (Sigma), sodium nitroprusside (Sigma), L-tryptophan (Sigma).

Results are expressed as mean ± s.e.mean and statistical analysis was by Student's unpaired t test. A probability (P) value of 0.05 or less was taken to indicate statistical significance.

Results

L-NMMA and L-NOARG on NANC relaxations

Field stimulation (10 Hz, 10 s train every 100 s) of the mouse anococcygeus caused reproducible NANC relaxations of carbachol-induced tone (Figure 1). These relaxations were reduced by L-NMMA (10–200 μM) in a concentration-dependent manner (Figures 1a,b and 2), although even at 200 μM L-NMMA, NANC relaxations were reduced by only 47%. Two hundred μM L-NMMA was the highest concentration studied in depth, but in two experiments it was found that raising the concentration to 500 μM produced no greater degree of inhibition of the NANC response. Arginine-reversibility was investigated using 50 μM L-NMMA (Figure 1a,b); inhibitions of NANC relaxations produced by this concentration of L-NMMA were unaffected by 100 μM D-arginine but were completely reversed by 100 μM L-arginine.

L-NOARG (1–50 μM) also caused concentration-related inhibition of NANC relaxations (Figures 1c,d and 2); it was some 30–50 times more potent than L-NMMA and 50 μM L-NOARG produced almost complete suppression of the NANC response. As with L-NMMA, the inhibitory effect of L-NOARG (10 μM) was unaffected by D-arginine (100 μM) but was completely reversed by L-arginine (100 μM, Figure 1c,d). In a further series of experiments we investigated the ability of some other compounds to reverse the effect of L-NOARG. In these experiments, 10 μM L-NOARG was used to produce a standard inhibition of the NANC relaxation (as in Figure 1); the reversing agent was then added to the organ bath and the degree of reversal measured (as % recovery) after four further trains of field stimulation. The results are shown in Table 1. Of the substances studied (all at 100 μM), L-arginine was the most effective reversing agent. Indeed, in the presence of L-

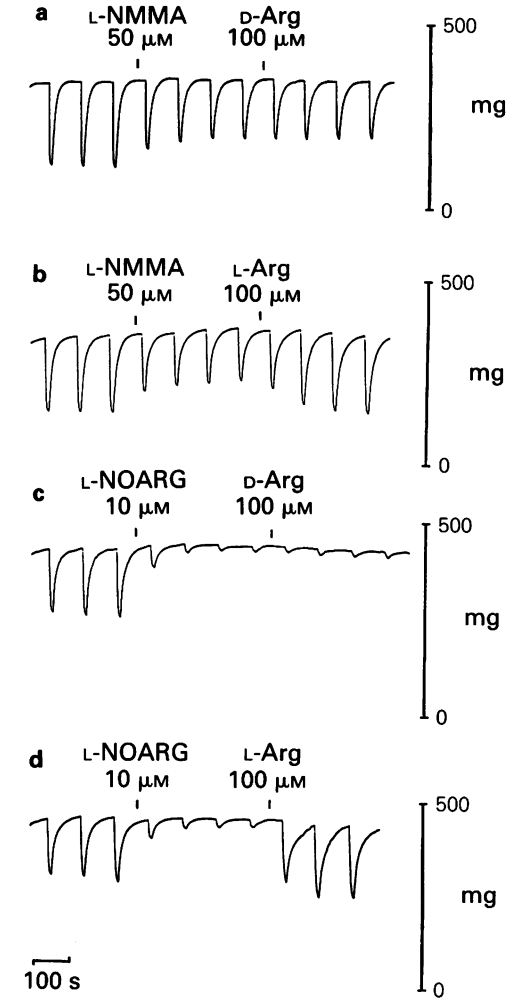

Figure 1 The effect of L-N^G-monomethyl arginine (L-NMMA; a and b) and of L-N^G-nitro arginine (L-NOARG; c and d) on non-adrenergic, non-cholinergic (NANC; 10 Hz, 10 s train every 100 s) relaxations of mouse anococcygeus muscles. Muscle tone was raised with 50 μM carbachol. Both drugs produced inhibitions of NANC relaxation which were unaffected by D-arginine (D-Arg) but completely reversed by L-arginine (L-Arg).

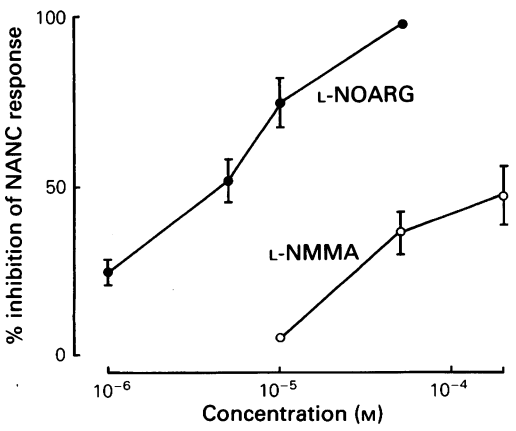

Figure 2 Concentration-response curves for the inhibitory effects of L-N^G-monomethyl arginine (L-NMMA; ○) and L-N^G-nitro arginine (L-NOARG; ●) on non-adrenergic, non-cholinergic (NANC) relaxations of the mouse anococcygeus muscle (protocol similar to that shown in Figure 1). Each point represents the mean from 6 individual muscle preparations; vertical lines show s.e.mean.

Table 1 Reversal of the inhibitory effect of 10 μM L-N^G-nitro arginine (L-NOARG) on NANC relaxations

Reversing agent (100 μM)	*% reversal of L-NOARG* (10 μM)
L-Arginine	131 ± 7
L-Citrulline	75 ± 7
L-Arginine methyl ester	46 ± 4
L-Homoarginine	22 ± 4
D-Arginine	0
L-Ornithine	0
L-Leucine	0
L-Cysteine	0
L-Tryptophan	0
L-Lysine	0
L-Phenylalanine	0
Glycocyamine	0
γ-Guanidino-butyric acid	0

$n = 6$ individual muscle preparations in each case. L-NOARG 10 μM reduced the NANC response by 80 ± 2% ($n = 80$).

arginine the NANC relaxation became greater than it had been before the addition of L-NOARG. Of the others, only L-citrulline, L-arginine methyl ester and L-homoarginine produced any significant degree of reversal.

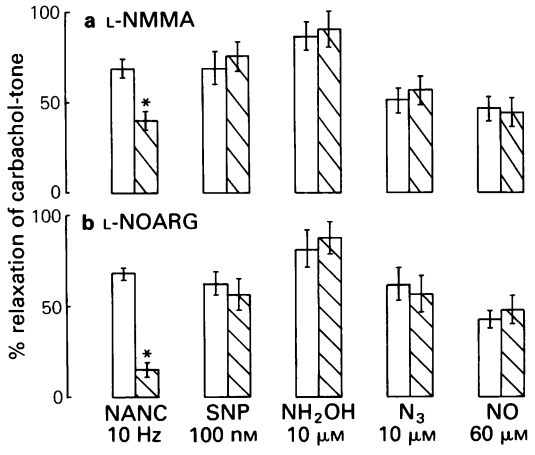

Figure 3 The effect of L-N^G-monomethyl arginine (L-NMMA; a) and of L-N^G-nitro arginine (L-NOARG; b) on relaxations of the mouse anococcygeus in response to non-adrenergic, non-cholinergic field stimulation (NANC), sodium nitroprusside (SNP), hydroxylamine (NH_2OH), sodium azide (N_3) and nitric oxide (NO). Muscle tone was raised with 50 μM carbachol. Open columns – control; hatched columns – in presence of either 50 μM L-NMMA (a) or 10 μM L-NOARG (b). Each column represents the mean from at least 6 individual muscle preparations; vertical bars show s.e.mean. $^*P < 0.05$, significant reduction of response. Both L-NMMA and L-NOARG reduced NANC relaxations but not those due to the nitrovasodilators.

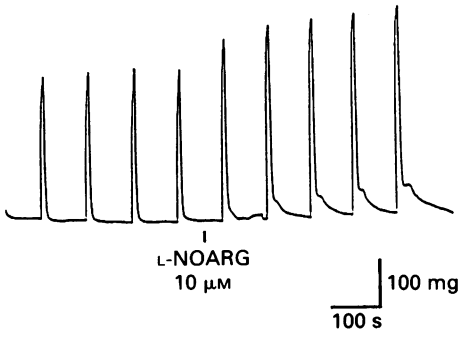

Figure 4 The effect of L-N^G-nitro arginine (L-NOARG) on motor responses of a mouse anococcygeus muscle in response to field stimulation (30 Hz, 10 s train every 100 s). These sympathetic responses were enhanced in the presence of L-NOARG.

Neither D-, nor L-, arginine (both at 100 μM) by themselves caused any significant change in background tone (Figure 1). In addition, D-arginine had no effect on NANC responses, but L-arginine (100 μM) produced a small but significant increase in NANC relaxations in the absence of either L-NMMA or L-NOARG, from 50 ± 5% to 70 ± 6% ($n = 7$, $P < 0.05$).

L-NMMA and L-NOARG on relaxations induced by nitrovasodilator drugs

The nitrovasodilator drugs sodium nitroprusside (SNP, 0.01–10 μM), hydroxylamine (0.5–100 μM), sodium azide (1–100 μM) and NO (3–120 μM) produced concentration-related relaxations of carbachol-induced tone; the effects of L-NMMA and L-NOARG were studied using concentrations of each relaxant which reduced tone by between 30–80% (Figure 3). Neither 50 μM L-NMMA nor 10 μM L-NOARG had any effect on relaxations induced by 100 nM SNP, 10 μM hydroxylamine, 10 μM sodium azide or 60 μM NO (Figure 3).

Since L-NOARG had proved to be a much better inhibitor of NANC responses compared with L-NMMA, we investigated its activity further.

L-NOARG on motor responses to sympathetic nerve stimulation

In these experiments, phentolamine was omitted from the Krebs solution and the muscle was not preincubated with guanethidine. Under these conditions, field stimulation of the atonic tissue (30 Hz, 10 s trains every 100 s) caused reproducible contractions due to noradrenaline released from sympathetic nerves (Gibson & Wedmore, 1981). These contractions were not inhibited, but were potentiated, by 10 μM L-NOARG (Figure 4); the time course of this potentiation mirrored that of the inhibition of NANC relaxations observed earlier (compare Figure 1c, d and Figure 4). Further, the potentiation was reversed by L-arginine but not D-arginine (both 100 μM; data not shown). When added to atonic tissues, 10 μM L-NOARG did not by itself cause muscle contraction (Figure 4). Although there appears to be a slight rise in baseline tension in Figure 4, this is likely to be due to the interval between trains of stimuli being short, such that nerve-evoked contractions are not completely relaxed before subsequent stimulation, due to the potentiated response.

Discussion

L-NMMA has been shown to inhibit the generation of NO-like material by a variety of tissues, including vascular endothelial cells, macrophages, brain cells and neutrophils (Moncada *et al.*, 1989). It is believed to be a specific inhibitor of the enzyme(s) which synthesize NO, or NO-like material, from L-arginine and it has been proposed as a valuable tool for the identification of physiological processes utilising this system (Moncada *et al.*, 1989). In the present study, L-NMMA markedly depressed NANC relaxations of the mouse anococcygeus. In addition, L-NOARG, which we have shown to be more effective than L-NMMA as an inhibitor of EDRF formation (Moore *et al.*, 1989), was also a more potent inhibitor of NANC relaxations in the mouse anococcygeus. The inhibitory effects of both L-NMMA and L-NOARG were reversed by L-, but not D-, arginine. Taken together, these observations provide strong evidence that NANC relaxations of the mouse anococcygeus result from the release of an endogenous nitrate, probably synthesized from L-arginine. Although we cannot rule out the possibility that this substance is released from some non-neural cell by the NANC transmitter, we favour the view that it is the transmitter itself and is released from the NANC nerves. The idea of the involvement of an endogenous nitrate in nerve function is not new; Garthwaite *et al.* (1989) have demonstrated the release of

a NO-like material from cerebellar cells and Moncada *et al.* (1989) have shown that L-NMMA inhibits NO production in brain cells. The anococcygeus muscles might therefore provide a useful model with which to investigate the role of endogenous nitrates in nerve function.

Some evidence that both L-NMMA and L-NOARG inhibit NANC relaxations by a presynaptic mechanism (presumably by interfering with the enzyme which synthesizes the transmitter from L-arginine) was obtained from experiments using nitrovasodilators. These drugs act postsynaptically to produce relaxation, probably by activation of guanylate cyclase with consequent generation of guanosine 3':5'-cyclic monophosphate (cyclic GMP, Bowman & Drummond, 1984; Waldman & Murad, 1987; Gibson *et al.*, 1989b). Drugs which act postsynaptically to block activation of guanylate cyclase, such as N-methyl-hydroxylamine (Deguchi *et al.*, 1978), should therefore inhibit relaxations to both nitrovasodilator drugs and NANC stimulation, as indeed is the case (Gibson & Mirzazadeh, 1989a). In contrast, drugs which act presynaptically should block NANC responses but not those to nitrovasodilators, as has been demonstrated for both L-NMMA and L-NOARG in the present study.

The inhibitory action of L-NOARG was specific against NANC transmission, since the drug did not block, but rather potentiated, motor responses of the anococcygeus. The potentiation probably results from removal of the opposing influence of the NANC transmitter during field stimulation, since it was reversed by L- but not D-arginine. Another interesting observation of these particular experiments was that L-NOARG did not by itself cause contraction, suggesting that the NANC transmitter does not contribute to the resting tone of the tissue, at least *in vitro*.

L-Arginine was found to be the most potent agent at reversing the inhibitory effect of L-NOARG on NANC transmission. This might be expected if L-arginine is the natural substrate for the enzyme presumably inhibited by L-NOARG. Indeed, even in the absence of L-NOARG, L-arginine produced a slight potentiation of NANC relaxations, although it did not by itself cause relaxation. This suggests that the enzyme is relatively inactive under resting conditions but is activated during nerve stimulation. Of the several other substances tested, only L-citrulline, L-arginine methyl ester and L-homoarginine caused reversal of L-NOARG. Palmer *et al.* (1988) found that L-citrulline enhanced the release of NO from endothelial cells, but suggested that this resulted from its prior conversion to L-arginine. Rees *et al.* (1989) showed that L-arginine methyl ester caused partial reversal of L-NMMA inhibition of EDRF function in aortic rings, although it appears not to be a substrate for the NO-generating enzyme (Moncada *et al.*, 1989). L-Homoarginine was neither a substrate for the enzyme nor did it reverse the functional inhibition of EDRF by L-NMMA (Rees *et al.*, 1989; Moncada *et al.*, 1989). It may be that these differences between the anococcygeus and endothelial cells will be resolved by experiments utilising the enzyme from the former. Alternatively, it could be that the two systems are similar but not identical. Certainly Gillespie has highlighted some important discrepancies between the properties of the inhibitory factor and those of NO (Gillespie, 1987; Gillespie & Sheng, 1988) and in some preliminary experiments we have found that hydroquinone completely blocks relaxations produced by NO in the mouse anococcygeus, but has no effect on NANC responses (Gibson & Mirzazadeh, 1989b). As mentioned previously, the inhibitory factor can be extracted from both retractor penis and anococcygeus (Gillespie & Martin, 1980) and other evidence has suggested that the NANC transmission processes in these two tissues are the same (Gillespie, 1987; Gibson & Mirzazadeh, 1989). Recently, however, Gillespie & Xiaorong (1989) have shown that L-NMMA did not inhibit NANC relaxations in the bovine retractor penis, although it did in the rat anococcygeus. In the present study, we found that L-NMMA was much weaker than L-NOARG as an inhibitor of NANC transmission in the mouse anococcygeus; it may be that L-NOARG will also prove more potent than L-NMMA in the retractor penis.

In conclusion, the results of this study provide evidence that NANC relaxations of the mouse anococcygeus are mediated by an endogenous nitrate substance, probably synthesized from L-arginine; the system is similar, but perhaps not identical, to that described for endothelial cells. The results also confirm that L-NOARG provides a very useful tool for the investigation of endogenous nitrates.

A.J.H. is an S.E.R.C. student.

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Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat

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1 The interactions between nitric oxide (NO), prostacyclin and sensory neuropeptides in the maintenance of gastric mucosal integrity have been investigated in the anaesthetized rat.

2 Administration of either N^G-monomethyl-L-arginine (L-NMMA) to inhibit endothelium-derived NO formation, indomethacin to inhibit prostanoid biosynthesis or chronic capsaicin pretreatment to deplete sensory neuropeptides, did not induce acute mucosal injury.

3 In capsaicin-pretreated rats, however, L-NMMA (12.5–100 mg kg^{-1} i.v.) dose-dependently induced acute mucosal damage, characterized as vasocongestion and haemorrhagic necrosis. The enatiomer D-NMMA (100 mg kg^{-1} i.v.) did not induce any detectable mucosal damage.

4 This mucosal injury induced by L-NMMA was inhibited by concurrent administration of L-arginine (300 mg kg^{-1} i.v.).

5 In indomethacin (5 mg kg^{-1} i.v.)-pretreated rats, L-NMMA also induced mucosal damage. Furthermore, following indomethacin administration in capsaicin-pretreated rats, L-NMMA induced widespread, severe haemorrhagic necrotic damage.

6 These findings suggest a role for endogenous NO formed from L-arginine, acting in concert with prostacyclin and sensory neuropeptides, in the modulation of gastric mucosal integrity.

Introduction

The maintenance of gastric mucosal function and integrity depends critically on the status of the microcirculation. Limitations to microvascular perfusion, either by direct vasoconstriction or by removal of endogenous vasodilator tone, can lead to the development of mucosal damage, erosion or ulceration (Whittle *et al.*, 1981; Whittle, 1986). Thus, local generation of thromboxane A_2 or intra-arterial administration of its stable mimetic U-46619, which induce vasoconstriction in the gastric microcirculation, lead to substantial mucosal damage (Whittle *et al.*, 1981; 1985; Esplugues & Whittle, 1988). More recently, close-arterial infusion of the endothelium-derived vasoconstrictor peptide, endothelin-1 (Yanagisawa *et al.*, 1988) has also been demonstrated to induce pronounced mucosal damage following local intra-arterial infusion (Whittle & Esplugues, 1988) and to potentiate ethanol-induced mucosal disruption (Wallace *et al.*, 1989).

One local vasodilator mediator that can be formed by endothelial cells is prostacyclin and its synthesis can be inhibited by cyclo-oxygenase inhibitors such as indomethacin (Moncada *et al.*, 1976). Inhibition of endogenous prostacyclin formation and a reduction in mucosal blood flow has been proposed to be a mechanism underlying the gastric damage induced by indomethacin (Whittle, 1977) and other non-steroid anti-inflammatory agents (see Whittle, 1986). Vascular endothelial cells also release a labile humoral vasodilator substance known as endothelium-derived relaxing factor or EDRF (Furchgott & Zawadzki, 1980; Furchgott, 1983). Nitric oxide (NO), formed by endothelial cells from the amino acid L-arginine, accounts for the biological properties of EDRF (Palmer *et al.*, 1987; 1988a). The formation of NO by endothelial cells is selectively inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989a). Studies in the rabbit and rat have demonstrated that L-NMMA increases systemic arterial blood pressure, suggesting that endogenous NO biosynthesis can modulate resting vascular tone *in vivo* (Rees *et al.*, 1989b; Whittle *et al.*, 1989). L-NMMA has therefore been used to investigate the role of endogenous NO in the regulation of gastric mucosal integrity in the rat.

The local release of vasodilator neuropeptides such as substance P or calcitonin gene-related peptide (CGRP) has also been implicated in the maintenance of mucosal integrity, since chronic pretreatment with capsaicin, which destroys primary sensory neurones and depletes their neuropeptide content (see Holzer, 1988), greatly augments mucosal damage induced by various challenging agents (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Esplugues *et al.*, 1989). In order to investigate the interactions between these distinct local vasodilator mediators, NO, sensory neuropeptides and prostacyclin, in the gastric mucosa, we have evaluated the pro-ulcerogenic actions of L-NMMA in rats pretreated with capsaicin or treated with indomethacin.

Methods

Gastric mucosal damage

Male Wistar rats (230–260 g), deprived of food but not water for 18–20 h before the experiments, were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a mid-line incision. After ligating the oesophagus and pylorus, 2 ml of acid saline (100 mM HCl) was instilled into the gastric lumen through the forestomach, followed by bolus intravenous administration of the agents under investigation. Forty-five min later, the stomachs were opened along the greater curvature, pinned to a wax block with the mucosal side up, immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically-visible damage was determined from these projected transparencies via computerized planimetry. The area of mucosal damage was calculated as the % of the total gastric mucosa showing macroscopically visible damage.

Two samples of the corpus (0.5 × 1.5 cm) were excised from standardized regions of the dorsal and ventral aspects of the corpus mucosa, 0.5 cm below the limiting ridge, and were processed by routine techniques before being embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and examined under a light microscope. A 1 cm length of each histological section was assessed for epithelial cell damage (a

score of 1 being assigned); glandular disruption, vasocongestion or oedema in the upper mucosa (a score of 2 being assigned); haemorrhagic damage in the mid to lower mucosa (a score of 3) and deep necrosis and ulceration (a score of 4). Each section was evaluated on a cumulative basis to give the histological index, the maximum score thus being 10. All determinations were performed in a randomized manner with both transparencies and histological sections coded to eliminate observer bias.

Gastric damage in capsaicin-treated rats

Similar experiments were performed in animals pretreated with capsaicin. Adult rats (190–210 g) were treated with capsaicin in increasing doses (20, 30 and 50 mg kg^{-1}, s.c.) on three consecutive days, in a regimen that has been shown to deplete neuropeptides in primary afferent neurones in adult rats (Martling, 1987) and to enhance platelet-activating factor (PAF)-induced gastric damage (Esplugues *et al.*, 1989). All capsaicin injections were made under halothane anaesthesia and, to counteract any respiratory impairment associated with the administration of capsaicin, the rats were pretreated with terbutaline (0.1 mg kg^{-1}, i.m.) and aminophylline (10 mg kg^{-1}, i.m.) prior to capsaicin injection. The animals were used 2 weeks after completion of the capsaicin treatment. In control studies, animals received a similar regimen of treatment with the vehicle, although no significant difference could be detected between any of the observations in vehicle-pretreated or non-pretreated rats.

Systemic arterial blood pressure

Mean systemic arterial blood pressure (BP) was measured from a cannula inserted into a carotid artery and connected to a pressure transducer (Elcomatic) and a chart recorder (Grass), with a resting value of 119 ± 2 mmHg (n = 30).

Materials

Indomethacin (Sigma Chemical Co) was freshly dissolved in 5% w/v $NaHCO_3$ solution and diluted to 1.25% with distilled water. L-Arginine (Sigma Chemical Co) was prepared as the acetate salt and dissolved freshly in isotonic saline. N^G-monomethyl-L-arginine acetate and its D-enantiomer, synthesized as described previously (Patthy *et al.*, 1977) in the Department of Medicinal Chemistry, Wellcome Research Laboratories by Dr H. Hodson, were freshly dissolved in isotonic saline before use. Capsaicin (Fluka Chemical AG) was prepared as a 50 mg ml^{-1} solution containing absolute ethanol, Tween 80 and isotonic saline (10:10:80: v/v/v).

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups of parametric data were made by Student's t test for unpaired data. Comparisons between groups of non-parametric data (histological index) were made by Mann-Whitney U-test. P values of less than 0.05 were taken as significant.

Results

Effects of L-NMMA or capsaicin pretreatment

Intragastric instillation of acid saline (2 ml; 100 mM HCl) did not lead to any macroscopically detectable mucosal damage over the 45 min study period (Figure 1). On histological examination, a minor degree of epithelial cell damage involving 13 ± 4% (n = 4) of the total section length was determined (Table 1). Following intragastric instillation of acid saline, intravenous administration of L-NMMA (12.5–100 mg kg^{-1}) did not induce any significant macroscopically detectable mucosal damage ($P > 0.05$; n = 3–5 per group), as shown for the higher doses of L-NMMA in Figure 1. Likewise, no significant increase in the minor histological damage was observed following administration of these doses of L-NMMA (Table 1).

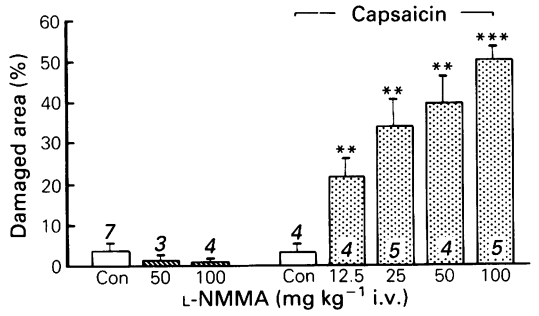

Figure 1 Effects of N^G-monomethyl-L-arginine (L-NMMA, 12.5–100 mg kg^{-1} i.v.) on the gastric mucosa of vehicle- or capsaicin-pretreated rats. Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage 45 min after administration, are expressed as mean of n (number in columns) experiments where vertical lines show s.e.mean. Significant difference from the respective acid-control group (Con; intragastric 100 mM HCl) is shown as $^{**}P < 0.01$, $^{***}P < 0.001$.

Capsaicin pretreatment alone did not induce any macroscopically apparent damage, following intragastric instillation of acid-saline (Figure 1). Furthermore, no histologically apparent damage could be located in the tissue sections following capsaicin pretreatment (Table 1).

Effect of L-NMMA following capsaicin pretreatment

In capsaicin-pretreated rats, L-NMMA (12.5–100 mg kg^{-1}) induced dose-related macroscopic damage to the mucosa (Figure 1). In a separate time-course study, distinct macroscopic damage could be detected in the corpus region 20 min after L-NMMA (100 mg kg^{-1}) administration (n = 3), while the degree of damage assessed 90 min after L-NMMA administration (65 ± 10% of total area, n = 3) was similar to that observed after 45 min. With increasing doses of L-NMMA, both the area of damage (Figure 1) and the severity of the mucosal injury were significantly increased, this damage being characterized macroscopically as surface cell sloughing, vaso-

Table 1 Effects of intravenous administration of N^G-monomethyl-L-arginine (L-NMMA), D-NMMA and indomethacin on the rat gastric mucosa, as assessed by histological evaluation

	Dose (mg kg^{-1})	*Histological Index*	(n)
Control	—	1.5 ± 0.5	(4)
L-NMMA	100	0.7 ± 0.3	(3)
Indo	5	1.6 ± 0.6	(3)
Capsaicin-pretreated			
Control	—	1.5 ± 0.5	(4)
D-NMMA	100	1.6 ± 0.6	(3)
L-NMMA	25	4.4 ± 1.0*	(5)
	100	5.8 ± 1.4*	(5)
L-NMMA + L-Arginine	100 + 300	0.7 ± 0.3†	(3)
L-NMMA + Indo	100 + 5	9.2 ± 0.8†	(5)

Results, shown as the histological index of damage (0–10 scale), are mean ± s.e.mean of n animals, where statistical difference from the corresponding group control (intragastric 100 ml HCl), is shown as $^*P < 0.05$ and from the L-NMMA (100 mg kg^{-1}) group in capsaicin-pretreated rats as $^\dagger P < 0.05$.

congestion and distinct areas of haemorrhagic necrosis. Such mucosal damage also involved the antral region.

In contrast, administration of the enantiomer, D-NMMA ($100\,mg\,kg^{-1}$, i.v.) in capsaicin-pretreated rats did not induce any detectable mucosal injury (Figure 2). In further studies, administration of L-arginine ($300\,mg\,kg^{-1}$, i.v.), immediately before L-NMMA ($100\,mg\,kg^{-1}$, i.v.) caused a significant reduction in the damage (by $86 \pm 2\%$, $n = 3$; $P < 0.01$) as shown in Figure 2.

Administration of L-NMMA (25 and $100\,mg\,kg^{-1}$ i.v.) dose-dependently increased the histological index of damage, which was inhibited by concurrent administration of L-arginine ($300\,mg\,kg^{-1}$ i.v.), as shown in Table 1. The damage induced by L-NMMA was characterized histologically as epithelial cell and glandular disruption, with vasocongestion and haemorrhagic damage being also noted. D-NMMA ($100\,mg\,kg^{-1}$ i.v.) did not induce any histological injury in capsaicin-pretreated rats (Table 1).

Bolus intravenous administration of L-NMMA (12.5, 25, 50 and $100\,mg\,kg^{-1}$) in vehicle-pretreated rats induced a dose-dependent increase in mean systemic arterial BP of 23 ± 2, 33 ± 3, 41 ± 3 and 44 ± 4 mmHg above the basal value, respectively ($n = 4$–6 for each), which reached its peak value within 10 min and which was maintained over the 45 min observation period. Administration of D-NMMA ($100\,mg\,kg^{-1}$) did not significantly increase systemic BP ($P > 0.05$; $n = 3$). The increase in BP induced by L-NMMA ($100\,mg\,kg^{-1}$) was significantly attenuated (from 44 ± 4 to 18 ± 3 mmHg above the basal value; $n = 4$; $P < 0.05$) by prior administration of L-arginine ($300\,mg\,kg^{-1}$, i.v.). There was no significant difference between the effects of L-NMMA (12.5 or $100\,mg\,kg^{-1}$ i.v.) on BP in vehicle or capsaicin-pretreated rats ($P > 0.05$; $n = 4$ for each group).

Effects of indomethacin

Administration of indomethacin ($5\,mg\,kg^{-1}$ i.v.) immediately before intragastric acid instillation, did not significantly damage the mucosa over the 45 min study period (Figure 3). However, following indomethacin administration L-NMMA ($100\,mg\,kg^{-1}$, i.v.) induced significant ($P < 0.05$) damage to the gastric mucosa (Figure 3).

In capsaicin-pretreated rats, indomethacin ($5\,mg\,kg^{-1}$, i.v.) induced distinct mucosal injury, this damage being characterized as localized areas of linear haemorrhage and necrosis (Figure 3). Furthermore, following indomethacin administration, the mucosal damage induced by L-NMMA ($100\,mg\,kg^{-1}$) in capsaicin-pretreated rats was substantially enhanced as shown in Figure 3, and involved virtually all of the mucosal surface. This damage was characterized macroscopically as both an increase in the number and area of the dark necrotic lesions, as well as the appearance of more widespread epithelial disruption, vasocongestion and haemorrhage, which was confirmed histologically (Table 1).

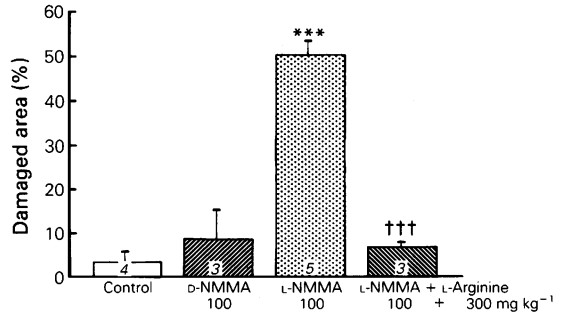

Figure 2 Prevention of the gastric mucosal damage induced by N^G-monomethyl-L-arginine (L-NMMA, $100\,mg\,kg^{-1}$ i.v.) by concurrent administration of L-arginine ($300\,mg\,kg^{-1}$ i.v.) in capsaicin-pretreated rats. D-NMMA ($100\,mg\,kg^{-1}$ i.v.) did not induce mucosal damage. Results, shown as % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (numbers in columns) experiments where vertical lines show s.e.mean. Significant difference from acid-control group (intragastric 100 mM HCl) is shown as $^{***}P < 0.001$ and from the L-NMMA group by $^{†††}P < 0.001$.

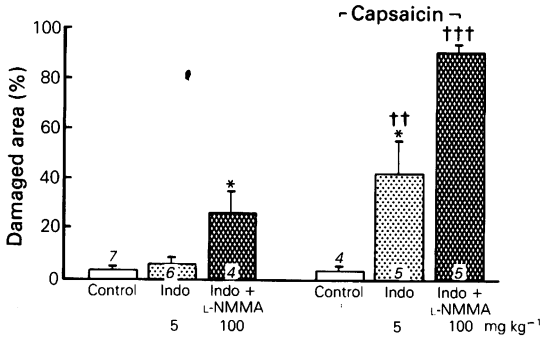

Figure 3 Effect of indomethacin (Indo, $5\,mg\,kg^{-1}$ i.v.), N^G-monomethyl-L-arginine (L-NMMA, $100\,mg\,kg^{-1}$ i.v.) or their combination, on the gastric mucosa of vehicle- or capsaicin-pretreated rats. Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (figures in columns) experiments, where vertical lines show s.e.mean. Significant difference from the appropriate acid-control group (intragastric 100 mM HCl) and from the corresponding non-capsaicin group is shown as $^{*}P < 0.05$ and $^{††}P < 0.01$, $^{†††}P < 0.001$, respectively.

Discussion

We have demonstrated that although the administration of L-NMMA alone or capsaicin-pretreatment did not lead to acute gastric damage, administration of L-NMMA in capsaicin-pretreated rats did induce substantial mucosal injury, determined by both macroscopic and histological techniques. Furthermore, indomethacin enhanced the damage induced by L-NMMA under both control conditions and in capsaicin-pretreated rats. These findings thus suggest an interaction between NO, prostanoids and sensory neuropeptides in the regulation of gastric integrity.

The release of NO from endothelial cells and vascular tissue, which can be detected by both bioassay and chemical techniques, accounts for the biological activity of EDRF (Palmer *et al.*, 1987; Khan & Furchgott, 1987; Ignarro *et al.*, 1987; Kelm *et al.*, 1988). Thus, in those vascular beds in which endogenous endothelium-dependent vasodilators regulate vascular tone, inhibition of NO formation would be expected to reduce blood flow. Indeed, in the rabbit coronary circulation, *in vitro*, L-NMMA increased vascular tone (Amezcua *et al.*, 1989) while *in vivo*, L-NMMA increased systemic BP in the rabbit and rat (Rees *et al.*, 1989b; Whittle *et al.*, 1989), as confirmed in the present study. The effects of L-NMMA in reducing blood flow in specific vascular beds *in vivo* have recently been reported (Gardiner *et al.*, 1989). It is also of interest that enzymic disruption of endothelial cells in the rat gastric microcirculation attenuated the vasodilator response to acetylcholine, suggesting the local involvement of an endothelium-derived factor (Kitagawa *et al.*, 1987). Furthermore, a reduction in gastric mucosal blood flow following intravenous administration of L-NMMA in the rat has recently been demonstrated, indicating a microvascular role of endogenous NO in the mucosa (Pique *et al.*, 1989b).

Intravenous administration of L-NMMA alone did not induce mucosal damage over the acute observation period employed in the current study in the rat. Whether more chronic treatment with L-NMMA or other more prolonged inhibitors of NO formation would induce gastric injury will require further evaluation. However, the macroscopic mucosal damage induced by L-NMMA in capsaicin-pretreated rats was readily apparent within 20 min of administration, and was near-maximally developed within 45 min. The specific nature

of this action of L-NMMA was demonstrated by the lack of injurious effect of D-NMMA, its enantiomer which does not inhibit NO synthesis by endothelial cells (Palmer *et al.*, 1988b). These findings thus implicate a contribution of endogenous NO to the protective mechanisms within the mucosa. Recently the nitrovasodilator, nitroprusside, has been demonstrated to inhibit mucosal damage following intravenous administration (Wallace *et al.*, 1989). The mechanisms of mucosal protection by endogenous NO or that released by nitrovasodilators remain to be explored, but are likely to involve actions on the microvasculature.

The severe mucosal injury induced by L-NMMA under these conditions of capsaicin-preatment, characterized macroscopically and confirmed histologically as epithelial cell sloughing, vasocongestion and haemorrhagic necrosis, was inhibited by concurrent intravenous administration of L-arginine. Previous studies have demonstrated that L-arginine can reverse the inhibitory actions of L-NMMA on NO synthesis by endothelial cells, in culture or on isolated vascular tissue, and by isolated neutrophils (Palmer *et al.*, 1988b; Rees *et al.*, 1989a; McCall *et al.*, 1989), suggesting the currently observed protective effect of L-arginine was not simply due to non-specific actions of this systemically administered amino acid. Furthermore, L-arginine attenuates the hypertension induced by L-NMMA in the rabbit and rat (Rees *et al.*, 1989b; Whittle *et al.*, 1989) as confirmed in the present study using comparable doses. These findings thus suggest that L-NMMA acts by competing for the uptake or utilization of L-arginine, the substrate for NO biosynthesis. The doses of L-NMMA that were found to provoke mucosal injury in the rat are similar to those previously shown to inhibit endothelium-dependent vasodilatation *in vivo* induced by such agents as acetylcholine or bradykinin in the rat and rabbit (Whittle *et al.*, 1989; Rees *et al.*, 1989b). Furthermore, these doses of L-NMMA have been shown to inhibit the *ex vivo* generation of NO by vascular tissue, detected by bioassay and chemiluminescence techniques (Rees *et al.*, 1989b). The present findings with L-NMMA thus suggest that the gastric mucosa can form endogenous NO derived from L-arginine, but its direct detection awaits further study.

Capsaicin-sensitive neurones containing vasoactive neuropeptides including substance P and CGRP have been located by immunohistochemical techniques in the rat stomach, particularly in association with the submucosal microvasculature (Sharkey *et al.*, 1984; Ekblad *et al.*, 1985; Green & Dockray, 1988). In previous studies in the rat, capsaicin pretreatment did not modify systemic arterial BP or resting gastric mucosal blood flow, and as also observed in the current study, did not induce damage to the gastric mucosa (Pique *et al.*, 1989a,b; Esplugues *et al.*, 1989). However, capsaicin pretreatment substantially potentiated the reduction in mucosal blood flow induced by the intravascular ulcerogen, PAF, which was accompanied by a significant increase in mucosal injury (Pique *et al.*, 1989a). Such increased gastric damage in capsaicin-pretreated rats has previously been observed following challenge with other ulcerogens (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986). Thus, the release of neuropeptides from capsaicin-sensitive primary sensory neurones appears to be involved in the regulation of a local microvascular protective response to challenge.

Substance P and CGRP have been demonstrated to induce endothelium-dependent vasodilatation *in vitro* (Furchgott, 1983; Brain *et al.*, 1985). Furthermore, the fall in systemic BP following intravenous administration of substance P in the rat can be attenuated by L-NMMA, suggesting a role of endogenous NO in the vasodilator responses of such neuropeptides *in vivo* (Whittle *et al.*, 1989). If such mechanisms operated in the mucosal microcirculation, L-NMMA would be expected to attenuate any vasodilator actions of these endogenous neuropeptides, as well as other endothelium-dependent vasodilators. Capsaicin pretreatment does not affect resting gastric mucosal blood flow (Pique *et al.*, 1989a), nor does it alter the increase in systemic BP induced by L-NMMA. However, it will be of interest to determine whether capsaicin pretreatment augments the changes in gastric blood flow induced by L-NMMA (Pique *et al.*, 1989b), since such microcirculatory changes could underlie the resulting mucosal damage.

Endogenous endothelium-independent vasodilators such as prostacyclin, whose systemic hypotensive actions are not modified by L-NMMA (Whittle *et al.*, 1989), may synergistically interact with these other vasodilator mediators in the local regulation of the microcirculation. Indeed, following intravenous administration of indomethacin, in a dose sufficient to induce near-maximal inhibition of mucosal prostacyclin formation (Whittle, 1986), the gastric injury induced by L-NMMA was significantly augmented, although not to the same degree as by capsaicin-pretreatment. In capsaicin pretreated rats, this low dose of indomethacin rapidly induced mucosal damage, as demonstrated previously following its intraperitoneal injection (Holzer & Sametz, 1986). Furthermore, the present findings show that in capsaicin-pretreated rats, indomethacin substantially augmented the damage induced by L-NMMA which involved virtually all of the mucosal area, including the antral region. Parenteral indomethacin has been demonstrated to limit blood flow in the gastric microcirculation (see Whittle, 1986) and whether such actions are substantially accentuated in capsaicin-pretreated rats or following NO inhibition will require evaluation.

Although the possibility that other actions of indomethacin may contribute to this enhanced injurious response cannot be entirely discounted, these present findings support a contribution of endogenous prostaglandins in the regulation of mucosal integrity, particularly under conditions where the synthesis or release of NO or the neuropeptides are concurrently reduced. Indeed, if these latter conditions occurred clinically, non-steriod anti-inflammatory agents would be expected to be considerably more injurious to the gastric mucosa.

The crucial importance of the gastric microcirculation is thus re-inforced by the current findings, that suggest that at least three chemically and physiologically distinct classes of local vasodilator mediators of diverse biochemical origins act in concert in the maintenance of mucosal viability. Thus, endogenous NO, sensory neuropeptides and a prostanoid, probably prostacyclin, all appear to subserve a modulator function in the regulation of gastric mucosal integrity. These mediators may not only exert local vasodilator actions on the microcirculation, essential for adequate microvascular blood flow under physiological conditions, but may act to enhance or preserve endothelial cell function and continuity, especially under conditions of challenge. It is therefore of relevance that both NO and prostacyclin can be synthesized by endothelial cells (Palmer *et al.*, 1988a), and it is possible that neuropeptides originating from the afferent sensory neurones in the vicinity of the microvessels are involved in the regulation of their release.

Since damage to the endothelial cell can be a prime event leading to mucosal injury induced by irritants (Guth *et al.*, 1984; Szabo *et al.*, 1985), this could reflect a derangement in the formation or release of these vasoactive mediators. Although selective inhibition of either mediator alone induced no acute mucosal injury, concurrent pharmacological interference with more than one of these mediators induced rapid and widespread tissue damage. It will therefore be necessary to consider the nature of the interactions of these vasoactive mediators when assessing the physiological regulation of gastric function and integrity, and the pathogenesis of mucosal damage and peptic ulceration.

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The effects of siguazodan, a selective phosphodiesterase inhibitor, on human platelet function

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1 The effects of siguazodan (SK&F 94836) a selective phosphodiesterase (PDE) inhibitor with inotropic and vasodilator activity, were studied on human platelets.

2 Siguazodan selectively inhibited the major cyclic AMP-hydrolysing PDE in human platelet supernatants. The inhibited enzyme has been variously termed cyclic GMP-inhibited PDE or PDE-III.

3 In platelet-rich plasma (PRP), siguazodan inhibited U46619-induced aggregation more potently than that induced by ADP and collagen. Treatment of the PRP with aspirin had no effect on the potency of siguazodan.

4 In washed platelets, siguazodan increased cyclic AMP levels and reduced cytoplasmic free calcium ($[Ca^{2+}]_i$). ADP decreased the ability of siguazodan to raise cyclic AMP and this may explain its lower potency in inhibiting responses to ADP.

5 Siguazodan has anti-platelet actions over the same concentration range that it is an inotrope and vasodilator.

Introduction

Siguazodan ((SK&F 94836) **R,S**-2-cyano-1-methyl-3-[4-(-methyl-6-oxo-1,4,5,6 tetrahydropyridazine-3-yl)phenyl]-guanidine), is a potent, selective inhibitor of guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-inhibited cyclic nucleotide phosphodiesterase, (cGI-PDE (Beavo 1988), sometimes referred to as phosphodiesterase III), that has positive inotropic and vasodilating actions in various laboratory animals and is orally active with a long duration of action in conscious dogs (Gristwood *et al.*, 1988). These properties suggested a utility in cardiac failure and siguazodan has undergone initial evaluation in man. Inhibition of cGI-PDE is known to raise adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in airways smooth muscle and thus to have a potential for bronchodilatation (Torphy *et al.*, 1988), and also to increase cyclic AMP in platelets with consequent anti-thrombotic effects (Muggli *et al.*, 1985). These actions on platelets have two main consequences for clinical studies: (1) 'anti-platelet' actions might prove additional benefit in patients with ischaemic heart disease; (2) reduced responsiveness of platelets taken from subjects after administration of a compound can provide a convenient pharmacodynamic monitor of drug effect. Additionally, the availability of potent isoenzyme selective inhibitors of phosphodiesterase allows an analysis of the role of these enzymes in platelet function.

We present here the effects of siguazodan on human platelets *in vitro*, including studies of separated, partially purified sub-types of platelet PDE, measurements of cyclic AMP concentration, effects on $[Ca^{2+}]_i$ transients evoked by adenosine 5′-diphosphate (ADP), and inhibition of aggregation in both washed platelets and platelet-rich plasma evoked by ADP and the thromboxane mimetic U46619. In the functional studies, effects over and above those attributable to interference with prostaglandin pathways were assessed by working with aspirin-treated cells. A preliminary account of part of this work has been communicated to the British Pharmacological Society (Simpson *et al.*, 1988a).

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Methods

Preparation of platelet-rich plasma and washed platelets and measurement of aggregation

Platelet-rich plasma (PRP) was prepared from whole blood, freshly drawn from healthy volunteers who gave informed consent, anticoagulated with 1/10 volume of acid-citrate dextrose containing: 7 mM citric acid, 93 mM sodium citrate and 139 mM detrose. The citrated blood was centrifuged at 700 *g* for 5 min and the PRP removed. The remaining red cells and plasma were centrifuged for a further 15 min at 900 *g* and the platelet-poor plasma removed. This was then mixed with the PRP giving a final platelet count of 2–3 × 10^8 cells ml^{-1}.

Washed platelets were prepared from whole blood anticoagulated with 1/6 volume of acid-citrate dextrose containing: 65 mM citric acid, 85 mM sodium citrate and 110 mM dextrose and centrifuged for 5 min at 700 *g*. The PRP was removed and then centrifuged at 350 *g* for 20 min and resuspended in N-2-hydroxyethyl piperazine-N-2 sulphonic acid (HEPES)-buffered physiological saline containing 10 mM glucose, and 0.05 u ml^{-1} hirudin (Simpson *et al.*, 1988b).

Aggregation was measured in a twin channel HU aggregometer or a 4-channel, PAP4 Biodata aggregometer. Aliquots were equilibriated to 37°C before being placed in the sample chamber, and were then incubated for 2 min with various concentrations of siguazodan. After the addition of the agonist, aggregation was determined by the change in absorbance monitored for 4 min.

Measurement of $[Ca^{2+}]_i$

This was as described by Rink *et al.* (1983). Briefly, washed platelets were prepared as described above except that the PRP was incubated with 20 μM quin 2 for 30 min at 37°C (and 100 μM aspirin, 40 μg ml^{-1} apyrase for the final 5 min) before the second centrifugation step. $[Ca^{2+}]_i$ was monitored in 0.8 ml aliquots of the quin 2-loaded, washed platelets in a 37°C thermostated Aminco Bowman fluorescent spectrophotometer; excitation was at 339 nm, emission was measured at 500 nm. Ca^{2+} and fibrinogen were added to the preheated aliquots to give final concentrations of 1 mM and 1 mg ml^{-1} respectively. Subsequently, siguazodan (or DMSO control)

was added for 2 min after which ADP or U46619 was added for a further 2 min.

Determination of cyclic AMP content in washed platelets

Washed platelets were prepared as described above and resuspended (ca. 10^8 cells ml^{-1}) in HEPES-buffered physiological saline containing 100 μM aspirin. Aliquots (480 μl) were equilibrated at 37°C, 10 μl siguazodan (0–5 mM) was added for 2 min after which 10 μl ADP (1 mM) or U46619 (0.5 mM) was added for a further 2 min; finally the incubation was terminated by the addition of 50 μl perchloric acid (3 M). Following centrifugation, acid was removed from a 400 μl aliquot of the supernatant by extraction with 500 μl of a 50/50 (v/v) mixture of tri-n-octylamine and freon (Sharps & McCarl, 1982). The cyclic AMP in the upper, aqueous layer was determined by use of commercial radioimmunoassay (Amersham, product number RPA 508). The number of platelets was measured by use of a Coulter counter and the results expressed as pmol cyclic AMP per 10^8 platelets (this is approximately equivalent to μmol per litre cell volume). Addition of [^{3}H]-cyclic AMP to platelets showed recovery by this procedure to be >95%; therefore the results have not been corrected for recovery.

Ion-exchange chromatography of platelet PDE

Washed human platelets were obtained from Addenbrookes Hospital Blood Bank and stored at −70°C until use. Homogenisation and anion-exchange chromatography on DEAE-Sepharose was as previously described (Simpson *et al.*, 1988b; Reeves *et al.*, 1987a). PDE activity was assayed by the method of Davis & Daly (1979) as modified by Reeves *et al.* (1987a).

Drugs

Siguazodan (Smith Kline and French Labs Ltd, Welwyn Garden City) was dissolved in DMSO to give a stock solution of 50 mM). The concentration of DMSO in platelet incubations was kept constant at 0.2% (v/v). U46619 (15S-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid; Upjohn, Kalamazoo, U.S.A.) was dissolved in absolute ethanol. ADP (Sigma) was dissolved in distilled H_2O. Quin 2 acetoxymethylester (Calbiochem, Cambridge U.K.) was made up as a 50 mM solution in DMSO. All other reagents were from Sigma Chemical Company Ltd, Poole.

Analysis of data

Concentration inhibition curves were fitted to the logistic equation by computer using the programme, ALLFIT (De Lean *et al.*, 1978).

Statistical analysis was by Student's *t* test. All results are shown as mean ± s.e.mean.

Results

Effects of siguazodan on platelet PDE activity

Figure 1 shows the profile of human platelet PDE activities following chromatography on DEAE-Sepharose. It can be seen that when assayed with 1 μM cyclic AMP there is one major cyclic AMP-PDE in human platelet supernatant. This activity has been referred to as the cyclic GMP-inhibited PDE (cGI-PDE) (Beavo, 1988) or PDE III (Weishaar *et al.*, 1986). This activity is inhibited by 100 μM siguazodan, whereas the earlier minor peak is unaffected. We have previously obtained similar results with another specific cGI-PDE inhibitor, SK&F 94120 (Simpson *et al.*, 1988b). The dose-inhibition curve showed that siguazodan produced a concentration-dependent inhibition of platelet cGI-PDE with an IC_{50} of 0.8 μM. For comparison, siguazodan inhibited cGI-PDE from human heart with the same IC_{50} (Table 1).

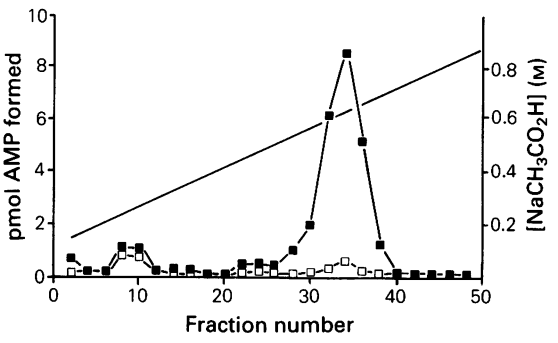

Figure 1 Separation of phosphodiesterase activities from human platelets on DEAE-Sepharose. A supernatant fraction was prepared from human platelets and applied to a DEAE-Sepharose column as described under 'Methods'. The column was eluted with a sodium acetate gradient (indicated by the unbroken line) and the fractions assayed with 1 μM cyclic AMP (■) or 1 μM cyclic AMP + 100 μM siguazodan (□).

Effect of siguazodan on cyclic AMP content in human platelets

Table 2 shows that siguazodan increased the measured content of cyclic AMP in intact human platelets resuspended in HEPES-buffered physiological saline. The first column shows the responses of platelets unstimulated by added agonist; statistically significant increases in cyclic AMP are apparent at concentrations of siguazodan above 0.3 μM. The increases in cyclic AMP at concentrations up to 3 μM are modest, around 20%, and are not dose-related. However, increasing concentrations of siguazodan produced further increments in cyclic AMP to more than double the basal level at the highest concentrations tested. Although a close quantitative comparison is clearly inappropriate, these results show that siguazodan does elevate cyclic AMP in intact platelets in a similar concentration range to that which inhibits the isolated cGI-PDE.

Effect of siguazodan on agonist-evoked increases in $[Ca^{2\pm}]_i$

Among the ways in which cyclic AMP inhibits platelet function is a reduction in signal transduction and in Ca^{2+} mobilization. We therefore examined the effect of siguazodan on stimulated increases in $[Ca^{2+}]_i$ in quin 2-loaded platelets resuspended in HEPES-buffered saline (Figure 2). Siguazodan, was able to inhibit almost completely the $[Ca^{2+}]_i$ elevation evoked by 20 μM ADP. The calculated IC_{50} for this effect was 1.5 μM. With 10 μM U46619 (a thromboxane mimetic) as the stimulus, the IC_{50} for the $[Ca^{2+}]_i$ response was 0.5 μM.

Table 1 Inhibition of human platelet and heart cGI-PDE by siguazodan

[*Siguazodan*] (μM)	*pmol AMP*	
	Platelet	*Heart*
0	2.08	4.16
0.1	2.29	3.71
0.3	1.33	2.97
1	0.95	2.06
3.2	0.55	1.30
10	0.14	0.61

Cyclic GMP-inhibited cyclic nucleotide phosphodiesterase (cGI-PDE) was prepared from human platelets as described under 'Methods' and from human ventricle as described by Reeves *et al.* (1987a). The results show pmol AMP formed per assay tube for each concentration of siguazodan and are the mean of two experiments; the concentration of cyclic AMP was 1 μM. Calculated $1C_{50}$ values were 0.84 μM (platelet), 0.81 μM (heart).

Table 2 The effect of siguazodan on cyclic AMP in intact, washed human platelets

Siguazodan μM	(pmol cyclic AMP per 10^8 cells) *Control*	*U46619* (10 μM)	*ADP* (20 μM)
0	1.10 ± 0.09	1.07 ± 0.07	1.25 ± 0.15
0.3	1.31 ± 0.20*	1.21 ± 0.09*	1.13 ± 0.09
1	1.31 ± 0.16*	1.19 ± 0.09*	1.38 ± 0.16
3	1.29 ± 0.15*	1.33 ± 0.12**	1.43 ± 0.27
10	1.71 ± 0.33*	1.52 ± 0.14**	1.33 ± 0.12
30	1.66 ± 0.17**	1.76 ± 0.11***	1.46 ± 0.10*
100	2.19 ± 0.26**	2.34 ± 0.28**	1.91 ± 0.17**

Washed human platelets were incubated with siguazodan for 2 min and variously with U46619 or ADP for a further 2 min. The reaction was terminated by the addition of perchloric acid and cyclic AMP determined by radioimmunoassay. For full details see 'Methods'. Results are shown as pmol cyclic AMP per 10^8 platelets and are expressed as means ± s.e.mean ($n = 5$ for all observations). Results were analysed by Students t test vs. control (no siguazodan) *$0.05 > P > 0.01$, **$0.01 > P > 0.001$. Platelets treated with ADP and 30 μM and 100 μM siguazodan had significantly lower cyclic AMP levels than cells treated with siguazodan alone ($P < 0.05$).

Effects of siguazodan on platelet aggregation

Siguazodan inhibited aggregation in response to U46619, ADP and collagen in washed platelet suspensions, PRP and aspirin-treated PRP with IC_{50}s in the range 1–10 μM (Table 3). The similarity of the values in washed platelets and PRP confirm the low binding of siguazodan to serum proteins (≃30% over the range 0.2–60 μM; SK&F file data). Siguazodan was most potent when U46619 was the agonist; the similarity of inhibition in PRP and aspirin-treated PRP suggest that this is not due to endogeneous thromboxane A_2 formation.

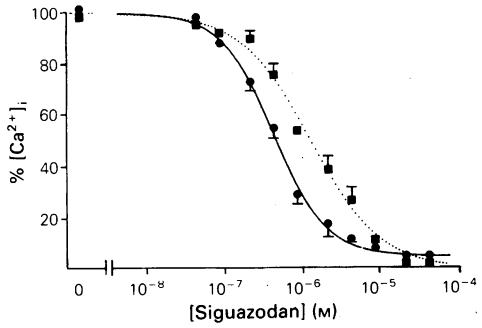

Figure 2 Inhibition by siguazodan of ADP- and U46619-stimulated increases in cytoplasmic free calcium. Platelets were loaded with quin-2 and $[Ca^{2+}]_i$ calculated as described under 'Methods'. Following a 2 min incubation with various concentrations of siguazodan the platelets were challenged with either 10 μM U46619 (●) or 20 μM ADP (■) and the $[Ca^{2+}]_i$ recorded after a further 2 min. The results are the % of the $[Ca^{2+}]_i$ recorded in the absence of siguazodan and are shown as mean ($n = 3$). Error bars for s.e.mean are only shown when they are significantly larger than the symbol.

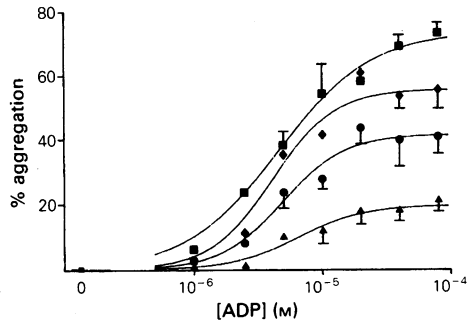

Figure 3 Effect of siguazodan on ADP-induced platelet aggregation. Platelet-rich plasma was prepared and incubated for 2 min with vehicle control (■), 2.5 μM (◆), 5 μM (●) or 10 μM siguazodan (▲). The platelets were challenged with the concentrations of ADP shown and the aggregation recorded after a further 2 min. Results are shown as mean ($n = 3$); error bars representing s.e.mean are shown only when they are significantly larger than the symbol.

Therefore, the ability of U46619 and ADP to modulate the siguazodan-stimulated increases in cyclic AMP were studied. Table 2 shows that neither of the agonists affected basal content, but that ADP decreased the ability of siguazodan to increase cyclic AMP content. This effect was most marked at the higher concentrations of siguazodan and may provide an explanation for the decreased potency when ADP is the agonist.

The proposed mechanisms of action of siguazodan would generate a functional inhibition that was not competitive with the agonist, and it seemed important to verify that this was the case. Figure 3 demonstrates clearly that the inhibitory effect of siguazodan on ADP-evoked aggregation of platelet plasma was not competitive. The dose-response curve for ADP showed a decrease in maximum effect with increasing concentrations of siguazodan without any detectable shift to the right.

Discussion

These results show that siguazodan inhibits the cGI-PDE separated from human platelets with a potency similar to that which it inhibits this PDE isoenzyme from human cardiac ventricles. In intact platelets, siguazodan increased cyclic AMP over a concentration range compatible with the observed effect on the separated enzyme. Siguazodan inhibited $[Ca^{2+}]_i$ and aggregation in washed platelets over a similar concentration range to that which produces positive inotropic effects in guinea-pig isolated ventricle strips (Reeves *et al.*, 1987b). The potency of the compound in inhibiting aggregation in platelet-rich plasma suggests that the presence of plasma protein does not influence the access to cell and tissues.

The profile of activity against different PDE sub-types, shown in Figure 1, indicates that cGI-PDE is the dominant cyclic AMP hydrolysing enzyme in human platelet supernatants. In other tissues, e.g. heart, cyclic AMP hydrolysis is more evenly balanced between various PDE isoenzymes (Reeves *et al.*, 1987a). These biochemical findings are reflected

Table 3 Inhibition of human platelet aggregation by siguazodan

	Stimulus *U46619*	*ADP* IC_{50} (μM)	*Collagen*
Washed platelets	1.4 ± 0.03 (3)	ND	ND
PRP	1.8 ± 0.07 (3)	4.0 ± 0.35 (3)	6.9 ± 0.70 (4)
PRP + 100 μM aspirin	1.8 ± 0.03 (3)	5.2 ± 0.28 (3)	10.9 ± 2.8 (4)

PRP or washed platelets were incubated with various concentrations of siguazodan for 2 min before the addition of 10 μM U46619, 20 μM ADP or 5 μg ml^{-1} collagen, aggregation was monitored for the subsequent 4 min. The values shown are the calculated IC_{50} (in μM) for the inhibition of aggregation. ND = not determined.

in the ability of siguazodan to elevate cyclic AMP in intact stimulated platelets, a finding which suggests that in the resting condition the steady-state cyclic AMP concentration is mainly determined by the relative activities of adenyl cyclase and cGI-PDE. The fact that 100 μM siguazodan only raised cyclic AMP 2 fold above the resting level suggests that another cyclic AMP hydrolysing enzyme is available. Possibly the modest cyclic AMP hydrolysing activity of one or more PDE 1 isoenzymes is sufficient to serve this purpose. The possibility of hydrolysis by PDEs associated with the particulate fraction of platelets must also be considered, as must activation of the supernatant cGI-PDE itself (Macphee *et al.*, 1988).

Modest increases in cyclic AMP levels appear to be a characteristic of cGI-PDE inhibitor action in a number of other tissues. In heart, increases in cyclic AMP of less than 50% are associated with significant increases in contractility (Evans, 1986; Murray *et al.*, 1987). Relaxation of vascular smooth muscle by cGI-PDE inhibitors results in a doubling of cyclic AMP levels (Kauffman *et al.*, 1987). In the heart (Murray *et al.*, 1987), smooth muscle (Silver *et al.*, 1988) and platelets (Seiler *et al.*, 1987), it has been shown that these small increases in cyclic AMP are associated with activation of cyclic AMP-dependent protein kinase. These results are in keeping with the known properties of isolated cyclic AMP-dependent protein kinase, which shows a high degree of positive cooperativity in its activation (Robinson-Steiner & Corbin, 1982).

The data clearly show that the inhibition of platelet function exhibited by siguazodan is not agonist specific and is, as expected, non-competitive with the agonist. There is a clearly reproducible distinction between the dose-inhibition curves for ADP and U46619, and the data in Table 2 suggest a partial explanation for this difference in terms of a smaller elevation of cyclic AMP in ADP-stimulated platelets. This might reflect some reduction of cyclase activity by ADP via its known action in activating G_i, the GTP binding protein associated with inhibition of adenyl cyclase (Tremblay & Hamet, 1987). Another possibility is that, because ADP and U46619 appear to use somewhat different signal transduction processes to cause aggregation, the stimulus-response coupling with U46619 is more susceptible to these modest elevations of cyclic AMP. Thromboxane receptor antagonism may be excluded, as 100 μM siguazodan had no effect on [^{125}I]-PTA-OH binding to washed human platelets (measured by the method of Mais *et al.*, 1985) (unpublished observations). The somewhat greater sensitivity of U46619-evoked responses, compared with the ADP or collagen responses, suggests that U46619 may be the ligand of choice for *ex in vivo* studies of siguazodan in human volunteers or patients. Indeed a detectable loss of responsiveness to U46619 was seen in *ex vivo* measurements on human volunteers, with doses of siguazodan giving plasma levels in excess of 0.1 μM (SK&F file data). From the *in vitro* work described here and previous studies on cardiac preparations there is no basis for expecting a dose separation for cardiac and platelet effects of this compound.

In conclusion, siguazodan has the general effects anticipated for a potent, selective cGI-PDE inhibitor in biochemical and functional studies of human platelets. All its actions can, in principle, be accounted for by inhibition of this enzyme and a consequent increase in cyclic AMP. Points of interest in terms of mechanisms of signal transduction are the apparent dominance by cGI-PDE of cyclic AMP hydrolysis in human platelets, the only modest increase in measured cyclic AMP that is associated with substantial functional antagonism, and the different dose-inhibition relations for different ligands. From a clinical perspective, the results show that *ex vivo* monitoring of platelet function should be a valuable measure of pharmacodynamics, and that an anti-platelet action is to be expected at doses that exhibit the inotropic and vasodilator action aimed at ameliorating the symptoms and signs of cardiac failure. Whether this anti-platelet action translates to clinical benefit can be determined only in controlled trials designed to answer this question.

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Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli

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1 Suramin, an inhibitor of several types of ATPase, was investigated for its ability to antagonize responses mediated via P_{2X}-purinoceptors in the guinea-pig urinary bladder and P_{2Y}-purinoceptors in the guinea-pig taenia coli.

2 In isolated strips of bladder detrusor muscle, suramin (100 μM–1 mM) caused a non-competitive antagonism of responses to α,β-methylene ATP with an estimated pA_2 of approximately 4.7, and inhibited responses to stimulation of the intramural purinergic nerves, with a similar pA_2 value. At a concentration of 10 μM, suramin had little effect, but at a concentration of 1 μM, suramin potentiated responses to α,β-methylene ATP, and potentiated responses to electrical stimulation of intramural purinergic nerves.

3 In isolated strips of taenia coli, in which a standard tone had been induced by carbachol (100 nM), suramin at 100 μM and 1 mM significantly antagonized relaxant responses to ATP (at an EC_{50} concentration) with an estimated pA_2 of 5.0 $\pm$ 0.82 and relaxant responses to electrical stimulation of the intramural non-adrenergic, non-cholinergic inhibitory nerves, either single pulses or trains of 8 Hz for 10 s, with estimated pA_2 values of 4.9 $\pm$ 0.93 and 4.6 $\pm$ 1.01, respectively. Suramin had no significant effect at 1 or 10 μM.

4 Suramin, at any of the concentrations tested, did not affect contractile responses to histamine (10 μM) or carbachol (10 μM) in the bladder detrusor preparations. In the taenia coli, suramin did not affect either the relaxant responses to noradrenaline (at an EC_{50} concentration) or the contractile responses to carbachol (100 nM).

5 Thus, suramin at concentrations above 10 μM blocked actions mediated via P_{2X}- and P_{2Y}-purinoceptors in the guinea-pig urinary bladder and taenia coli respectively. Potentiation of purinoceptor-mediated activity was seen only at a low concentration of suramin (1 μM) and only in the urinary bladder (P_{2X}-purinoceptor). For its antagonistic activity suramin did not discriminate between P_{2X}- and P_{2Y}-purinoceptors, but it was selective for P_2-purinoceptor-mediated activity rather than that mediated via cholinoceptors, adrenoceptors or histamine receptors.

Introduction

The compound sym-bis (*m*-aminobenzoyl-*m*-amino-*p*-methyl benzoyl-1-naphthylamino-4,6,8-trisulphonate) carbamide, commonly called suramin, has been used extensively as a therapeutic agent for trypanosomiasis and onchocerciasis (see Bowman & Rand, 1980). Suramin inhibits various types of ATPases (Fortes *et al.*, 1973; Calcaterra *et al.*, 1988; Moriyama & Nelson, 1988; Hourani & Chown, 1989), GTPase (Butler *et al.*, 1988), DNA and RNA polymerases (Ono *et al.*, 1988; Offensperger *et al.*, 1988), reverse transcriptase (Mitsuya *et al.*, 1984; Broder *et al.*, 1985; Faber *et al.*, 1987; Offensperger *et al.*, 1988) and can also inhibit the binding of platelet-derived growth factor and fibroblast growth factor to their receptors (Williams *et al.*, 1984; Neufeld & Gospodarowicz, 1988; Sturani *et al.*, 1988).

It has also recently been reported that suramin can antagonize responses mediated via the P_2-purinoceptor in the mouse vas deferens (Dunn & Blakeley, 1988). With this in mind, the current study was undertaken in order to evaluate the antagonistic properties of suramin in tissues with P_{2X}- and P_{2Y}-purinoceptors (guinea-pig urinary bladder and taenia coli, respectively) as defined by Burnstock & Kennedy (1985). In the bladder there is a purinergic excitatory nerve supply (Kasakov & Burnstock, 1983; Moss & Burnstock, 1985; Katsuragi *et al.*, 1986; Fujii, 1988); while in the taenia coli it has been claimed that there is a purinergic inhibitory nerve supply (see review by Hoyle & Burnstock, 1989). Suramin was examined for interactions with exogenous purinoceptor agonists and for effects on non-adrenergic, non-cholinergic (purinergic) neuromuscular transmission in these two preparations.

Methods

Male guinea-pigs, 380-680 g were stunned and bled. The bladder was removed and mucosa-free strips of muscle approximately 2 by 15 mm were dissected from the corpus. The strips were mounted in 5 ml organ baths for isometric recording of mechanical activity; an initial load of 1 g was applied. Electrical field stimulation was applied via two platinum-wire rings 2.5 mm in diameter, 5 mm apart, through which the preparation was threaded. The Krebs solution for these experiments had the following composition (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.4, $MgSO_4$ 0.6, NaH_2PO_4 1.4, $CaCl_2$ 2.5 and glucose 7.7, and was gassed with 95% O_2/5% CO_2 (pH 7.3–7.4) and was maintained at 36 $\pm$ 0.5°C. The non-specific peptidase inhibitor bacitracin was routinely present in the Krebs solution (2000 u l^{-1}): this is because suramin contains several peptide linkages and inclusion of bacitracin would prevent putative degradation. In some experiments atropine (1 μM) was also included in the Krebs solution. Frequency-response relationships were examined in preparations before and after incubation with suramin (1 μM–1 mM, minimum 20 min) and in either the absence or presence of atropine. Electrical field stimulation was applied at a given frequency (0.5–32 Hz) with a pulse width of 0.3 ms, until a maximum contraction had been reached and the tone had declined by about a third (i.e. for approximately 5 s). Carbachol (10 μM) and histamine (10 μM) were used as control

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contractile agents in the absence and presence of atropine respectively. Intervals of at least 30 min were allowed between consecutive applications of α,β-methylene ATP in order to avoid tachyphylaxis, otherwise at least 10 min was allowed for washout of drugs.

Strips of longitudinal muscle (taenia coli) were removed from the caecum, and segments approximately 15 mm long were mounted in the organ baths as for the bladder; an initial load of 1 g was applied. A standard tone was induced by addition of carbachol (100 nM). When the contraction evoked by carbachol reached a plateau, electrical field stimulation was applied (one pulse, 0.3 ms, 60 V and at 8 Hz for 10 s), followed by ATP or noradrenaline. After a maximum response had been observed, the preparation was washed repeatedly and an interval of 10 min was allowed before the next applications of carbachol, electrical field stimulation and ATP or noradrenaline. Suramin was added to the organ bath (1, 10, 100 or 1000 μM) and a minimum initial equilibration period of 20 min was allowed.

It is not feasible to construct concentration-response curves from cumulative application of α,β-methylene ATP in the bladder preparations, or of ATP and noradrenaline in the taenia coli: therefore, because only a limited amount of suramin was available, full concentration-response curves were constructed for α,β-methylene ATP in the presence of suramin at 1, 10 and 100 μM, and only three concentrations of α,β-methylene ATP were tested in the presence of suramin at 1 mM. In the taenia coli, suramin was tested in the range 1–1000 μM against a concentration of ATP or noradrenaline that caused an approximately 50% relaxation of the carbachol-induced contraction.

Drugs used

Atropine sulphate was obtained from Antigen. Adenosine 5'-triphosphate sodium salt (ATP), α,β-methylene ATP lithium salt, carbamyl-β-methyl choline chloride (carbachol) and histamine dihydrochloride were obtained from Sigma. Suramin was obtained as a generous gift from ICI Pharmaceuticals, courtesy of Dr M.G. Collis.

Analysis of results

Means were compared by Student's *t* test (paired or unpaired, as stated). The 5% fiducial was used to establish significance. Data are presented as mean ± standard error (*n*), unless stated otherwise. Sigmoidal concentration-response curves were fitted to the data following probit transformation and horizontal averaging (Bliss, 1935; Finney, 1971; Hoyle & Greenberg, 1988).

Results

Guinea-pig urinary bladder

In isolated strips of detrusor muscle, α,β-methylene ATP (0.1–100 μM) caused concentration-dependent contractions (Figure 1). In many preparations the relationship was not monotonic, with submaximal contractions being evoked by higher concentrations (generally above 30 μM). The pD_2 value (i.e. negative log EC_{50}) for the contractile responses was 5.7 ± 0.25 (13). In the presence of suramin at 1 μM, responses to α,β-methylene ATP were enhanced (Figures 1, 2a), the maximal response was increased by 65 ± 19.7% (7), which was a significant effect ($P < 0.02$, paired *t* test), and the equivalent value, in the presence of suramin (1 μM), was 6.2 ± 0.18 (7) and this represents a significant increase ($P < 0.02$, paired *t* test). At 10 μM, suramin had no significant effect but at 100 μM there was a significant antagonism of responses to α,β-methylene ATP (Figure 1), with a decrease in the maximum response of 32 ± 8.0% (7) ($P < 0.01$, paired *t* test) and the negative log

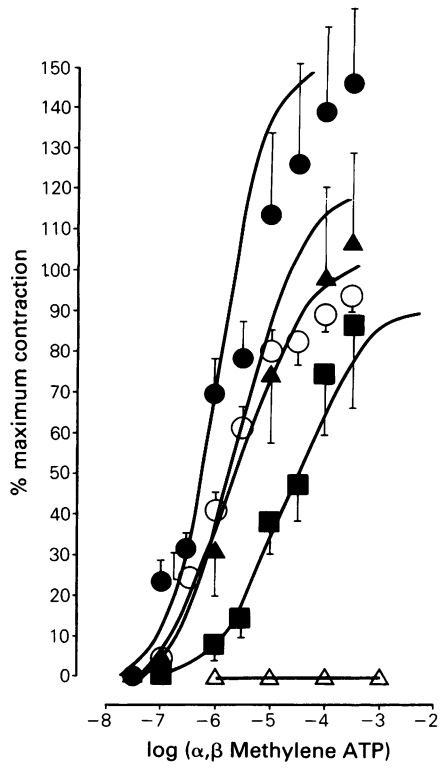

Figure 1 Guinea-pig urinary bladder, isolated detrusor muscle: responses to α,β-methylene ATP in the presence and absence of suramin. Responses (ordinate scale) are expressed as a percentage relative to the maximum contraction obtained in the absence of suramin (○, $n = 23$), in the presence of suramin (1 μM, ●, $n = 9$), (10 μM, ▲, $n = 7$), (100 μM, ■, $n = 9$), (1 mM, △, $n = 8$). Points show mean with s.e. indicated by vertical bars; solid lines show fitted curves.

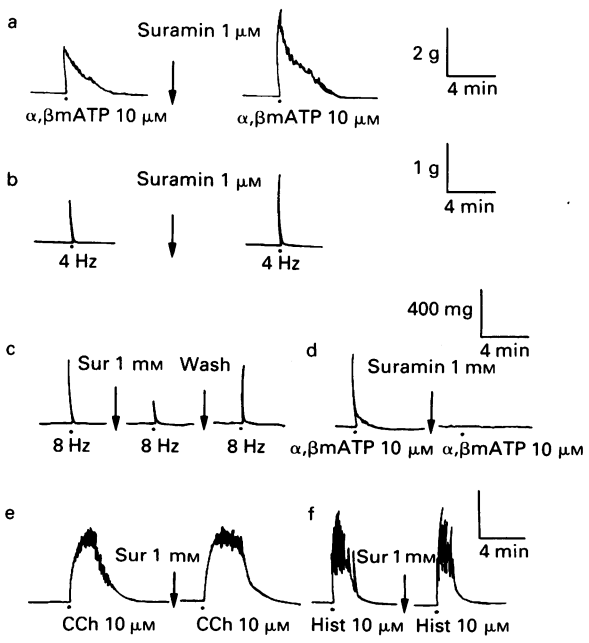

Figure 2 Guinea-pig urinary bladder, isolated detrusor muscle. (a) Response to α,β-methylene ATP (α,β mATP, 10 μM) was potentiated in the presence of suramin (1 μM). (b) Similarly the response to electrical field stimulation (4 Hz, 0.3 ms, supramaximal V) was potentiated. (c) The response to electrical field stimulation (8 Hz, 0.3 ms, supramaximal V) was inhibited by suramin (Sur, 1 mM) but recovered on washout, and (d) the response to α,β-methylene ATP (10 μM) was abolished at this concentration of suramin (1 mM). (Calibration bars also apply to c.) (e) Responses to carbachol (CCh, 10 μM) and (f) histamine (Hist, 10 μM) were unaffected by suramin (1 mM). Calibration bar represents 1 g in (e) and 400 mg in (f).

[α,β-methylene ATP], producing a response in the presence of suramin at 100 μM equivalent to that produced by the control pD_2 value, was 4.5 ± 0.16, and this was significantly lower ($P < 0.01$, paired t test). Suramin (1 mM) abolished the contractile responses to α,β-methylene ATP at 1 and 10 μM (Figure 2d), which were 0.34 ± 0.110 g (8) and 0.76 ± 0.210 g (8) respectively. Even at 100 μM, α,β-methylene ATP was devoid of apparent action when suramin was present at this concentration (Figure 1).

In the presence of atropine (1 μM), the frequency-response relationship was altered by suramin in a manner similar to its effects on responses to α,β-methylene ATP. At a concentration of 1 μM suramin potentiated responses evoked at frequencies from 0.5 to 4 Hz (Figures 2b, 3); the potentiation was greatest at 2 Hz where suramin caused an increase in amplitude of contraction of 85 ± 30.9% (9) ($P < 0.02$, paired t test). At a concentration of 10 μM suramin had no significant actions but at 100 μM it caused an inhibition of contractile responses evoked at frequencies from 4 to 32 Hz (Figures 2c, 3). At 32 Hz the reduction in contraction due to suramin (100 μM) was 48 ± 7.0% (9) ($P < 0.001$, paired t test). At a concentration of 1 mM, suramin inhibited the neurally evoked purinergic contractile responses at all frequencies from 0.5 to 32 Hz (Figure 4). In the absence of atropine, suramin at 1 mM caused inhibition of neurally evoked contractions but to a lesser extent than in the presence of atropine, at all frequencies tested (Figure 4). Because of the nature of the interaction of suramin with α,β-methylene ATP, it was not possible to calculate a pA_2 value. However, from an interpolation of the data shown in Figures 1 and 2, the −log [suramin] which produces a rightward shift of the 50% point of the control curve for α,β-methylene ATP of 0.3 log units, would be approximately 4.7. Likewise, interpolation of Figure 3 yields a similar 'pA_2' for antagonism of purinergically mediated contractions.

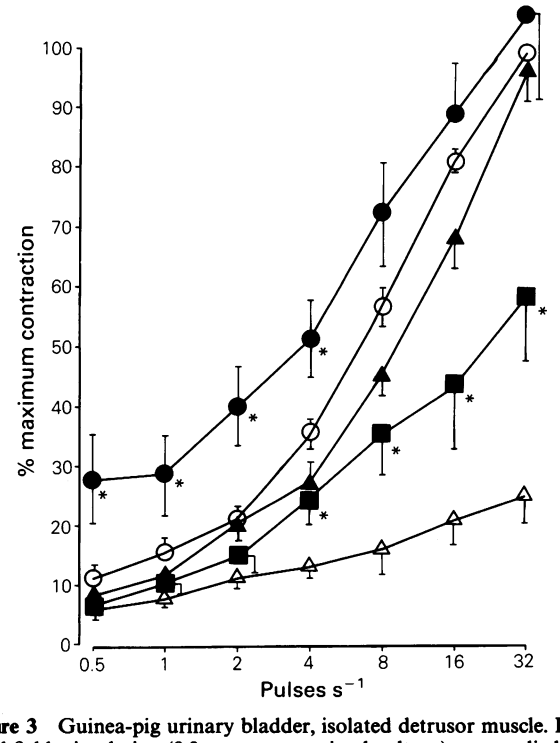

Figure 3 Guinea-pig urinary bladder, isolated detrusor muscle. Electrical field stimulation (0.3 ms, supramaximal voltage) was applied at a given frequency until a maximum response had been observed. Frequency-response relationship in the absence of suramin (○). At 1 μM (●), suramin significantly enhanced this response at frequencies from 0.5 to 4 Hz (*$P < 0.05$). In the presence of suramin (10 μM, ▲) there was no significant difference, at 100 μM (■) suramin significantly inhibited responses from 4 to 32 Hz (*$P < 0.05$) and at 1 mM (△) at all frequencies the responses were significantly reduced ($P < 0.05$). All frequency-response relationships were determined in the presence of atropine (1 μM). Responses were related to the maximum contraction obtained at 32 Hz, in the absence of suramin. Points show mean with s.e. indicated by vertical bars. The abscissae are on a logarithmic scale.

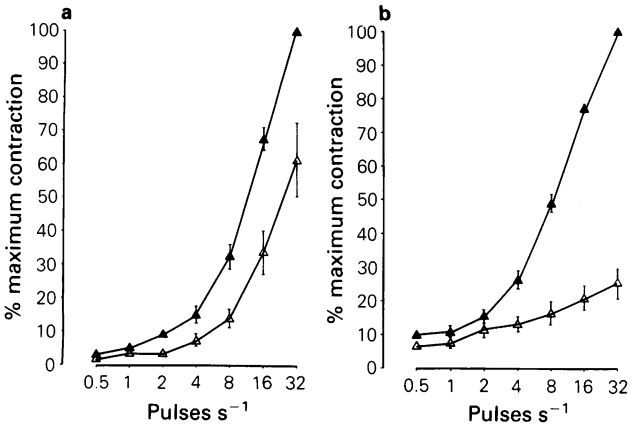

Figure 4 Actions of suramin in isolated strips of guinea-pig urinary bladder. (a) Frequency-response relationships established in the absence of atropine before (▲) and after (△) incubation with suramin (1 mM). Electrical field stimulation was applied until a maximum contraction had occurred and tone had declined by about 33%. Responses were related to contractions evoked at 32 pulses per second (0.3 ms, supramaximal V). An ordinate scale value of 100% was equivalent to 3.7 ± 0.56 g ($n = 6$). (b) Frequency-response relationships established in the presence of atropine (1 μM) before (▲) and after (△) incubation with suramin (1 mM). On the ordinate axis, 100% was equivalent to 2.1 ± 0.31 g ($n = 8$). For (a) and (b), points show mean with s.e. indicated by vertical bars, unless occluded by the symbol. Stimulation frequency is expressed on a log scale.

Guinea-pig taenia coli

In the carbachol-contracted taenia coli, ATP in the range of 0.1–10 μM (mean 2.8 μM) caused a relaxation of 53 ± 7.1% (10). Suramin at concentrations of 1 and 10 μM had no significant effect, but at 100 μM and 1 mM there was an antagonism of responses to ATP (Table 1, Figure 5). The antagonism at 1 mM was greater than that at 100 μM.

Single pulses of electrical field stimulation caused a transient relaxation of the carbachol-induced tone with a mean amplitude of 29 ± 4.6% (17), while trains of 8 Hz caused relaxations with a mean amplitude of 88 ± 4.2% (17). Responses to electrical field stimulation were affected in parallel with those to ATP. At 1 and 10 μM suramin had no significant action, but at 100 μM and 1 mM it caused a concentration-dependent inhibition (Table 1, Figure 5).

The pA_2 for suramin against the action of ATP and electrical field stimulation was estimated from an extrapolation of a plot of % inhibition versus log [suramin] (at 100 μM and 1 mM) to determine the log [suramin] which would shift the concentration-response relationship for ATP by 0.3 log units. For the action of suramin against ATP the estimated pA_2 value was 5.0 ± 0.82 (10), and against responses to single pulses and trains of 8 Hz of electrical field stimulation the values were 4.9 ± 0.93 (17) and 4.6 ± 1.01 (17) respectively. Amongst these values there were no significant differences.

Specificity of suramin

In the preparations from the urinary bladder, responses to neither carbachol (10 μM) nor histamine (10 μM) were significantly affected by any concentration of suramin (1 μM–1 mM) (Figures 2e, f). The contractions evoked by histamine were 0.75 ± 0.09 g before and 0.70 ± 0.14 g ($n = 8$) after incubation with suramin (1 mM). These responses were matched by α,β-methylene ATP at 10 μM and by the response to field stimulation of purinergic nerves at 8 Hz. The responses to car-

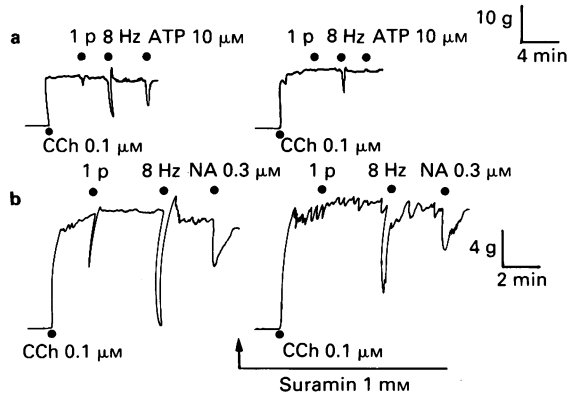

Figure 5 Guinea-pig isolated taenia coli. Standard tone was induced by application of carbachol (CCh, 0.1 μM). (a) Responses to electrical field stimulation (single pulse, 1p), 8 Hz for 10 s, and ATP (EC_{50} concentration) in the absence (left-hand panel) and presence of suramin (1 mM) (right-hand panel). (b) Responses to electrical field stimulation (as in a) and noradrenaline (EC_{50} concentration) in the absence (left-hand panel) and presence of suramin (1 mM) (right-hand panel).

bachol (10 μM) were 1.7 ± 0.41 g and 1.9 ± 0.5 g in the absence and presence of suramin (1 mM) respectively ($n = 6$). These responses were matched by contractions evoked by electrical field stimulation of 16 Hz. Suramin *per se* had no obvious effect on the bladder preparations at any concentration, causing no change in tone and no change in spontaneous activity.

In the preparations of the taenia coli, suramin had no significant effect on the relaxant responses to noradrenaline (Figure 5), which caused a 50 ± 8.7% (8) relaxation of control preparations at a mean concentration of 11.7 μM (range 3–100 μM) (Table 1). Also, the carbachol-induced contractures were unaffected by suramin at any concentration.

Discussion

The results show that suramin can block responses mediated via P_{2X}-purinoceptors in the guinea-pig urinary bladder and via P_{2Y}-purinoceptors in the guinea-pig taenia coli. The antagonism in the urinary bladder was not competitive, since there was a depression of the maximal response as well as a rightward shift of the concentration-response relationship for α,β-methylene ATP. The interaction of suramin with the P_{2X}-receptor was further complicated by the potentiation of P_{2X}-receptor-mediated activity seen at the lowest concentration tested. In the bladder the action of suramin seemed to be specific for the P_2-purinoceptor, since there were no changes in responses to histamine or carbachol, with neither a potentiation nor inhibition of their responses at any concentration. Suramin affected responses to purinergic nerve stimulation in parallel with responses to α,β-methylene ATP, which would be expected since the purinergic transmitter acts on the same receptor as α,β-methylene ATP (Kasakov & Burnstock, 1983). It therefore appears that the effects of suramin on responses to electrical field stimulation are mediated purely at postjunctional sites.

Suramin also antagonized responses mediated via the P_{2Y}-purinoceptor in the guinea-pig taenia coli. The nature of the antagonism was not evaluated, but suramin was selective for P_2-purinoceptors as it did not significantly alter the responses to noradrenaline. This implies that suramin is not acting as a blocker of the potassium ion channels which are operated by α-adrenoceptors and P_2-purinoceptors and which can be blocked by apamin (Vladimirova & Shuba, 1978; 1980; Banks *et al.*, 1979; Shuba & Vladimirova, 1980; Maas *et al.*, 1980).

All the estimated 'pA_2' values in both the bladder and the taenia coli were similar, which indicated that suramin was not discriminating between P_{2X}- and P_{2Y}-purinoceptors, whether activated by exogenous or endogenous agents. However, potentiation of responses by suramin at the lowest concentration (1 μM) was peculiar to the P_{2X}-purinoceptor system.

Suramin inhibits the binding of some growth factors to their receptors (Williams *et al.*, 1984; Hosang, 1985; Itoh *et al.*, 1988; Neufeld & Gospodarowicz, 1988) with IC_{50} values in the range of 10–100 μM but it is not clear whether this is because suramin binds to the same sites as the growth factor or is due to an allosteric effect. However, suramin binds to the catalytic unit of H^+-ATPase in chromaffin granules (Moriyama & Nelson, 1988) and with a pD_2 value of approximately 4.7. As an inhibitor of DNA and RNA polymerases, suramin is more potent with K_i values of 0.2–2.6 μM (pK_i range 7.7–5.6), but acts competitively or non-competitively according to the functional state of the enzyme and the substrate (Ono *et al.*, 1988). In inhibiting GTPase activity of certain G-proteins, suramin has an IC_{50} of 21 μM (pIC_{50} 4.7) (Butler *et al.*, 1988), substantially a less potent action than the inhibition of GTP-binding to DNA-primase (Ono *et al.*, 1988). However, under some conditions suramin increases the maximum velocity of GTPase activity (Butler *et al.*, 1988), although at the concentration tested there is no leftward shift in the [agonist]-response profile.

From the data quoted above it seems that suramin has an equal affinity for recognition sites on these ATPases and GTPase as it does for P_{2X}- and P_{2Y}-purinoceptors. This may reflect similar structural domains (presumably basic groups lining a pocket) within these disparate groups of enzymes and receptors. However, as an inhibitor of ecto ATPase in guinea-pig urinary bladder, suramin does not have significant activity until 10 mM (Hourani & Chown, 1989).

In conclusion, suramin at concentrations above 10 μM can act as an antagonist of P_{2X}- and P_{2Y}-purinoceptors in the guinea-pig urinary bladder and taenia coli respectively; at a

Table 1 Effect of suramin on relaxant responses to ATP, noradrenaline (NA) and electrical field stimulation (EFS) in the carbachol-contracted guinea-pig taenia coli

	Change in amplitude of response (%)			
[*Suramin*] (μM)	*ATP* ($n = 10$)	*NA* ($n = 8$)	*EFS single pulse* ($n = 17$)	*EFS 8 Hz* ($n = 17$)
1	+21 ± 26.2	−3 ± 19.0	+14 ± 10.0	+2 ± 2.8
10	+6 ± 26.4	−6 ± 24.6	+2 ± 13.0	+2 ± 3.9
100	−40 ± 11.6*	−26.7 ± 13.2	−42 ± 10.0**	−12 ± 4.6*
1000	−80 ± 5.5***	−15.4 ± 31.8	−92 ± 1.5***	−26 ± 9.0**

*$P < 0.02$; **$P < 0.01$; ***$P < 0.001$ from paired Student's t tests, relative to control responses in the absence of suramin. + Denotes an increase in response, − denotes a decrease. Concentrations of ATP and NA were adjusted for each preparation such that a 50% relaxation was obtained. EFS was applied as a single pulse (0.3 ms, 60 V) or as a train at 8 Hz for 10 s (0.3 ms, 60 V). The mean maximum response to a single pulse of EFS was a relaxation of 32 ± 4.5% and that of EFS at 8 Hz was 88 ± 4.2%, in the absence of suramin.

micromolar concentration it potentiated activity mediated via P_{2X}- but not P_{2Y}-purinoceptors. In these preparations suramin appeared to be specific for P_2-purinoceptors rather than cholinoceptors, adrenoceptors or histamine receptors.

Further, the selective blockade of non-adrenergic, non-cholinergic neurogenic responses and P_2-purinoceptors in the bladder and taenia coli lends support to the hypothesis that both these organs are innervated by purinergic nerves. However, in view of its divers actions amongst other systems, suramin may have limited use as a pharmacological tool for investigating purinoceptor-mediated activity. Nevertheless, suramin may prove to be a parent of a useful family of compounds with greater selectivity.

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Effects of chain-length and unsaturation on affinity and selectivity at muscarinic receptors

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1 Lengthening the chain in diphenylacetylcholine decreases affinity for muscarinic cholinoceptors in guinea-pig ileum. Diphenylacetoxypropyldimethylamine and its quaternary trimethylammonium salt are roughly equiactive: the dimethylamine and the piperidine have some selectivity for ileum compared with atria, but are not as active nor as selective as 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) methobromide (MeBr). With the weaker diphenylacetoxybutyl compounds the base is more active than the quaternary salt.

2 The diphenylacetoxybutyl-, *cis*-butenyl and *trans*-butenyl compounds have similar affinities. The quaternary salts are less active than the tertiary bases, but they are less selective than the butynyl analogues studied in earlier work.

3 1,1-Diphenyl-1-hydroxy-2,4-hexadiynyl dimethylamine and its trimethylammonium salt are inactive in concentrations below 100 μM, as are the (+)-camphor-sulphonyl ester of 4-hydroxy-*N*-methyl piperidine and its methiodide. The (±)-phenyl*cyclo*pentylacetyl ester of 4-hydroxy-*N*-methylpiperidine methobromide is more active than its *cyclo*hexyl analogue and than 4-DAMP MeBr but it is less selective than 4-DAMP MeBr.

4 The high selectivity of *p*-fluoro-hexahydrosila-diphenidol is confirmed but this compound has relatively low affinity (for ileum log $K = 7.8$).

5 The results indicate steric constraints to binding at muscarinic receptors which could be used to check molecular modelling of the receptor based on its known amino acid sequence. The group binding the charged nitrogen is probably at the mouth of a cavity which can accommodate two large rings (as in 4-DAMP MeBr) but with a depth less than about 7 Å so that the rod-like hexadiynes cannot fit. Differences between types of receptor may only involve small changes in geometry secondary to differences in amino acids not directly involved in binding and the production of selectivity depends upon finding substituents which interfere with binding more at one type of receptor than at another.

Introduction

The binding of 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) methobromide (MeBr) and of other antagonists at muscarinic receptors appears to involve contributions from at least three parts of the molecule, the charged (onium) nitrogen atom and the two aromatic rings. The part of the molecule linking these groups may also contribute to binding but it may be more important in determining that the rings and the onium nitrogen are held at suitable distances and suitably arranged in space. The distances will depend on the number of atoms present and the spatial arrangement will depend on whether they form part of a ring and whether the bonds are saturated or unsaturated. Results obtained with some acetylenic analogues of 4-DAMP MeBr (Barlow *et al.*, 1988) suggested that whereas 4-DAMP MeBr is more active than 4-DAMP base, the tertiary base was more active than the quaternary salt with longer compounds. Diphenyl-acetoxybutynyl dimethylamine was more active than its trimethylammonium analogue and although it was very much less active than 4-DAMP MeBr it had similar selectivity, being about 10 times as active on ileum as on atria. In these but-2-ynes the four carbon atoms lie in a straight line and the structure is rod-like. It is possible that this makes the compound less well able to fit the receptor and that this unfavourable effect is greater for receptors in atria than for those in ileum.

This paper describes results obtained with (i) saturated compounds with different chain length, diphenylacetoxypropyl and butyl compounds (results for diphenylacetyl choline are already published), (ii) the *cis* and *trans* butenyl compounds and (iii) the diacetylenes, 1,1-diphenyl-1-hydroxy-2,4-hexadiynyl-dimethylamine and its trimethylammonium salt.

Results were also obtained with the phenyl*cyclo*pentyl acetoxy and (+)-camphorsulphonyl esters of 4-hydroxy-*N*-methyl piperidine and with (±)-*p*-fluoro hexahydro-sila-diphenidol, which is known to be much more active at muscarinic receptors in ileum than at those in atria (Eglen *et al.*, 1989).

Methods

Guinea-pig isolated ileum

The guinea-pig ileum was dissected out and set up as described by Edinburgh Staff (1974). The responses were recorded isotonically with a load of about 0.5 g. The tissue was suspended in Krebs solution (Edinburgh Staff, 1974) aerated with a mixture of 95% O_2 and 5% CO_2 containing *nor*phenylephrine, 5 μM, and experiments were done at 29.8 ± 0.3°C and 37.0 ± 0.1°C.

The agonist, carbachol, was allowed to act for 30 s and added once every 90 s by relays controlled from a PET microcomputer. Alternate small and large control responses were obtained, usually to 0.1 and 0.2 μM carbachol. When these were regular the tissue was exposed to a solution of the antagonist and the concentration of the agonist was increased to try to obtain responses which roughly matched the controls. When these were regular the approximate dose-ratio was given by the ratio of the concentrations of the agonist used in the presence and in the absence of the antagonist and an exact dose-ratio was calculated from the size of the responses by a calculation similar to a 4-point assay (Edinburgh Staff, 1974; Barlow, 1983). Usually a second concentration of antagonist was tested on each preparation and where possible a fresh set of control responses was obtained.

Guinea-pig isolated atria

The atria were dissected out as described by Edinburgh Staff (1974) and set up in Krebs solution, aerated with a mixture of

Table 1 Log affinity constant (mean ± s.e.mean and number of estimates) for the compounds and the concentrations tested

	Atria		*Ileum*	
Compound	*Rate*	*Force*	30°	37°
(1) Diphenylacetoxypropyl dimethylamine HBr				
1 μM	6.79 ± 0.01 (4)	6.67 ± 0.07 (4)	7.53 ± 0.05 (4)	7.46 ± 0.02 (4)
5 μM	6.81 ± 0.03 (4)	6.76 ± 0.08 (4)	7.52 ± 0.05 (4)	7.44 ± 0.05 (4)
(2) Diphenylacetoxypropyl trimethylammonium bromide				
1 μM	7.03 ± 0.04 (5)	6.97 ± 0.03 (5)	7.40 ± 0.01 (4)	7.31 ± 0.02 (4)
5 μM	7.04 ± 0.06 (4)	6.99 ± 0.06 (4)	7.39 ± 0.02 (4)	7.29 ± 0.02 (4)
20 μM	7.06 ± 0.06 (2)	7.05 ± 0.04 (2)	7.35 ± 0.01 (2)	7.24 ± 0.02 (2)
(3) Diphenylacetoxypropyl pyrrolidine HBr				
1 μM	6.56 ± 0.08 (3)	6.42 ± 0.07 (5)	7.21 ± 0.03 (7)	7.22 ± 0.04 (6)
5 μM	6.71 ± 0.07 (4)	6.34 ± 0.07 (5)	7.03 ± 0.05 (6)	7.09 ± 0.06 (5)
(4) Diphenylacetoxypropyl *N*-methylpyrrolidinium bromide				
1 μM	6.72 ± 0.09 (5)	6.57 ± 0.07 (5)	7.16 ± 0.09 (6)	7.14 ± 0.06 (6)
5 μM	6.79 ± 0.11 (4)	6.54 ± 0.07 (5)	7.04 ± 0.11 (6)	7.04 ± 0.06 (6)
(5) Diphenylacetoxypropyl piperidine HBr				
0.5 μM			6.74 ± 0.17 (4)	6.79 ± 0.22 (3)
2.5 μM	6.00 ± 0.07 (4)	5.61 ± 0.07 (5)	6.66 ± 0.14 (3)	6.64 ± 0.15 (4)
(6) Diphenylacetoxypropyl N-methylpiperidinium bromide				
0.5 μM	6.31 ± 0.16 (4)	6.43 ± 0.09 (5)	7.03 ± 0.13 (5)	7.21 ± 0.14 (6)
2.5 μM	6.52 ± 0.14 (3)	6.42 ± 0.11 (4)	6.85 ± 0.06 (4)	6.90 ± 0.04 (6)
(7) Diphenylacetoxybutyl dimethylamine HBr				
1 μM	6.48 ± 0.10 (4)	6.47 ± 0.05 (4)	6.92 ± 0.06 (6)	6.75 ± 0.04 (5)
5 μM	6.44 ± 0.07 (5)	6.43 ± 0.07 (5)	7.01 ± 0.05 (6)	6.77 ± 0.08 (5)
(8) Diphenylacetoxybutyl trimethylammonium bromide				
10 μM	6.08 ± 0.03 (3)	6.03 ± 0.07 (3)	6.44 ± 0.04 (3)	6.44 ± 0.02 (3)
30 μM	6.09 ± 0.12 (3)	6.07 ± 0.09 (3)	6.55 ± 0.09 (3)	6.49 ± 0.02 (3)
(9) *cis* Diphenylacetoxy-but-2-enyl dimethylamine HBr				
1 μM	6.78 ± 0.03 (3)	6.70 ± 0.05 (7)	7.37 ± 0.05 (7)	7.32 ± 0.06 (7)
5 μM	6.72 ± 0.15 (2)	6.75 ± 0.05 (5)	7.34 ± 0.08 (4)	7.30 ± 0.05 (6)
(10) *cis* Diphenylacetoxy-but-2-enyl trimethylammonium bromide				
5 μM		5.79 ± 0.08 (5)	6.26 ± 0.02 (7)	6.15 ± 0.04 (7)
25 μM		5.72 ± 0.05 (4)	6.27 ± 0.05 (6)	6.20 ± 0.03 (6)
(11) *trans* Diphenylacetoxy-but-2-enyl dimethylamine HBr				
1 μM	6.61 ± 0.05 (4)	6.59 ± 0.05 (5)	7.31 ± 0.02 (4)	7.33 ± 0.02 (5)
5 μM	6.64 ± 0.08 (4)	6.64 ± 0.05 (5)	7.39 ± 0.04 (4)	7.41 ± 0.01 (5)
(12) *trans* Diphenylacetoxy-but-2-enyl trimethylammonium bromide				
5 μM	5.94 ± 0.04 (5)	5.86 ± 0.08 (6)	6.07 ± 0.07 (9)	6.04 ± 0.07 (9)
25 μM	5.78 ± 0.05 (5)	5.70 ± 0.04 (5)	6.20 ± 0.08 (3)	6.21 ± 0.02 (5)
(13) 4-Phenyl-4-*cyclo*pentylacetoxy-*N*-methyl-piperidine MeBr				
0.1 μM	9.06 ± 0.07 (5)	8.94 ± 0.07 (5)	9.69 ± 0.10 (5)	9.56 ± 0.08 (5)
(14) 4-(+)-Camphorsulphonyl-*N*-methylpiperidine HCl inactive below 100 μM				
(15) 4-(+)-Camphorsulphonyl-*N*-methylpiperidine MeBr inactive below 100 μM				
(16) 1,1-Diphenyl-1-hydroxy-2,4-hexadiynyl-dimethylamine HBr inactive below 50 μM				
(17) 1,1-Diphenyl-1-hydroxy-2,4-hexadiynyl-trimethylammonium bromide inactive below 50 μM				
(18) (±)-*p*-Fluoro-hexahydro-sila-diphenidol				
0.1 μM			7.77 ± 0.05 (5)	7.77 ± 0.06 (5)
1.0 μM	5.91 ± 0.10 (6)	5.56 ± 0.19 (8)	7.53 ± 0.05 (8)	7.53 ± 0.05 (8)

95% O_2 and 5% CO_2 containing *nor*phenylephrine, 5 μM (the same solution as was used for the ileum). The temperature was 29.8 ± 0.3°C and the spontaneous contractions were recorded isometrically with a load of about 0.2 g: action potentials were also recorded and the time required for 50 beats was continuously printed out (Barlow & Shepherd, 1986).

The agonist, carbachol, was added by relays operated from a Commodore 128 microcomputer and allowed to act for 5 min: doses were given once every 15 min, with a second wash 10 min from the start of the cycle. The effects of the agonist were expressed as the percentage inhibition of the force of contraction and the percentage increase in the time for 50 beats. As with the ileum the control responses were usually obtained with 0.1 and 0.2 μM carbachol. The preparation was then exposed to the antagonist, and the experiment continued as with the ileum.

These methods are the same as those used by Barlow & Shepherd (1986) and Barlow *et al.* (1988).

Compounds

The synthesis of the diphenylacetoxy propyl compounds (1, 2, 3, 4, 5 and 6) started from diphenylacetyl chloride and 3-chloro-propan-1-ol. The diphenyl-acetoxypropyl chloride formed was converted to the iodide which was treated with dimethylamine, pyrrolidine or piperidine.

The butyl compounds (7 and 8) were prepared similarly, starting from diphenylacetyl chloride and tetrahydrofuran, which gave diphenylacetoxy butyl chloride.

The *cis* butenyl compounds (9 and 10) were prepared from *cis* but2-ene-1,4-diol (Aldrich). This was converted into the mono-chloro compound and esterified with diphenylacetylchloride. The 4-diphenylacetoxy-*cis*-but-2-enyl chloride formed was treated directly with the amine (without first converting it to the iodide).

The *trans* butenyl compounds (11 and 12) were prepared from fumaric acid which was reduced to give *trans* but-2-ene-1,4-diol and the synthesis was then similar to that for the *cis* compounds.

The phenyl*cyclo*pentyl compound (13) was obtained from phenyl*cyclo*pentylacetic acid, which was converted into the acid chloride and esterified with 4-hydroxy-*N*-methylpiperidine. The (+)-camphor-sulphonyl esters (14 and 15) were also obtained from the acid chloride (Aldrich) and 4-hydroxy-*N*-methylpiperidine.

The hexadiynes (16 and 17) were obtained starting from but-2-yne-1,4-diol. This was converted into the 1,4-dichloro-compound and when treated with sodamide in liquid ammonia this formed the sodio derivative of buta-1,3-diyne which was reacted with ethanethiol, forming 1-thioethyl-but-1-ene-3-yne. This was treated with dimethylaminomethanol in the presence of cupric acetate to form the 3-dimethylamino-methyl compound, which was converted back to a diacetylene by removal of the thioethyl group with sodamide in liquid ammonia. The 4-dimethylaminomethyl-but-1,3-diyne was isolated and treated with butyl-lithium in ether and the lithio-derivative was reacted with benzophenone, as in previous work (Barlow *et al.*, 1988) to form 1,1-diphenyl-1-hydroxy-2,4-hexadiynyl dimethylamine.

All the final products were recrystallized from combinations of ethanol, butanone and ether. Their structures were checked by nuclear magnetic resonance (n.m.r.) and they gave satisfactory analyses. A table of melting-points and analyses is available from the authors.

(±)*p*-Fluoro hexahydro-sila-diphenidol was a gift from Dr R.M. Eglen. Carbachol chloride was bought from Sigma.

Results

The results are summarized in Table 1. The (+)-camphor sulphonic esters (14 and 15) were inactive in concentrations up to 100 μM so their log affinity constants must be less than 4 and the diacetylenes (16 and 17) must have log K less than 4.3. Most of the compounds were only tested at two concentrations but the mean estimates of log K are similar, which is consistent with the idea that the antagonism is competitive. The propyl compound (2) was tested at three concentrations and the slopes (±s.e.mean and number of degrees of freedom) of the Schild plots were 1.02 (±0.06; 8) for atrial rate, 1.06 (±0.05; 8) for atrial force, 0.97 (±0.02; 9) for ileum at 30°C and 0.95 (±0.02; 9) for ileum at 37°C.

The results with *p*-fluoro-hexahydro-sila-diphenidol (18) on ileum suggested that a check should be made that it acts competitively, as reported by Eglen *et al.* (1989). It was not possible to test a wide range of concentrations, so 1.0 μM solutions were tested together with 0.01 μM 4-DAMP methobromide. On ileum these concentrations separately produce dose-ratios of about 36 (DR_1) and 10 (DR_2); thus, the expected combined dose-ratio should be 45 ($DR_1 + DR_2 - 1$) if both were acting competitively at the same site (Paton & Rang, 1965; Abramson *et al.*, 1969). The results are shown in Table 2 and confirm that this is so.

The selectivity has been calculated from the difference between the log K for effects on ileum (the overall mean for all results at 30°C) and log K for effects on atria (the overall mean for all results, both effects on atrial rate and effects on atrial force). The effects of chain length are shown in Figure 1a in which the butyl compounds are compared with the corresponding propyl compounds and with diphenylacetylcholine (Barlow *et al.*, 1976). Lengthening the chain decreases the affinity but whereas the butyl base is stronger than its metho salt this is not true for the corresponding propyl compounds and the propyl base has some selectivity. Increasing the size of the onium group from dimethylamino to pyrrolidino, however, reduces affinity. This is similar to what is seen with the corresponding butynes (Barlow *et al.*, 1988) but unlike what is observed with choline derivatives (Abramson *et al.*, 1969). These compounds all have much less affinity than 4-DAMP methobromide. The dimethylamine had some selectivity but less than that of 4-DAMP methobromide. It is interesting that the piperidine also had some selectivity because this ring occurs in diphenidol and its derivatives.

The effects of saturation can be seen in Figure 1b, which compares the butyl, butenyl and butynyl compounds. With all these the base is consistently more potent than the quaternary salt but there is remarkably little difference between the compounds, except in selectivity. It is surprising that the *cis* and *trans* compounds are so similar but there is no reason to doubt their chemical identity. The *trans* compound was prepared from fumaric acid; the *cis* compound was obtained from the *cis* diol by methods which should not lead to isomerization and the base would not crystallize as the hydrobromide. The n.m.r. spectra are quite distinct.

The effects of replacing one phenyl ring in 4-DAMP methobromide by *cyclo*pentyl are shown in Figure 1c. There is an increase in affinity (more than obtained by replacing phenyl by *cyclo*hexyl; Barlow & Shepherd, 1986), but there is a loss of selectivity. The very high selectivity of *p*-fluoro-hexahydro-sila-diphenidol can also be seen but the compound has relatively low affinity, less than that of silabenzhexol or silaprocyclidine (Barlow & Shepherd, 1986). The value for receptors in ileum (log $K = 7.6$) is comparable with that of diphenylhydroxymethyl-prop-3-ynyl-trimethyl-ammonium (log $K = 7.3$; Barlow *et al.*, 1988), which has a C—C≡C—C chain instead of Si—C—C—C, but this compound has no selectivity (for atria log $K = 7.4$).

Discussion

The propyl base (1) can be regarded as 4-DAMP MeBr with an incomplete piperidine ring, which might explain why it is the most selective of the series ethyl, propyl, butyl (Figure 1a) but it is very much weaker. For ileum it has log K about 1.5 log units less than that of 4-DAMP MeBr, which indicates a difference of 2.1 kcal (8.7 kJ) mol^{-1} in the free energy of adsorption. This does not seem to be too big a contribution to be expected from two methylene groups (Abramson *et al.*, 1974) but it suggests that the introduction of these groups does not greatly disturb existing binding (Abramson *et al.*,

Table 2 Test for competition: dose-ratios (mean ± s.e.mean and number of estimates) for the solution indicated

Atria		*Ileum*	
Rate	*Force*	30°C	37°C
Dose-ratios with (±)-*p*-fluoro-hexahydrosiladiphenidol (1.0 μM)			
1.91 ± 0.16 (6)	1.51 ± 0.10 (8)	36.8 ± 4.11 (8)	36.0 ± 3.51 (8)
Dose-ratios with (±)-*p*-fluoro-hexahydrosiladiphenidol (1.0 μM) + 4-DAMP MeBr (0.01 μM)			
4.42 + 0.49 (3)	3.66 ± 0.24 (4)	48.2 ± 5.03 (5)	41.7 ± 3.42 (4)

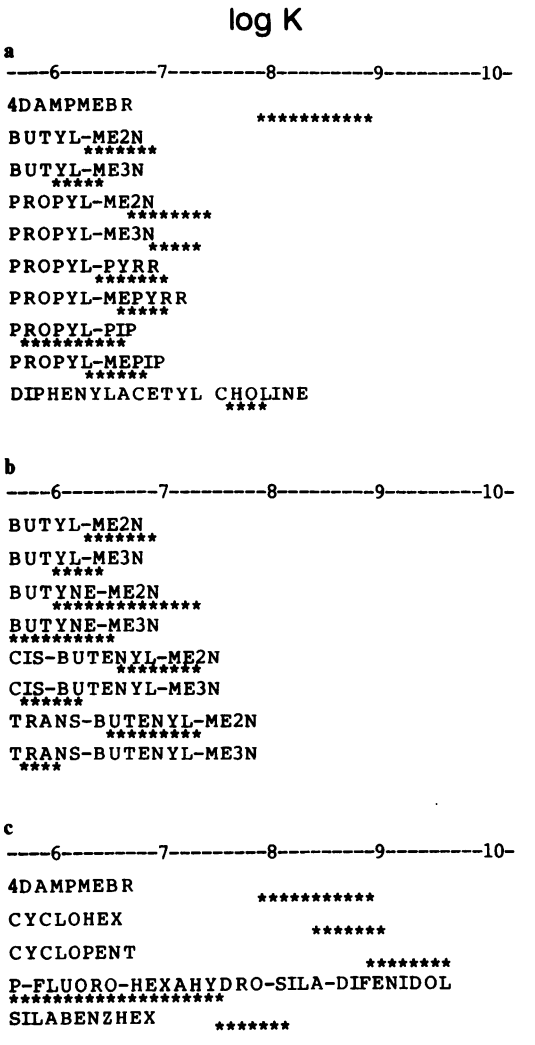

Figure 1 Selectivity, indicated by the difference between mean log affinity constant for atria (pooled results for effects on rate and effects on force at all concentrations) and mean log affinity constant for ileum (pooled results at all concentrations): temperature 30°C. With all compounds the affinity is lower for atria. The scale indicates log K and each asterisk (*) indicates a difference of 0.1 log unit.

(a) The effect of chain length. The esters of aminobutanol (BUTYL-Me_2N = compound 7, BUTYL-Me_3N = compound 8) have lower affinity than the esters of aminopropanol (1, 2, 3 and 4), which have lower affinity than diphenylacetylcholine (Barlow *et al.*, 1976), which is weaker than 4-DAMP methobromide (4DAMPMEBR). Increasing the size of the onium group in the propyl esters decreases activity (PROPYL-PYRR = compound 3, PROPYL-MEPYRR = compound 4) but the piperidine appears to have some selectivity (PROPYL-PIP = compound 5, PROPYL-MEPIP = compound 6).

(b) Effects of unsaturation. The butyl compounds (7 and 8) are compared with their *cis* analogues (9 and 10), their *trans* analogues (11 and 12) and the butynes (Barlow *et al.*, 1988). Their overall affinities are similar but the butyne base (BUTYNE-Me_2N) has appreciable selectivity.

(c) Replacing phenyl by *cyclo*pentyl in 4-DAMP methobromide (4DAMPMEBR) (compound 13) increases affinity more than replacing phenyl by *cyclo*hexyl (Barlow & Shepherd, 1988). High selectivity is found with *p*-fluoro-hexahydro-sila-diphenidol (compound 18) but it has relatively low affinity (lower than sila-benzhexol). The results with this compound are similar to those of Eglen *et al.* (1989).

1969), i.e. that binding of the diphenylacetyl and onium parts of these molecules is very similar but with 4-DAMP MeBr there is an additional large contribution from the ethylene bridge which forms one side of the piperidine ring. The maximum observed effect of a phenyl group on affinity in these compounds (Abramson *et al.*, 1974; Barlow, 1980) is an increase of 3.5 log units so with two phenyl groups log K might be around 7. The charge might contribute 1 log unit to binding (Barlow & Tubby, 1974; Barlow, 1980) and, with 1.5 log units from the two methylene groups (see above), log K would be 9.5 for 4-DAMP MeBr. This is only 0.5 log units more than the experimental value for ileum, suggesting that these groups are so arranged that they can exert almost their maximal effects and the structure of 4-DAMP MeBr may be a good template for the structure of part of the muscarinic receptor in ileum.

The absence of a difference between the *cis* and *trans* butenyl compounds (Figure 1b) could be explained if these are not bound in an extended form. If the N—C—C—C—C— chain has N—C—C—C *trans* and C—C—C—C *cis*, the length will be similar if the arrangement is N—C—C—C *cis* and C—C—C—C *trans*. From the low affinity of these compounds compared with 4-DAMP, it seems likely that these parts of the molecule are not much involved in binding and that they merely link the main binding groups, the onium atom and the two large (phenyl/*cyclo*alkyl) rings. From measurements on Dreiding models the nitrogen to (end) carbon distance is around 4 Å. In the amino-butynes, which are held extended by the acetylenic bond, this distance is nearer to 5 Å.

In the hexadiynes (16 and 17) the end-to-end carbon distance is almost 7 Å and the nitrogen-to-(end) carbon distance is greater than this. Their inactivity suggests a definite limit to the size of group which can be accommodated: in 4-DAMP methiodide the distance between the nitrogen and the carbon of the acetyl group is only 6.5 Å in the crystal (Barlow *et al.*, 1987). The inactivity of the camphor sulphonates also suggests that there is a limit to the bulk of the ring which is acceptable. It has long been known that the combination of phenyl and cyclohexyl rings leads to high affinity but the phenyl*cyclo*pentyl-acetoxy analogue of 4-DAMP MeBr is actually more potent than its phenyl*cyclo*hexyl analogue (Figure 1c). The carbon-carbon distances in a phenyl ring are approximately 2.8 Å lengthways and 2.3 Å across (from the crystal structure for 4-DAMP methiodide (MeI), Barlow *et al.*,

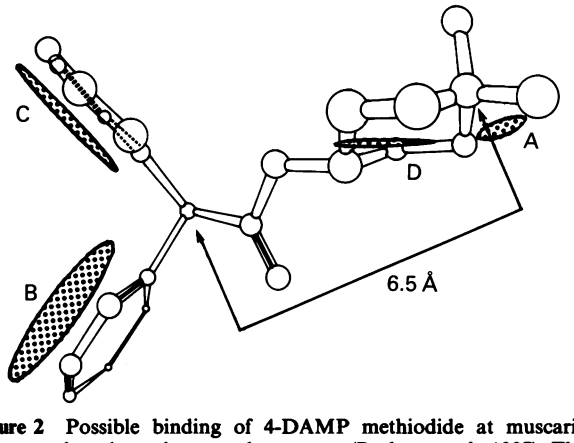

Figure 2 Possible binding of 4-DAMP methiodide at muscarinic receptors, based on the crystal structure (Barlow *et al.*, 1987). There are possible contributions from: (A) the onium nitrogen atiom, (B) one large (phenyl or *cyclo*hexyl) ring, (C) a second ring and (D) the two ethylene groups in the piperidine ring. As drawn the postulated binding groups in the receptor (indicated by shading) are towards the viewer so the drug will approach from the rear. The distance between the onium and acetyl groups in the crystal is 6.5 Å: in the diacetylenes, which are inactive, the comparable distance is about 7.0 Å. It is suggested that the onium binding site (A) is on the outer side (more easily accessible from solution) and that with longer molecules a better fit is obtained with the smaller dimethylamines than with trimethylammonium salts. There are probably size limits (possibly about 3Å) to groups binding at sites B and C, because the camphorsulphonate esters, in which some distances in the ring are around 3.8 Å, are inactive. The relative positions of sites B and C are important. The 'claw-like' structure of many muscarinic antagonists was commented on by Hamor (1977) and the replacement of tetrahedral carbon by trigonal nitrogen in the carbamate analogue of 4-DAMP MeBr produces a compound which appears to bind only to one site, not to both (Barlow & Johnson, 1989). Reasons for believing that site D is particularly important in the binding of 4-DAMP are discussed in the text.

1987): for a *cyclo*hexyl group the corresponding distances are approximately 2.9 Å and 2.5 Å (from the crystal structure for phenyl*cyclo*hexyl glycolloyl ethyl piperidine methiodide, Hamor, 1977); for camphor-10-sulphonic acid the distance between the 8 (or 9) methyl group and carbon atoms on the far edge (2, 3, 5 or 6) is nearer 3.8 Å (from the crystal structure, Koch *et al.*, 1975), again suggesting that there are limits to the size of the group which can be accommodated. In Figure 2 an attempt has been made to collect these ideas together, based on the crystal structure of 4-DAMP MeI.

Although this picture can only be conjecture, the size limits should help to check molecular modelling of the muscarinic receptor based on its amino acid sequence which is now known (Kubo *et al.*, 1986a; 1986b; Bonner *et al.*, 1987; Peralta *et al.*, 1987; 1988). It is possible to speculate about the actual groups involved in the binding of muscarinic antagonists and work with propylbenzilyl choline mustard by Curtis *et al.* (1989) suggests that the binding of the onium group involves an aspartate residue (D, 105) in transmembrane helix 3. There are tryptophan (W) and tyrosine (Y) residues nearby which might be involved in binding phenyl or *cyclo*alkyl rings in the antagonists, but the aminoacids present in this part of the sequence are very similar (CDLWL**DYV*) in all four receptor types studied by Bonner and colleagues. Although the relative positions of these groups is not known (or even whether they are the ones actually involved), this may explain why it is difficult to achieve selectivity. Differences in the geometry of the receptors, which allow a drug to be selective by fitting one shape much better than another, may depend on differences in the arrangements of the same groups caused indirectly by differences in other groups in the sequence.

In these compounds it seems that there are definite steric constraints and that selectivity is achieved by introducing groups which reduce binding but have a greater adverse effect at receptors in atria than at those in ileum.

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